KINETIC BEHAVIOR OF A PECTINASE COCKTAIL AT HIGH HYDROSTATIC PRESSURE CONDITIONS

By

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To my family

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HHP	High Hydrostatic Pressure
PG	Polygalacturonase
PL	Pectin Lyase
PME	Pectin Methyl Esterase

Abstract of Dissertation Presented to the Graduate School
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Apple juice is one of the most popular fruit juices consumed in the United States. Pectinase cocktails, containing pectinases, hemicellulases, and cellulases are used in the production of commercial apple juice to reduce juice viscosity, increase yield, and to clarify the final product (Jayani and others 2005). High hydrostatic pressure (HHP) at moderate levels has stabilized and activated some enzymes. This project focused on testing a high hydrostatic pressure process to stabilize pectinase enzymes at raised temperatures above levels customarily used for clarification for possible economic benefits to traditional thermal processing.

In the first set of experiments, the kinetics of inactivation of a commercial pectinase formulation was studied at 0.1 to 400 MPa and 55-85 °C. High pressure slowed the rate of inactivation of the pectinase cocktail treated at inactivating temperature conditions. Samples treated at temperatures of 77 °C or 85 °C and high pressures (200 – 400 MPa) retained activity for at least 8 times longer than samples treated at atmospheric pressure.

In the second set of experiments, the stabilization of a commercial pectinase formulation at high hydrostatic pressures (200 and 300 MPa) at moderate temperatures (42.4 to 62.4 °C) was studied. The rate of viscosity reduction increased with temperature with a maximum of 0.0960 $Pa^{-1} \cdot s^{-2}$ occurring at 62.4 °C and 300 MPa. Negative apparent activation volumes of -0.22 to -5.21 $cm^3 \cdot mol^{-1}$ demonstrated that pressure favored the increase in the rate of viscosity reduction with pressure having the greatest effect at 57.1 °C. Apparent activation energies suggest that temperature had greater effects at high pressure (200-300 MPa) than at atmospheric pressure.

In the third set of experiments, the effects of high hydrostatic pressure on the shift in pH of pH indicator solutions were explored. Absorbance spectra for color acid/base indicators (pH 1.2 to 6.8) in the visible light range were used to quantify the change in pH induced by high pressures up to 600 MPa. Increases in pressure produced an apparent acidic pH shift in acid/base color indicators.

CHAPTER 1
BACKGROUND INFORMATION AND LITERATURE REVIEW

Apple juice is the second most popular fruit juice consumed in the United States only behind orange juice. In 2007-2008, over 535,000 metric tons of concentrated apple juice were consumed (USFAS 2008). Americans drank an average of 8.34 L of apple juice (single strength equivalent) per capita in 2006/07, while consuming only 3.79 L of all other non-citrus juices such as grape, pineapple, cranberry and prune juice (Pollack and Perez 2008). Improved processing for fruit juices can lead to higher quality products. Consistent high quality products are more likely to see increased popularity among juice drinkers and greater economic profits over competitors.

The objective of this chapter was to give an overview of clarified fruit juice production focusing specifically on clarification and pasteurization. The effects of high hydrostatic pressure (HHP) on the stabilization of enzymes and inactivation of microorganisms will be reviewed in detail. Also it is known that HHP induces shifts in pH which in turn may be responsible for some of the effects of HHP on juice processing. A review on the thermodynamics and methods of assessing effects of pH in food processing are provided. This chapter describes the current methods of juice clarification processing, the behavior of enzymes with HHP treatments, and identifies relevant gaps of knowledge in industrial juice processing. Finally, the specific objectives of this research are presented.

Apple Juice Production

Apple Juice Production Overview

Apple juice consumption has increased with the rise of orange juice prices. Because most apple juice in the U.S. is imported from other countries such as China,

Chile, and Argentina, apple juice has a large economic impact on the U.S. In 2007, the U.S. spent a half billion dollars importing over 90% of its consumed apple juice (USFAS 2008).

The U.S. produces apples for fresh consumption and mostly cull apples are sent to juice processing. While the amount of fruit for fresh consumption has increased over the past thirty years, apples for processing (juice and cider, canned, frozen, and fresh apple slices) have fluctuated. In 1980 the amount of apples processed was 3,866 million tons, and this amount increased to over 4,000 million tons in the 1990s. However, since the 1990s the amount of apples processed decreased and in 2007 was down to 2,979 million tons. The grower's price, defined as "average price received by producers from the first level of sales for the commodity" has not shown a clear trend over the past thirty years. From the period of 2000-2007, four seasons produced a grower's price of below \$0.093/kg for juice and cider processing and four seasons produced a grower's price over \$0.110/kg. Growers received more for a season when lower quantities of apples were produced. Apple juice consumption increased slightly for each season. In the early 1980s consumption per person was under 5.68 $L \cdot yr^{-1}$ and has increased to 8.33 $L \cdot yr^{-1}$ for the 2006/07 year (Pollack and Perez 2008).

Processing

Lea (1990) and Lozano and SpringerLink (2006) have reviewed in detail the processing of apple juice. A summary is provided here with a visual flow diagram of apple juice processing in Figure 1-1. Raw apples, typically culls, are processed into juice products that range from clear single strength juice to cloudy or natural juice, cider or juice concentrates. The juice concentrates will be made into juice blends or diluted back to single strength after shipping. Apple juice can be made into several different

final products, but all the juices have the same initial process. The front end operation is the portion of the processing that involves the collection and classification of the fruit. The apples are sorted, washed and then milled before pressing. The fruit is washed with rotatory brushes that remove unwanted contaminants such as rot, soil, pesticides, and microorganisms. A variety of mills can be used, fruit grinding mills, rasp or grater mills, or fixed blade hammer mills to reduce fruit size. During pressing, the juice is extracted and the pomace is removed. The pressing process varies slightly depending on equipment; popular equipment includes the rack and cloth press, horizontal pack presses, screw presses, and continuous belt presses. After pressing, the production process normally depends on the type of juice to be produced: cloudy single strength juice, clear single strength juice, or clear juice concentrate.

Concentrated products have the aromas stripped off before the clarification process. Methanol created from the depectinization with pectin methylesterases (PME) in the clarification process can damage the aroma essences and decrease the recovered aroma volume. Cloudy juice only goes through a rough filtration or centrifugation to remove large solid particles and defects before it is pasteurized and packaged. The clear juice products go through a clarification step, in which polysaccharides, starch, and cellulose molecules that cause the juice haze are broken down with enzymes and allowed to settle over a period of time. Clarification can include fining, centrifugation, and several filtration steps before the juice is pasteurized and packaged. Fining agents, such as gelatin and bentonite, are used in the fining process to remove haze causing particles from the juice. The fining agents cause particles to sink out of solution by either promoting attractive charges or increasing the particles'

weight. Next pasteurization can be performed with different methods; two popular methods include high temperature short time pasteurization (15 s at 72 °C) or ultrahigh temperature (0.1 s at or greater than 121 °C). Pasteurization is covered in greater detail in a future section of this review. For concentrated products, centrifuged and filtered juice is evaporated to remove the water and increase the concentration of soluble solids to around 70 °Brix. The concentrate can be shipped then reconstituted back to single strength and/or blended with other juices, and filtered before the product is pasteurized and packaged.

To maintain quality and confidence of apple juice customers, producers must have strict quality control. The Code of Federal Regulations (CFR) states 100% single strength apple juice must have a minimum soluble solids content (SSC) of 11.5 °Brix (21CFR101.30). The Food and Drug Administration (FDA) has guidelines in the form of a Hazard Analysis Critical Control Point (HACCP) plan with requirements for juice manufacturers to ensure consumer safety (USFDA 2004). The hazards that concern the FDA in apple juice production are patulin, microorganisms, and metal fragments. Patulin is a toxin produced by molds such as *Penicillium*, *Aspergillus* and *Byssochylamys* that can grow on apples and appears in higher quantities on damaged fruit (rotting or bruised fruit caused by falling, birds, insects, etc). The FDA recommends removing damaged fruit or fruit pieces before processing and having the fruit supplier guarantee the quality of fruit harvests (not from damaged fruit). The pertinent microorganisms for apple juice are *Escherichia coli* O157:H7 and *Cryptosporidium parvum,* and a standard 5-log reduction of these organisms during pasteurization is required. Pasteurization conditions including temperatures and holding times will vary from company to company

due to the difference in process equipment, production size and pasteurization temperature. Finally, the FDA requires using screens during processing to ensure the metal fragments from processing equipment are not found in the final juice product (USFDA 2004). A juice company must guarantee consumer safety for a profitable product.

Haze molecules

The molecules that cause haze formation and increase juice viscosity come mainly from the fruit cell wall. These molecules include polysaccharides (pectins, starches, and gums), proteins, polyphenols, polyvalent cations, and lipids. Starch is mainly found in young fruit and therefore is not as prominent as pectin in the juice haze from the production of mature apples. Pectin however is the leading cause of the juice haze and it is specific to the middle lamella of fruit, the section between cells walls (Jayani and others 2005). Pectin and other cell wall components are responsible for providing support and texture to the fruit (Alkorta and others 1998). Pectin's negative charge repels one molecule from the other (Binning and Possmann 1993). Pectin amounts vary in fruit and vegetables. Pectin amounts can be as high at 10-30% dry matter in sugar beet pulp. Fresh apple tissue can have between 0.5 to 1.6% pectin, which is similar to bananas, but more than strawberries and cherries (Jayani and others 2005). Pectin molecular weights can vary in apples and lemons (200-360 kDa), while in other fruits such as pears and prunes (25-35 kDa) or oranges and sugar beet pulp (40-50 kDa) the molecular weight has little variation.

Apple pectin molecules are large multifaceted molecules that are highly methoxylated (Lea 1990). Though pectin is not a uniform substance, the core chain of molecules is made up of anhydrogalacturonic acid (Jayani and others 2005). The linear

portion of pectin chains is termed the smooth region (Pedrolli and others 2009). The galacturonate of the core chain are connected by alpha 1-4 linkages. The methyl groups common in apple pectin are attached to the carboxyl group of the galacturonic acid. Besides the esterification or of the carboxyl group or acetylation of the hydroxyl groups of other carbons (C_2 or C_3), sodium, potassium and ammonium ions can neutralize the carboxyl groups by removing the final hydrogen and replacing it with the ion. Pectin is highly variable and can have side chains of arabinan, galactan, arabinogalalactin, xylose, and fructose (Jayani and others 2005). Besides the long smooth linear portion of pectin, there are portions of the pectin molecule with side chains made up of Rhamnogalacturonan I and II. These portions of pectin are termed the hairy region. Sections of rhamnogalacturonan I (RG I) have disaccharide rhamnose in between the galacturonic units in the pectin chain and can contain the previously mentioned side chain. The other portion of the hairy region of pectin contains rhamnogalacturonan II (RG II) have the linear galacturonan chain with the side chains (Pedrolli and others 2009).

Pectin has been separated into four main groups by the American Chemical Society: protopectin, pectic acid, pectinic acids, and pectin (Jayani and others 2005). Protopectins are the insoluble form in the fruit. The remaining three groups differ in the amount of methyl groups on the esterified carboxyl groups: Pectic acid has very little esterification, pectinic acid esterification is below 75%, and pectin chains contain carboxyl groups that at least 75% have been esterified with methyl groups (Jayani and others 2005). Often pectin molecules can interact with proteins to create proto-pectin

(Kashyap and others 2001). Apple juice contains highly methylated pectin, which must be taken into account when selecting a suitable blend of enzymes for clarification.

Clarification

During juice extraction, particles from the cell wall form a cloudy haze in the juice making it viscous and difficult to filter (Binning and Possmann 1993). These substances also decrease the juice yield from the fruit's pulp. For clear juice products the clarification step, i.e. the removal of haze causing particles, is performed after the apples are pressed and before filtration, centrifugation, fining, and evaporation.

The haze-causing particles are removed by addition of enzymes that break down pectin and suspended insoluble molecules allowing the particles to settle. Enzymes are used during fruit mashing and clarification to decrease viscosity and improve juice yield (Binning and Possmann 1993). In white grape juice clarification, the commercial product Pectinex™ (Novo Industries, Denmark) had the highest degrading activity for pectin and starch of commercial products and removed 98-99% of turbidity. The other commercial enzyme preparation Celluclast™ (Novo Industries, Denmark) and a laboratory *A, niger* pectinase only removed 50-60%. These results illustrate the difference in activities of selected commercial products (Sreenath and Santhanam 1992). After enzymatic treatment, small insoluble particles remain in the fruit juice and cause a turbid haze (Gutiérrez-López and others 2008). Therefore other processing steps including decanting, centrifugation, filtration, and fining are often necessary.

Clarification can be monitored by measuring the turbidity of the juice and has been studied in various dark and clarified juices. Turbidity is measured in different units depending on the observation methods; two notable units are Formazinain Nephelometric units (FNU) or Nephelometric Turbidity units (NTU). Formazinain

Nephelometric unit measurements are more common in Europe and made with infrared light, while NTU measurements are made with white light. Stable clarified juices are those having low turbidity of less than 2 NTU (Araya-Farias and others 2008). As seen with the turbidity values from several clarified fruit juices in Table 1-1, enzymatic treatments play a critical role in clarification.

Clarification enzymes

There are several classes of enzymes used in the apple juice clarification process, including pectinases, lyases or trans-elminases, protopectinases, and proteases among others. Pectinases have been described elsewhere (Alkorta and others (1998), Jayani and others (2005), and Pedrolli and others (2009)) therefore, this section only briefly summarizes the findings of enzymes specific to the pectin molecules. The first main enzyme pectin methyl esterase (PME, E.C, 3.1.1.11) is a hydrolase that demethyloxylates the galacturonic acid in apple pectin shown in Figure 1-2(a) (Lea 1990). Figure 1-2 was adapted from (Pedrolli and others 2009). Pectin methyl esterase belongs to the group of enzymes that de-esterify pectin. This enzyme goes by many names: pectinase, pectin methoxylase, pectin demethoxylase, and pectolipase. The molecular weight of PMEs range from 35-60 kDa and have an optimum temperature range of 40-50 °C. Though the average pH range for pectin PMEs is from 4 to 8, fungal PMEs are more active at an acidic pH and bacterial PMEs are more active at a basic pH.

These enzymes are carboxylic acid esterases that hydrolyze methyl groups of methylated pectin. The final product of the de-esterification is pectic acid seen in Figure 1-2(a).The side product of the de-esterification is methanol. If the pectin is esterified with an acetyl group, there are similar enzymes called pectin acetyl esterases that

remove acetyl group from the pectin to make pectic acid and acetate. De-esterifying enzymes are found in fungi, yeast and bacteria, and they are also involved in the aging and ripening process in fruits and vegetables. Pectin methyl esterases from fungi randomly remove the methyl groups, while plant PMEs have a single chain mechanism in which the enzyme reacts with non-reducing end or next to a free carboxyl group. In fruits and vegetables, PMEs are part of the changes that occur in the cell wall during growth. A gel diffusion assay is common for PME activity determinations: as PME removes the methyl groups, more ruthenium red (used to stain for spectroscopy) can bind to the pectin thus greater ability to measure pectin in solution (Downie and others 1998). The pH changes are another way to determine PME activity due to the release of the hydrogen atom from water during the ionization of the carboxyl group (Whitaker 1984). Pectin methyl esterase's effect on viscosity is minimal, but allows the pectin molecule to be broken down into smaller chains by enzymes such as polygalacturonase.

Polygalacturonase (PG) and pectin lyase (PL) are depolymerizing enzymes .The depolymerizing enzymes break down pectin in two ways: hydrolysis or β-elimination. Polygalacturonases, endo-PG (E.C. 3.2.1.15 and exo-PG (E.C. 3.2.1.67), use hydrolysis by using water to break the galacturonic acid α-1-4-glycosidic bond (oxygen bridge) shown in Figure 1-2(b). The enzymes that use hydrolysis to break down pectin molecules are varied; polygalacturonases are used with pectate while polymethylgalacturonases break down pectin (highly esterified), and endo-enzymes attack randomly while exo-enzymes focus on the terminal ends of the molecule. Exo-polygalacturonases are more common in fruit and vegetables, and endo-galacturonases

are found in other higher plants as well as fungi, bacteria, yeasts, and nematodes. Microbial endo-PGs have been cloned and studied for research. Fungal and bacterial exo-galacturonases produce different products; monogalacturonic acid and digalacturonic acid respectively. In apple juice clarification, PG can only break down galacturonic acid units, but not methylated galacturonic acid. Polygalacturonase causes the proto-pectin complexes to settle out by partially removing the negative pectin charge, but the PG enzyme cannot demethoxylate the pectin (Lea 1990). The method used to measure PG activity involves 3,5-dinitrosalicylate reagent method or arsenomolybdate-copper reagent method to determine the rate of increase in reducing sugars with activity being described as 1 $\mu mol \cdot mL^{-1} \cdot min^{-1}$ galacturonic acid (Somogyi 1952; Miller 1959). Another method to measure PG activity uses the flow rates of water, a test solution, and test solution with enzyme to determine the reduction in viscosity with the activity being described as amount of enzyme required to produce a specific decrease in viscosity per unit of time (Roboz and others 1952). Polygalacturonases vary greatly from one another; molecular weights range from 38-496 kDa (most range from 38-79 kDa), optimum pH range from 3 to11, and optimum temperatures range from 30 to 69 °C. Common microbial PGs are most active in the acidic range (pH 3.5-5.5) and warm temperatures (30-50 °C). *Aspergillus*, *Bacillus*, *Fusarium*, *Penicillium*, and *Termoascus* species are some of the producers of PG.

Lyases or trans-eliminases are the second major group of enzymes classified as depolymerizing enzymes. Lyases, endo-PL (E.C. 4.2.2.2) and exo-PL (E.C.4.2.2.9), use trans-elimination instead of water to break the α-1-4-glycosidic bond as shown in Figure 1-2(c). When lyases cleave the α-1-4-glycosidic bond, hydrogen from the C-5 and

oxygen are moved to create a hydroxyl group on one product and double bond on the second galacturonic acid.

Similar to the polygalacturonase enzymes, lyases can break down highly esterified pectin or pectate. Polymethylgalacturonate lyases (PMGL) do not remove the methyl group from the esterified galacturonic acid. While the enzymes that are specific for pectate (polygalacturonate lyases or PGLs) can be cleave random (endo) or at terminal ends (exo), the enzymes specific to pectin (PMGLs or PLs) are mainly endo-enzymes. For PGLs, the endo form of the enzyme is more common than the exo form. Another difference between PGL and PMGL are that PGLs require calcium ions, while PGMLs do not. Calcium and other ions can increase PGMLs behavior and chelating agents can inhibit PGL. Lyases can be made by bacteria and pathogenic fungi. For apple juice clarification, PL degrades polygalacturonate chains of methylated pectin (Lea 1990). There are several ways to determine the activity of lyases. A common way is to observe the production on the double bonds in the product with the increase of absorbance (235 nm wavelength), and activity is described at 1 μmol of unsaturated product per minute. Other methods for measuring PL activity are through reducing group methods, reduction in viscosity, and HPLC or GC analysis. Pectin lyases are mainly found in microorganisms such as bacteria and fungi, but rarely in higher plants. Lyases from bacteria are often associated with plant pathogenicity to break into fruit and vegetables. Lyases have an average molecular weight of 30-40 kDa, but some PLs have large molecular weights greater than 50 kDa. Also, this enzyme tends to be active at higher temperatures than other pectinase enzymes as the optimum ranges is between 40 and 70 °C and the optimum pH range is 7 to 10. Although, a few PMGLs from species

Aureobasidium pullulans LV-10 and *Pichia pinus* have lower optimum pH of 5 and 4.5, respectively. *Aspergillious*, *Bacillus*, *Debaryomyces*, and *Penicillium* species are some of the main producers of PL.

The final group of enzymes is specific to protopectins and is therefore named protopectinases. The enzymes can also be called pectinosinases, and these enzymes use water while converting protopectin to pectin. The enzymes are classified by the site they cleave to break insoluble protopectin into pectin; type-A enzymes are specific to the polygalacturonic acid region and type-B enzymes are specific to the polysaccharide chains of protopectin. Protopectinases are usually produced by yeasts and fungi similar to yeast.

Other enzymes are used to break down the remaining chains found in the pectin molecule. There are enzymes that are specific to the rhamnogalacturonan chains and chains containing xylose molecules. The rhamnogalacturonan enzymes that hydrolyze the rhamnogalacturonan chain are rhamnogalacturonan rhamnohydrolases, galacturonohydrolases, and hydrolases that produce rhamnose, monogalacturonate, and oligogalacturonates respectively. Other enzymes include rhamnogalacturonan lyases, rhamnogalacturonan acetylesterases, and xylogalacturonan hydrolases.

For apple juice clarification, pectin is the main target molecule because apple pectin molecules are highly esterified. As the particles are removed from solution by PME, PG, and PL, the juice becomes less viscous and yield increases. Without the pectin, the juice appears clear and after fining is appealing to the customers. Though clarification is an established technology, new research continues to focus on different enzymes in respect to their sources, production methods, and behavior under different

conditions in order to improve juice processing and clarification. Research focusing on clarification enzymes will be covered more in-depth within the innovations portion of this review.

Clarification processing conditions

After pressing, apple juice and pulp are stirred at least 1 to 20 min for fruit oxidases to oxidize the polyphenols, which are enzyme inhibitors. Sometimes the juice is centrifuged to remove large particles before clarification (Kashyap and others 2001). The clarification process is carried out between 15 and 55 °C (Lea 1990). As mentioned, polysaccharides (pectins, starches, and gums), proteins, polyphenols, polyvalent cations, and lipids are some of the particles that can create the juice cloud, and enzymes such as pectinases, amylases, and proteases are used to treat these particles (Binning and Possmann 1993).

Industry uses specific tests to ensure their process has removed the necessary amount of pectin from the apple juice. One of the standard methods is the alcohol precipitation test, in which one part juice and two parts ethanol are combined. If flocculation does not occur in about 15 min, the process was successful (Lea 1990). However this test provides results that are subjective and hard to quantify. As researchers, we have more conclusive ways to analyze the clarification than industry. For clarification, it is desirable to determine the effect of temperature on enzyme cocktail activity to optimize the process for each juice. One way to observe the activity of a pectinase cocktail is to measure its effect on the viscosity of a pectin solution, and this method will be used for this research. As enzyme activity increases, then a greater decrease in a treated pectin solution viscosity will be observed. The rate constants of enzyme inactivation or viscosity reduction can be used with the Arrhenius equation to

calculate the activation energy (Ceci and Lozano 1998; Ortega and others 2004; Eisenmenger and Reyes-De-Corcuera 2009b). The activation energy value establishes the effect of temperature on enzyme activity with low values having negligible temperature effects on enzymes; activation energies are important in characterizing enzyme behavior at different conditions. The extent of clarification can also be monitored by the transmission of light through a centrifuged sample of juice. Clarified juices will absorb less light than cloudy juices achieving a higher transmission value.

Pasteurization

Apple juice consumers desire a safe product with organoleptic and nutritional properties that are comparable to fresh juice. Pasteurization is important to inactivate microbial populations that would either cause product spoilage or consumer illness. A low pH in many juices (orange, apple, and grapefruit) due to naturally present organic acids helps prevent a large variety of microorganisms from growing in fruit juices; yet, some microorganisms can survive, and some may become acid-adapted (Mosqueda-Melgar and others 2008). Pathogenic microorganisms that can survive and grow in apple juice are the focus of producers and the FDA to create a safe product for consumers.

Microbes pertinent to apple juice

The acidic pH of apple juice (typically between pH 3.0 to 4.5) decreases the types of microorganisms that can survive in the juice and many human pathogens do not actively grow in fruit juice. But some pathogens such as *E. coli* O157:H7 and some *Salmonella* species can survive. The microorganisms that have adapted to acidic environments are termed acid-adapted (Mazzotta 2001). The FDA recognizes that pathogens occurring in nature and from animal manure can contaminate acidic juices

(pH below 4.6) and low acid juices (pH above 4.6) and cause foodborne illness in humans. For acidic juices, *E. coli* O157:H7, *Listeria monocytogenes*, *Cryptosporidium parvum*, and a variety of *Salmonella* species can taint juice; and low acid juice, like carrot juice, can host *Clostridium botulinum* (USFDA 2004).

One of the earliest noted outbreaks of apple products was apple cider containing *Salmonella* Typhi in 1922 (Paquet 1923). *Escherichia coli* O157:H7 was noted later with cases in fresh apple cider in Canada (1980) and the USA (1991) (Steele and others 1982; Besser and others 1993). Cases continued to appear in the 1990s, and *Cryptosporidium parvum* caused an outbreak in New York in fresh apple cider in 1996 (USCDC 1997). These outbreaks were mainly illnesses with limited deaths. Illness associated *E. coli* O157:H7 have symptoms of bloody diarrhea and hemolytic uremic syndrome (HUS) (Luedtke and Powell 2000). Many of these cases were due to inadequate sanitation or fecal contamination of apples. The possibility of contamination of pathogenic microorganisms during apple production and juice processing presents the reason for which proper pasteurization is a necessity for safe human consumption.

For each juice, the FDA has requirements for the treatment of the pertinent microorganism or microorganism associated with any of the specific juice outbreaks. The FDA considers *Cryptosporidium parvum* and *E. coli* O157:H7 to be the pertinent microorganisms for apple juice (USFDA 2004). However, juice processors must be mindful of all pathogens that can contaminate juice including *Salmonella* Eenteritidis which has also been found in grapefruit juice and lemonade (Mosqueda-Melgar and others 2008) and the commonly occurring *Listeria monocytogenes* (USFDA 2004).

In addition to pathogenic strains, producers must also be concerned about spoilage microorganisms that deplete shelf life and cause economic losses with destroyed products. Spoilage microorganisms cause unwanted sensory characteristics and resist thermal processing. A major juice spoilage microorganism of concern for apple juice producers is the spore former *Alicyclobacillus acidoterrestris* that is a thermo-acidophilic bacterium. *Alicyclobacillus acidoterrestris* found in soil, thrives in optimal conditions of pH 4.5 to 5 at 36 to 53 °C, and can survive in the pH range of 2.5 to 5.8 at 20 to 70°C. Currently, no strains of *A. acidoterrestris* are considered pathogenic. Spoilage is noted as off flavors, odors, as well as possible sedimentation or change in color or cloudiness. Guaiacol, and halophenols 2,6-dichlorophenol and 2,6-dibromophenol are responsible for the off flavors and odors creating medicinal, antiseptic and smoky notes. *Alicyclobacillus acidoterrestris* spores are resistant to thermal treatments, but vegetative cells are responsible for spoilage (Smit and others 2011).

Pasteurization standards

The FDA has mandated a 5-log reduction of the pathogens pertinent or relevant to each juice for each pasteurization process. A study cited by the FDA recommends treating juice for 3 s at 71.1 °C for a 5-log reduction of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes (*Mazzotta 2001*)*. The pasteurization conditions in apple juice are often recommended for the pathogen *E. coli* O157:H7, because of the lack of information on the pathogen *C. parvum*. The FDA makes recommendations for a 5-log reduction of *C. parvum*, which is more heat resistant than other microbes, by thermal processes ranging from 71.1 °C for 6 s to 82.2 °C for 0.3 s (USFDA 2004).

Juice nutrients

A major focus of juice production is creating safe shelf stable products while maintaining vital juice nutrients. A section is included on juice research focused on optimizing juice processing to retain important nutritional characteristics of juice such as antioxidants and vitamins. This section provides examples of juices notably high in antioxidants: blueberry and pomegranate juices.

Anthocyanins, beneficial nutrients commonly found in plants such as blueberries, eggplants and grapes, are part of juice antioxidants and contribute to the color of the fruit and its juice (Buckow and others 2010). Thermal treatments of blueberries and its juice have to be higher than 80 °C to deactivate polyphenol oxidases (PPO) which can breakdown anthyocyanins in blueberry juice; however, thermal degradations of nutrients and flavors are an unwelcome side effect. The anthocyanin concentrations in juices and reactions to processing vary due to different types and amounts of anthocyanins, pH, presence and concentration of degrading enzymes, free radicals, and ascorbic acid. With this complicated matrix, experimental degradation of blueberry anthocyanins gave a thermal degradation that acted as a 1.4^{th} order reaction. Though high pressure and high temperature slowed the degradation of blueberry anthocyanins in storage when compared to untreated juice; thermal processing had the slowest degradation and highest concentration of anthocyanins after storage. The total anthocyanins half-life was highest for a storage temperature of 4 °C with a half-life of 251.7 d for the thermal treatment of 90 °C. For storage at 4 °C, the juice processed at 600 MPa at 90 °C, had a half-life of 245.7 days. Half-lives decreased with higher storage temperature blocks (25 and 40 °C), and thermal processing had higher half-lives than pressure/thermal treatments with warmer storage. Pressure/thermal and thermal processing both

degrade ascorbic acids; however, the increased free radicals and oxygen concentration during pressurization could lead to the faster degradation rates than thermal processing (Buckow and others 2010).

Antioxidants and vitamins are also crucial to the popularity of pomegranate juice. Hydrolysable tannins are the main antioxidant of pomegranate juice, but anthocyanins and phenols also contribute to the antioxidant composition. These molecules are affected by light, pH, temperature and oxygen content. High pressure is being considered an alternative to control microbial populations while limiting the loss of antioxidants and nutrients. Pomegranate juice with an initial microbial load of 10^4 cfu/mL was reduced to a population at or below 1 cfu/mL by high pressure processing for 400-600 MPa, 5-10 min, and 25-50 °C. Temperature and process time had the greater effects on color and aroma of pomegranate juice than high pressure. Turbidity decreased with high pressure processing. Anthocyanins decreased with high pressure processing, except when processed at 500 MPa for 5-10 min and 600 MPa at 10 min (both with the processing temperature of 50 °C). Phenols increased in concentration for samples treated at 400 MPa and 50 °C; the untreated juice polyphenol content was 1.36 g/L of gallic acid and treated samples had a phenol content 1.48 g/L of gallic acid and 1.92 g/L of gallic acid for 5 and 10 minutes processes respectively (Ferrari and others 2010).

Current Innovations

Juice producers and researchers continue to search for and develop processes that make juice production more economical and more consistent with fresh pressed juice. This section will highlight some of the recent research involving clarification

enzymes juice production/pasteurization, and new clarification techniques that will improve future production and that is relevant to this research project.

Innovation in Clarification enzymes

Juice clarification is a current area of study due to its importance in the juice processing. Juice clarification reduces viscosity and cloudy haze for ease of downstream processing and visual desirability; however, the clarification step is a time-consuming phase with the added expense of heating and the addition of enzymes. Current research focuses on improving clarification by decreasing clarification times and reducing expenses. Enzyme immobilization to retain and reuse enzymes during processes as well as new sources of pectinases and mixtures could decrease clarification time and enzyme requirements.

Researchers have looked at immobilizing enzymes for enzyme retention during apple juice clarification and have found immobilized pectinases performed better with pan (polyacrylonitrile) beads over glass and nylon beads. Immobilized pectinases have higher optimum pH (pH 6.2) than free enzymes pH 5.6, but do not perform as well as free enzymes at acidic conditions. Diano and others (2008) found the optimum temperature range of 48 to 65 °C for apple juice clarification for immobilized enzymes was similar to that of free enzymes (43 to 65 °C).

Clarification research compares new enzymes from different sources to concentrated commercial products traditionally used in industry. Table 1-2 highlights some of the new enzymes in juice clarification research. The fungi *Aspergillus niger* is popular in research due to its high pectinase activity and commercial safety for food products. Sandri and others, (2011) observed the effect of *A. niger* T0005007-2 (TE1) and *A. oryzae* IPT 301 (TE2) to compare commercial clarification enzymes

Pectinex®Clear (PC) and Pectinex®BeColour (PB) for light and dark clarified juices. Viscosity reduction has been used for total pectinase activity with one unit of activity stated as the necessary amount of enzyme required to reduce the viscosity by 50% (Sandri and others 2011). Pectinex®Clear and PB had total pectinase activities of 919 $U \cdot mL^{-1}$ and 1150 $U \cdot mL^{-1}$ respectively, and TE1 and TE2 had activities of 75 $U \cdot mL^{-1}$ and 44 $U \cdot mL^{-1}$. However, the new enzyme extract TE1 had a larger exo-PG activity then PB and performed comparably in clarification studies to commercial products. For apple juice clarification, based on turbidity reduction, TE1 and PC had similar results of about 60% clarification with treatments with PC for 60 min at 30 °C, and TE1 treated samples at 50 °C for periods of 30 and 60 min. *A. niger* T0005007-2 had comparable or greater clarification results than PC in Butia palm juice and PB in blueberry and grape juice. However, TE2 did not produce the largest turbidity reduction for any of the four juices. Though the new extract TE1 had lower total activity, its PG and PL activities were higher resulting in similar results as commercial products (Sandri and others 2011). The research shows that the ratio of individual enzymes (PG, PME, and PL) in products is a large factor in overall pectinase behavior.

Another area in innovation in enzymes is the potential to clone plasmids with PGs specific to yeast such as *Pichia pastoris.* One of the main purposes of these experiments is to produce enzymes that work best in acidic pHs such as those found in juices. Polygalacturonases from *Penicillium* sp. CGMCC 1699 and *Bispora* sp. MEY-1 have been successfully produced by *Pichia pastoris. Penicillium* sp. CGMCC 1699 had optimal activity of pH 3.5 at 40 °C and has been studied with commercial pectinases and lyases to reduce intrinsic viscosity (Yuan and others 2011). The viscosity was

measured with a glass capillary viscometer as a function of flow time, density and the same conditions observed with water; viscosity reductions were obtained from the difference in viscosity of treated and untreated juice divided by the viscosity of the control sample of untreated juice (Yang and others 2011; Yuan and others 2011). Optimum activity in apple juice was obtained when the PG (0.1% v/v) was mixed with an unnamed commercial PL product (0.05% w/w); this mixture had higher viscosity reductions than a commercial pectinase product (also unnamed in the study) and the commercial PL product alone. The optimum mixture of endo-PG and the commercial PL product obtained intrinsic viscosity reductions of 22.7% and 33.1% in apple juice for 180s and 240s at 40 °C (Yuan and others 2011). Viscosity reduction was also assessed for endo-PG from *Biospora* sp. MEY-1; with an amount of 10 U/mL, the viscosity of apple juice was reduced by 7.7% and transmittance increased more than 84% (Yang and others 2011). Viscosity reduction is an additional factor in determining pectinase relevance in juice clarification for viscosity is an important parameter in processing and filtering juice. Viscosity reduction can be used as a measurement of total enzyme activity of enzyme mixtures. These studies demonstrated that a solution of multiple enzymes achieved higher clarification and viscosity reduction than a solution with an individual product.

Shelf life is another factor important and should be characterized for enzymes used in juice clarification. The ability to maintain enzyme activity for long storage periods is beneficial for researchers and industry alike in that enzymes are often expensive and are often not used immediately after their production. For example, polygalacturonase from *A. niger* (ATCC9642) maintained its initial activity when stored

for 40 d in low temperatures of 4, -10, and -80 °C (Gomes and others 2010). Therefore, the identification of new, highly active, stable enzymes at juice processing condition could lower costs for juice processing.

Juice pasteurization

Pasteurization is a critical step to insure safety of consumers and increase shelf lives of products. Pasteurization research is at present focused on techniques that minimize thermal degradations of nutrients while inactivating pathogenic and spoilage microorganisms. The consumer desire for “fresh” and safe juice has led researchers to study pasteurization processes that do not include high temperatures and are termed “non-thermal” processing. Researchers are also combining other processing techniques called “assisted thermal processing” to minimize the effect of high temperatures on quality while inactivating pathogenic and spoilage microorganisms. Non-thermal processes, such as UV irradiation and pulsed light treatments, must be approved by the FDA. Unlike other novel non-thermal processing techniques, high pressure processing (and other processes not using radiation or chemical agents) does not need FDA approval for validation because it has already been proven effective and safe as a pasteurization technique (USFDA 2004). High pressure, pulse electric fields, ultraviolet light and several other non-thermal pasteurization techniques are being investigated to enhance fruit juice processing.

High-intensity pulsed electric fields (HIPEF) have been proven to be successful at pasteurization and is more applicable with the addition of organic acids, enzymes, bacteriocins, and spices. The technique has also been combined with generally recognized as safe products (GRAS) such as citric acid and cinnamon bark in apple, pear, orange, and strawberry juices. Non-linear microbial population reductions were

noted with most of the inactivation occurring at the beginning of treatment. Tailing, the decrease in the rate of microbial reductions, can be caused by several possibilities such as heterogeneous sensitivities among populations or shielding by juice matrix and dead cells. Electric pulsed fields have had FDA approval and is starting to be used on a commercial scale (Mosqueda-Melgar and others 2008).

Another alternative to thermal processing, ultraviolet light (UV) can be applied at low temperatures, is not known to produce any toxic effects on products, and requires very little energy. One disadvantage of UV processing is its lack of penetration in solid or on opaque surfaces; thus air, direct surfaces, and clear liquids have the greatest sensitivity to UV light treatment. For juices, the typical penetration depth is 1 mm with 90% of the light absorbed and the type of liquid, soluble solids and suspend matter can affect penetration. Turbulent flow is often used to address the stated issues to expose as much of the juice to the UV light and prevent clustering of microorganisms. UV light is effective for inactivating microorganisms by causing cross-linkages in DNA (between thymine and cysteine) which causes cell death. Radiant exposure dosages can be stated as energy per area or volume. For example, clarified apple juice inoculated with *E. coli* K12 had 3.5-log reductions in aerobic plate counts while yeast and molds were reduced by 3-log reductions after UV treatments (Keyser and others 2008).

Lastly, supercritical gases are capable of pasteurizing fruit juices as an alternative to thermal processing. Supercritical gases require lower temperatures and pressures than thermal and high pressure processing, respectively. In apple juice with initial concentrations of natural microflora of 5×10^2 cfu·mL^{-1} and a treatment of 36 °C, 100 bar (CO_2 or N_2O), 300 rpm (stirring), the microbial populations were reduced to 8.01%

and 7.42% by CO_2 and N_2O, respectively. Both gases completely inactivated natural microflora populations after 10 minutes of the same treatment (Gasperi and others 2009).

HHP alternatives

As discussed previously, the three important factors in clarified juice production are maintaining vitamins and nutrients, clarifying juice in a timely and an inexpensive manner, and inactivating pertinent microorganisms. All three of these factors are critical to a successful juice product. HHP processing is being highlighted as a viable process to perform all three of these steps. The remaining portion of this review will explore HHP research in the clarified juice field to demonstrate its potential benefits to the food processing industry.

High Hydrostatic Pressure Processing

General History and Economics

In recent years, consumers have started to demand more fresh-like products that have minimal processing and that have little to no artificial stabilizers in them (Deliza and others 2005). Fruit juice as a whole has seen a decrease in consumption in the past several years (Pollack and Perez 2008). Improved processing techniques leading to a fresher tasting product could help provide a boost to the public's perception of fruit juice, and high pressure processing is a current topic of research to improve fruit juice quality. High hydrostatic pressure processing as a non-thermal alternative (below 40 °C) or as a pressure-assisted thermal treatment are two techniques that offer improved benefits to thermal processing by retaining juice nutrients, antioxidants, coloration, and clarity after pasteurization.

At the end of the nineteenth century, experimentation important for food processing began to occur. Demazeau and Rivalain (2011) have reviewed the history of high pressure processing, and the key research is summarized in the following section. One of the first major high pressure discoveries relevant to food processing was that 300 MPa did not inactivate *Staphylococcus aureus*, however the same pressure could inactivate other *Streptococcus* species. Enzymatic studies involving high pressure began when Büchner induced fermentation with brewer's yeast using pressures of 40 to 50 MPa. In 1899, Hite investigated high pressure as an alternative to thermal processing, and established that a pressure over 463 MPa (for 1 h) could slow milk souring by 24 h. From this initial research, high pressure studies have been performed focusing on vaccines, protein denaturation, enzyme activity, and its effect on microorganisms for food preservation. The Japanese were the first to commercially produce food products with HHP processing. These products were jams and were available in market in the early 1990s (Demazeau and Rivalain 2011). Other foods pasteurized by high pressure processing expanded to include squid and rice cakes in Japan, orange and apples juice in Europe, and oysters and guacamole in the American market (Rastogi and others 2007). Pressure-treated juices available today include orange, grapefruit, and apple juice (Patterson and others 2007).

The initial equipment and operational costs of HHP continue to decrease as research and popularity of this non-thermal option grows. The HHP can be performed by either a batch process or a semi-continuous process in which the food product and its package are treated or where the juices are processed with high pressure before being packaged. For semi-continuous, a pressure medium and piston are used to

pressurize the fluid product in two separate vessels. An additional pressure vessel can be added (three in total), so while one product vessel is being pressurized the other product vessel is being filled or discharged. The typical system consists of a pressure vessel, seal closures, pumps, and systems to monitor control and transport product during processing (Balasubramaniam and others 2008). Commercial manufacturers of high pressure equipment include Avure Technologies (Franklin, TN), NC Hyperbaric (Burgos, Spain), and Uhde (Dortmund, Germany). Food companies that have incorporated high pressure into their production line include Hormel (Austin, MN), Kraft (Northfield, IL), Perdue (Salisbury, MD), Foster Farms (Livingston, CA) and Wellshire Farms (Swedesboro, NJ). Around 11% of commercial products processed by high pressure are fruit juices and beverages, and 80% of the commercial equipment was put into processing in 2000 or later (Balasubramaniam and others 2008).

High pressure equipment can range from $500,000 to $2.5 million and the vessels can typically hold from 30 to over 600 L of product (Balasubramaniam and others 2008). An estimate of cost of HHP processing ranges from $ 0.05 and $ 0.50 per liter or kilogram with the lowest end of the range being near thermal processing costs (Rastogi and others 2007). With research and the acceptance by mainstream consumers, commercial HHP processing could become a more feasible option for juice production.

Technological Challenges and Opportunities

High pressure treatments offer many opportunities for the food and fruit juice industries. Some HHP treatments can be performed at room temperature, thus it can be used as a pasteurization process that may eliminate the need for heat and preservatives (Rastogi and others 2007). Thermal treatments can result in temperature gradients that can be detrimental if products do not have the same exposure to the

required temperature throughout the process. In extremes, this gradient can lead to incomplete microbial reduction due to lack of uniformity in heating of the product and cause a decrease in quality of the product. As pressure increases, the resulting compression can change the pH of juice with an acidic shift of 0.2 pH units per 100 MPa as seen with apple juice in 1995 (Rastogi and others 2007). Also with this compression, molecules shift to the most compact form. An increase of temperature is observed with an increase in pressure on the order of generally around 3 °C per 100 MPa increase for liquid mediums (Rastogi and others 2007).

Another challenge with HHP is its effects on enzymes and other proteins which can cause changes in activity or configuration. Luckily, these effects are normally reversible at pressures between 100 and 400 MPa and this reversibility is not found in most cases with thermal processing. One of the benefits of HHP is that the processing does not appear to affect desirable qualities of food (flavor, nutrients, an color) as is often found with thermal processing (Rastogi and others 2007).

High pressure homogenization (HPH) has also been linked with the ability to pasteurize juice while keeping fresh qualities. For example, when fresh clear Annurca apple juice was pasteurized with HPH processing (20 °C, 150 MPa, 3 passes), the juice maintained a longer storage life and color than untreated juice. The soluble solids content and color stayed constant in treated juice for 28 days, while the Brix fluctuated in unprocessed apple juice throughout the 28 day storage time. The HPH treated apple juice maintained a minimum shelf life of 28 days under refrigerated conditions that met European Commission regulations no. 2073/2007 of a microbial load under 100 $cfu \cdot g^{-1}$ with no coliform contamination (Maresca and others 2011).

Sometimes perceived as a challenge, the larger molecules with non-covalent bonds such as proteins and polysaccharides are affected during high pressure processing (Rastogi and others 2007). The costs of equipment and unknown effects of high pressure on food components are the major limiting factors affecting the adoption of high pressure processing. In addition, the pressure vessel size is limited to around 25 L with intended pressures over 400 MPa, and the pre-stressed, wire-wound vessels needed are expensive. For commercial use, pressure vessels are limited to less than 680 MPa. The vessel limitations make some processing (oyster shucking at 200-400 MPa) more economical to process with HHP than guacamole salsa (around 600 MPa). Equipment manufacturers are working on designing pressure vessels that can withstand and exceed these pressures and higher temperatures (Torres and Velazquez 2005). The final technological hurtle is the unknown behaviors of many reactions (enzymatic and microbial) that have to be explored for the HHP technology to become widespread and effectively used in industry.

Enzymes in HHP

Most of the work on enzyme reaction rates in food processing has focused on inactivating a specific enzyme of concern. Conversely, for some industrial enzyme processes, it is desirable to activate and stabilize the enzymes as in the case of fruit juice clarification for a combination of pectinases and cellulases. However, little is known about pressure activation of enzymes or the reasons for the increase in stability at higher pressures. It has been hypothesized that activation and stabilization is a result of changes in the enzyme structure, reaction mechanism, or the substrate/solvent physical properties. Even with advancement of mathematical modeling, actual data need to be acquired to accurately predict the outcome affected by pressure and

temperature processing (Eisenmenger and Reyes-De-Corcuera 2009b). Recent studies that focused on enzyme stabilization focused on individual enzymes. More research needs to be conducted to characterize the effect of pressure on enzymes mixtures in apple juice clarification.

High pressure does not have the same effect on all enzymes. Some enzymes are inactivated with high pressure while others can be stabilized or activated with HHP at temperatures that would normally inactivate the enzymes (Hendrickx and others 1998; Eisenmenger and Reyes-De-Corcuera 2009b). Enzymes such as PME in cloudy juices like orange juice can break down pectin and other cloud forming molecules in the juice leading to undesirable clearer product. Some PMEs are more resistant to heat, and high pressure can be effectively used to decrease PME activity in orange juice (Nienaber and Shellhammer 2001). The condition of pulp, pasteurized or unpasteurized, affects the activity of PME for much of the PME is found within the juice pulp. Samples with 8.5% of unpasteurized pulp had higher PME activity than samples with the same amount of pasteurized pulp treated at room temperature and high pressures (Nienaber and Shellhammer 2001). The residual activities of PME in unprocessed samples for unpasteurized and pasteurized pulp were 100% and 64.8%, respectively. After treatments using 500 MPa at 50 °C for 5 min the PME residual activity reduced to 19.9% in samples with unpasteurized pulp down to 11.3% in samples with pasteurized pulp. Higher pressures of 800 MPa at 25 °C for 1 min showed even further decreases in PME activity with a drop to 6.4% and 3.9% in unpasteurized and pasteurized pulp, respectively (Nienaber and Shellhammer 2001).

High pressure homogenization can be performed under a variety of different pressures, temperatures, and number of passes and is another non-thermal alternative to inactivate PME in orange juice. For example, a linear inactivation of PME was seen for different temperature treatments for orange juice (e.g., 22 °C, 35 °C, and 45 °C) as the pressure increased for a single pass (Welti-Chanes and others 2009). The fastest inactivation was at 45 °C with a rate of -0.262 %·MPa^{-1}, while at 22 °C and 35 °C there were similar inactivation rates of -0.192 %·MPa^{-1} and 0.195 %·MPa^{-1}, respectively. The number of passes through the homogenizer did not have a significant effect on PME activity at 100 MPa, but led to a constant increase in inactivation for high pressure of 250 MPa. PME activity increased with storage time at 4 °C, most likely due to isoenzymes that were split during processing and reacted with juice later. Higher pressures and increased number of passes slowed the PME activity rate in storage, and all samples treated with high pressure retained the cloud during the 12-day storage while untreated juice lost the cloud after 6 h of storage. It was hypothesized that the resulting smaller haze molecules from sieving and homogenization contributed to the juice cloud.

Fruit juice smoothies are another area in which high pressure processing is being explored for its potential as a minimal process treatment. The research focused on this method includes smoothies containing apples, apple juice concentrate, bananas, strawberries, and oranges. Specifically these studies were looking at the effects of HHP processing on antioxidants, phenols, and color compared to thermal and untreated juices. For example, smoothies that were treated at 450 MPa at 20 °C for 5 minutes or less were compared with thermal processing that reached 70 °C for at least 10 minutes

(Keenan and others 2010). Interestingly, smoothies treated with a thermal process had higher antioxidants than samples treated with HHP or untreated samples, while HHP had higher phenol concentrations than thermally processed samples. It is believed that thermal processing increases extractability of antioxidants while inactivating enzymes that would degrade antioxidants (Keenan and others 2010). While HHP also has the capability to disrupt cell membranes and increase extractability of antioxidants and enzymes, the HHP processing at room temperature doesn't inactivate the enzymes at shorter industrial process times. Chilled storage conditions at 4 °C also led to a decrease in antioxidant concentrations in all samples. Polyphenol oxidase (PPO) enzymes from the bananas and apples also lead to browning of samples (Keenan and others 2010).

While much of the high pressure research focused on inactivation of microorganisms and enzymes, some has focused on the stabilization and activation effects that high pressure can have on enzymes. For example, tomato PME was activated at 300 MPa, and carrot PME was stabilized around 500 MPa (Ly-Nguyen and others 2003; Hsu 2008). Reports on the stabilization of PMEs and PGs with HHP processing are prevalent in the literature due to the improved texture results seen in fruit and vegetables with this method. The inactivation of PG and the stabilization of PME with or without calcium ions lead to higher quality in texture.

Though most studies report that PME is pressure-stable below 400 to 500 MPa, PG has been reported as sensitive to pressure processing. Pressure and temperature behavior of two tomato polygalacturonases, PG1 and PG2, have been reported. Polygalacturonase 1 was heat stable while PG2 was heat labile, and both were

inactivated at pressures of 300-500 MPa at room temperature for 15 min. The same process failed to inactivate tomato PME (Rodrigo and others 2006). Tomato PME was stable up to 700 MPa and still retained activity of 50% after treatment at 850 MPa for 15 min. Some researchers have focused on the behavior of tomato PME and PG in the presence and absence of each other at high pressure. Polygalacturonase in the presence of highly methoxlyated pectin and PME at pH 4.4 had maximal activity at 50 °C and 200 MPa. Samples treated at 100 and 200 MPa at 50 °C had higher activity than samples treated at atmospheric pressure or above 300 MPa. PME activity was optimal at 60 °C, yet the presence of PG affected the activity at high pressures with 0.1 MPa having activity slightly higher than 200 MPa with PG present. All high pressures obtained higher activity than 0.1 MPa with the absence of PG (Verlent and others 2007). High pressure has shown to decrease inactivation rates in tomato PME at ambient and inactivation temperatures, though citric acid buffer with a pH of 3.8-4-5 decreased the antagonistic effect pressure had against temperature. Finally, PME activation has been achieved at different high pressures. Activation of tomato PME was found at temperatures of 59-60 °C at 100 MPa (without calcium ions) or 400 MPa (with calcium ions) (Hendrickx and others 1998) whereas others report tomato PME activation at 300 MPa (Hsu 2008).

In addition to tomato, carrot PME has been widely studied in HHP since this method can improve carrot texture quality. Carrot PME was stabilized around 500 MPa (Ly-Nguyen and others 2003). Reported activation volumes at 30 to 55 °C for purified carrot PME were between -7.8 and - 5.73 $cm^3 \cdot mol^{-1}$, and increased with temperatures greater than 40 °C (Sila and others 2007). The small activation volumes confirmed the

stability of the enzyme to high pressure treatments. Activation energy (E_a) reported for carrot PME at thermally degrading temperatures showed no apparent trend with pressure with values that ranged from 31.6 to 81.4 $kJ \cdot mol^{-1}$ for 0.1 to 500 MPa treatments with no significant decrease in E_a with high pressure treatment at 200 to 500 MPa (31.6-81.4 $kJ \cdot mol^{-1}$) than from 0.1 MPa treatments (48.9 $kJ \cdot mol^{-1}$) (Sila and others 2007).

Ortega and others, (2004) also investigated inactivation kinetics for the commercial blends of pectinase. For temperatures of 40-60 °C inactivation plots of residual polygalacturonase activity (natural log) versus time did not produce first order inactivation for commercial cocktails Pectinex 3XL® (Novozyme), Pectinase CCM (Biocon), and Rapidase® C80 (Gist-Brocades). Therefore, a multi-fraction, first-order model was fitted for the PG enzyme in the commercial products likely due to heat-labile and heat-stable forms in the mixtures. For Pectinex 3XL®, the same commercial product used in this research project, the heat-labile form of PG was inactivated too quickly for inactivation rate calculation. The heat-stable form of PG in Pectinex 3XL® in thermal process studies had an activation energy of 160 $kJ \cdot mol^{-1}$, while the PG for heat-labile and heat-stable forms had energies of 92.4 and 145 $kJ \cdot mol^{-1}$ for Rapidase® C80 and 166 and 76.6 $kJ \cdot mol^{-1}$ for Pectinase CCM (Ortega and others 2004). Of the three commercial products studied, Rapidase® C80 was the most heat tolerant due to the lowest activation energy for the heat-stable form of PG. Taken together, thermal treatments have varying effects on the activation energies of commercial pectinase products.

Comparison HHP with thermal processing

As described, HHP treatments can reduce the time or temperature of the thermal processing by stabilizing enzymes. For microbial pectinases, treatments at 200 MPa and 45°C and 300 MPa at 50 °C have been proven optimal in *A. aculeatus* PME activity for rate of de-esterifying apple pectin, pH 4.5. At 0.1 MPa, the activity decreased with the increase in temperature above the optimal 45 °C (Fraeye and others 2007). Also, researchers have found samples of *A. aculeatus* PME treated at 55 °C and pH 4.5, at high pressures (400 and 700 MPa) for 30 min retained higher than 90% enzyme activity, while samples treated at the same parameters and atmospheric pressure retained less than 10% after 10 min treatment. At atmospheric conditions and pH 4.5, microbial *Aspergillus aculeatus* PME followed first order kinetics for inactivation at temperatures 46-56 °C. However, inactivation kinetics varied for enzyme source. Apple, tomato, and banana also followed first order, while strawberry and carrot demonstrated a biphasic thermal inactivation (Duvetter and others 2005).

Maximum activity for commercial pectinase cocktails has been studied for thermal processing. The activity of PG in the commercial product was determined with the reducing sugar method. Pectinase 3XL® (Novozyme) had the maximum activity at 50 °C. Pectinex 3XL® only retained 10% of initial activity after being thermally treated at 50 °C for 1 hr; other commercial pectinases, Pectinase CMM (Biocon) and Rapidase® C80 (Gist-Brocades), retained 57 and 5 % activity (Ortega and others 2004).These results demonstrate that at moderate temperatures, pectinases normally retain activity for an hour or less and activity can be stabilized with the application of HHP.

HHP and microorganisms

Until recent years, a majority of the HHP research for all foods has centered on microbial inactivation. High pressure inactivates *E. coli, Salmonella*, and *Vibrio* strains and is also an approved method for pasteurization by the USDA of *L. monocytogenes* in processed meats (Balasubramaniam and others 2008). Current research is still being conducted with pathogenic and spoilage microorganisms pertinent to fruit juices and the spores that are resistant to both thermal and pressure pasteurizations.

Yeast organisms can be inactivated by pressures ranging from 300-600 MPa, but bacterial spores and pressure-resistant bacteria are harder to destroy (Hendrickx and others 1998). Microbial spores are problematic due to their resistance to heat (moist and dry), radiation, and chemicals. One of the common mechanisms believed to inactivate spore concentrations is using high pressure and temperatures to cause germination and force spores to lose their resistance to processing (Black and others 2007). Injury to the cell membrane is considered to be the main mechanism to vegetative microbial inactivation with pressure pasteurization. Cells that are actively growing are more sensitive to pressure than those in the stationary phase (Patterson 2005). *Clostridium botulinum*, *Clostridium perfringens*, and *Bacillus cereus* are all spore formers that can cause food borne illness in humans. *Alicyclobacillus acidoterrrestris* is a spoilage spore former that can grow in acidic foods like fruit juices (Black and others 2007). Many spore-forming microorganisms cannot be treated with pressure alone, and thermal processing alone requires high degradation conditions for antioxidants and nutrients. Pressure assisted thermal sterilization is a focus for many researchers studying food preservation. *Alicyclobacillus acidoterrestris* spores can survive within a pH of 2.5-6 and temperatures of 25-60 °C. These bacterial spores have D-values (90%

reduction times) of 15-23 min at 90 °C or 2.4-2.8 min at 95 °C. When tested with a pressure pasteurization process, the bacteria were reduced to less than 1 cfu/mL (5.5-log reduction) with a process of 414 or 621 MPa at 71 °C for 10 min (Lee and others 2002).

Pressure assisted thermal pasteurization of a 5-log reduction has been seen for *C. botulinum* at conditions of 827 MPa, 5 min, 50-55 °C (type E); 600 MPa, 70 min, 80 °C (proteolytic type B); and 600 MPa, 6 min, 80 °C (proteolytic type A) (Rendueles and others 2011). For less thermally resistant microorganisms, a pressure process of 400 MPa or higher and at 20 °C or less can be used for fruit juice pasteurization and can inactivate *E. coli* O157:H7, a common pathogen in apple and orange juice (Patterson 2005).

Researchers have tested pressure-resistant *E. coli* and found that storage after the pressure pasteurization helped destroy the bacteria. After a 15 minute process at 20 °C and 300 MPa, there was only a 1.1-log reduction of the pressure-resistant bacteria *E. coli* LMM1010. However, after the same treatment and a 2-d storage period, the bacteria were reduced by almost 5-log. The optimum process with storage afterwards, 15 min at 20 °C and 500 MPa, in a juice of pH 4 had a 5-log reduction in the bacteria count (Garcia-Graells and others 1998). These results show that pressure can be used as an adequate pasteurization process.

E. coli O157:H7 strains also show variation in their susceptibility to high pressure treatment. Strain C9490 is more resistant to pressure than *E. coli* NCFB 1989, which was completely inactivated with pressure treatment of 350 MPa. An additional storage

period of 24 h and high pressure treatments of 300 MPa, destroyed all survivors (Jordan and others 2001).

Comparison with thermal pasteurization

Commercial thermal pasteurization processes are used to destroy pathogenic and spoilage microorganism with high temperatures, but the thermal processing can damage antioxidants and vitamins. Spore forming microorganisms are the most difficult to treat with heat treatments. *Alicyclobacillus acidoterrestris* thermal resistance decreased without linearity with the increase in temperature. High temperatures also have been shown to decrease the effect of pH and soluble solids on the bacteria's resistance. Resistance to thermal processing varied between strains of *A. acidoterrestris* with D values for 90 °C ranging from 7.38 to 20.80 min. The D value decreased with increasing temperature, with D values for 95 °C ranging from 2.3 to 2.8 min (Smit and others 2011).

Acid adapted *E.coli* O157:H7 showed higher resistance to thermal processing than stationary non-acid adapted microorganisms in apple, orange, and white grape juice. However as temperature increased, the D-values for both acid adapted and stationary phase samples decreased; a low temperature of 56 °C produced D-values of 7.0 and 4.1 min for acid adapted and stationary *E. coli* respectively and a high temperature produced D-values of 1.5 and 0.8 min respectively in apple juice. The z-value was slightly higher for acid adapted *E. coli* O157:H7 (z = 5.9 °C) than stationary phase *E. coli* O157:H7 (z = 5.6 °C). *Escherichia coli* O157:H7 had higher heat resistance than *Salmonella* and *L. monocytogenes*; and a complete thermal pasteurization for all three microbes could be performed with a thermal process of 3 s at 71.1 °C for a 5-log reduction (Mazzotta 2001).

Pressure alone is not always effective in destroying resistant endospores. However, a pressure-assisted thermal pasteurization has the ability to lower the pasteurization temperature to reduce possible thermal degradation and/or heating expenses.

Effects of High Hydrostatic Pressure on pH

General History

The final portion of this research involves the investigation of the shift in pH with HHP processing. During HHP treatment, pH should be monitored because it is a physical factor that affects the processing and food quality. The role of pH in high pressure processing is critical due to effects on proteins, microorganisms, as well as chemical reactions (Hayert and others 1999; Stippl and others 2004). Currently there are no commercial pH sensors for pressurized vessels available. Common pH sensors with glass and reference electrodes cannot withstand HHP processing. Studies on the pH behavior of solutions at HHP will aid in producing accurate measurements for any future pH sensors designed for pressurized vessels.

Acid and base ionization is affected by pressure and Le Chatalier's thermodynamic principle demonstrates the relationship between the reaction volume, temperature, and ionization constant (Bruins and others 2007).The study of dissociation constants of weak electrolytes is important not only to food processing with high pressure, but the study of the science of high pressure. Researchers have conducted studies to determine the effects of HHP on dissociation constants with concentration cell emf (electromagnetic force or voltage), optical density, as well as density and fluorescent measurements (Hayert and others 1999). Method and models have been developed for prediction of pH with equilibrium constants that are often unknown for

food systems. Yet pressure will affect pH with the volume change that directly impacts equilibrium constants (Stippl and others 2004).

An increase in the ionization constant with pressure can lead to an acidic pH shift (El'yanov and Hamann 1975). Reports vary on the exact amount of pressure required to affect pH. The effect of 100 MPa at room temperature caused a shift in the pH of water from -0.16 pH units to -.073 pH units (Hayert and others 1999). Compressibility and density are two physical factors that are associated with pH and their behaviors under pressure are more commonly noted than pH behavior (Min and others 2010). The NIST Chemistry WebBook provides the compressibility of water up to at least 900 MPa (USNIST 2008).

Another factor affected by the change in pH with pressure is a solution's absorbance levels. Absorbance levels are affected with an increase in pressure as HHP compresses and decreases the volume (Hayert and others 1999). Color indicators absorb light at a specific wavelength, and produce single or multiple peaks in the visual light range of 400-700 nm. Acid/base color indicators have a pH range in which colors shift with the shift in pH; some examples are Bromocresol Purple that shifts from yellow at pH 5.2 to purple at pH 6.8 and Metanil Yellow that shifts from hot pink at pH 1.2 to orange at pH of 2.4. The combination of compression of water and the change in the absorbance of light by color acid/base indicators together can be used to create pH calibration charts for determining the change in pH with the application of pressure. Color acid/base indicators are solutions that have been used to observe the effect of pressure on pH with their change in the transmission of light (Stippl and others 2004).

The buffer's sensitivity to pressure and temperature and thus its potential shift in pH at high pressure are important considerations when choosing a buffer for high pressure studies. The need for use of barostable buffers in high pressure research is often overlooked. Some buffers are temperature sensitive yet resistant to pressure, such as ACES (N-(2-acetamido)-2-aminoethane-sulfonic acid) and TRIS (2-amino-2-hydroxymethyl-1,3-propanediol). Yet other buffers are sensitive to pressure; phosphate buffer (initial pH of 7) drops to pH 5.4 at 600 MPa (Bruins and others 2007). Researchers have used a wavelength intensity ratio of Fluorescein to determine the change in pH with the increase in pressure for water and buffers (Hayert and others 1999). An acidic shift, or decrease in pH, was seen for all solutions tested except for MES buffer; the largest shift was seen with orthophosphoric acid with a change of -0.92 pH units at 200 MPa. Distilled water and acetic acid started with initial pH of 5.8 and 4.1 respectively. Shifts in pH of -0.30 and -0.22 pH units at 100 MPa and -0.31 and -0.40, respectively at 200 MPa were observed (Hayert and others 1999). These results were similar to values seen in previous literature (Owen and Brinkley 1941; Distèche 1972).

Current Research

Thermodynamic models that considered molar volumes, equilibrium constants and activity coefficients have been used to predict change in pH in CO_2 systems as well as orange juice at high pressure. The models accurately predicted a pH decrease of a water and CO_2 solution of 2.53 pH units when pressure was increased from 0 to 5.516 MPa. However, the model did not accurately predict the behavior of orange juice, (Meyssami and others 1992). Color indicator systems with up to 16 indicators encompassing a pH range of 1-10 have been used with chemometric models (principal component regression and partial least squares) to predict pH behavior at high

pressures. With the large pH indicator system, pH at high pressures was predicted with the spectrometric predictions at HHP minus the spectrometric prediction for atmospheric pressure and pH measured value at atmospheric pressure (Stippl and others 2004). Other indicator systems to observe the effect of pressure on pH behavior have been developed. Dual-wavelength indicators LysoSenser™ Yellow/Blue DND-160 as well as seminaphthofluorescein and seminapthorhodafluor utilize ratio of two wavelengths to determine pH at HHP (Salerno and others 2007; DePedro and Urayama 2009). Though several systems and models have been produced to quantify the shift in pH with high pressure, variations in predictions show that research is still needed to confirm actual shifts.

Gap of Knowledge

Juice Processing

Clarification at atmospheric pressure and thermal pasteurization of fruit juices are well established industrial technologies. New techniques beyond thermal processing to retain nutritional value, organoleptic properties and aromas are being explored to improve apple and fruit juice quality. However, there are still unknown factors that should be investigated before novel research can move into commercial practice. Mechanisms for microbial and enzymatic inactivation and activation need to be elucidated for HHP and other non-thermal processing.

While knowledge has advanced on how to retain nutritional qualities of juice, further research is needed to improve antioxidant and vitamin retention during the complete juicing process while manufacturing a safe and quality product. Finally, the effects of high pressure thermal-assisted processing on nutritional and flavor characteristics of fruit juice need to be further documented.

High Hydrostatic Pressure Processing

There are several gaps in knowledge that remain for HHP juice processing. First, the activity of pectinase enzymes varies greatly. The formula of the enzyme mixture, pressure applied, temperature, pH and matrix (whether it is food or buffer) are some of the important factors to consider when using HHP. For industrial processing, the behavior of commercial pectinases at moderate and high temperatures has yet to be explored in combination with high pressure processing.

Secondly, the need exists for an enzymatic activity quantification of the overall activity of a mixture of enzymes to determine the effect of high pressure treatments on the enzyme mixture as a whole. The rate of viscosity reduction is a parameter that can be used for enzymatic activity. Also, the few studies using viscosity reduction all have variations in assay technique. Further experimentation with viscosity reduction to ensure an accurate and consistent representation of enzyme activity for a commercial pectinase cocktail is needed.

Lastly, assessing antioxidant and vitamin stability in a pressure assisted thermal pasteurization processes is required to determine whether the cost-benefit of a non-thermal technology justifies the investment.

Shift in pH with HHP

Theoretical equations have been derived and experimentation has been performed to predict pH shift at high pressures. However, without a high pressure vessel equipped with a pH sensor all pH shifts can only be estimated with simple solutions. Determination of pH shifts *in situ* is crucial in helping characterize this phenomenon. Colored pH indicators have been studied to quantify this shift in pH,

however, their respective dissociation constant behavior in HHP treatments has not been studied.

Predictions of pH shift with any absorbance or reflectance values can only be applied to optically clear solutions which is rare in most food systems. Models for pH shift can be limited when applied to food systems due to the complex nature of food matrixes that affect the pH. Additionally, the combined effects of pressure and temperature on food systems pH should be further evaluated for equilibrium constants of organic and weak acids, which vary in concentration and could be affected differently with HHP processing.

Summary

Over the past several decades a great deal of progress had been made to propel HHP processing from a novel technology into a commercially viable process used worldwide. Research has highlighted relevant effects of high pressure on microbial activity, enzymatic activity and pH behavior, all of which are important factors for commercial food processing. With advances in research, high pressure is a suitable treatment for apple juice that could allow the combination of juice clarification and pasteurization with a high pressure-assisted thermal process. Without studies that optimize juice clarification and pasteurization with pressure assisted thermal processing, a true comparison to thermal processing cannot be made for each specific juice product. Juice clarification experiments must be performed with commercial pectinase blend for valid HHP results, since industry does not process juice with only individual pectinases. Finally, without a constant quantitation of shift in pH with HHP processing, high pressure sensors will be difficult to manufacture without accurate calibration charts

available. The use of high pressure should be further investigated to improve and enhance commercial food processing.

Objectives

This project focused on testing a HHP process to stabilize pectinase enzymes at raised temperatures above levels customarily used for clarification. Kinetic behavior of enzyme inactivation and viscosity reduction can be used to optimize HHP treatments for juice clarification. This high pressure, high temperature process could be used to clarify and pasteurize apple simultaneously. The data from this project could benefit the apple juice producers and consumers alike by testing the feasibility of combining the clarification and pasteurization processes.

The project will focus on the clarification process with enzymes specific for the polysaccharide pectin molecules. The purpose of this study is to find a pressure, temperature, and processing time combination that will stabilize and activate a mixture of commercial pectinases to reduce the clarification time and required amount of enzyme used in apple juice processing. In virtue of the increased temperature, we also anticipate reduction of pathogenic microbial populations to a safe level. This research is expected to translate into operating costs savings and increased product quality. The final portion of the project is to observe and quantify the change in pH with the absorbance of color acid/base indicator water solutions.

Specific Objective 1

To determine the effect of HHP on the stability of a commercial pectinase cocktail at selected high temperatures for pectinases used for juice clarification.

Specific Objective 2

To maximize the activity of a pectinase cocktail at HHP for optimal clarification processing that minimizes thermal degradation of pectinase enzymes. As preliminary results for future experimentation, inactivation of *E. coli* K12 will be performed at inactivating conditions to determine if complete pasteurization is obtainable with clarifying treatments.

Specific Objective 3

To assess pH behavior under high pressure conditions with the visible spectrum produced by acid/base color indicators and monitored with a high resolution spectrometer.

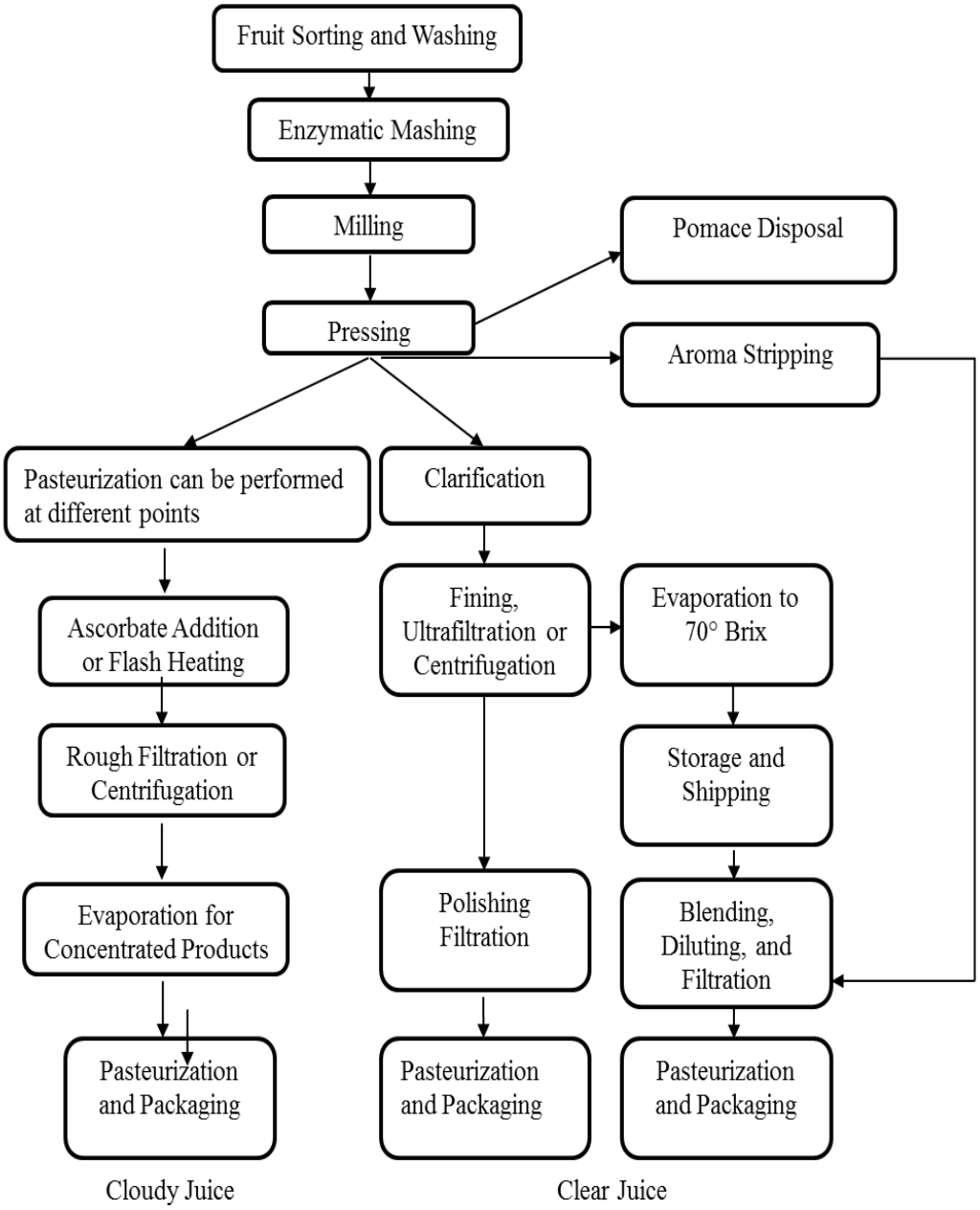

Figure 1-1. Overview of apple juice production to obtain either cloudy or clear juice.

Table 1-1. Turbidity values for processed juices

Juice	Process	Turbidity	Reference
Apple Juice	Fresh centrifuged	259-436 NTU	(Araya-Farias and others 2008)
	Depectinized centrifuged	8.9 – 9.5 NTU	(Araya-Farias and others 2008)
	Depectinized centrifuged, and electrofloatation	7.9-10.2 NTU	(Araya-Farias and others 2008)
	Depectinized centrifuged, and electrofloatation with gelatin	3.4 NTU	(Araya-Farias and others 2008)
Banana	Enzyme treated and centrifuged	600-5000 NTU	(Vaillant and others 2008)
Blackberry	Grinded/sieved and enzyme treated	9600-16200 NTU	(Vaillant and others 2008)
	Pressed and enzyme treated	350-750 NTU	(Vaillant and others 2008)
Cherry	Non Clarified	55.6 FNU	(Pinelo and others 2010)
	Clarified	11.0 FNU	(Pinelo and others 2010)
	Clarified centrifuged	5.79 FNU	(Pinelo and others 2010)
	Clarified, centrifuged, and filtered	2.75 FNU	(Pinelo and others 2010)
Pineapple	Non treated	1500-4000 NTU	(Vaillant and others 2008)
	Enzyme treated and centrifuged	150-600 NTU	(Vaillant and others 2008)

Notes: Standard clarification and processing vary slightly by juice and research team for specific processing conditions; please note original research paper noted in the reference section.

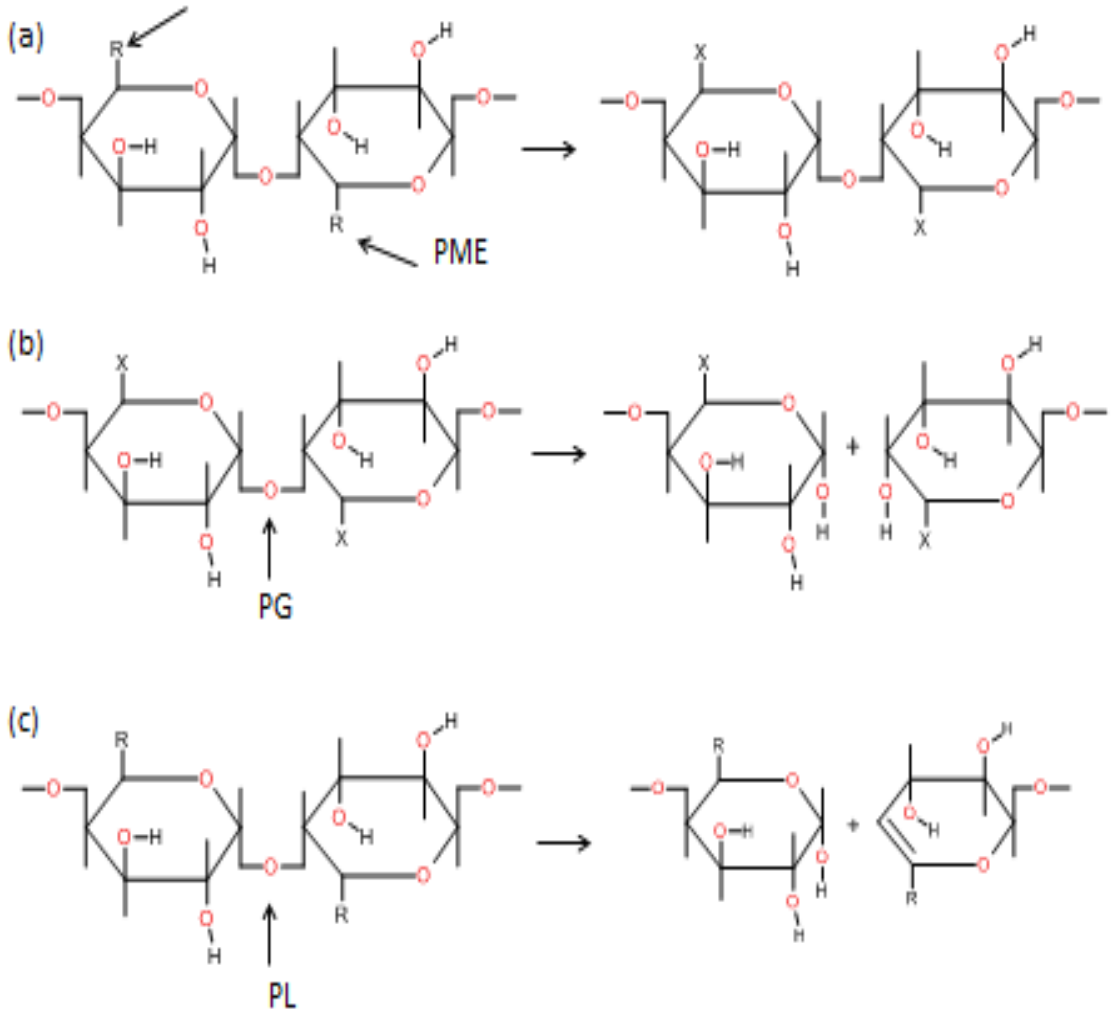

Figure 1-2. Basic summary of pectinase behavior: (a) R = COOCH3 and X = COOH, (b) X = COOH, (c) R = COOCH3 or COOH. Enzymes cut pectin molecule at position highlighted with arrow. PME, pectin methyesterase; PG, polygalacturonase; PL, pectin lyase.

1

Table 1-2. Enzymes in juice clarification research

Origin	Enzyme	Assay	Assay Conditions (°C)	Activity	Optimum Conditions	Comments	Reference
A. niger T0005007-2	Exo-PG	VR[a]	30	75 U·mL^{-1}	40 °C		(Sandri and others 2011)
	PME	RS[b]	35	142 U·mL^{-1}			
	PL	T[c]	30	0.03 U·mL^{-1}			
		A[d]	40	432 U·mL^{-1}			
A. oryzae IPT 301	Total Pectinase Activity	VR[a]	30	44 U·mL^{-1}	40 °C		(Sandri and others 2011)
	Exo-PG	RS[b]	35	40 U·mL^{-1}			
	PME	T[c]	30	0.29 U·mL^{-1}			
	PL	A[d]	40	180 U·mL^{-1}			
A. niger ATCC9642	PG	DNS[e]	40	51.82 U·mL^{-1}	pH 5.5 and 37 °C		(Gomes and others 2010)
Penicillium sp. CGMCC 1699	Endo-PG	DNS[e]	40	815.5 U·mg^{-1}	pH 3.5 and 40 °C	Expressed in the yeast *Pichia pastoris*	(Yuan and others 2011)
Bispora sp. MEY-1	Endo-PG	DNS[e]	55	1520 U·mg^{-1}	pH 3.5 and 55 °C	Expressed in the yeast *Pichia pastoris*	(Yang and others 2011)

[a] Viscosity Reduction, [b] Reducing Sugars Method, [c] Titration of Carboxylic Groups, [d] Absorbance Readings at 235 nm, and [e]Dinitrosalicylic acid absorption method

CHAPTER 2
HIGH HYDROSTATIC PRESSURE DECREASED THE RATE OF THERMAL INACTIVATION OF A PECTINASE COCKTAIL

To the best of our knowledge, the effect of HHP on the stability of commercial microbial pectinases has not been studied. We hypothesize that using a HHP stabilized pectinase cocktail for fruit juice clarification in which temperature can be increased above the levels currently possible at atmospheric pressure could allow several processing benefits. These possible benefits include shortening production times; reducing required amounts of enzyme needed for clarification; and if combined with juice pasteurization, could lead to economically viable higher quality products. The objective of this study was to determine the effect of HHP on the stability of a commercial pectinase cocktail at selected high temperatures.

Materials and Methods

Materials

Pectinases from *Aspergillus niger* in an aqueous solution (Pectinex 3XL®, Product No. P2736, Novozymes, Napa, CA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The commercial cocktail of enzymes contains pectintranseliminase, polygalacturonase, pectinesterase, and smaller portions of cellulases and hemicellulases. Sodium Citrate and Citric Acid were both obtained from Fisher Scientific (Pittsburg, PA, USA). Pectin (Product No. P-9135) from citrus fruit was obtained from Sigma-Aldrich (St. Louis, MO USA) and dissolved to make a 1.5% (w/v) solution in ultra-filtered water.

Equipment

All high pressure equipment was from Unipress Equipment (Warsaw, Poland). Samples were submerged and treated in a high hydrostatic pressure cell Model U111

with silicone oil serving as the pressure medium. The high pressure cell was pressurized with a micropump model MP5 and controller. The high pressure chamber was jacketed to control temperature with alternating water baths model Isotemp 3016D from Fisher Scientific (Pittsburg, PA, USA) and pinch valves described previously in (Eisenmenger and Reyes-De-Corcuera 2009a). One water bath was set at 10 °C and the other at desired process temperature. A computer program written in LabVIEW v8.5 and data acquisition board model DAQ Card 6062E from National Instruments (Austin, TX USA) were used to control and record pressure, temperatures and processing time. A temperature-controlled cone and plate viscometer-rheometer, with a Wells-Brookfield Cone and Plate and CP-40 cone spindle, model LVDV-II+Pro and Rheocalc software from Brookfield Engineering Laboratories, Inc. (Middleboro, MA USA) were used to record the viscosity reduction of pectin solutions at 45 °C. The jacketed cup was temperature controlled with a water bath also from Fisher Scientific. A photo of the laboratory setup is provided in Figure 2-1.

Methods

Sample preparation and HHP processing

The pectinase solution was diluted in citrate buffer to 0.5% w/v (15 unit/mL) and aliquots were place in 1-mL plastic pouches, heat sealed, and placed on ice until treated. Enzyme solutions were made with 0.1 M citrate buffer pH 3.5. Buffers and the enzyme cocktail were held at separately 4 °C before use, and placed on ice when enzymes were added to citrate buffer. For treatment, a pectinase aliquot was placed in the high pressure cell, held at 10 °C, and the pressure cell was closed. Pressure was raised to the process set point. Then, temperature was raised to the incubation set point and when 90% of the change in temperature was reached, processing time started.

After processing, the pressure cell was cooled back to 13 °C, depressurized, and then the sample was placed on ice. Residual enzyme activity was assayed on the same day as the HHP treatment.

Processing conditions

Samples were treated at 0.1 MPa (control) or 200 to 400 MPa at 50 MPa increments, 55 to 85 °C at 7 to 8 °C increments, and for a processing time of 0 to 45 min. The processing temperatures were 55 °C, 62 °C, 69.3 °C, 77 °C, and 85 °C; temperature increments were chosen for even distribution of T^{-1} for the calculation of activation energy with the Arrhenius equation. Ramp-up and ramp-down times were accounted for as follows. Ramp-up time included pressurizing and heating the cell to 90% of set point temperature. Ramp-down time included cooling the pressure cell to 13 °C followed by depressurization to atmospheric pressure. To determine 100% residual activity with an incubation time of 0 min, samples were cooled and depressurized immediately after reaching the set point pressure and 90% of the set point temperature. Figure 2-2 shows the pressure and temperature profile for a sample treated at 69.3 °C, 250 MPa, and 15 min. Samples of this time series data were processed in duplicate. The study was performed in a randomized block design with temperature treated as blocks. Pressure and process times were randomly selected.

Activity measurements

To assess the activity of the enzyme cocktail, 132 µL of the treated enzyme sample was added to 1,865 µL of pectin solution and stirred for 40 s with a miniature magnetic bar and stirrer. The mixture was then immediately withdrawn with a syringe and injected into a three-way valve attached to a viscometer cup port, in order to record the viscosity reduction as soon as the mixing period had been completed. The viscosity

of pectin was recorded every 1.2 s for 10 min with a maximal viscometer rotational speed of 20 rpm as the treated enzyme cleaved pectin molecules reducing the viscosity. Samples that contained pectin only or pectin with only citrate buffer were run for 5 min to check pectin consistency.

The rate of viscosity reduction was used as the method to determine the treated enzymes' residual activity after high pressure processing. The initial rate of viscosity reduction was not used as the parameter to measure enzyme activity because of the inconsistencies that occurred during the mixing process. Therefore, a pseudo-second order rate of reaction was used to determine the rate of viscosity reduction. To determine the maximum rate of viscosity reduction with a pseudo-second order reaction, the inverse viscosity was plotted against assay time as seen in Figure 2-3. The maximum slope for a period of 60 s was taken as the maximum rate of viscosity reduction (the rate constant for viscosity reduction). The maximum rate constant of viscosity reduction (Pa^{-1}•s^{-1}) for each sample was used as measurement of the cocktail's residual activity. Figure 2-4 shows a predicted viscosity from the pseudo-second order rate of viscosity reduction plotted with the observed viscosity reduction. The percent residual activity was calculated with respect to process time of 0 min (k_0).

Rate of enzyme inactivation

The rate of enzyme inactivation was determined from the relationship between residual activity and HHP treatment time. Orders of reactions 0, 1^{st}, and 2^{nd} were analyzed for pectinase inactivation. No order was a definitive fit for all temperature blocks and pressures. However, a pseudo-first order reaction was the best fit for most treatments or sometimes second best fit for all pressure levels. Therefore, pseudo-first order was used to calculate the rate constant of inactivation as discussed in the results

section. This selection of order of inactivation was also pertinent for the purpose of comparing our results with those in the literature. Indeed, most enzyme inactivation studies are reported as first or pseudo-first order reactions.

With the rate of inactivation, Eyring's equation (Equation 2-1) was used to estimate the activation volume

$$\ln(k_o) = \left(-\frac{\Delta V \neq}{RT} \times P\right) + \ln(k_{Po}) \quad (2\text{-}1)$$

where k_o [min^{-1}] is the rate constant of enzyme inactivation of a specific pressure, $\Delta V \neq$ the activation volume [$cm^3 \cdot mol^{-1}$] , R the universal gas constant (8.3145 $J \cdot mol^{-1} \cdot K^{-1}$) , T the absolute temperature [K], P the pressure [MPa], and P_0 the reference pressure of 0.1 MPa.

Arrhenius equation (Equation 2-2) was used to estimate the activation energy:

$$\ln(k) = \left(-\frac{E_a}{R} \times \frac{1}{T}\right) + \ln(k_{To}) \quad (2\text{-}2)$$

where k [min^{-1}] is the rate constant of enzyme inactivation, E_a the activation energy [$kJ \cdot mol^{-1}$] and T_0 is the reference temperature.

Results and Discussion

Activity Measurements

With the addition of pectinase cocktail, the viscosity of the pectin solution decreased as seen with Figure 2-4. Viscosity reduction was selected as a measurement of the overall activity of the cocktail because in practice, in the clarified juice process, it is the desired effect. Also, the activity of individual enzymes contained in the cocktail was not determined because the intent of this research was to assess the impact of HHP on a commercially available cocktail. The maximum rate of viscosity reduction was

chosen as the parameter to quantify residual activity. Orders of reactions 1, 1.7, 2, 2.3 and 3 were analyzed to fit the rate of viscosity reduction. Van't Hoff and Powell methods were used to determine the best fit (Smith 1981; Masel 2001). None of the proposed orders fitted the data as per Powell's criteria because with a mixture of enzymes, the viscosity reduction is complex reaction. However, a pseudo-second order reaction fitted best the maximum rate of viscosity reduction with high correlation coefficients (r^2 > 0.762) for samples that maintained activity and best predicted the initial viscosity better than previously tested orders. The predicted viscosity reduction using this rate constant is shown by the continuous line in Figure 2-4. The prediction from the pseudo-second order only accounts for the viscosity reduction, not the initial mixing period. A linear relationship between the changes in viscosity of pectic acid and the logarithm of the amount of enzyme, pectin depolymerase has been reported (Roboz and others 1952). Viscosity reduction had also been fitted to exponential models (Ceci and Lozano 1998). However our results did not fit an exponential trend. Despite their practical relevance these methods are not often used in current research methods, because a correlation has not been found between the reduction in viscosity and hydrolysis of glycosidic bonds by polygalacturonases (Jayani and others 2005). Yet, the quantification of the hydrolysis of glycosidic bonds is not a useful parameter for industry as it does not directly relate to the characteristics of fruit juices or pastes such as viscosity or turbidity.

Residual Enzymatic Activity

For this study, enzyme activity was reported as residual activity after treatment or as the rate of enzyme inactivation in order to compare results to previously published literature. Temperature treatments produced some irreversible inactivation of the pectinase cocktail in that no pressure/treatment combination maintained 100% activity

through the longest process time for each temperature block. At lower temperatures of 55 °C and 62 °C, activity over 22.5% was maintained for all samples treated at atmospheric or high pressure. For temperatures of 69.3 °C to 85 °C, samples treated at high pressure maintained activity for longer process times than samples treated at atmospheric pressure as seen in Figure 2-5. At 69.3 °C, samples treated at atmospheric pressure for 4.5 min only retained an average activity of 33.2%, while high pressure samples retained at least 11.2 % activity for 45 min. High temperatures of 77 °C and 85 °C samples treated at 0.1 MPa maintained only an average of 2.5% and 10% residual activity for 1.5 min and 0.75 min respectively, in contrast to samples treated at 200 MPa that had residual activity of at least 2.2% and 7.4% for 15 and 6 min, respectively.

As process time increased for each temperature block, residual activity tended to decrease. Variation from this general decrease was seen for lower temperatures of 55 °C and 62 °C. At 55 °C, activation of enzymes can be seen for 200, 250 and 300 MPa at the 15 min process times as seen in Figure 2-6. Residual activity reached 106.4%, 119% and 102% for 200, 250 and 300 MPa, respectively for 15 min. However, after longer incubation times up to 45 min, an average of 49% residual activity was observed for samples treated at 0.1 MPa, and 57.8% for high pressure samples. Stabilization also occurred at lower temperatures, such as 62 °C 400 MPa and 45 min, in which activity was higher than the previous 30 min time point. This is an indication that the enzymes in the cocktail could be affected by different pressure and time conditions. The difference between high and lower temperatures of the study was illustrated in Figure 2-4 for temperature blocks 55 °C and 85 °C. Residual activity did not change greatly with the increase of pressure. From 200 to 400 MPa, residual activity fluctuated with a 0.61 to

50.28% difference between samples with no defined trend; this could be explained by the large experimental error in processing and being located at edge of the elliptical pressure-temperature diagram in which enzymes begin to denature. The general pressure-temperature diagram for enzymes has been previously summarized (Eisenmenger and Reyes-De-Corcuera 2009b).

For 55 °C and 62 °C, samples treated at atmospheric pressure maintained steady activity after the initial decrease of 49.0% to 56% and 22.6% to 32.0% respectively for the entire process time from 15 to 45 min. Atmospheric pressure samples however had less residual activity than samples treated at high pressure. For samples treated at higher temperatures (69.3 °C and greater), residual activity decreased faster for atmospheric pressure samples. Samples treated with high pressure maintained activity 10 times longer than atmospheric pressure samples for 69.3 °C and 77 °C, and 7.9 times longer for 85 °C. High pressure has a stabilization effect on the pectinase cocktail; the effect is more pronounced for higher temperatures. Moderate temperatures and high pressure combinations, that do not produce thermal degradation, provide the best results for industrial processing. Pressure processing resulted not only in a stabilization effect but in an activation effect for short process times, 15 min in the case of this research.

Most pectinase pressure and temperature stability studies focus on PME and PG (Hendrickx and others 1998; Duvetter and others 2009; Eisenmenger and Reyes-De-Corcuera 2009b). Tomato polygalacturonases was inactivated at 300 to 500 MPa, while tomato PME was stable up to 700 MPa (Rodrigo and others 2006). The activity of orange juice PME decreased with increased pressure, temperatures and processing

times. Samples treated at atmospheric pressure for 15 min at 37.5 °C had a residual activity of 89.9%, while samples treated for 12 min at 50 °C only retained 65%. Samples treated at 400 MPa, 50 °C for 12 min had a residual activity of 50.1%. Orange PME inactivation increased with the increase in temperature as well as the increase in pressure (Nienaber and Shellhammer 2001). The two tomato enzymes, PME and PG, also affect the behavior of each other. Verlent and others, (2007) reported that tomato polygalacturonase in the presence of highly esterified pectin and PME at pH 4.4 had peak activity at 50 °C and 200 MPa, after which activity declined. This research confirms tomato PGs can be inactivated at pressures greater than 300 MPa. Tomato PME also affected the activity of PG. Maximum PME activity was seen at 60 °C and ambient pressure or at 50 °C and 200 MPa with the presence of PG; however, with the absence of PG, PME peak activity was seen at 60 °C and 400 MPa. Researchers have hypothesized the that shorter pectin chains created by tomato PG, may make better substrates for PME and increase its activity (Verlent and others 2007). These results differ from our inactivation study results in which high pressure treatments had greater activity than samples treated atmospheric pressure above 55 °C. There are multiple enzymes within the pectinase mixture; the behavior of each enzyme could be affecting mixture as a whole creating an overall stabilization effect on total activity.

Studies have also focused on the activity of commercial enzymes and microbial PME. For microbial PME, similar conditions of 200 MPa at 45°C or 300 MPa at 50 °C were reported as optimal in *A. aculeatus* PME activity for de-esterifying apple pectin. At 0.1 MPa, the activity decreased with the increase in temperature above the optimal 45 °C (Fraeye and others 2007). Maximum activity for commercial pectinase cocktails has

been studied for thermal processing at atmospheric conditions; Pectinase 3XL®, the same cocktail used for this study had the maximum activity at 50 °C (Ortega and others 2004). Pectinex 3XL® only retained 10% of initial activity when treated at 50 °C for 1 h; other commercial pectinase Pectinase CMM and Rapidase® C80 retained 57 and 5% activity (Ortega and others 2004).These studies agree with our research that high pressure is stabilizing pectinase enzymes that are sensitive to high pressures and temperatures.

Rate of Enzyme Inactivation

The rate of pectinase inactivation was fitted to a pseudo-first order rate of reaction. Zero, first and second orders were compared with first order rate providing the best fit for most experiments or second best fit for all temperature and pressure combinations. First order kinetics for enzyme inactivation have been used to describe orange PME as well as *A. niger* PME in apple and cloudberry juices (Nienaber and Shellhammer 2001; Wilinska and others 2008). Ortega and others, (2004) also investigated inactivation kinetics for the commercial blends; for temperatures of 40-60 °C inactivation plots of the natural logarithm of residual polygalacturonase activity versus time did not produce linear results but rather the combination of two separate linear regions due to different forms of PG present. A multi-fraction first-order model was not suitable for the PG of Pectinex 3XL® inactivation; a rate constant could not be calculated for heat-labile form due to the sudden inactivation, but the heat-stable form of PG was fitted with a first order model. Rapidase® C80 and Pectinase CCM were fitted with two fractional first order models for polygalacturonase activity (Ortega and others 2004). With viscosity reduction as an indirect form of the total pectinase activity, our experimental data showed a linear first order model could be used to fit inactivation rates for the pectinase

cocktail with temperatures as low at 55 °C. Research pertaining to specific enzymes, such as PG from Pectinex 3XL®, showed that only one form of PG may be contributing to the overall pectinase activity.

Rate constants of enzyme inactivation were shown in Table 2-1. At atmospheric pressure, the rate of enzyme inactivation increased as temperature increased. The same general trend was noted for high pressures. Rates of inactivation at 55 and 62 °C were under 0.03 min^{-1} at all pressures and rates of inactivation increased with the increase of temperature for 69.3, 77, and 85 °C. Figure 2-7 shows the increase in inactivation rate for samples treated at 250 MPa. The larger inactivation rates at higher temperatures showed the effect of temperature inactivation increases for temperatures from 69.3 to 85 °C for all pressures. The coefficient of correlation for pressures treated at 69.3 °C and 77 °C were equal or greater than 0.80, while R^2 values for 85 °C were equal or greater than 0.90.

For atmospheric pressure, the rate of enzyme inactivation was faster than all samples treated at high pressure. This result indicates that high pressure stabilizes the pectinase cocktail at all temperatures studied in the results presented. Figure 2-7 graphs natural logarithm of residual activity versus process time to obtain rates of inactivation shown in Table 2-1. High pressure slowed the rate of inactivation by 40.4%, 81.9%, 43.9%, 20.0% or 37.7% at 200, 250, 300, 350 or 400 MPa respectively compared to samples treated at atmospheric pressure at 55 °C. The rate of inactivation was slowed by over 83% for all high pressure samples treated at 69.3 to 85 °C. The rate of inactivation for orange PME was reported to increase with temperature and pressure. Samples treated a 400 MPa and 25 °C were inactivated at a rate of 0.0197

min^{-1}, while an increase in pressure to 600 MPa increased the rate to .3308 min^{-1}. The largest rate of inactivation was seen with the highest pressure-temperature combination of 600 MPa and 50 °C, with a rate of 0.50 min^{-1} (Nienaber and Shellhammer 2001). Although these results appear to contradict ours, the pressure levels used by (Nienaber and Shellhammer, (2001) were higher which explains the pressure-induced enzyme inactivation. The rate of inactivation of PME from *A. niger* increased with the increase in temperature from 52-66 °C following first order kinetics in apple juice and cloudberry juices. The rate of inactivation in 0.1 M sodium acetate buffer with a pH 3.6 was 0.010 to 0.870 min^{-1} from the previously stated temperatures while in fresh and industrially produced apple juice the inactivation rate constants were 0.022 to 1.143 min^{-1} and 0.012 to 0.654 min^{-1} for temperature range of 54 to 66 °C (Wilinska and others 2008). Orange PME at 400 MPa and PME from *A. niger* in apple juice had similar rates of inactivation for temperatures of those tested in this research study (55 to 85 °C). The pectinase cocktail also had smaller inactivation rates at 62 and 69.3 °C than those observed for *A. niger* at 66 °C, which showed that the pectinase cocktail was more stable at atmospheric and high pressure than PME alone.

Effect of Pressure on the Rate of Inactivation

The Eyring plot (Figure 2-8) shows the natural logarithm of the rate constant of enzyme inactivation (K_{inact}) plotted against pressure to obtain an apparent activation volume for each temperature. The trend is not linear throughout the pressure range. At pressure above 250 MPa, the curve levels off suggesting that beyond that pressure, pressure no longer has an effect on the overall stability of the enzyme cocktail in slowing thermal inactivation. This is in agreement with the earlier studies at 400 MPa and above on PME from different sources in which the rate of inactivation increased

with pressure. A pressure-induced inactivation could be anticipated from the trend shown in Figure 2-8 in higher pressures (> 400 MPa). Therefore, the activation volume of stabilization can only be calculated for up to 250 MPa. The calculated apparent activation volume ($\Delta V^{\neq}$) had a small decrease from 55 °C to 62 °C, and increased with temperature reaching a maximum at 77 °C; pressure had a greater effect on the enzyme as the temperature increases to 77 °C with volume of 33.72 $cm^3 \cdot mol^{-1}$ indicated in Figure 2-9. With positive activation volumes, pressures up to 250 MPa slow thermal inactivation, with the greater effect seen at 77 °C. Also, the pressure range studied (200-400 MPa) was narrow, thereby inducing only small changes in activity between individual high pressure treatments. Future research should include lower pressures to better correlate the effect of pressure on the stability of the pectinase cocktail.

Reported activation volumes at 30 to 55 °C for purified carrot PME were between - 7.8 and - 5.73 $cm^3 \cdot mol^{-1}$, and increased with temperatures greater than 40 °C (Sila and others 2007). Inactivation volumes for orange PME were - 30.9 to - 35 $cm^3 \cdot mol^{-1}$ with an increase in volume with the increase in temperature 25-50 °C (Nienaber and Shellhammer 2001). The order of magnitude of these results was similar to this research. Even though we did not observe negative activation volumes, apparent activation volumes increased with temperature for carrot PME, orange PME and the results presented here, for temperatures up to 77 °C. With a positive activation volume pressure slows thermal inactivation of the pectinase cocktail; the opposite trend was seen for carrot and orange PME.

Effect of Temperature on the Rate of Inactivation

The effect of temperature on the rate of inactivation of the pectinase cocktail was studied using Arrhenius approach as shown in Figure 2-10, in which K_{inact} stands for the

rate constant for enzyme inactivation. At all pressures, apparent Arrhenius behavior was observed as confirmed by the linear relationship between the logarithm of the rate constant and the reciprocal of temperature. The apparent activation energy of inactivation at atmospheric pressure was 195.6 $kJ \cdot mol^{-1}$. Temperature had less effect at high pressure with apparent activation energies of 107.3 – 154.4 $kJ \cdot mol^{-1}$ than at atmospheric pressure, in particular at 300 to 400 MPa where the lowest activation energies were calculated. Activation energies at selected temperatures are shown in Figure 2-11. Error was reported using the standard error of the slope from the linear regression of Arrhenius plots. Therefore, in the ranges of temperature and pressure studied here, these two variables have antagonistic effects on the rate of inactivation of the pectinase cocktail.

Activation energy reported for carrot PME showed no apparent trend with values that ranged from 31.6 to 81.4 $kJ \cdot mol^{-1}$ for 0.1 to 500 MPa with no significant decrease in E_a with high pressure treatment at 200 to 500 MPa (31.6-81.4 $kJ \cdot mol^{-1}$) than from 0.1 MPa (48.9 $kJ \cdot mol^{-1}$) (Sila and others 2007). Activation energies of orange PME, 13.5 - 30.1 $kJ \cdot mol^{-1}$ decrease with the increase in pressure, 400 to 600 MPa (Nienaber and Shellhammer 2001). Carrot and orange PME activation energies were generally lower for those obtained for this pectinase study (107.3-195.5 $kJ \cdot mol^{-1}$) which can be explained by the use of a cocktail with multiple enzymes. The heat-stable form of polygalacturonase in Pectinex 3XL® in thermal process studies had an activation energy of 160 $kJ \cdot mol^{-1}$, while the PG for heat-labile and heat-stable forms had energies of 92.4 and 145 $kJ \cdot mol^{-1}$ for Rapidase® C80 and 166 and 76.6 $kJ \cdot mol^{-1}$ for Pectinase CCM (Ortega and others 2004). Apparent activation energy for Pectinex 3XL® calculated by

Ortega and others (2004), is in the same order of magnitude as seen with the apparent activation energy for 0.1 MPa samples with this research. This result was expected; though activity assays were different for each study, the reaction was accelerated in the same way. Apparent activation energies for individual enzymes are smaller than those seen for commercial products indicating that the collective or one specific enzyme of the pectinase was more affected by temperature. For the pectinase product, an increase in temperature can affect the activity of one enzyme, which in turn affects the overall viscosity reduction and activation energy.

The activity and stabilization of lipase in hexane was recently studied in our laboratory using the same HHP system and similar conditions (Eisenmenger and Reyes-De-Corcuera 2009a). At low pressure (below 10 MPa) an E_a of 42.7 $kJ \cdot mol^{-1}$ was reported, while at 400 MPa E_a of 42.36 $kJ \cdot mol^{-1}$ was obtained showing no significant difference from low pressure. Activation energies for lipase were lower than apparent activation energies found in this study for pectinases by an order of 10. Observing variation between apparent activation energies from atmospheric pressure and high pressure, but little variation in lipase in hexane at different pressures indicates the necessity of observing each system's processing conditions. Enzyme behavior under high pressure conditions is dependent not only on temperature but dependent on the specific enzyme, solvent, and mobilization conditions as well.

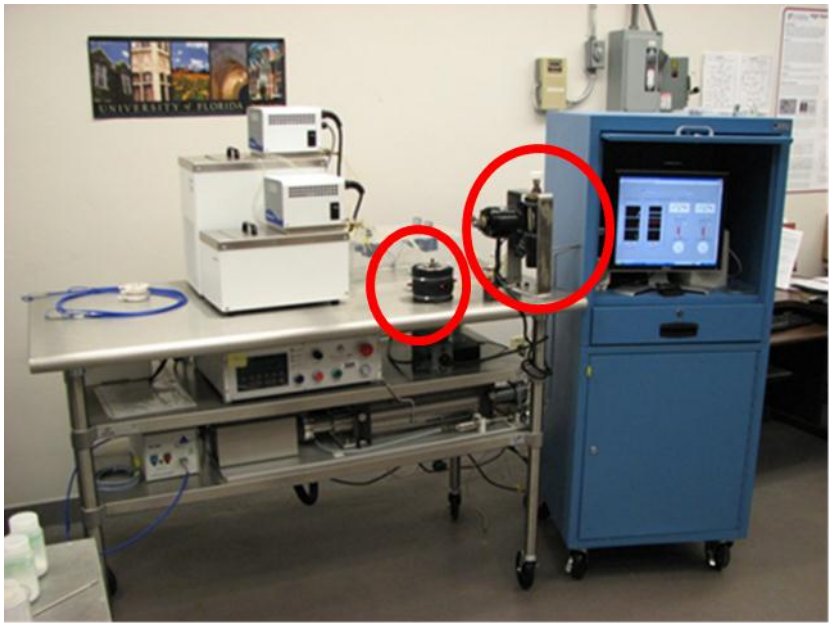

Figure 2-1. High pressure laboratory equipment set-up. The high pressure piston pump is located on the bottom shelf of the cart, the HHP controller is located on the middle shelf, and the water baths are on the top of the cart. The two HHP cells are located of the top of the cart with the smaller optical cell circled on the left and the larger blind cell circled on the right. The HHP setup is controlled and monitored with the computer on the right.

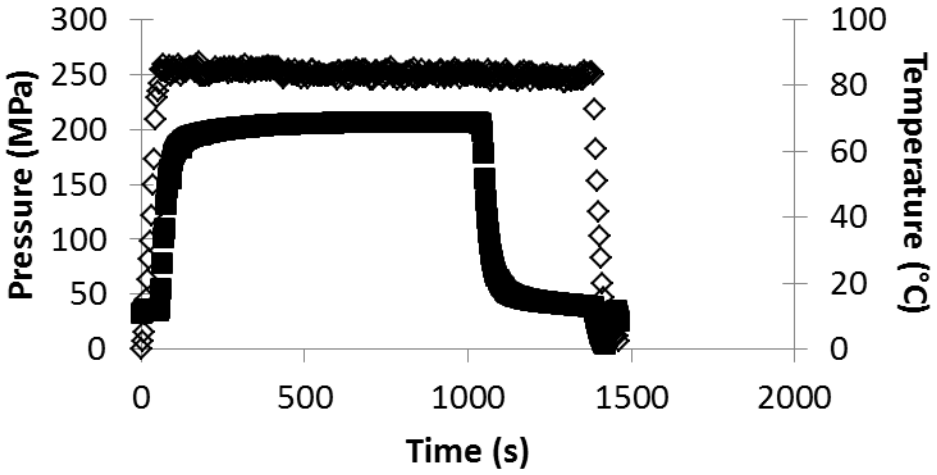

Figure 2-2. Pressure (◊) and temperature (■) profile in high pressure cell for sample treated at 69.3 °C, 250 MPa, 15 min.

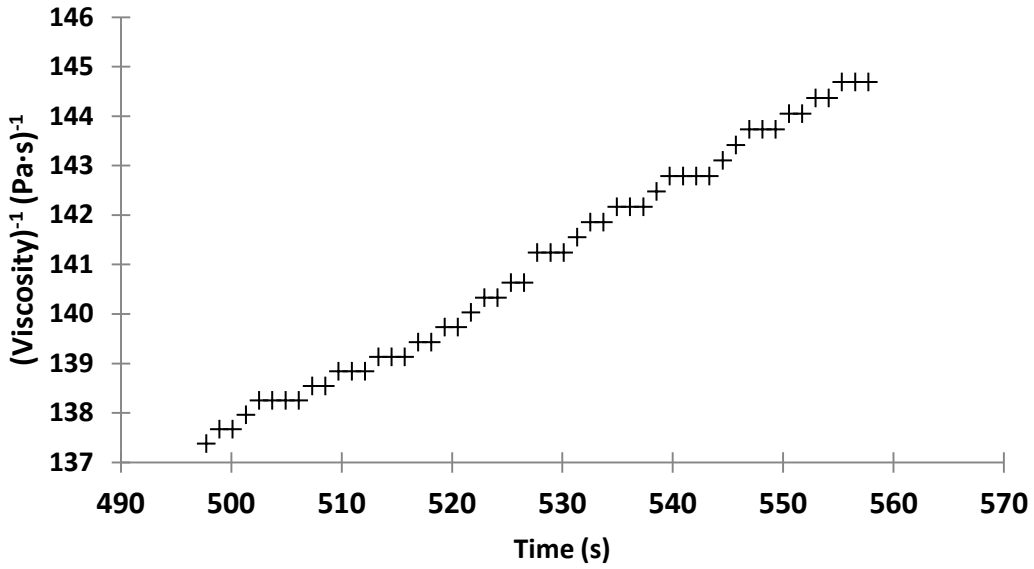

Figure 2-3. Maximum rate of viscosity reduction with a pseudo second order rate of reaction plot for pectinase treated at 69.3 °C, 250 MPa, 30 min with a rate constant for viscosity reduction of 0.127 $Pa^{-1} \cdot s^{-2}$ and $R^2 = 0.993$.

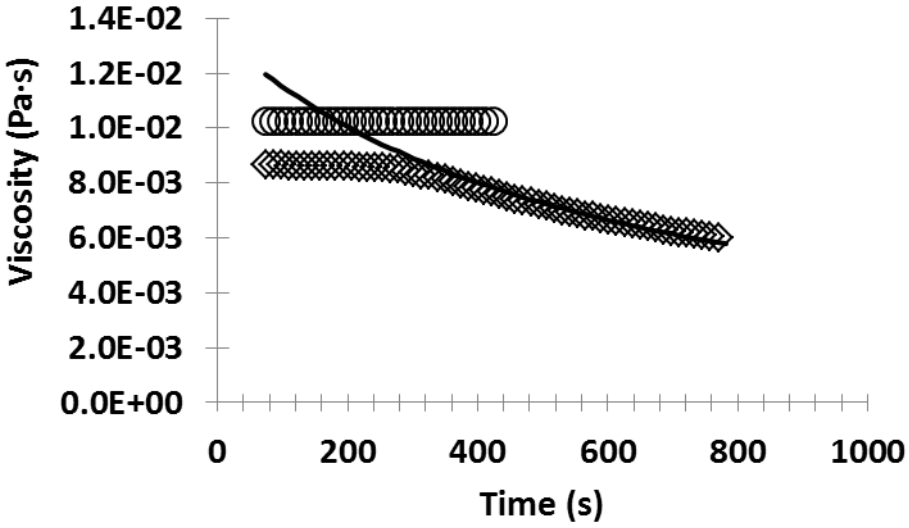

Figure 2-4. Observed and predicted viscosity for sample treated at 69.3 °C, 250 MPa, 30 min. (◊) pectin + treated samples, (-) predicted viscosity, and (○) and pectin + citrate buffer .

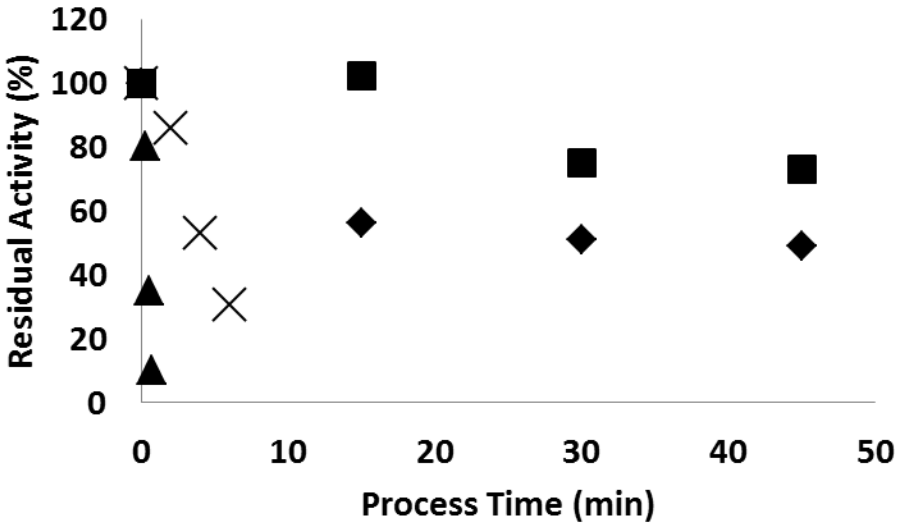

Figure 2-5. Average pectinase residual activity for samples treated at 55 °C and 0.1 MPa (♦), 55 °C and 300 MPa (■), 85 °C and 0.1 MPa (▲), and 85 °C and 300 MPa (X).

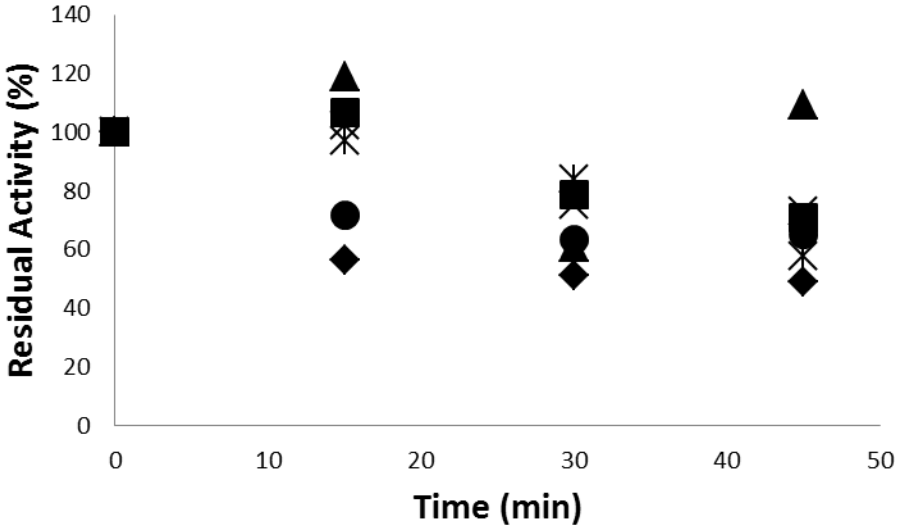

Figure 2-6. Average pectinase residual activity for samples treated at 55 °C and 0.1 MPa (♦), 200 MPa (■), 250 MPa (▲), 300 MPa (X), 350 MPa (∗), and 400 MPa (●).

Table 2-1. Rate constant of enzyme inactivation (min^{-1}) ± standard error (n = 2) for six pressure and five temperature treatments.

Pressure	Temperature				
(MPa)	55 °C	62 °C	69.3 °C	77 °C	85 °C
0.1	0.015 ± 0.006	0.029 ± 0.011	0.281 ± 0.100	2.331 ± 0.743	3.067 ± 0.644
200	0.009 ± 0.003	0.008 ± 0.004	0.028 ± 0.007	0.244 ± 0.066	0.433 ± 0.091
250	0.003 ± 0.011	0.010 ± 0.003	0.028 ± 0.008	0.125 ± 0.021	0.282 ± 0.020
300	0.008 ± 0.003	0.007 ± 0.006	0.024 ± 0.006	0.120 ± 0.030	0.201 ± 0.032
350	0.012 ± 0.004	0.011 ± 0.004	0.047 ± 0.011	0.194 ± 0.018	0.205 ± 0.049
400	0.009 ± 0.004	0.012 ± 0.009	0.026 ± 0.002	0.080 ± 0.018	0.221 ± 0.064

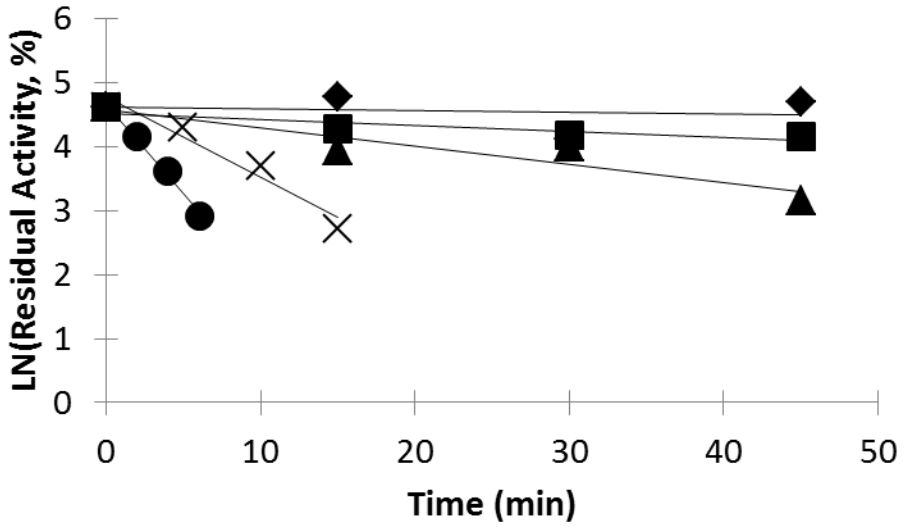

Figure 2-7. Rate of pectinase inactivation with lines representing linear regression behavior for samples treated at 250 MPa and 55 °C (♦), 62 °C (■), 69.3 °C (▲), 77°C (X), and 85 °C (●).

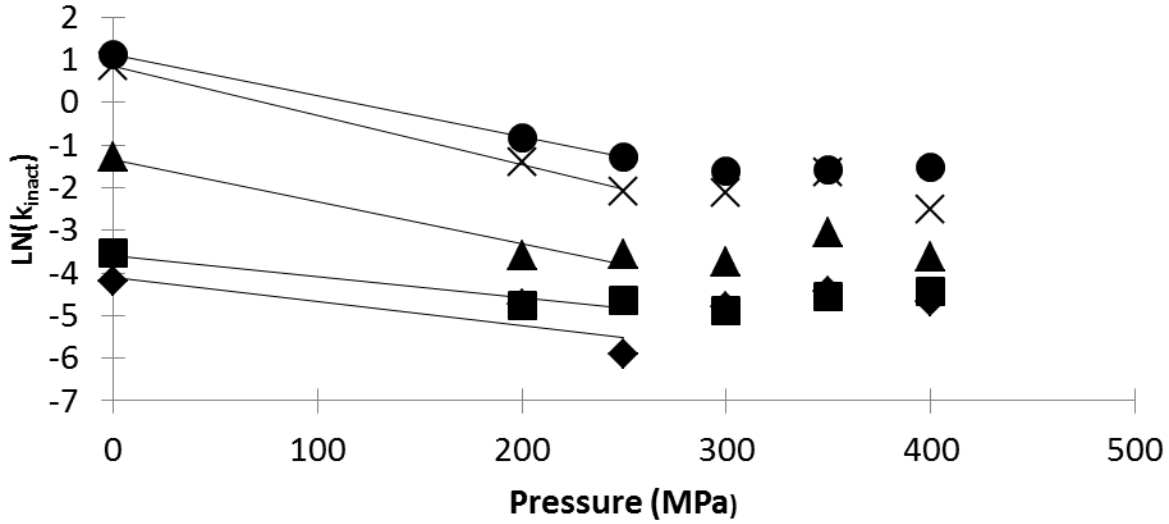

Figure 2-8. Eyring plot for enzyme inactivation for samples treated at 55 °C (♦), 62 °C (■), 69.3 °C (▲), 77°C (X), and 85 °C (●).

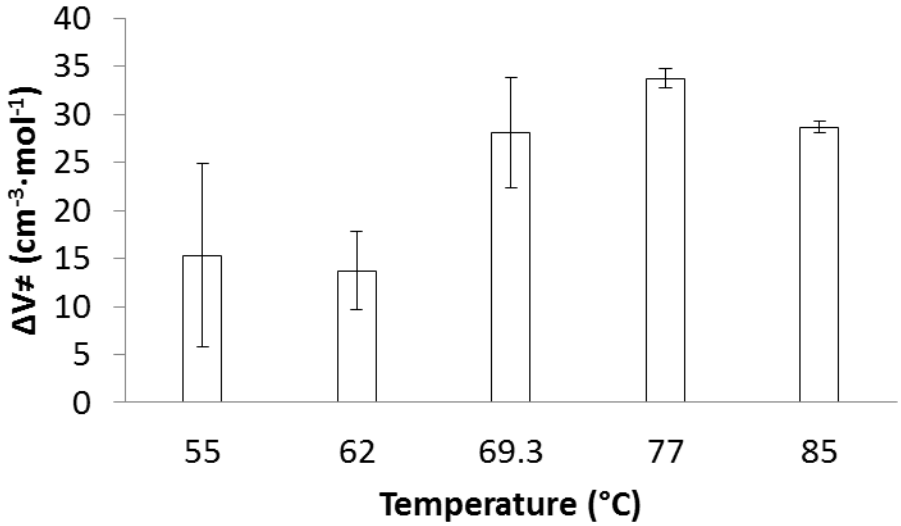

Figure 2-9. Activation volumes for pectinase samples treated at 0.1 MPa and 200 to 400 MPa. Error bars represent standard error of linear regression (n=2)

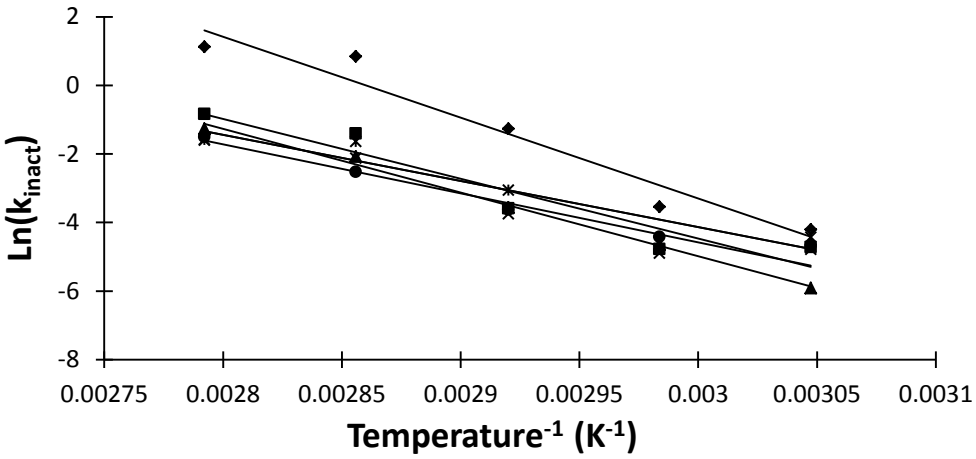

Figure 2-10. Arrhenius plot for viscosity reduction at 0.1 MPa (♦), 200 MPa (■), 250 MPa (▲), 300 MPa (X), 350 MPa (∗), and 400 MPa (●).

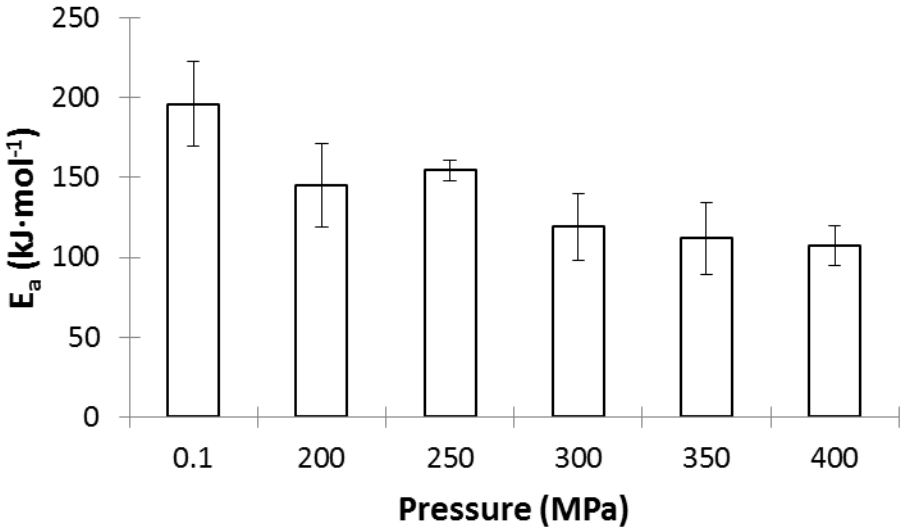

Figure 2-11. Activation energies for pectinase samples treated at high hydrostatic pressure. Error bars represent standard error of linear regression (n=2)

CHAPTER 3
INCREASED RATE OF VISCOSITY REDUCTION OF A PECTINASE COCKTAIL AT HIGH HYDROSTATIC PRESURE

Chapter 2 reported the effects of HHP and high temperature on the kinetics of inactivation of a pectinase cocktail. Based on the results from the inactivation study, this chapter describes the treatment of the cocktail moderate temperatures and high pressure. The objective of this study is to maximize the activity of a pectinase cocktail at HHP for optimal clarification processing that minimizes thermal degradation of pectinase enzymes. Only a summary of the preliminary microbial inactivation results is provided in this chapter; for greater detail, see Appendix.

Materials and Methods

Materials

The pectinase cocktail, citrate buffer and pectin products were the same as those used in Chapter 2. Sodium acetate, glacial acetic acid, and sodium chloride were all purchased from Fisher Scientific (Pittsburg, PA, USA).

Equipment

The equipment used for this experimental study was the same as detailed in Chapter 2 with the addition of a third Isotemp 3016D water bath from Fisher. The three water baths were set for cooling at 4 °C, the processing condition, and finally an inactivation temperature of 95 °C. The remainder of the HHP setup and rheometer equipment were described Chapter 2.

Methods

Sample preparation and HHP processing

Pectin was diluted to 1.5% (w/v) solution in 0.2 M acetate buffer pH 4.5, and ionic strength of 0.1 M. The pectinase solution was diluted in 0.5 M citrate buffer (pH 4 and

ionic strength of 0.844M) to 0.05% (1.5 unit·mL^{-1}) and placed on ice before treatments. Of the untreated enzyme sample 132 µL were added to 1,800 µL of pectin solution and stirred for 40 s with a miniature magnetic bar and stirrer in an ice bath. The pectinase and pectin solution were placed in 1-mL plastic pouches, heat sealed and placed in the high pressure cell held at 4 °C to minimize enzyme activity. For treatment, the HHP cell was closed and a modified LabVIEW program was started exactly 2 min and 30 s after the pectinase had been added to the pectin solution. The constant hold time was to minimize variation in viscosity reduction that occurred before treatment.

Pressure was raised to the process set point. Then, temperature was raised to the incubation set point and when 90% of the change in temperature was reached, processing time started. After processing, the pressure cell was heated to over 89 °C for a three-minute period while the cell was depressurized to inactivate the enzymes. After the inactivation period, the cell was cooled to 8 °C and the sample was placed on ice before viscosity analysis. Enzyme activity was assayed on the same day as the HHP treatment.

Processing conditions

Samples were treated at 0.1 MPa (control) or 200 to 300 MPa at 25 MPa increments, 42.4 to 62.4 °C with increments close to 5 °C, and for a processing time of 0 to 30 min with 7.5 min increments. The processing temperatures were 42.4 °C, 47.1 °C, 52.1 °C, 57.1 °C, and 62.4 °C; temperature increments were chosen for even distribution of T^{-1} for the calculation of activation energy with the Arrhenius equation. Come-up and come-down times were accounted for as follows. Ramp-up time included pressurizing and heating the cell to 90% of set point temperature. Ramp-down time included inactivation, depressurization, and cooling of the cell. Figure 3-1 shows the

pressure and temperature profile for a sample treated at 62.4 °C, 250 MPa, and 15 min. Samples were processed in duplicates. The study was performed in a randomized block design, with temperature blocks. Pressure and process times were randomly selected.

Activity measurements

To assess the activity of the enzyme cocktail, the pectinase and pectin solution were placed in a water bath to raise the sample temperature to 20 °C. The viscosity of pectin was recorded every 1.2 s for 5 min with a maximal viscometer rotational speed of 20 rpm and at a temperature of 20 °C. Samples that contained pectin only or pectin with only citrate buffer were run for 10 and 5 min respectively to check pectin consistency.

The rate of viscosity reduction measurements differ from the ex-situ measurements made for the viscosity reduction observed in Chapter 2. For this experiment, the viscosity reduction occurred inside the pressure cell (*in-situ*). Therefore the extent of *in-situ* viscosity reduction was determined after processing at selected temperatures followed by enzyme inactivation. Then viscosity was determined *ex-situ*. Full enzyme inactivation was verified by a constant viscosity of the reaction mixture after treatment. An average viscosity measurement of the last 50 measurements of each viscosity reading was used as the viscosity value for each time point. For this study, the rate of viscosity reduction at high pressure was determined again with same pseudo-second order rate. Error was reported using the standard error of the slope from the linear regression from the pseudo-second order rate of viscosity reduction plot of inverse viscosity versus process time.

Additional calculations for this experiment include the percent viscosity reduction (Equation 3-1).

$$Viscosity\ Reduction\ (\%) = 100 - \left(\frac{\mu_{0\ min}}{\mu_{30\ min}} \times 100\right) \quad (3\text{-}1)$$

Eyring and Arrhenius equations were used as in Chapter 2 to determine the apparent activation volumes and activation energies of viscosity reduction. Experimental error derived from linear regression experiments was reported as standard error.

Results and Discussion

Viscosity Reduction

Pectin viscosity decreased with the increase of processing time as seen in Figure 3-2. The initial mean pectin viscosity for initial processing time of zero minutes was 0.0120 Pa•s with an error of ± 0.0014 Pa•s. The rate of reduction slowed at longer processing periods of 22.5 and 30 min suggesting the enzymes has cleaved a majority of the pectin molecules. The lowest viscosity obtained after 30 min treatment was achieved for each pressure by the highest temperature block of 62.4 °C with the exception of 57.1 °C temperature block for 200 MPa. However, the difference from lowest viscosity values at 57.1 and 62.4 °C of 0.00454 and 0.00462 Pa•s i probably can be attributed to experimental error. During the experimentation there were some slight variations between pectin solutions as well as mixing due to the high viscosity. Also a small temperature gradient was likely to occur during mixing and could have also affected enzyme activity before the sample was treated with high pressure.

All samples treated at high pressures, 200 to 300 MPa, for 30 min at 62.4 °C achieved at least a 60% viscosity reduction. At atmospheric pressure only a 54.6% reduction was achieved. The reduction at atmospheric pressure was 14.4% smaller than greatest reduction of 69% at 200 MPa and 62.4 °C. The average viscosity reduction for all temperatures for 30-min treatments is summarized in Table 3-1. At

lower temperatures of 42.4 °C or 47.1 °C, atmospheric conditions produced similar viscosity reduction in which the stabilization of pressure is not yet observable with the short processing times. Figure 3-2 plots the viscosity reduction for the studied extreme temperatures of 42.4 °C and 62.4 °C. Temperatures of 52.1 to 62.4 °C, achieved greater percent viscosity reduction results with high pressure treatment (≥ 55.2%) than seen with atmospheric conditions (≤ 54.6%).Samples that were treated with 0.1MPa, 200, or 300 MPa, with 62.4 °C showed high percentages of viscosity reduction compared to other treatments, while samples at 225 MPa to 275 MPa showed high viscosity reductions at temperatures of 52.1 or 57.1 °C. The variation in these results between temperature blocks is likely due to the differences in the initial viscosity between samples and possibly due to inadequate mixing, this ended up in the process of using the rate of viscosity reduction as the parameter for measurement of pectinase activity.

Rate of Viscosity Reduction

In our previous study (Chapter 2), pectin viscosity reduction with the addition of pectinase cocktail was best fitted as pseudo-second order reaction. For this study, the same pseudo-second order model was used with inverse viscosity plotted against time and the linear relationship showed with a regression line as seen in Figure 3-3. The rate of viscosity reduction for atmospheric and high pressure conditions increased with the increase in processing temperatures from 42.4 °C to 62.4 °C. The second-order rate constant of viscosity reduction along with the standard error of the average of the two replicates is shown in Table 3-2. Deviations that occurred from the general increase were at 52.1 °C for 300 MPa, 57.1 °C for 0.1 MPa, 250 MPa, and 300 MPa, and finally 62.4 °C with 300 MPa. There was no obvious trend of the rate of viscosity reduction with

pressure. Larger variations were observed at higher temperatures of 52.1 to 62.4 °C. However, at those temperature blocks samples treated at atmospheric pressure had higher rates of viscosity reduction than at lower temperatures. The largest rate of viscosity reduction of 0.0960 $Pa^{-1}•s^{-2}$ occurred at 62.4 °C and 300 MPa. The variation could be due to inconsistencies in initial pectin viscosity, mixing, or to the difference in behavior of the different enzymes in the cocktail with temperature.

Pressure has been reported to have different effects on individual pectinase enzymes. While tomato PME has been noted to be stable up to 700 MPa at room temperature, two forms of tomato PG, a heat labile and heat stable form, have been inactivated within 15 min at pressures from 300 to 500 MPa (Rodrigo and others 2006). For orange juice the activity of PME has been reported to decrease with increased pressure, temperature and processing time. For example, samples treated at atmospheric pressure for 15 min at 37.5 °C had a residual activity of 89.9%, while samples treated for 12 min at 50 °C only retained 65% of residual activity where samples treated at 400 MPa, 50 °C for 12 min had a residual activity of 50.1% (Nienaber and Shellhammer 2001). The two enzymes, PME and PG, also affect the behavior of the other enzymes. Verlent and others, (2007) reported that tomato PG in the presence of highly esterified pectin and PME at pH 4.4 had peak activity at 50 °C and 200 MPa. Tomato PME had an optimum activity at 60 °C and 400 MPa without PG present, however with the presence of PG, optimal PME activity was at 0.1 MPa and at lower temperatures (Verlent and others 2007). While high pressures inhibit activity of some forms of pectinases, HHP increased activity of the pectinase cocktail at moderate temperatures as it had done with other fungal pectinases. Fungal pectin methylesterase

(PME) has been stabilized with high pressure. Optimal conditions for PME from *A. aculeatus* were found at 200 MPa and 45 °C and 300 MPa and 50 °C, while activity decreased for atmospheric conditions over the optimal temperature of 45 °C (Fraeye and others 2007). Taken together, it seems that environmental factors (pressure, temperature and process time), type of enzymes, and the enzyme composition of the solution all interact to determine the extent that high pressure stabilize enzyme activity.

The increase in viscosity reduction can also be quantified with the percent increase of activity with respect to samples treated to atmospheric pressure and at 45 °C, which are the recommended conditions for use of the cocktail used in this study. The rate of viscosity reduction at 45 °C and 0.1 MPa was 0.0471 ± 0.0026 $Pa^{-1} \bullet s^{-2}$. High pressure started generating an increase in activity with respect to activity at 45 °C and 0.1 MPa, at 47.1 °C for pressures of 200 to 250 MPa. The greatest increase in activity with respect to the recommended conditions was at 62.4 °C with all pressures having increase in activity from 60 to 104%. However, the standard error calculated from the linear regression used to calculate the rate constants, suggests that there was no significant difference among all high pressure levels at 62.4 °C. The largest rate of viscosity reduction obtained at atmospheric pressure was also at 62.4 °C; the percent increases in activity for high pressures in comparison to the 0.1 MPa sample at the same temperature were between 15.7 to 47%.

At temperatures greater than 52.1 °C, activity generally increases with temperature compared to the standard of 45 °C and 0.1 MPa. High pressure stabilized samples from temperature inactivation as shown in Table 3-2 and previously discussed in Chapter 2. At atmospheric pressure, the percent increase was from 3.3% to 38.6%

from 47.1 to 62.4 °C. At high pressures of 275 or 300 MPa at 47.1 °C did not increase the rate of viscosity reduction compared to the standard; however, percent activity increases were seen in all remaining pressures at 47.1 °C and all high pressures at temperatures from 52.1 to 62.4 °C. For tomato PG in the presence of tomato PME activity was 71.9% at 200 MPa and 50°C in comparison to samples treated at 40 °C and 0.1 MPa, while tomato PME in the presence of tomato PG had the greatest percent increase of 33.7% at the same conditions (Verlent and others 2007). Tomato PG did not have an increase in activity at 50 °C or 60°C for pressures over 200 MPa, while PME had increased activity up to 300 MPa. The pectinase cocktail had greater increased activity than tomato PME and PG; showing that pectinase enzymes as a cocktail were more affected than tomato PG and PME. *A. aculeatus* PME, however, had larger increases in activity with respect to samples treated at 45 °C and 0.1 MPa than the pectinase cocktail. For pressures of 200 to 300 MPa and temperatures of 50 and 55 °C, *A. aculeatus* PME had increased activity of 146.8% to 198.9%, while the pectinase cocktail had increased activity of 29.4 to 66.7% for pressures of 200 MPa and 300 MPa and temperatures of 52.1 to 57.1 °C. Yet at higher temperatures and 200 to 300 MPa, the pectinase cocktail had greater increases in activity of 60.4 to 103.8% at 62.4 °C, while *A. aculeatus* PME increased 15.0% at 200 MPa and 70.6% for 300 MPa at 60 °C. Temperature and pressure combinations affect pectinases differently; this data shows the necessity of optimization of processing conditions for each enzyme or set of enzymes. Tomato PG and PME, *A. aculeatus* PME, and the pectinase cocktail all had increased activity with moderate high pressure (<300 MPa) and moderate temperatures of 50 to 60 °C with respect to standards at atmospheric pressure.

Maximum activity for commercial pectinase cocktails has been studied for thermal processing at atmospheric conditions; Pectinase 3XL®, the same cocktail used for this study, had maximum activity at 50 °C and had an active range from 30 to 70 °C (Ortega and others 2004). Pectinase 3XL® had a wider activity range than two other commercial products tested: Rapidase® C80, optimal condition at 55 °C and Pectinase CCM, optimal condition at 50 °C. Pectinex 3XL® only retained 10% of initial activity when thermally treated at 50 °C for 1 h; other commercial pectinase Pectinase CMM and Rapidase® C80 retained 57 and 5 % activity (Ortega and others 2004). These studies support our findings that high pressure can stabilize pectinase enzymes that are sensitive to high pressures and temperatures. Viscosity reduction has also been used to characterize total pectinase activity of commercial products Pectinex®Clear (PC) and Pectinex®BE Colour (PB) and extracts from *A. niger* and *A. oryzae* for the clarification of apple, butia palm, grape and blueberry juice (Sandri and others 2011). Viscosity reduction was used for total pectinase activity with one unit of activity stated the necessary amount of enzyme required to reduce the viscosity by 50% (Sandri and others 2011). A lower optimal temperature of 40 °C for commercial products was observed than the stated manufacturer optima of 50 °C for PC and 54 °C for PB (though manufacturer's processing conditions were not known to researchers). For apple juice clarification, based on turbidity reduction, *A. niger* T0005007-2 and Pectinex®Clear had similar results of about 60% clarification measured by optical density with treatments of 30 °C for 60 min for Pectinex®Clear and samples treated at 50 °C for periods of 30 and 60 min for *A. niger* T0005007-2. Sandri and others, (2011) reported that increasing process time, rather than temperature, promoted greater clarification. Our results

indicate that raising process temperature (42.4 to 62.4 °C) and pressure (200 to 300 MPa) increases the rate of viscosity reduction.

Activation Volume

The effect of pressure on enzyme activity was quantified by calculating the apparent activation volume. Only pressures of 0.1, 200, and 225 MPa were used for the estimation. Pressures higher than 225 MPa deviated from linearity. Therefore, Eyring's equation no longer applies for pressures from 200 to 300 MPa (Figure 3-4). Error was likely to be increased without an even distribution between pressures points of 0.1, 200, and 225 MPa. Immobilized lipase in hexane had similar results of change in sign; apparent activation volume with negative values for pressures below 200 MPa and positive apparent activation volumes values from 300 to 500 MPa (Eisenmenger and Reyes-De-Corcuera 2009a). At higher pressures from 250 to 300 MPa, temperature inactivation and pressure stabilization of the pectinase cocktail were less predictable at temperatures of between 42.4 and 62.4 °C. The use of pressures of 0.1, 200 MPa or 225 MPa, allowed for a calculated apparent activation volume that clearly showed increased rates of viscosity reduction and pressure stabilization shown in Figure 3-4.

The activation volumes varied from -0.22 ± 0.18 to -5.21 ± 0.39 $cm^3 \bullet mol^{-1}$ for the studied temperatures of 42.4 °C to 62.4 °C as shown in Figure 3-5. Apparent activation volumes increased in magnitude as temperature increased to 57.1 °C, then decreased in magnitude to 62.4 °C. High pressure had the smallest effects on lower temperatures 42.4 and 47.1 °C, and slightly greater effects on higher temperatures of 52.1°C to 62.4 °C. The negative apparent activation volumes show that viscosity reduction was favored with high pressure treatments for all temperatures. The apparent activation volumes

indicate that that pressure had the most favorable effects at the temperature range of 52.1 to 62.4 °C, with the peak effect of stabilization at 57.1 °C.

Previously reported apparent activation volumes typically fall into the range of -70 to 60 $cm^3 \cdot mol^{-1}$ for enzymes (Michels Peter and Clark Douglas 1992). Apparent activation volumes have also been studied for carrot and orange PME. Purified carrot PME had similar apparent activation volumes of -7.80 to -5.73 $cm^3 \cdot mol^{-1}$ for temperatures of 30 to 55 °C; the smallest volume was seen at 40 °C and the volume increased as temperature increased to -5.73 $cm^3 \cdot mol^{-1}$ (Sila and others 2007). Orange PME apparent activation volumes also increased from -35 to -30.9 $cm^3 \cdot mol^{-1}$ as temperature increased from 25 to 50 °C (Nienaber and Shellhammer 2001). Results from our experiment differed from carrot and orange PME, in that, apparent activation volume increased in magnitude as temperature increased from 42.4 to 57.1 °C. High pressure appears to have had less effect on the pectinase cocktail than orange PME.

Activation Energy

The apparent activation energy was also calculated to observe the effect of temperature on the pectinase cocktail at different pressures. The apparent activation energy generally increased with pressure from 15.03 to 32.22 $kJ \cdot mol^{-1}$ at 0.1 to 225 MPa, then slightly decreased to 25.71 $kJ \cdot mol^{-1}$ at 250 MPa, then again increased with pressure to 42.4 $kJ \cdot mol^{-1}$ at 300 MPa (Figure 3-6). Though there was a small decrease in apparent activation energy at 250 MPa, the decrease was not significant for the values for 225 MPa and 250 MPa (32.22 $kJ \cdot mol^{-1}$ and 31.66 $kJ \cdot mol^{-1}$ respectively). For high pressure samples, the effects of temperature from 200 to 275 MPa were similar (25.7 to 32.2 $kJ \cdot mol^{-1}$) with temperature having greater influence at 300 MPa (42.4 $kJ \cdot mol^{-1}$). The similarity between activation energies was expected due to the narrow

range of pressures selected from the results of the previous experiment at inactivating conditions (Chapter 2). The error range was 0.7 to 4.6 kJ•mol^{-1} for pressures of 0.1 to 275 MPa, and was larger for 300 MPa with 11.3 kJ•mol^{-1}.

Also, temperature had a greater effect when enzymes were treated at high pressure versus atmospheric pressure with an increase of 71.0% at 250 MPa to 182.1% at 300 MPa. Temperature with the addition of high pressure appears to be the main stimulus of the increased activity of the pectinase cocktail at moderate temperatures of 52.1 to 62.4 °C.

Reported activation energies for carrot PME were similar but slightly higher to data obtained in this experiment. Apparent activation energies for carrot PME at 0.1 MPa was 48.9 kJ•mol^{-1} and 31.6 to 81.4 kJ•mol^{-1} for pressures 200 to 500 MPa, with no trend associated with increase in pressure for temperatures of 30 to 55 °C (Sila and others 2007). Apparent activation energies of orange PME, 13.5 - 30.1 kJ•mol^{-1} had the opposite general trend seen in our experiment as apparent activation energy decreased with the increase in pressure of 400 to 600 MPa (Nienaber and Shellhammer 2001). The pectinase cocktail, with multiple enzymes, had less variation in activation energies than the carrot PME indicating the mixture may be more stable than individual enzymes alone.

Preliminary Microbial Inactivation

E. coli K12 was inactivated with treatments ranging from 250 to 350 MPa, 60 to 80 °C, and a process time of 15 min. A 1 to 2 log reduction was also seen for ramp-up and ramp-down times (process time of zero minutes). The experiment is detailed in the Appendix.

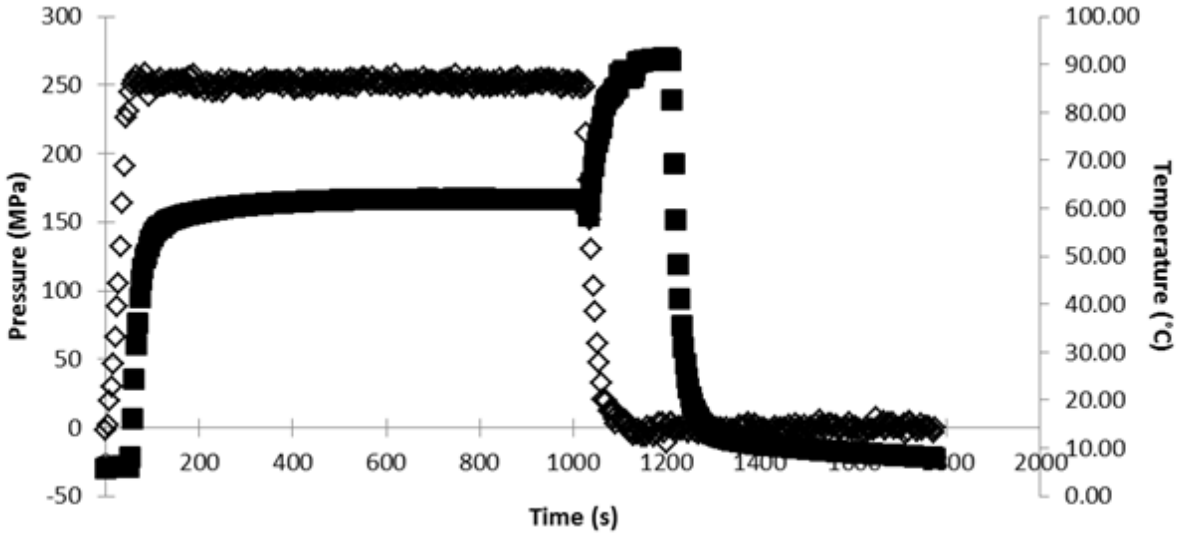

Figure 3-1. Pressure (◊) and temperature (■) profiles in high pressure cell for a sample treated at 62.4 °C, 250 MPa, 15 min.

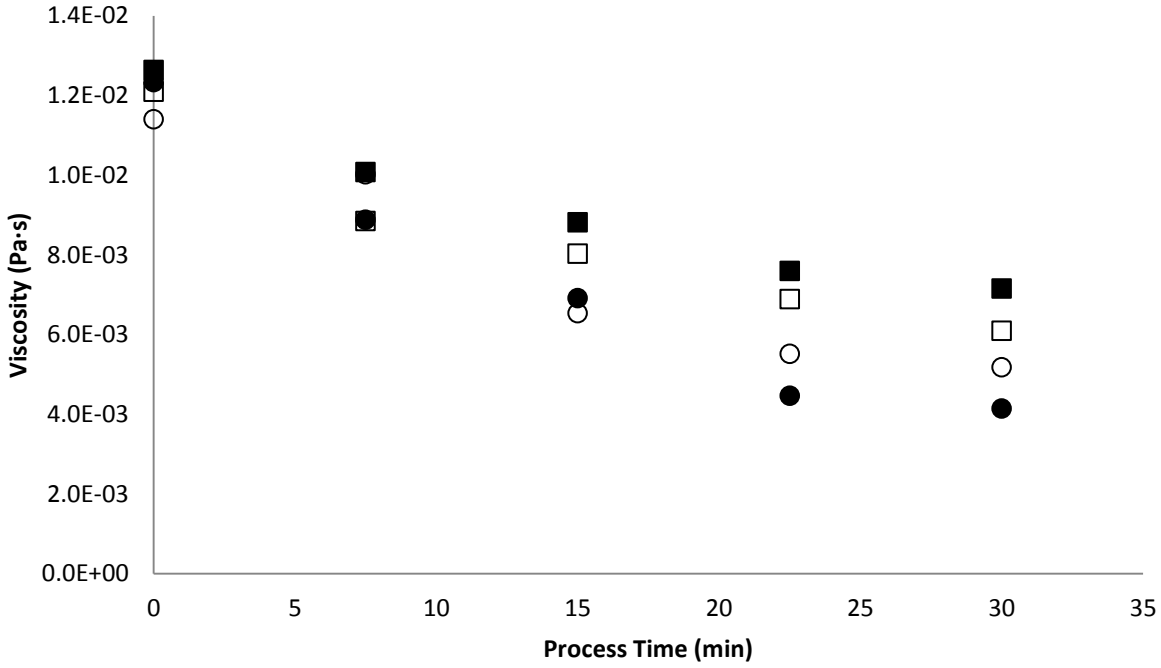

Figure 3-2. Viscosity reduction for samples treated at (□) 42.4 °C and 0.1 MPa, (■) 42.4 °C and 300 MPa, (○) 62.4 °C and 0.1 MPa, and (●) 62.4 °C and 300 MPa

Table 3-1. Average viscosity reduction (%) of a 30-min process time relative to t = 0 min.

	Temperature					
Pressure (MPa)	42.4 °C	45 °C	47.1°C	52. 1°C	57.1°C	62.4 °C
0.1	49.6	51.8	51.1	46.0	52.7	54.6
200	50.8		52.5	60.5	59.9	69.0
225	47.4		50.2	58.8	63.3	62.3
250	49.3		49.7	65.3	56.6	62.2
275	45.5		49.5	55.2	61.1	61.0
300	43.4		47.8	61.2	55.2	66.4

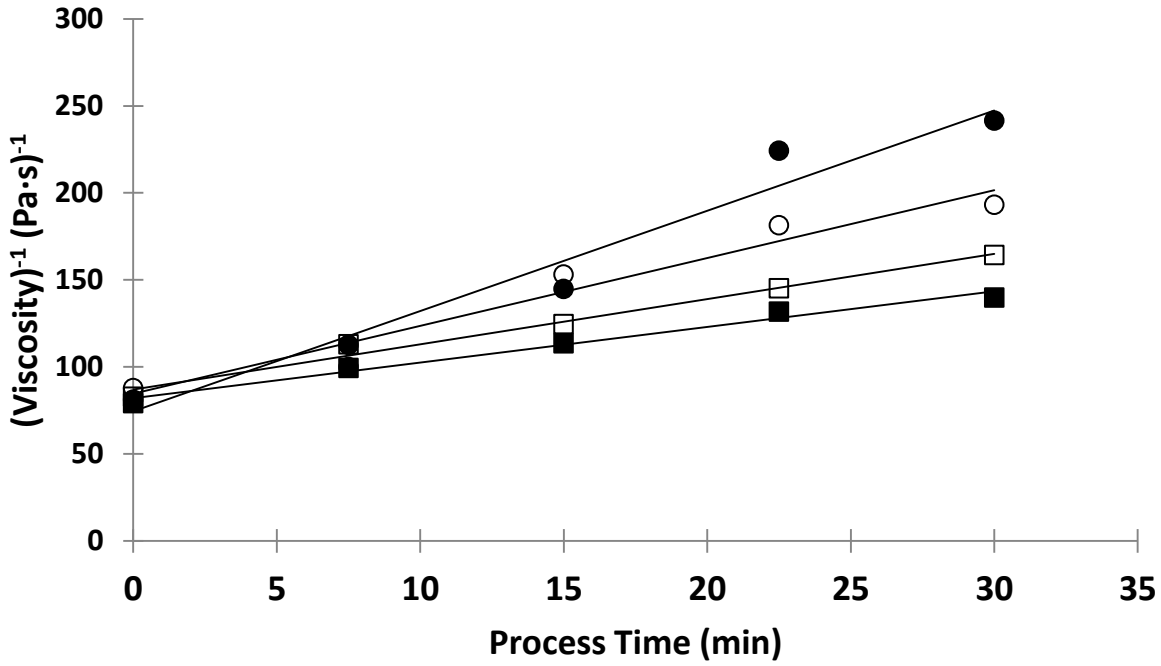

Figure 3-3. Rate of viscosity reduction with a pseudo second order rate of reaction plot for pectinase treated at (□) 42.4 °C and 0.1 MPa, (■) 42.4 °C and 300 MPa, (○) 62.4 °C and 0.1 MPa, and (●) 62.4 °C and 300 MPa. Rates are supplied in Table 3-2.

Table 3-2. Rate of viscosity reduction ± standard error ($Pa^{-1} \cdot s^{-2}$), n = 2 with R^2 values for different pressure treatments.

Temperature (°C)	0.1 MPa	R^2	200 MPa	R^2	225 MPa	R^2
42.4	0.0434 ± 0.0032	0.984	0.0446 ± 0.0023	0.992	0.0439 ± 0.0006	0.999
47.1	0.0487 ± 0.0022	0.994	0.0531 ± 0.0028	0.992	0.0506 ± 0.0015	0.997
52.1	0.0487 ± 0.0062	0.954	0.061 ± 0.0056	0.975	0.0677 ± 0.0044	0.987
57.1	0.0505 ± 0.0051	0.970	0.0723 ± 0.0047	0.988	0.0785 ± 0.0039	0.993
62.4	0.0653 ± 0.0084	0.952	0.0829 ± 0.0101	0.958	0.088 ± 0.0095	0.966
(°C)	250 MPa	R^2	275 MPa	R^2	300 MPa	R^2
42.4	0.0437 ± 0.0026	0.990	0.0388 ± 0.0038	0.972	0.0342 ± 0.0025	0.984
47.1	0.0495 ± 0.0028	0.991	0.0457 ± 0.0046	0.970	0.0426 ± 0.0035	0.981
52.1	0.0676 ± 0.0059	0.977	0.0619 ± 0.0043	0.986	0.0763 ± 0.0113	0.938
57.1	0.0653 ± 0.0062	0.974	0.0727 ± 0.0041	0.991	0.0599 ± 0.007	0.961
62.4	0.0789 ± 0.005	0.988	0.0755 ± 0.0069	0.976	0.096 ± 0.0108	0.963

Note: Each replicate was calculated from the linear regression of the reciprocal of viscosity vs. time of at least five incubation times.

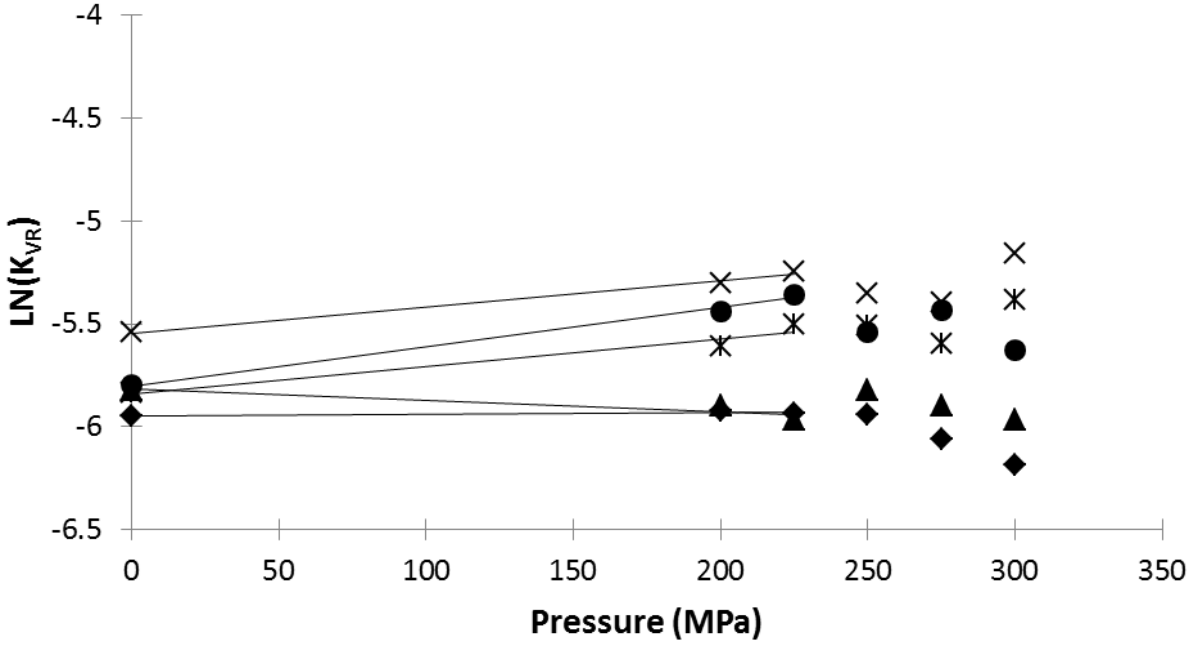

Figure 3-4. Eyring plot for viscosity reduction for samples treated at 42.4 °C (♦), 47.1°C (▲), 52.1°C (*), 57.1°C (●), and 62.4 °C (X).

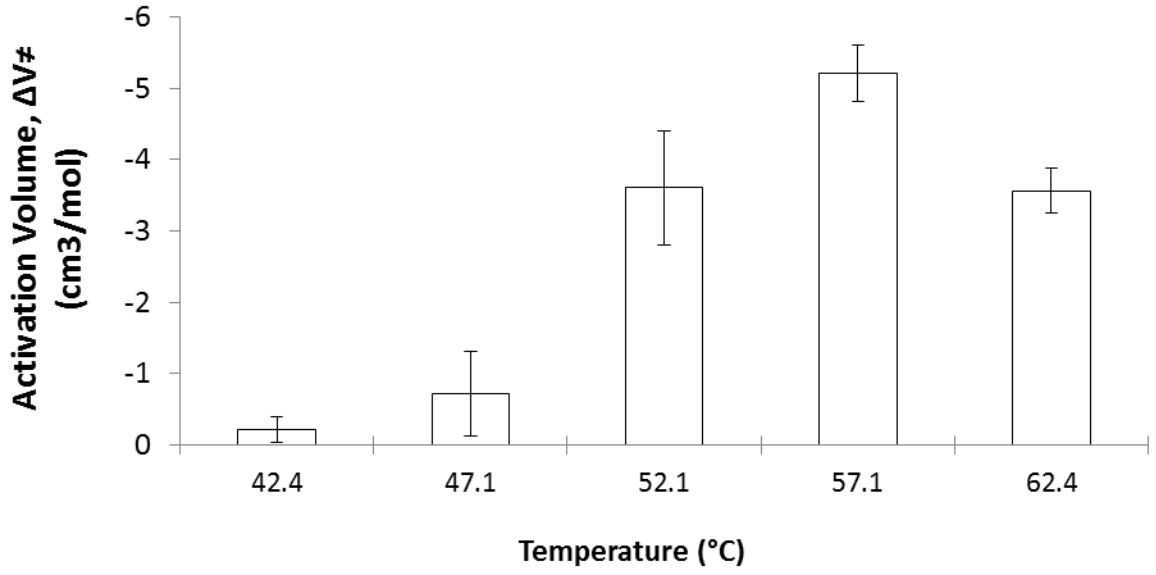

Figure 3-5. Effect of pressure on the activation volume of the rate of viscosity reduction of pectin solutions. Error bars represent standard error of linear regression.

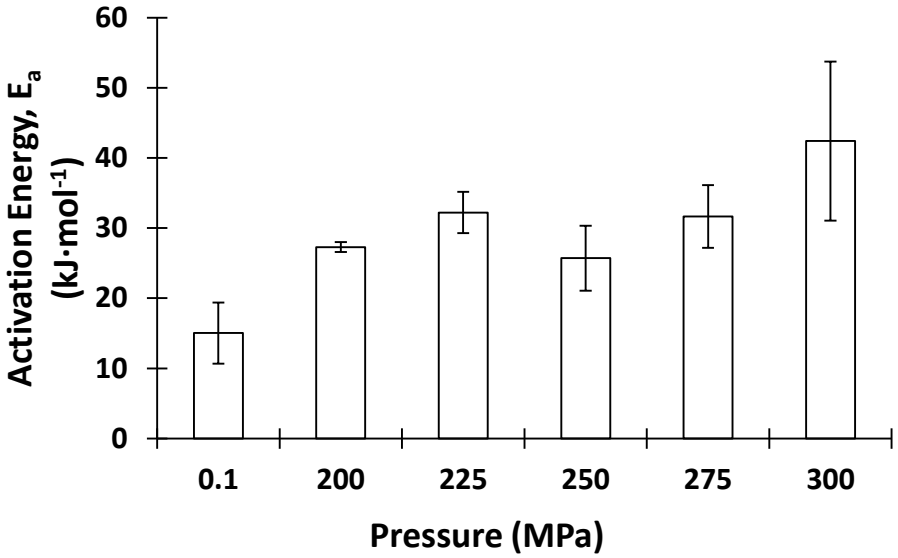

Figure 3-6. Effect of temperature on the activation energy of the rate of viscosity reduction of pectin solutions. Error bars represent standard error of linear regression.

CHAPTER 4
EFFECT OF HIGH PRESSURE ON PH COLOR INDICATORS IN SOLUTION

As high pressure becomes more prominent as an alternative to thermal processing in the food industry, its effects on physical factors such as the pH of a food must be considered. Knowledge of food pH during processing is important due to its effect on the behavior of enzymes, microorganisms and other parameters crucial to the food quality. The final portion of this research focused on the pH shift of acid/base color indicators in response to HHP. This research is significant because pH probes for high pressure are not commercially available; therefore, an alternative method must be established to determine shifts in pH with HHP. The objective of this study was to assess color changes of pH indicators at pressures up to 600 MPa for five acid/base indicators and compare to calculated pressure shifts.

Materials and Methods

Materials

Five color acid/base indicators were used to provide a color array for acidic pH range ($1.2 < pH < 6.8$). Table 4-1 provides a list of the five indicators used, their pH range, and the colors associated with the pH range. Bromocresol Purple, Bromophenol Blue, Metanil Yellow, Methyl Orange and Thymol Blue were purchased from Fisher Scientific (Pittsburg, PA, USA). The color indicators were each dissolved in distilled water to make a 0.1% (w/v) stock solution.

Equipment

The laboratory high pressure setup was similar to the equipment setup detailed in Chapters 2 and 3. For this experiment, the Unipress (Warsaw, Poland) High-Pressure Optical Vessel U103 with sapphire windows was used instead of Model U111 pressure

cell. A DH-2000 UV-VIS-NIR light source (Mikropak) with deuterium and halogen lamps was used as the light source and a HR4000CG-UV-NR high resolution spectrometer from Ocean Optics, Inc. (Dunedin, FL, USA) was used to collect visible spectra. The light source and the spectrometer were connected to the high pressure optical cell through fiber optic cables. The temperature of the jacketed high pressure chamber was controlled with a water bath model Isotemp 3016D from Fisher Scientific (Pittsburg, PA, USA); the water bath was used to keep the optical vessel and indicator solution at 25 °C. OOIBase32 software (Ocean Optics, Inc) was used to measure and collect absorbance spectra from 200 to1200 nm.

Methods

Sample preparation

The concentrated indicator stock solution was added to distilled water to produce a lighter dilute indicator/water solutions to be used for absorbance measurements. The final concentrations are as followed: Bromophenol Blue was 5.9 $x10^{-6}$ M, Methyl Orange 9.5 x 10^{-6} M, Thymol Blue 2.0 x 10^{-5} M, Bromocresol Purple 3.6 x 10^{-5} M, and Metanil Yellow 5.1 x 10^{-5} M. Distilled water was used as the blank. The dilute indicator solution was adjusted to the desired pH with addition of HCl or NaOH. The sample was enclosed in a cylindrical quartz cuvette with two Teflon® stoppers. The vial was plugged avoiding the entrapment of air bubbles, and then levelly submerged into the pressurization silicon fluid contained in the optical cell to avoid refraction of the light beam and provide a consistent path length through the cuvette.

Processing conditions

The spectrum of each dye at selected pH was recorded at atmospheric pressure at 25 °C (Table 4-1). Dyes were pressurized up to 600 MPa in 100 MPa increments at

25 °C. Spectra of two replicates were collected for each indicator solution. The study was performed in a randomized block design, blocked by indicator. Pressure was randomly selected.

Peak area measurements

Figures 4-1 and 4-2 display the spectra of the acid or basic form of each indicator respectively. The data was normalized by adjusting the average data from 750 nm to 800 nm in the infra-red region to the origin of absorbance data. The peak area was calculated for each peak with the adjusted spectrum by integrating absorbance values against wavelength using Microsoft Excel.

Calibration curves and compression compensation

As pressure was applied, volume decreased causing an increase in concentration of the dyes, resulting in increased large absorbance values. The change in the peak area with pressure is therefore due to both an increased concentration and a shift in pH with applied HHP. The effect of temperature on the pH was not compensated for due to the sample being kept at room temperature with the jacketed cell and water bath. The effect of pressure compression was compensated for by using the known compressibility of water. This adjustment allowed us to determine the pH shift due to HHP alone. The effect of compression of HHP on water was sourced from the NIST Standard Reference Database 69: NIST Chemistry WebBook (USNIST 2008). The compression factor was the percent decrease of water density with HHP treatments. The peak area at atmospheric pressure was divided by the water compression factor to compensate for the concentration effect at each selected pressure as described in equation 4-1.

$$PA_{comp} = \frac{PA_{0.1\ MPa}}{CF} \quad (4\text{-}1)$$

Where PA_{comp} is the pressure compensated peak area, $PA_{0.1MPa}$ is the peak area at atmospheric pressure, and *CF* is the compression factor.

The difference between the peak area compensated for compression and the observed peak area, PA_{obs} with pressure was used to assess the shift in equilibrium (PA_{shift}) for each indicator as expressed in equation 4-2

$$PA_{shift} = PA_{obs} - PA_{comp} \quad (4\text{-}2)$$

With the change in color, adjusted peak area, and the pH calibration curves established for each indicator, an apparent pH shift was calculated.

The compression factor decreases with the increase in pressure with a value of 1 at atmospheric pressure, 0.96 at 100 MPa and continued to decrease to 0.85 at 600 MPa.

Results and Discussion

Peak Area

For each acid/base indicator, a shift in pH within its specific indicator range led to a color change that was monitored by absorbance in the visible spectrum. Some pH indicators had one large absorbance peak that shifted with pH, such as Methyl Orange and Metanil Yellow, while others like Bromocresol Purple, Bromophenol Blue, and Thymol Blue had two peaks that changed in size with the change in pH (Figure 4-3). For Bromocresol Purple the peak from 400 to 485 nm was used, while for Bromophenol Blue and Thymol Blue the peaks from 500 to 650 nm and 400 to 490 nm were used respectively for pH correlation. With the application of pressure, not only the height of the peak increased, but the peak absorbance wavelength shifted. The peak area

calculated provided the best relationship at atmospheric pressure with the change in pH because peak area accounted for changes in peak absorbance as well as shifts in peak absorbance.

Calibration Curves

Peak area correlated linearly with pH at atmospheric pressure in the studied range as shown in Figure 4-4 and Table 4-2. Table 4-2 provides the parameters of the linear relationships used to correlate pH with peak area found with linear regression. In some cases, deviations from linearity occurred at edges of the reported pH indication range for each indicator as shown for Bromophenol Blue Figure 4-3. At the edge of the color indication range, change in color is minimal in comparison to the working pH indicator's range and either the acidic or basic form of the indicator dominates the solution limiting color change. This explains the leveling off from the linear relationship. The correlation coefficients support the adequacy of the calibration curves for estimation of pH shifts.

Effect of Pressure on pH

With the application of pressure, a reversible change was seen in peak areas for each indicator. For indicators that produced two distinct peaks, the peak with the area that increased in size with pressure was chosen to represent the indicator. As pressure was applied, volume decreased causing an increase in concentration due to compression.

After compensation for compression, predictions of the pH shift were obtained using the correlation of peak area and pH at atmospheric pressure. For all dyes, experimental results indicated an apparent acidic pH shift when pressurized between 0.1 and 600 MPa, as shown in Figure 4-5. Research has reported increases in water ionization constant that create an acidic shift pH (El'yanov and Hamann 1975). At 600

MPa, four indicators had smaller apparent pH shifts of 0.12 to 0.29 pH units than Bromocresol purple which had an apparent shift of 0.98 pH units as shown in Figure 4-6. Although this may indicate that the pH shifts are more pronounced at a higher pH, closer to neutral pH, the largest difference suggests that the equilibrium of the dye is itself affected by pressure.

Literature reports the decrease in pH with HHP as a common effect for water and some buffers with fluorescence and acid/base indicators (Stippl and others 2004; Quinlan and Reinhart 2005). For example, fluorescence has been used to determine the effect of pressure on buffers (Stippl and others 2004). Carboxylate buffers showed a decrease in pH with the application of pressure, while cationic buffers had an increase in pH. Acetate pH decreased at a rate of -0.08 pH units/100 MPa, from an initial pH 7, while other carboxylate buffers were more sensitive to pressure. Blending buffers that have opposing pH responses to pressure has led to pressure stable combinations, such as Tris/tricarballylate and Tris/phosphate, which had an estimated ΔpH of less than -0.025 pH units/100 MPa (Quinlan and Reinhart 2005). Yet reported changes in pH with pressure do vary. Acetic acid, an acidic buffer with initial pH of 4.1, also has been reported to drop by -0.22 and -0.40 pH units at 100 and 200 MPa respectively. Distilled water, with an initial pH of 5.8, had pH shifts of -0.30 units at 100 MPa and -0.31 units at 200 MPa (Hayert and others 1999). These results were similar to values seen in previous literature (Owen and Brinkley 1941; Distèche 1972) but higher than predicted by our apparent shift reported here suggesting that the shift in the equilibrium constant of the color indicator is antagonist to the dissociation of water. An acid shift has also been noted in CO_2 pressurized systems; the observed pH of a CO_2-water system

decreased from the initial pH by 2.53 pH units with an increase in pressure of 0 to 5.516 MPa. However, the observed pH only decreased an additional 0.14 pH units with an increase in pressure of 5.516 to 34.49 MPa indicating that the solvation of CO_2 is the main cause in the pH shift not pressure. Systems that contained ascorbic acid and/or citric acid showed even smaller initial decrease in pH with the increase in pressure. This result shows that the systems component concentrations also have an effect on the pH of a system (Meyssami and others 1992). The acid shift in pH with water and carboxylates acids observed in these studies agrees with the apparent decrease of acid/base indicators in water.

Table 4-1. Acid/base color indicators

Indicator	pH Range	Low Acid Color	High Acid Color	pH Points
Bromocresol Purple	5.2-6.8	Purple	Yellow	5.2, 5.5, 6, 6.5, 6.8
Bromophenol Blue	3-5	Purple	Yellow	3, 3.5, 4, 4.5, 5
Metanil Yellow	1.2-2.4	Orange	Hot Pink	1.2, 1.8, 2.4
Methyl Orange	3.1-4.4	Orange	Red-Orange	3.1, 3.5, 4, 4.4
Thymol Blue	1.8-2.8	Orange	Pink	1.2, 2, 2.8

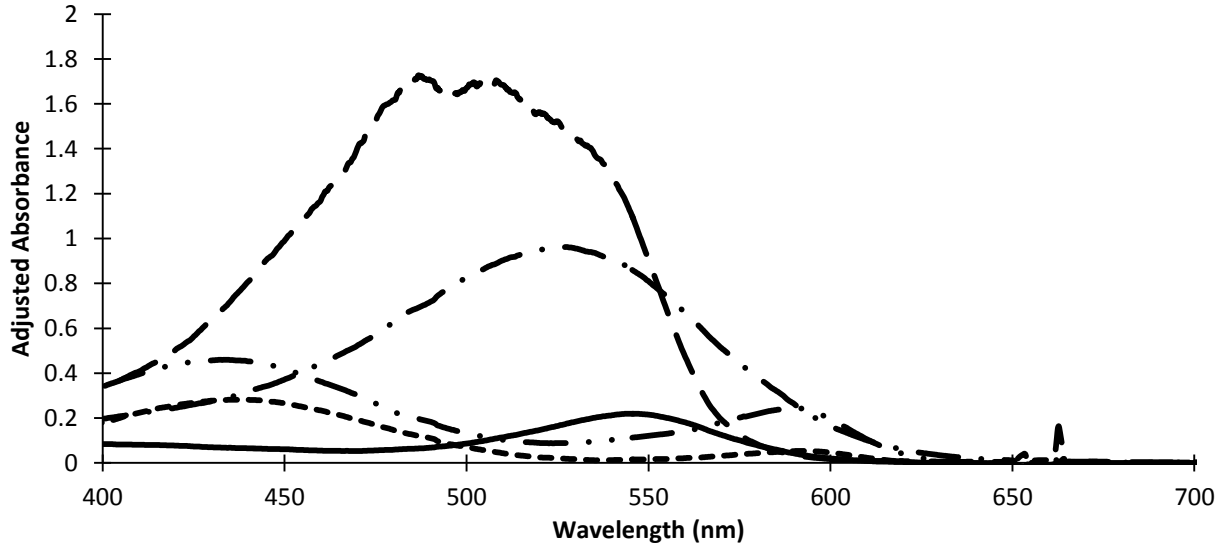

Figure 4-1. Adjusted absorbance spectra for acidic pH points. A) Bromocresol Purple (- -- - - ----) pH 5.2, B) Bromophenol Blue (- - - - -) pH 3, C) Metanil Yellow (---

- --- -) pH 1.2, D) Methyl Orange (--- ---) pH 3.1, and E) Thymol Blue (------) pH 1.2.

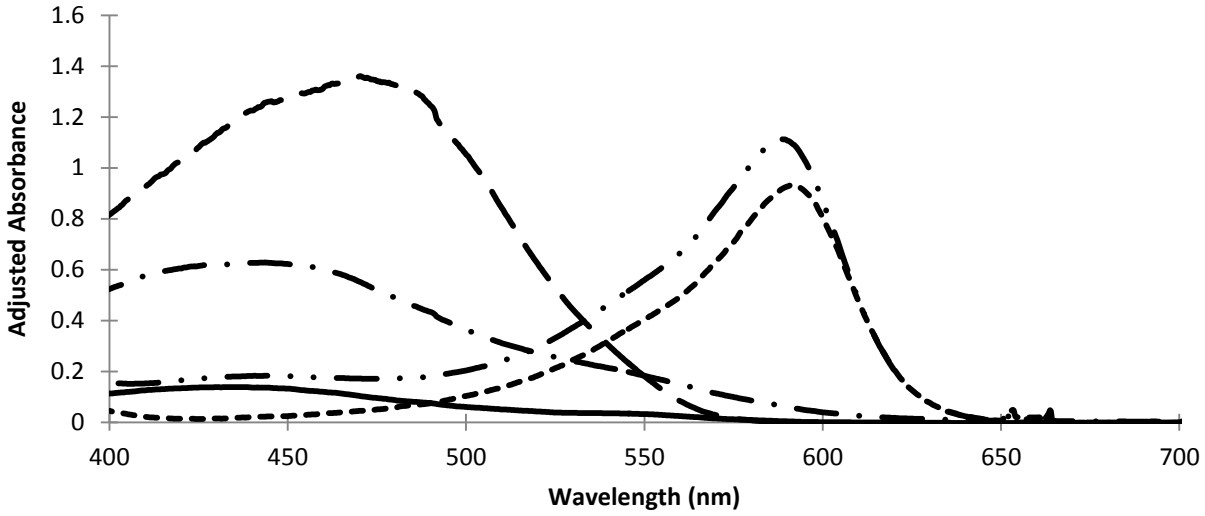

Figure 4-2. Adjusted absorbance spectra for low acid pH points. A) Bromocresol Purple (--- - - ----) pH 6.8, B) Bromophenol Blue (- - - - -) pH 5, C) Metanil Yellow (-- - --- -) pH 2.4, D) Methyl Orange (--- ---) pH 4.4, and E) Thymol Blue (------) pH 2.8.

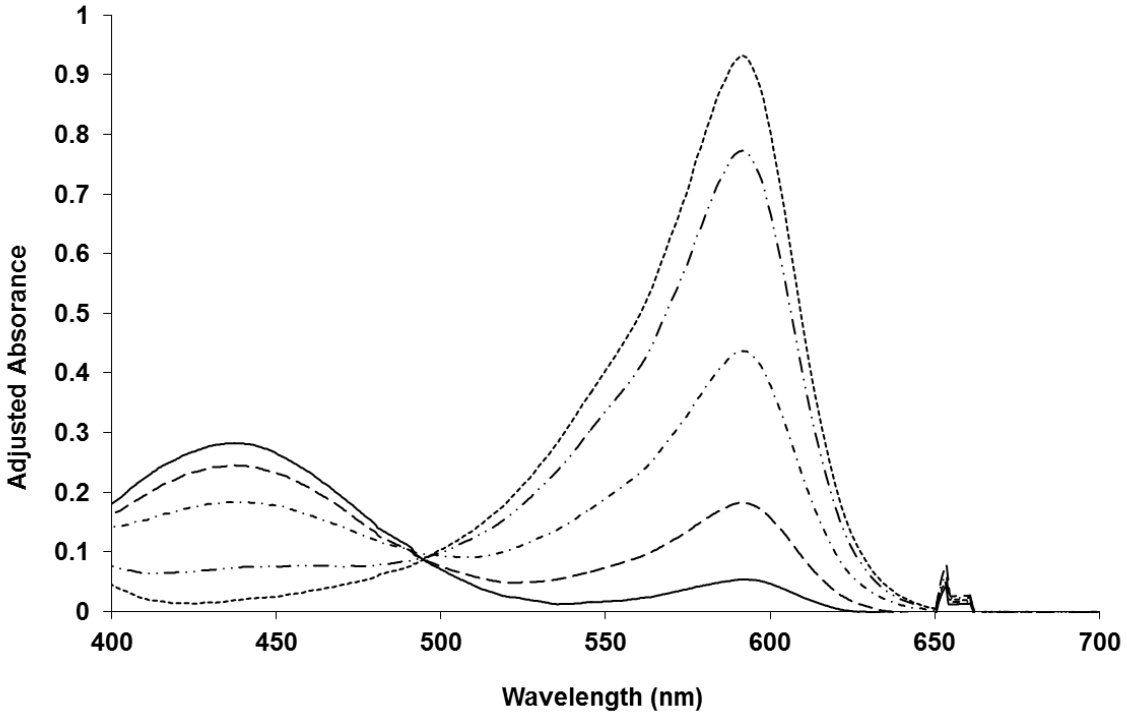

Figure 4-3. Adjusted absorbance spectra for Bromophenol Blue at 0.1 MPa. A) pH 5 (- - - - -), B) pH 4.5 (--- - - ---), C) pH 4 (--- - --- -), D) pH 3.5 (--- ---), and E) pH 3 (------).

Table 4-2. Parameters for linear relationships correlating pH vs. peak area

Indicator	Slope (nm^{-1})	Intercept	Coefficient of Determination
Bromocresol Purple	-0.0813 ± 0.00113	8.7515	.9996
Bromophenol Blue	0.034 ± 0.00412	2.8513	.9578
Metanil Yellow	-0.0246 ± 0.00564	3.951	.9994
Methyl Orange	-0.0275 ± 0.06092	8.3029	.9224
Thymol Blue	0.3398 ± 0.00059	-.9301	.9689

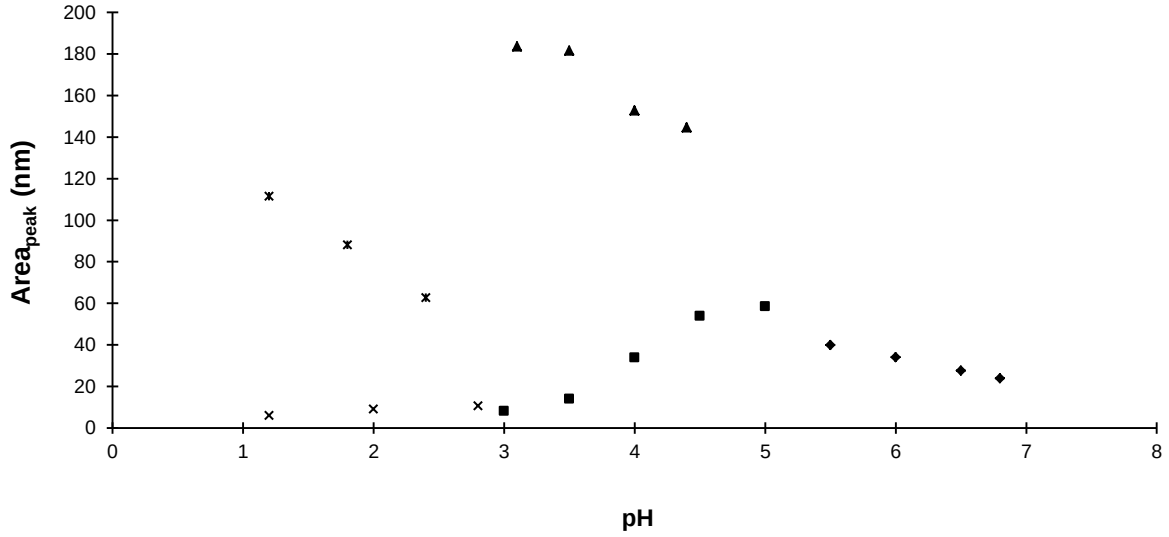

Figure 4-4. The pH calibration plot. A) Bromocresol Purple (♦), B) Bromophenol Blue (■), C) Metanil Yellow (*), D) Methyl Orange (▲), and D) Thymol Blue (X).

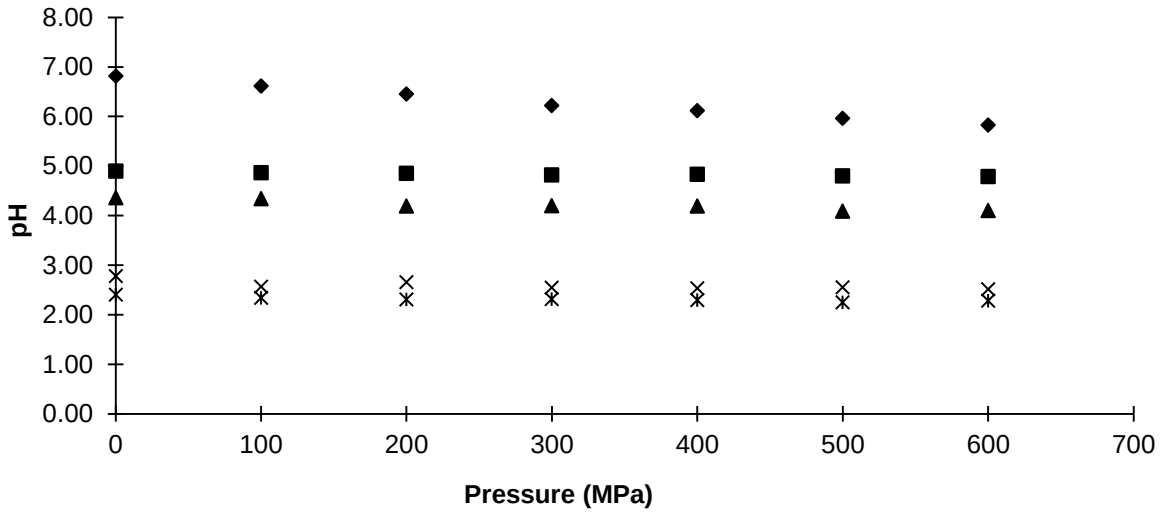

Figure 4-5. Apparent effect of pressure on pH. A) Bromocresol Purple (♦), B) Bromophenol Blue (■), C) Metanil Yellow (*), D) Methyl Orange (▲), and D) Thymol Blue (X).

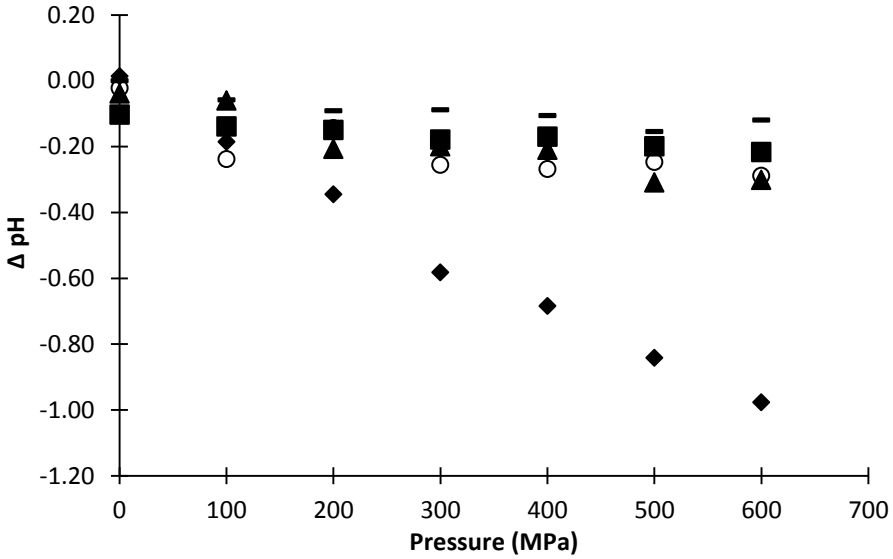

Figure 4-6. Apparent pH shift with pressure. A) Bromocresol Purple (♦), B) Bromophenol Blue (■), C) Metanil Yellow (-), D) Methyl Orange (▲), and D) Thymol Blue (○).

CHAPTER 5
FINAL COMMENTS

Overview

High pressure treatments of 200-400 MPa stabilized the commercial pectinase cocktail which maintained activity longer than the pectinase cocktail did at atmospheric pressure at temperatures of 69.3 to 85 °C. Pressure slowed thermal inactivation. At moderate temperatures and high pressures of 200-300 MPa, the rate of viscosity reduction and pectinase activity increased with temperatures for 42.4 to 62.4 °C. High pressure can be used to stabilize enzymes used in fruit juice clarification which might allow the use of higher temperatures than for treatments at atmospheric pressure while minimizing thermal degradation. High hydrostatic pressure-assisted thermal processing would be an alternative that could stabilize clarification enzymes at higher temperatures than that possible at atmospheric pressure. Preliminary results confirmed that HHP treatments at these temperatures can inactivate *E. coli* K12*.* Finally, acid-base color indicators demonstrated an apparent change in pH with HHP treatments at 25 °C. High hydrostatic pressure processing is a relevant alternative to be explored for improved food and beverage processing techniques.

Future Work

The pectinase cocktail was stabilized with high pressure and moderate temperature treatments. Future experimentation can be performed to further optimize clarification and pasteurization conditions at HHP to make accurate economic comparisons to thermal processing. Because the highest rates of reduction were seen with the highest temperature block tested, 62.4 °C, research at higher temperatures (>62.4 °C) would be beneficial and provide more inclusive results. Also, research testing the individual

enzymes of the pectinase solution under the same conditions would be appropriate to help explain some of the variation in this experimentation which could not be attributed to individual variation of replicates and/or deviation from second order plots. Future studies at lower pressures should be considered to improve the accuracy of the apparent activation volumes and would allow for better quantification of the effects of HHP on the pectinase cocktail. Experimentation at pressures between 0.1 MPa and 350 MPa with 50 MPa increments, and temperatures from 60 to 80 °C should be performed to determine optimal conditions for juice clarification with HHP. Optimal conditions found for pectin solutions should be confirmed in apple juice and with particular attention paid to the microbes pertinent to apple juice such as *E. coli* O157:H7 and *A. acidoterrrestris*. Lastly, HHP research on the change in pH result should be expanded to include the behavior of acid/base indicators at HHP. A possible experiment could be to use indicators with known peak absorbance wavelengths for the acid and basic forms of the compound; and a change in the absorbance at these wavelengths with HHP could be correlated with pH and then used to determine if the ionization constant K_a changed with the application of pressure.

APPENDIX
INACTIVATION OF *E.COLI* K12 WITH HHP TREATMENT

Preliminary work was done with *E. coli* K12 to confirm inactivation of *E. coli* at processing conditions for juice clarification with HHP processing. The *E. coli* K12 was used as a substitute for the pathogenic *E. coli* O157:H7.

Materials and Methods

The *E. coli* K12 (L.E. Barrett) was inoculated in 10 mL of tryptic soy broth and incubated at 37 °C for 24 hours. Cloudy apple juice, pH of 4.2, was made with red delicious apples crushed in a hydraulic press from Norwalk Juicers (Lowell, AR, USA). The *E. coli* broth was mixed with boiled cloudy apple juice to dilute the cells to an initial concentration in the order of 10^8 CFU·mL^{-1} (Mean = 6.01 x CFU·mL^{-1}). Two milliliters of apple juice and *E. coli* were heat-sealed in plastic pouches. Controls included pouches of untreated samples. Broths and agar for this experiment were purchased from Fisher Scientific (Pittsburg, PA, USA).

The high pressure equipment had the same setup as the experiment design described in Chapter 2. Two milliliters of inoculated juice were sealed in plastic pouches and submerged in HHP cell. Water baths kept the high pressure reactor at either 25 °C or the selected incubation temperature.

Inoculated juice samples were treated between 250 and 350 MPa with 50 MPa increments, at 60 to 80 °C with 10 °C increments, and process times of 0 or 15 min. Untreated samples were used as controls. *E. coli* and apple juice solutions were kept on ice until and after processing. After treatments, samples were serially diluted with Bacto peptone and plated on MacConkey agar. Plates were placed in an incubator at 37 °C for at least 24 h. After incubation, plate counts were performed to estimate the

number of cells after treatment; plate counts were limited by the number of colonies. Plates with more than 250 colonies were noted as “too many to count” and plates with less than 25 colonies were labeled “too few to count”. All experiments were run in triplicate. A picture of *E. coli* colonies grown on a MacConkey agar plate from an untreated sample is shown in Figure A-1.

Results and Discussion

One to two decimal reductions of *E. coli* K12 populations occurred during process come-up and come-down time. An 8-log reduction in *E. coli* K12 was observed with pressure-temperature combinations from 250 to 350 MPa and 60 to 80 °C after 15-min incubation. Because initial population was on the order of 10^8 CFU·mL^{-1} (the standard deviation between initial concentrations was between 1.5 and 3.1 x10^8 CFU·mL^{-1}), a 5-log reduction required for pasteurization can be ensured. Garcia-Graells and others (1998) reported treating pressure-resistant *E. coli.* A treatment of 15 min at 20 °C and 500 MPa, followed by 2-day refrigeration in a juice at pH 4 resulted in a 5-log reduction of microbial populations.

Conclusions

Operation under these conditions have the potential to simultaneously pasteurize and clarify fruit juices as seen with pasteurization confirmed with this experiment. This study was a preliminarily study, that should be followed by more thorough microorganism pasteurization studies. Once optimization of clarification processing is completed, pasteurization experiments with *E.coli* O157:H7 and *A. acidoterristris* will be critical to confirm pasteurization under specific HHP clarifying conditions. Pasteurization experiments with these microorganisms after acid adaption are needed to test the ability of HHP to pasteurize juices under the proposed conditions.

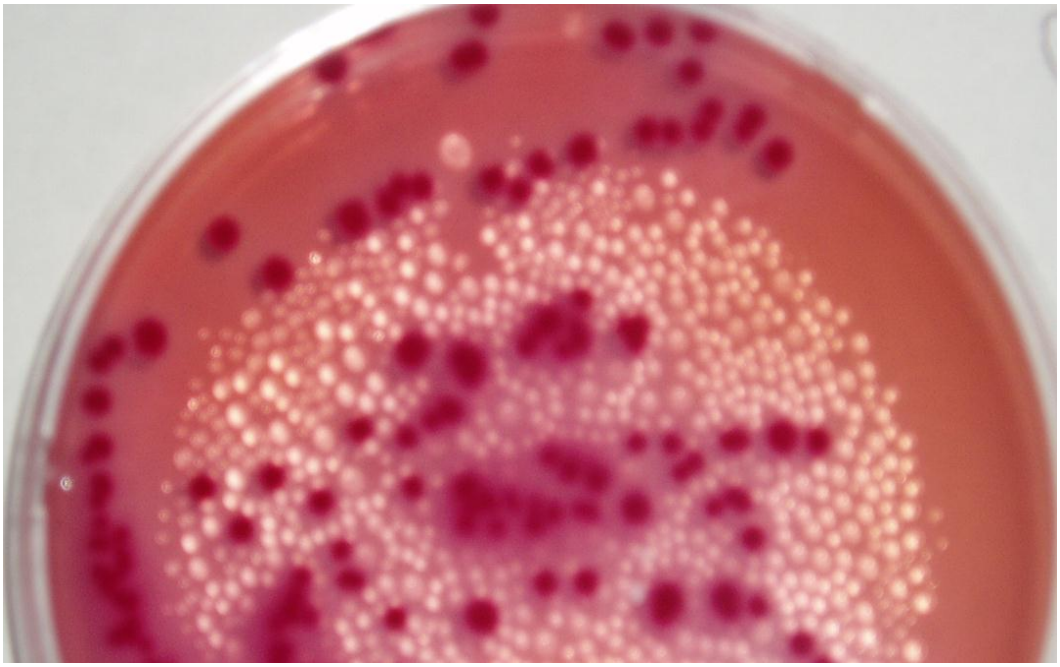

Figure A-1. *E. coli* K12 colonies growing on a MacConkey agar plate. Samples was not treated with pressure or heat. The pink colonies represent the *E. coli* K12 colonies, while the appearance of white circles is the condensation and reflection on the plate.

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BIOGRAPHICAL SKETCH

Brittany was born in Birmingham, Alabama and attended high school in Gulf Breeze, Florida. She obtained a Bachelor of Agricultural and Biological Engineering from the University of Florida, and continued her studies in the ABE graduate program at Florida. Brittany presented research at the 6th International CIGR Technical Symposium in Nantes, France, 61st Annual Citrus Processors' and Subtropical Technology Conference Meeting in Lake Alfred, FL, National Annual IFT Meeting in Chicago, IL and New Orleans, La, and Florida ASABE Conference in Jupiter Beach, FL.

Brittany’s interests are in food and biological research, development, and processing. Her master’s research focused on novel high pressure processing of fruit juice clarification and pasteurization. Brittany’s professional career goal is to join the industrial workforce in a research and development unit.