BREE 495 ENGINEERING DESIGN 3

# Production of Apple cider from apple pomace using an enzymatic pre-treatment process

Group 7

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# Abstract

The goal of this project was to design a process that would allow for the production of apple cider with an acceptable alcohol content from apple pomace. Since the quantity of alcohol produced by yeast during fermentation depends on the quantity of sugars present in the juices being fermented, our main objective was to extract the maximum amount of sugars from the apple pomace in order to achieve a cider with an alcohol content between 3 and 5%. This objective was achieved through the use of pectinase, cellulase, hemicellulase and α-amylase in the pre-treatment of the apple pomace prior to pressing and fermentation. Pectinase, cellulase, hemicellulase and α-amylase enzymes hydrolyse pectin, cellulose, hemicellulose and starch, respectively, present in the apple pomace which significantly increases the juice yield and the amount of sugars extracted from the pomace. Treating the apple pomace with these enzymes would produce a theoretical yield of apple cider with a 4.75% alcohol content in ideal conditions.

# Acknowledgements

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# Introduction

The main by-product of apple juice production is a press-cake composed of apple flesh, peel and seeds known as apple pomace. Apple pomace is a rich source of carbohydrates, fiber, nutrients and minerals (Shalini and Gupta, 2010). Waste apple pomace is typically thrown away or used as compost, thus disposing off a commodity that has potential to become a valuable food product.

According to Statistics Canada, Quebec produces around 98 Million kilograms of apples per year, or 28% of Canada's total apple production. Therefore, there is a good and plentiful source of apple pomace. A method for developing a food product from waste apple pomace would benefit apple juice producers, especially small-scale producers, who may wish to increase their productivity by adding economic value to both their products and by-products.

Last semester (Design 2), we studied potential uses of apple pomace to find the best way to add value to waste apple pomace. Our search led us to our decision to design a process that would allow for the production of hard apple cider from apple pomace. Craft-style alcoholic beverages and locally produced products are popular. Since the Régie des alcools, des courses et des jeux (RACJ, Quebec's alcohol regulating body) began issuing permits to produce craft cider in 1988, the market for craft cider in Quebec has grown and now there are over 40 cider producers making over 100 apple juice-based ciders (ACAQ, 2008). According to an article written by the Montreal Gazette in 2013, cider sales in Quebec are estimated to be over $20 million dollars per year, and sales increased by over 40% between 2011 and 2012 alone. Growth in production and the introduction of brand extensions of existing producers shows the vast growth and further potential of the craft cider industry. Therefore, there is currently a good market for cider products in Quebec.

For our Design 2 project, we conducted a lab experiment in order to determine some properties of apple pomace from a local small-scale apple juice processing facility. We found that the apple pomace was composed of 54% apple flesh, 33% apple peel and 13% seeds. It had an 80.4% moisture content and a 12.8% Brix value (sugar content). Strength tests were also performed on the pomace using the Instron machine, and from these tests, we were able to see that there was close to 20 grams of apple juice remaining in 100 grams of the apple pomace. This lab study showed us that the apple pomace still contained a good amount of juice and sugars that could be extracted and fermented into cider.

Hard apple cider can be made from apple pomace using a "water cider" method. This is a unique method of producing cider which involves steeping the apple pomace in water and allowing it to ferment (Watson, 2013). This differs from how cider is traditionally made, which is by fermenting apple juice. However, if an adequate amount of juice and sugars is extracted from the pomace, the traditional method could be used to make the apple cider. Cider available in Quebec have 3-5% alcohol by volume (ABV) and therefore goal of using either one of these methods would be to produce a cider with an alcohol content between 3 and 5% ABV.

Under the guidance of our mentor, Dr. Valérie Orsat, Chair and Associate Professor in the Department of Bioresource Engineering at McGill University, we have designed process that would allow

for the production of apple cider from apple pomace. The process was designed with the main objective of maximizing sugar and juice extraction from the apple pomace by treating the pomace with enzymes in an a pre-treatment step. This will enhance the fermentation process by providing more sugars for the yeast to convert to ethanol, and thus producing a hard apple cider with an acceptable alcohol content of at least 3% ABV.

# Literature Review

## Composition of Apple Pomace

Apple pomace consists of apple flesh, seeds, skins and core, composed of carbohydrates, proteins and lipids and other polymeric compounds. These compounds are responsible for the apple's structure, textures and, nutritional and sensory properties as well as other factors such as ease of juice extraction. Understanding the composition of the apple cultivar is also important in determining the best treatments to apply to the apple in order to derive the most benefit and maximum quality of the final food product being produced. The main treatments utilized in the apple juice industry before the fruit is pressed and in order to improve juice yields, are heat treatments and enzymatic treatments. The main objective of both treatments is to break down the carbohydrates and the cell walls in the apple in order to release more liquids and sugars during extraction.

In 1989, Wang and Thomas studied the composition of freeze-dried apple pomace. The apple pomace used in the study contained the entire insoluble portion of the apple i.e. it included the seeds, skins and cores of the whole apple. They found that the freeze-dried apple pomace contained 11.16% Sucrose, 13.35% Glucose, 30.05% Fructose, 54.34% Total sugars, 6.09% Pectin, 4.69% Hemicellulose and 16.67% Cellulose. (Wang & Thomas 1989) Singhal et al. found that 17.89% dried Apple pomace was composed of starch (Singhal K.K. et al. 1991; Roelofsen 1956).

Last semester, we conducted a lab study at the Post Harvest Drying Lab at McGill's Macdonald Campus, in order to analyse the composition of the apple pomace sourced from Quinn Farm's juicing facility. The apple pomace consisted of about 54% apple flesh, 33% apple peels and 13% seeds. The pomace was found to have an 80.4% moisture content, 12.8% Brix value or sugar content, and a pH of 3. Furthermore, strength tests conducted on the pomace using the Instron machine showed that close to 20 grams of apple juice could be pressed from 100 grams of the apple pomace using forces of 700 N or greater.

The most important carbohydrates in the apple for juice and sugar extraction, are simple sugars, such as glucose, fructose and sucrose, and polysaccharides, such as starch, pectin cellulose and hemicellulose. Pectin, cellulose and hemicellulose fibers are important components of cell walls. Cellulose and hemicellulose form a network in which hemicelluloses are tightly bound to the surface of celluloses though hydrogen bonding, cross-linking microfibrils. This network interacts with pectin polysaccharides (Grassin & Coutel 2009) and the combination of pectin and cellulosic polysaccharides in plant cell walls acts as an intercellular cementing material (Towle & Christensen 1973).

Pectins are a family of complex heteropolysaccharides that all contain α-1,4 D-galacturonic acid (GalpA) residues which are referred to as galacturonans (Ridley et al. 2001). They are three groups of pectins; homogalacturonan, rhamnogalacturonans and substituted galacturonans (Grassin & Coutel 2009). The composition of pectins depend on many factors including its plant source, the plant's developmental stage, and environmental conditions. This makes identifying and extracting specific types of pectins difficult. However, pectin is extracted from food products such as citrus peels and apple pomace, for commercial purposes. Depending on their characteristics, pectins have found many applications in industry. In the food industry, pectin is used as a gelling agent, thickener, stabilizer, and emulsifier (Grassin & Coutel 2009)

Another very important polysaccharide in the fruit processing industry is starch. Starch is composed of amylose, a linear polymer of glucose units linked together by α-1,4 glycosidic bonds, and amylopection, a heteropolymer of glucose units linked via α-1,4 and α-1,6 glycosidic bonds. Amylose and amylopectin are packaged into starch granules which vary in size and shape depending on the plant source. (van der Maarel 2009)

Heat treatments and enzymatic treatments are geared towards hydrolysing the polysaccharides especially those that make up the cell wall. Enzymatic treatments have been found to be most effective in hydrolyzing cell wall components and other polysaccharides present in the apples which increases the juice yield and the amount of sugars and/or other valuable compounds extracted.

## Enzymatic treatment

Enzymes are used by fruit processors to enhance the extraction of juices, sugars and other desired compounds from fruit. Some of the enzymes used in the apple industry are pectinases, cellulases, hemicellulases and starch-hydrolyzing enzymes such as amylases. These enzymes hydrolyze starch, pectin, cellulose and hemicellulose polysaccharides into smaller units, oligosaccharides, including simple sugars.

For example, enzymes can be used in the saccharification of starch in order to produce simple sugars glucose (monomer) and maltose (dimer). Enzymes that hydrolyze starch are categorized into two major groups; hydrolases and glucanotransferases. glucanotransferases hydrolyze α-1,4 bonds and form new α-1,4 or α-1,6 bonds. Hydrolases can be endo- or exo- acting. They hydrolyze the α-1,4 and/or α-1,6 glycosidic bonds. The hydrolase group are most well-known starch-hydrolyzing enzymes. Examples of hydrolases include amylases, glucoamylases and pullulunases.[1] (van der Maarel 2009)

[1] Source: "Starch processing enzymes" by Marc van der Maarel. Chapter 14 in Enzymes in Food technology.

## Enzyme kinetics: Michaelis-Menten model

Enzymes are soluble proteins that act as catalysts. As catalysts, enzymes speed up the rate of reactions such as the hydrolysis of polysaccharides. The Michaelis-Menten model is a simple model used to explain how enzymes enhance reaction rates or kinetics i.e. the product of the reaction is produced. It is based on the relationship between the rate of the reaction and the concentration of the substrate (the substance the enzyme acts on) for a system where the enzyme (E) binds reversibly to a substrate (S) molecule to form the enzyme-substrate (ES) complex, which then reacts irreversibly to generate the product.[2] This system is described by equation below.

$$E + S \leftrightarrow ES \rightarrow E + P$$

The Michaelis-Menten equation for an enzyme-catalysed reaction system is

$$v = \frac{V_{max}[S]}{K_M + [S]}$$

Where v is the reaction rate, $V_{max}$ is the maximum reaction rate; $K_M$ is the Michaelis-Menten constant, [S] is the substrate concentration. The Michaelis-Menten constant, $K_M$, is the substrate concentration when the rate of the reaction has reached half the value of the maximum reaction rate, $V_{max}$. The maximum reaction rate, $V_{max}$, is the speed of the reaction when the system is saturated with substrate and it is directly proportional to the concentration of enzyme and its specific activitity, $k_{cat}$.

The relationship between substrate concentration and the rate of the reaction can be described using the following graph plotted using the Michealis-Menten equation.

[2]Michaelis-Menten Kinetics and Briggs-Haldane Kinetics
Abhinav Nath (Atkins Group), Departments of Medicinal Chemistry and Pharmaceutics Univerity of Washington
https://depts.washington.edu/wmatkins/kinetics/michaelis-menten.html

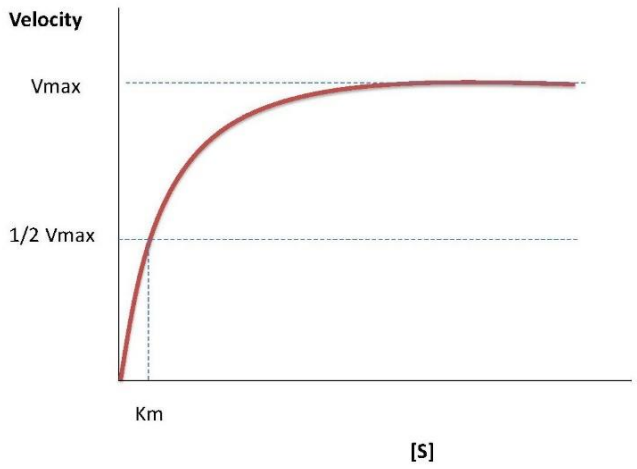


Figure 1: Michaelis-Menten plot.

Source https://depts.washington.edu/wmatkins/kinetics/michaelis-menten.html

The Michaelis-Menten model has been used in this project to estimate the amount and concentration of enzymes that would be needed in the enzymatic pre-treatment process.

Some researchers have studied the properties, activities and kinetic behaviour of enzymes produced by a variety of organisms including certain species of bacteria and fungi. Examples of such include the work of Colonna et al., Ikram Ul-Haq, Rodriguez et al. and Chang et al. These researchers have studied the types of α-amylase enzymes that will be used in the proposed design.

In 1988, Colonna et al. studied the kinetics of an enzyme-catalysed reaction reaction using scanning electron microscopy, differential scanning calorimetry and X-ray diffractometry. The reaction studied was for the degradation of starch granules, in pre-gelatinized starch, by the α-amylase enzyme *Bacillus subtilis*. In this reaction, α-amylase hydrolyzes amylose and amylopectin polysaccharides composing starch, and breaks them down into oligosaccharides. Colonna et al. studied the kinetics of the reaction. They proposed a two stage mechanism where the α-amylase enzyme would further hydrolyze large oligosaccharides (LS) produced from starch (S) degradation, into smaller oligosaccharides (SS). (Komolprasert & Ofoli 1991; Colonna et al. 1988)

$$S + E \leftrightarrow (E|S) \rightarrow E + LS$$

$$LS + E \leftrightarrow (E|LS) \rightarrow S + LS$$

Here, (E|S) represents the complex formed by the enzyme and the starch components. (E|LS) represents the complex formed by the enzyme and the large olisaccharides.

Ikram-Ul-Haq et al. studied the kinetics of α-amylase produced from a mutant form of *Bacillus licheniformis* bacterium. The enzyme had an optimum temperature range of 60-70$^{o}$C and an optimum pH of 7.0. Enzyme activity decreased above the optimum temperature and above and below the optimum pH. The study found the enzyme had a $K_M$ value of 8.3 mg/mL, $V_{max}$ of 2778 U/mg/min and a $k_{cat}$ value of 152.8 $min^{-1}$ with a specific constant ($k_{cat}/K_M$) of 184.09. (Ikram-Ul-Haq et al. 2010)

Similarly Rodriguez et al. studied the enzymatic hydrolysis of soluble starch with *Bacillus licheniformis* α-amylase (commercial enzyme Termamyl 300 L Type DX) at a pH of 7.5 within a temperature range of 37-75$^{o}$C. The kinetics of the reaction were described using a Michaelis-Menten equation and the kinetic constants of the equation were found to be 734.9 g/L for the $K_M$ value and $1.74x10^{8}$ $min^{-1}$ for the $k_{cat}$ value. (Bravo Rodríguez et al. 2006)

In their study of the purification and properties of α-amylase from *Aspergillus oryzae*, Chang et al. found that the purified enzyme had an optimal pH between 4 and 5, an optimal temperature pf $50^{0}$C and a $K_M$ value of 0.22% for the hydrolysis of starch. Using gel filtration, they determined that the enzyme has a molecular weight of 52 kDa. The enzyme was found to exhibit a specific activity of 410 μmol/min/mg. However, 80% of the enzyme activity was lost after a 30 min incubation at 50$^{o}$C. (Chang et al. 1995)

## Heat Treatment

In 2015, Pedras studied the valorization of grape pomace through hot compressed water extraction and hydrolysis. He used a colorimetric method, which quantifies all the recovered sugars, using a glucose calibration curve, followed by a High-performance liquid chromatography (HPLC) analysis. Results showed that from ambient temperature to 50°C, the main process taking place was the extraction of free sugars. Furthermore, for temperatures above 100°C, the corresponding increase in yield with time was much more moderate. This, he believed, is because polymers such as cellulose and hemicellulose must be hydrolyzed in order to release other structural carbohydrates. Heat-induced hydrolysis of biomass only resulted in the depolymerization of lignocellulose and mainly released disaccharides and oligosaccharides. Sugars bound in oligomers were protected from degradation. Moreover, he noted the importance of enzymatic treatment to obtain complete hydrolysis. (Pedras, 2015)

Furthermore, decomposition of the discarded wine grape pomace consumed free sugars present in the pomace thus decreasing final yield of sugars extracted. Rapid freezing of pomace or instant processing is therefore very important. When the pomace is not bound for human consumption, hot compressed water extraction is performed.

Guidelines for the pasteurization of apple juice suggest limiting heat treatment of regular apple juice, to 80°C. Juices are thought to have a burnt after-taste when heated above this temperature (Pnr-Millevache). These guidelines helped us set a limit to the temperature used in our practical study of a heat treatment of the apple pomace.

## Fermentation

Fermentation is a metabolic process that converts natural renewable substrates into value-added products such as enzymes, organic acids, alcohols and polymers, and the formation of such end -products is dependent on microbial strain and the environmental conditions employed (Clark & al. 2014). Yeasts are facultative anaerobes. This means that they respire when oxygen is present, and revert to fermentation in the absence of oxygen.

The selection of the microbial strains used in fermentation depends on the desired product. S cerevisiae bayanus (EC-1118) produce ethanol from sugars. It is a popular yeast strain that is used in the production of a wide variety of products such as sparkling wines, fruit wines and ciders. This strain is genetically stable and it is stable in relatively harsh environmental conditions. For instance, it can survive low temperatures and a relatively wide range of pH. Moreover, it can display good levels of flocculation and excellent alcohol tolerance. According to its specification sheet (LALEVIN), EC-1118 is added at 0.4g/L and grows between 15°C to 25°C.

Formation of by-products is also dependent on environmental conditions, such as nutrients, pH, and presence of oxygen (Shuler & Kargi, 2008). Therefore, optimizing the nutrient available for a yeast culture can enhance both growth and production rates.

Saccharification refers to the process in which polysaccharides are hydrolysed into monosaccharides. Yeast cells use these monosaccharides, or simple sugars, for energy. Once taken up by yeast cells, monosaccharides such as glucose, are transformed into pyruvate through metabolic process known as glycolysis. The pyruvate molecules are further processed into ethanol and carbon dioxide during anaerobic respiration by the yeast cells (Lefsrud, 2016).

Yeasts require macronutrients and micronutrients for growth and productivity. Macronutrients include nitrogen, oxygen, sulfur, phosphorus, magnesium, and potassium compounds, while micronutrients include iron, zinc, manganese, copper, cobalt and calcium (Clark et al. 2014). Furthermore, environmental conditions such as pH and temperature, are important factors that affect the fermentation process. The best range of pH for yeast is typically between pH 3 and 6 (Shuler & Kargi, 2008).

## Pasteurization

Pasteurization of the substrate solution (e.g. juice) before inoculation with the yeast is important in preventing the growth of spoilage organisms. Traditional batch pasteurization for fruit juices, cream, soy milk, yogurt, wine, soups and other products is accomplished using holding time of 30 mins at 63°C (Singh & Heldman, 2014). An example of a method used in the food industry to pasteurize food products is high temperature short-time (HTST) pasteurization. HTST, also known as flash pasteurization, is a processing method that allows processors to heat the food product to very high temperatures in a continuous process. It typically involves heating the product to 71°C and holding it at this temperature for 15 seconds. The

product passes through holding tubes at rates that ensures the required holding time. HTST method is used only in continuous processes for rapid heating and cooling. (Singh & Heldman, 2014).

# METHODOLOGY

## Using Enzymes

### Pectinase treatment

The aim of the Pectinase treatment step was to disrupt the cell walls by hydrolyzing the pectin polysaccharides which are present in the cell walls and which play an important structural role. This would make it easier to extract cell contents and juice during pressing. Pectin present in the fruit juice is also one of the main contributors to juice viscosity. The hydrolysis of pectin in decreases the viscosity of the juice extracted from the fruit.

Pectinase from *Aspergillus niger* would be added to the pomace-water mixture in this step and the mixture is allowed to sit at room temperature for 2 hours. The amount and concentration of the pectinase used in the process is estimated using the Michaelis-Menten model with the assumption that the maximum rate of the reaction can be estimated by dividing the total amount of product expected by the total reaction time (2 hours). As mentioned above, in 1989, Wang and Thomas studied the composition of freeze-dried apple pomace and found that it contains 6.09% Pectin (Wang & Thomas 1989). Pectin, also known as Poly-D-galacturonic acid methyl ester, is the methylated ester of polygalacturonic acid (Sriamornsak 2003). In their study of the quantification of the methoxyl content using enzymes, Tsan-Piao Lin et al. studied commercial apple pectin from the Sigma Chemical Co. This apple pectin consisted of 78% galacturonic acid and 7.4% methoxy content (Lin, P.L., Teng, Y.F., Yuan, H.C., & Ching 1990).

It is therefore assumed that the pomace consists of 6.09% pectin and this pectin consists of 78% polygalacturonic acid. Therefore, dry pomace is assumed to contain 4.75% polygalacturonic acid of which 100% would be hydrolysed into its galacturonic acid units by the pectinase enzymes. From these assumptions, the total mass of galacturonic acid produced from the hydrolysis of pectin by pectinase can be assumed to 4.75 grams for every 100 grams of dried apple pomace. Apple pomace has moisture content of about 80%. Therefore, 500 grams of fresh apple pomace is assumed to contain 6.09 grams of pectin of which 4.75 grams is polygalacuronic acid.

Pectinase hydrolyses polygalacturonic acid to produce galacturonic acid residues. One unit of the enzyme will liberate 1.0 µmole of galacturonic acid from polyglacturonic acid per minute at pH 4.0 at 25°C (Sigma Aldrich n.d.).[3] Commercial pectinase enzyme purchased from LD Carlson has an activity of 75 Units/g and therefore, a specific activity of 0.075 µmol/min/mg at pH 4.0 and 25°C. Using the Michaelis-

[3] Sigma Aldrich: Pectinase from *Aspergillus niger*
http://www.sigmaaldrich.com/catalog/product/sigma/p4716?lang=en&region=US

Menten model and the assumptions above, the following calculations were used to estimate the amount and concentration of pectinase enzyme required to treat a 500 Kg batch of fresh apple pomace. A 1:2 ratio of pomace to water mixture is prepared; 1000 L of water is added to 500 Kg of pomace

$$\text{Maximum reaction rate} = V_{max} = \frac{\Delta[P]}{\Delta t} = \frac{4.75\% * \left(20\% * \frac{500\ \text{Kg}}{1000\ \text{L}}\right) * 1000\frac{\text{g}}{\text{Kg}} * 0.001\frac{\text{L}}{\text{mL}}}{2\ \text{hours} * 60\frac{\text{min}}{\text{hour}}}$$

$$\therefore V_{max} = 3.95 \times 10^{-5}\frac{\text{g}}{\text{mL}*\text{min}}$$

Here, [P] is the product concentration in the reaction mixture and t is the time taken by the reaction.

Galacturonic acid has a molecular weight of 194.139 g/mol (or 1.94139 x $10^{-4}$ g/µmol), therefore;

$$V_{max} = 3.95 \times 10^{-5}\frac{\text{g}}{\text{mL} * \text{min}} = 0.20\frac{\mu\text{mol}}{\text{mL} * \text{min}}$$

The specific activity, $k_{cat}$, of *Aspergillus niger* pectinase at room temperature and pH 4.0 is 0.075 µmol/min/mg and

$$V_{max} = k_{cat} * [E_T]$$

Where $[E_T]$ is the total enzyme concentration in the reaction mixture.

$$\therefore [E_T] = \frac{V_{max}}{k_{cat}} = \frac{0.20\frac{\mu\text{mol}}{\text{mL} * \text{min}}}{0.075\frac{\mu\text{mol}}{\text{min} * \text{mg}}} = 2.72\frac{\text{mg}}{\text{mL}}$$

A pectinase enzyme (from *Aspergillus niger*) concentration of 2.72 mg/mL is required to treat 500 Kg of fresh apple pomace. A 1.0 L pectinase solution is prepared by dissolving 2.72 grams in 1.0 L of water.

## Amylase treatment

The goal of the Amylase treatment step is to catalyze the hydrolysis of the starch present in the apple pomace into simple sugars thus increasing the amount of sugars extracted. In this step, α-amylase from *Aspergillus oryzae* would be added to the pomace and the mixture would be allowed to sit at room temperature for 3 hours.

*Aspergillus oryzae* α-amylase enzyme, also known as 4-α-D-glucan glucanohydrolase, catalyses the hydrolysis of the α-1,4 glycosidic bonds and breaks down soluble starch, into oligosaccharides (short-chained glucose polymers) –predominantly maltose – and dextrins. For every glycosidic bond broken, a

water molecule is consumed. Therefore, starch hydrolysis consumes water and increase the dry matter content of the reaction mixture.

In 1991, Singhal et al. studied the composition of dried apple pomace and found that it contained 17.89% starch (Singhal K.K. et al. 1991). It is assumed that the apple pomace treated in this process, also contains 17.89% starch and that 100% of this starch is broken down to form maltose (a disaccharide of glucose). This would mean that every 500 Kg batch of fresh apple pomace would contain 17.89 Kg of starch and that this would be broken down during the amylase treatment into 17.89 Kg of maltose

The Commercial α-amylase enzyme (from *Aspergillus oryzae*) used by Cheirslip et al. to process banana-based wine had an activity of 35 Units/mg (Cheirsilp & Umsakul 2008). According to Sigma Aldrich, at a pH of 6.0 and a temperature of 25ºC, one unit of *Aspergillus oryzae* α-amylase enzyme liberate 1.0 µmol of maltose per minute. In this case, *Aspergillus oryzae* α-amylase enzyme has a specific activity of 35 umol/min/mg.

Using these assumptions and the Michaelis-Menten model, the amount and concentration of the enzyme needed to treat a 500 Kg batch of fresh apple pomace (80% moisture content), was estimated. A 1:2 ratio of pomace to water mixture is prepared; 1000 L of water is added to 500 Kg of pomace

$$\text{Maximum reaction rate} = V_{max} = \frac{\Delta[P]}{\Delta t} = \frac{17.89\% * \left(20\% * \frac{500\ \text{Kg}}{1000\ \text{L}}\right) * 1000\frac{\text{g}}{\text{Kg}} * 0.001\frac{\text{L}}{\text{mL}}}{3\ \text{hours} * 60\frac{\text{min}}{\text{hour}}}$$

$$\therefore V_{max} = 9.94 \times 10^{-5}\frac{\text{g}}{\text{mL}*\text{min}}$$

Maltose has a molecular weight of 342.3 g/mol (or 3.423 x $10^{-4}$ g/µmol), therefore;

$$V_{max} = 9.94 \times 10^{-5}\frac{\text{g}}{\text{mL} * \text{min}} = 0.29\frac{\mu\text{mol}}{\text{mL} * \text{min}}$$

The specific activity, $k_{cat}$, of *Aspergillus oryzae* pectinase at room temperature and pH 6.0 is 35 µmol/min/mg and

$$V_{max} = k_{cat} * [E_T]$$

Where $[E_T]$ is the total enzyme concentration in the reaction mixture.

$$\therefore [E_T] = \frac{V_{max}}{k_{cat}} = \frac{0.29\frac{\mu\text{mol}}{\text{mL} * \text{min}}}{35\frac{\mu\text{mol}}{\text{min} * \text{mg}}} = 8.30 \times 10^{-3}\frac{\text{mg}}{\text{mL}}$$

A solution of α-amylase enzyme (from *Aspergillus oryzae*) with a concentration of 8.30 g/mL is required to treat 500 Kg of fresh apple pomace. A 1.0 L solution of amylase is prepared by dissolving 8.30 grams of α-amylase in 1.0 L of water.

### Summary of Enzymatic pre-treatment materials: Treating 500 Kg batch at room temperature.

The calculations made in the sections above allowed us to estimate the amount of pectinase and α-amylase that would be required to treat a 500 Kg batch of fresh apple pomace. The results of the calculations are summarized in the recipe below.

Materials required to treat 500 kg of fresh apple pomace include;

- 2.72 g of Pectinase (75 Units/g) from *Aspergillus niger*
- 8.30 g of α-Amylase (35 Units/mg) from *Aspergillus oryzae*
- 1000 L of water
- 500 Kg of fresh Apple pomace

This is the recipe initially proposed for the enzymatic pre-treatment of Apple Pomace.

## Testing heat and enzymatic treatments

Two series of tests were performed to study the nutrient transfer of pomace to the soaking water using a heat treatment and an enzymatic treatment at room temperature. The following two sections describe the methods used for both experiments; experiment 1 and experiment 2.

### Experiment 1

1. Prepare 3 solutions in beakers.

   Solution1: 100g pomace, 200ml of water.

   Solution 2: 100g pomace, 400ml of water.

   Solution 3: 50g pomace, 400ml of water.

2. Place the beakers in a water bath at 80$^{o}$C.

3. Measure Brix value of the liquids in the beakers at 15-30 min intervals.

### Experiment 2

1. Prepare 9 solutions in beakers with all the same ratio (100g pomace, 200ml of water)
2. Add the following amounts of pectinase enzyme to the respective beakers.

    Solution 1: 0g

    Solution 2: 0.025g

    Solution 3: 0.1g

    Solution 4: 0.4g

    Solution 5: 2

    Solution 6: 4g

    Solution 7: 4g

    Solution 8: 8g

    Solution 9: 8g

3. Measure Brix index straight form the beaker at 15-30 min intervals
4. After 2 hours, add the following amounts of amylase to the respective beakers

    Solution 7: 4g

    Solution 9: 8g

5. Measure Brix value of the liquids in the beakers at 15-30 min intervals.

## Results of the experiments

The first series of tests were performed at a high temperature (80C-100C) and with different mass ratios of pomace to water (0.5; 0.25 and 0.125) to study the effects on extracted sugar and juice. The objective was to develop a graphical representation of the required mass ratio to obtain a specific ethanol theoretical yield and the juice yield obtained without pressing the pomace. Results of the experiments were tabulated. These tables have been provided in the appendix

The second series of tests were performed to study the nutrient and liquid extraction after treating the pomace with enzymes (pectinase and amylase) at room temperature. A brix indicator was used in the hopes of measuring the sugars released during this process. The results of this experiment were inconclusive because a) the brix sums all dissolved solid including enzymes and results only in an approximation, and

b) there was very low difference (13%) in the juice extracted with or without enzymes as it the treated pomace was not pressed. Moreover, there is a strong chance that the enzymes used were inactivated or denatured before use e.g. during storage.

The following graph illustrates the results of the second experiment using the enzymatic treatment.

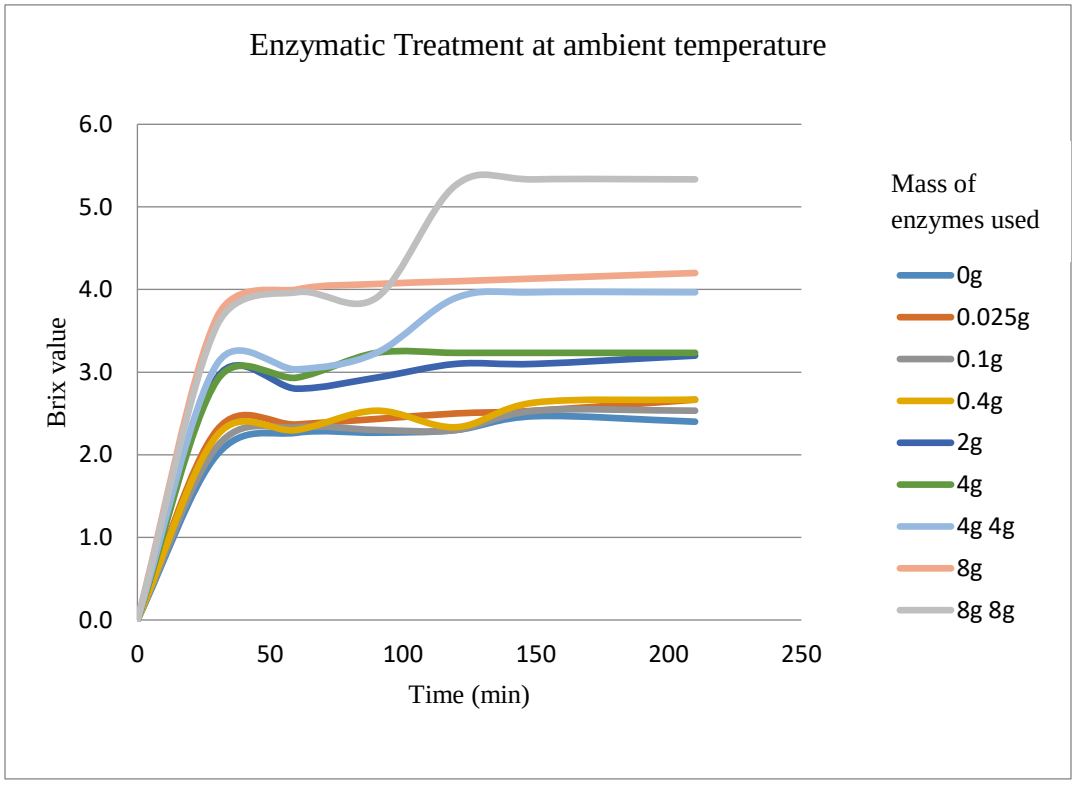


Figure 2: Results of the enzymatic treatment of the apple pomace at room temperature - Brix value over time.

High-performance liquid chromatography (HPLC) would be required to analyse dissolved compounds before and after treatment in order to accurately determine the effectiveness of the treatment in extracting compounds and to identify the compounds present in the solution. However, HPLC is expensive to use and its calibration requires an excessive amount of time. We therefore decided to rely on the results of previous studies and scientific publications for further use of enzymes.

Heating the pomace to a high temperature of 80$^{o}$C did not result in an increase in the Brix value. However, it did results in a decrease in the difference in the volume of liquid before and after the treatment and thus a decrease in the juice yield. When testing sample of the same water-to-pomace ratio, the sample heated to 80-100$^{o}$C yielded 200 mL of liquid while the sample left at room temperature yielded 350 mL of liquid. Therefore, heating the pomace to high temperatures results in about a 1.4-fold decrease in juice yield. This is because the apple pomace absorbed a lot more water at high temperatures. Pressing the pomace would extract the absorbed water as well as the juice.

Using the results of this experiment, a graph was developed to estimate water-to-pomace ratio that would result in a certain juice yield at room temperature, and without pressing, and a water-to-pomace ratio that would produce a certain ethanol yield.

## Calculating potential ethanol yield from fermentation

The following calculations were made in order to determine the amount ethanol that could result from the fermentation of sugars extracted from different water-to-pomace ratios **without the use of an enzymatic pre-treatment**.

Theoretical yield on a mass basis:

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2CO_2$$

$$M_{C_6H_{12}O_6} = 180.18 g/mole$$

$$M_{CH_3CH_2OH} = 46.08 g/mole$$

$$yield\left(\frac{g_{CH_3CH_2OH}}{g_{C_6H_{12}O_6}}\right) = \frac{2 \times 46.08 g/mole}{180.18 g/mole} = 51.1\%$$

Theoretical yield on a mass basis:

$$yield\left(\frac{ml_{CH_3CH_2OH}}{g_{C_6H_{12}O_6}}\right) = \frac{\left(\frac{2 \times 46.08 g/mole}{0.7893 g/ml}\right)}{180.18 g/mole} = 64.8\%$$

Therefore, Theoretical Alcohol Potential would be:

$$C_{CH_3CH_2OH}: Volume\ concentration\ of\ ethanol\ ml/ml$$

$$C_{C_6H_{12}O_6}: Concentration\ glucose\ g/ml$$

$$r = mass\ ratio\ pomace/water$$

$$C_{C_6H_{12}O_6} = \frac{C_{CH_3CH_2OH}}{0.648}$$

$$C_{C_6H_{12}O_6} = 0.0245 ln(r) + 0.0636$$

$$\frac{C_{CH_3CH_2OH}}{0.648} = 0.0245 ln(r) + 0.0636$$

$$\frac{\left(\frac{C_{CH_3CH_2OH}}{0.648} - 0.0636\right)}{0.0245} = ln(r)$$

$$r = e^{\left(\frac{\left(\frac{C_{CH_3CH_2OH}}{0.648} - 0.0636\right)}{0.0245}\right)}$$

The equations developed were then used to plot a graph describing the relationship between the pomace-to-water ratio and the resulting sugar concentration.

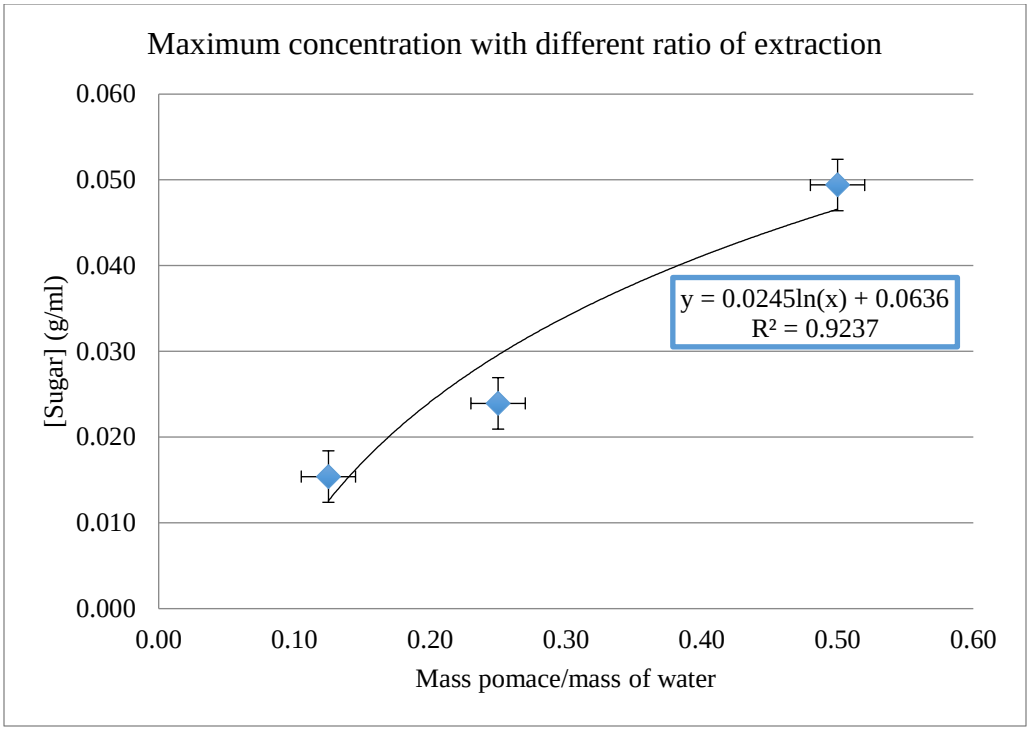


Figure 3: Relationship between the pomace-to-water ratio and the resulting sugar concentration.

The following calculations were then used to develop an equation that can be used to estimate the sugar concentration in g/mL, using the Brix value.

$$SG = 0.9977 + 0.0043 \times {}^{\circ}Brix = \frac{\rho_{mixture}}{\rho_{H_2O}}$$

$$m_{mixture} = V_{mixture} \times \rho_{mixture}$$

$$m_{mixture} = V_{mixture} \times SG \times \rho_{H_2O}$$

$$m_{mixture} = V_{mixture} \times (0.9977 + 0.0043 \times {}^{\circ}Brix) \times \rho_{H_2O}$$

$$[C_{sugar}] = \frac{m_{sugar}}{V_{mixture}} = \frac{Brix/100 \times m_{mixture}}{V_{mixture}}$$

$$[C_{sugar}] = \frac{Brix/100 \times \left(V_{mixture} \times (0.9977 + 0.0043 \times {}^{\circ}Brix) \times \rho_{H_2O}\right)}{V_{mixture}}$$

$$[C_{sugar}] = Brix/100 \times \left((0.9977 + 0.0043 \times {}^{\circ}Brix) \times \rho_{H_2O}\right)$$

The following graph describes the relationship between the pomace-to-water ratio and the volume of juice extracted.

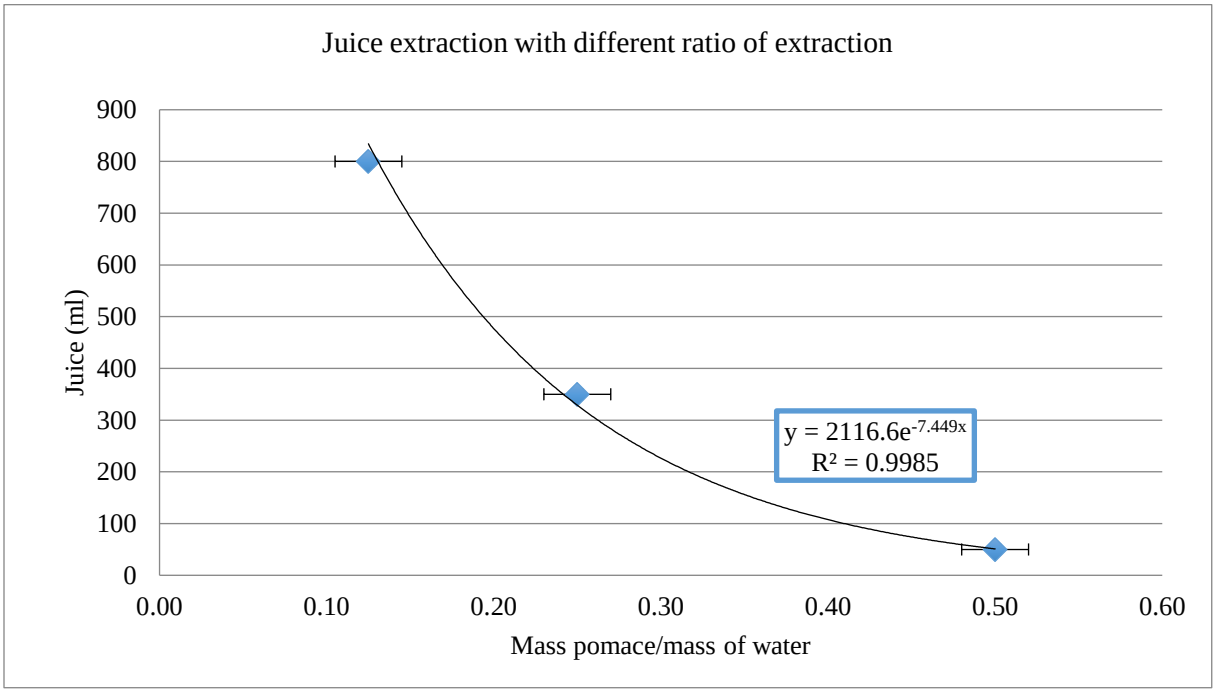


Figure 4: Graph describing the relationship between the pomace-to-water ratio and the volume of juice extracted.

The graph above, showing pomace-to-water ratio versus the volume of juice extracted, was developed using the equation below.

$$V = 2116.6e^{-7.449r}$$

Or

$$V = 2116.6e^{-7.449e^{\left(\frac{\left(\frac{C_{CH_3CH_2OH}}{0.648} - 0.0636\right)}{0.0245}\right)}}$$

Furthermore, a graph illustrating the relationship between the potential ethanol yield and the pomace-to-water ratio was developed.

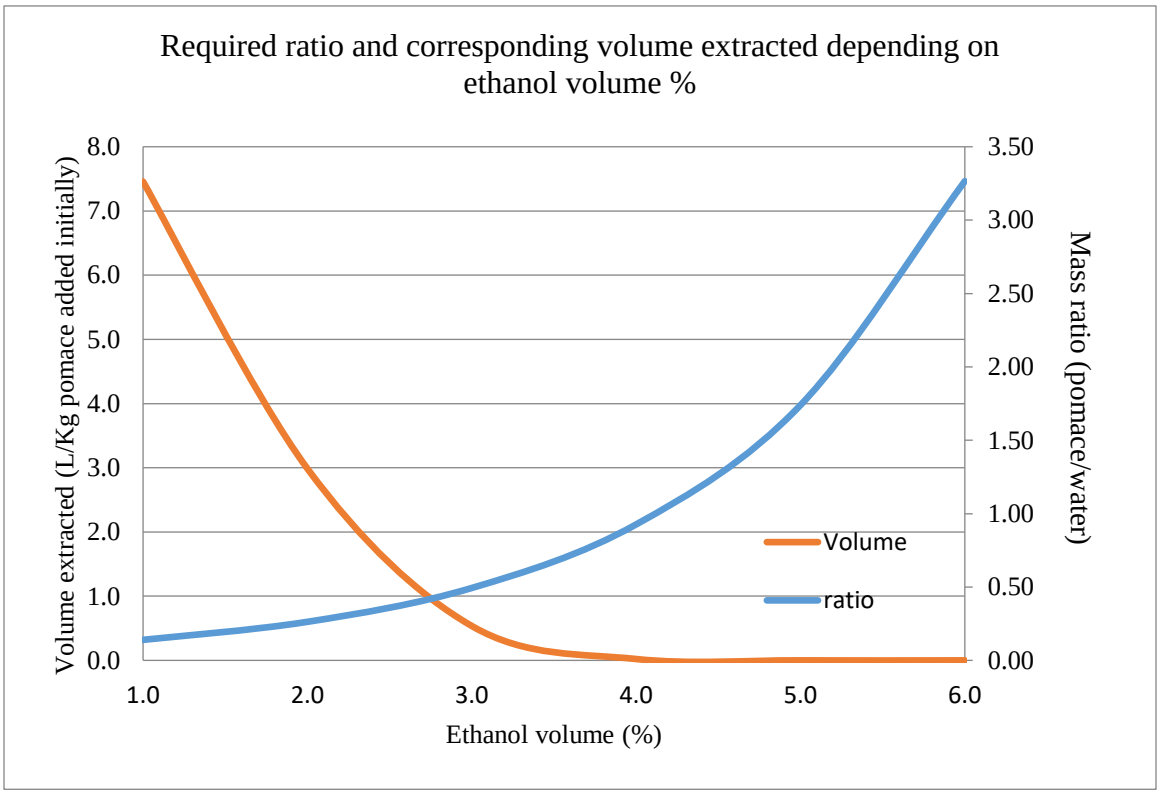


Figure 5: Graph showing the relationship between the juice yield, the pomace-to-water ratio and potential ethanol volume obtained.

The graph above can be used to estimate the pomace-to-water ratio that would be required to achieve a certain alcohol content in the cider. For example, producing a cider with a 3.0% alcohol content from a 500 Kg batch of apple pomace would require the preparation of a 0.5 pomace-to-water ratio in the initial step. Using the graph, it is estimated that a 0.5 pomace-to-water ratio would result in a 0.54 L juice yield for every kilogram of pomace. Therefore;

$$r = \frac{m_{pomace}}{m_{water}}$$

$$m_{water} = \frac{m_{pomace}}{r} = \frac{500kg}{0.5} = 1000kg$$

$$V_{water} \approx 1000L$$

$$V_{extracted} = \frac{0.54L}{kg} \times m_{pomace\ initially} = \frac{0.54L}{kg} \times 500kg$$

$$V_{extracted} = 270L$$

It is therefore estimated that a pomace-to-water ratio would result in a 270L yield with a potential alcohol content of 3.0%.

A higher yield and ethanol content is anticipated when an enzymatic pre-treatment is used, followed by pressing. Using enzymes will greatly improve the yield.

# Prototype: Initially proposed process design

A Pugh chart was used to help select the pomace-to-water ratio, the temperature and whether or not to use an enzymatic pre-treatment step.

Table 1: Pugh chart for selection of pomace-to-water ratio, temperature and use of enzymes.

| | Pomace-to-water ratio | | | Heat (w/o enzyme) | | | Enzyme (ambient temperature) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1:1 | 1:2 | 1:4 | AT | 25-80 | 80+ | None | P | A | Both |
| Yield [sugar] | | ++ | 0 | | 0 | 0 | | 0 | +++ | +++ |
| Yield juice (ml) | | 0 | + | | - | -- | | +++ | 0 | +++ |
| Taste | | 0 | 0 | | 0 | - | | 0 | 0 | 0 |
| Cost | | 0 | 0 | | - | --- | | - | - | -- |
| Ease of use | | 0 | 0 | | - | - | | - | - | - |
| Simplicity | | 0 | 0 | | 0 | --- | | - | - | - |
| Safety | | 0 | 0 | | -- | --- | | 0 | 0 | 0 |
| Sum of (+) | | 2 | 1 | | 0 | 0 | | 3 | 3 | 6 |
| Sum of (-) | | 0 | 0 | | 5 | 13 | | 3 | 3 | 4 |
| **Total Weighted Score** | | **2** | **1** | | **-5** | **-13** | | **0** | **0** | **2** |

*AT = Ambient temperature; P = Pectinase and A = Amylase

From the Pugh Chart, we found that it would be best to use a pomace-to-water ratio and to treat the pomace with both pectinase and amylase in a process carried out at room temperature.

The proposed design included an enzymatic treatment step that involved the use of Pectinase and α-Amylase commercial enzymes at room temperature. The enzymatic pre-treatment process would be divided into two major steps; pectinase treatment and α-amylase treatment. The chart below illustrates the enzymatic pre-treatment process that we had initially proposed.

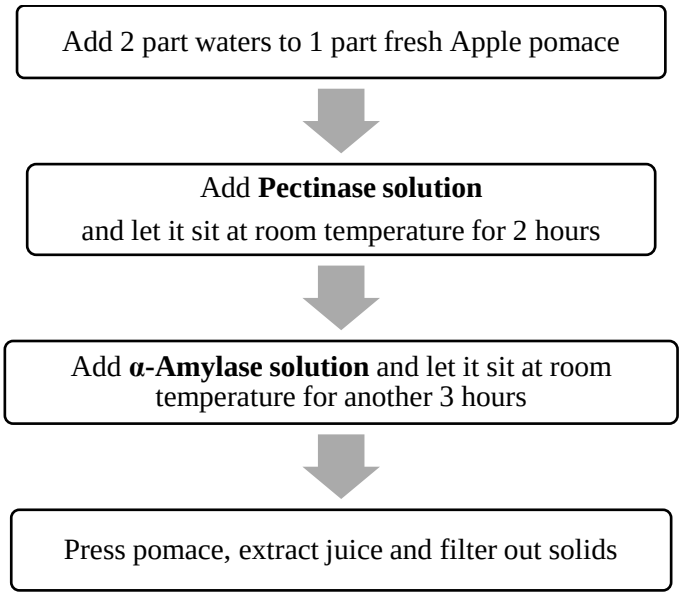


Figure 6: Flow chart of Initial Process Design of Enzymatic Pre-treatment of Apple Pomace.

The enzymatic pre-treatment process was followed by pasteurization of the liquid extracted, inoculation with yeast and finally, fermentation into cider. The graph below illustrates the steps of this process.

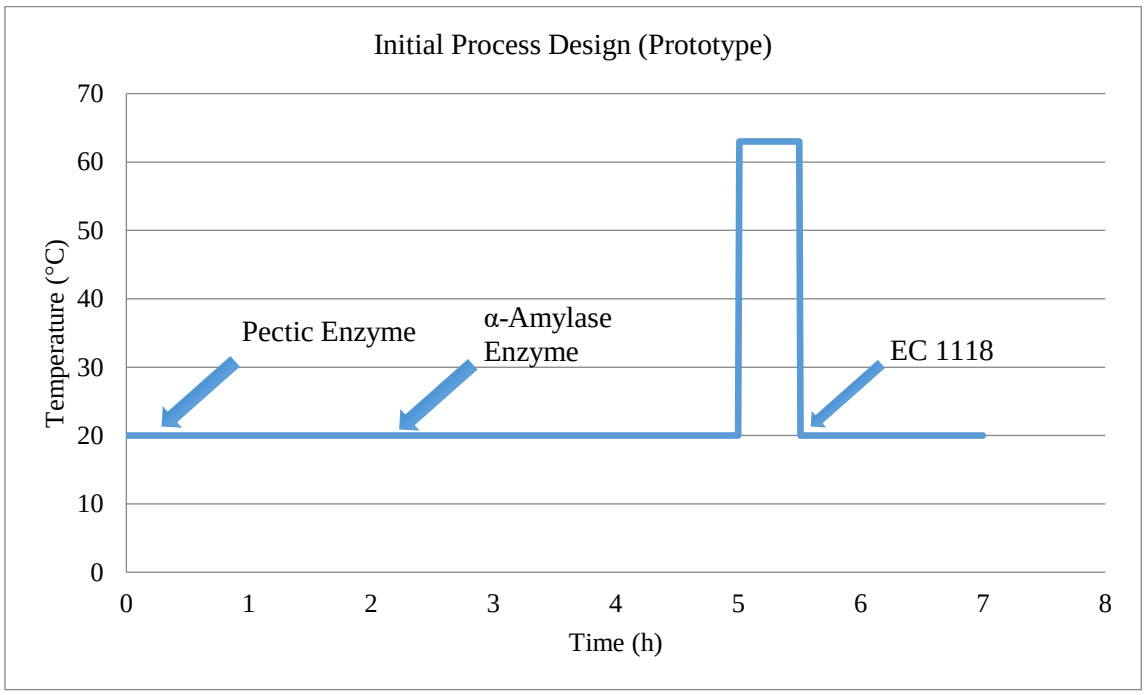


Figure 7: Summary of the initially proposed process deesign (prototype)

Processing steps as illustrated in the graph above

T=0 At ambient temperature, add Pectinase enzymes.

T=2h Add α-Amylase Enzymes.

T=5h Filter mixture and heat to 63°C for 30 minutes.

Cool down to ambient temperature, add EC-1118 and let ferment.

Materials for processing 500 kg of fresh apple pomace

- 2.72 g of Pectinase (75 Units/g) from *Aspergillus niger*
- 8.30 g of α-Amylase (35 Units/mg) from *Aspergillus oryzae*
- 1000 L of water

## Testing the initially proposed process (prototype)

In order to test the initially proposed design (prototype), apple cider was brewed from 2 Kg of apple pomace. The pomace was first treated with pectinase and α-amylase purchased from a local brewing supplies store.

### Materials used for 2 Kg of Apple pomace

Note: Pomace-to-water ratio of 1:2

Enzymatic pre-treatment

- 5 g of Pectic Enzyme; 5% Pectinase (75 Units/g) from *Aspergillus niger*
- 4 mg of α-Amylase (35 Units/mg) from *Aspergillus oryzae*
- 4 L of water

Fermentation

- 0.4g/L of Yeast, EC 1118

The amount of enzymes used was estimated using the Michaelis-Menten model and a number of assumptions (also listed in the sections above) including the fact that the enzymes are 100% active.

## Test Results and Analysis

The Brix values of the liquid from the pomace and the fermented cider were measured in triplicates and recorded. The recorded Brix values were then used to calculate the specific gravities of the solutions, which were then used to estimate the alcohol content of the cider produced.

Brix before fermentation: 4.8; 5.1; 4.8

Average: 4.9

$$SG = 0.9977 + 0.0043 \times {}^{\circ}Brix$$

$$SG = 0.9977 + 0.0043 \times 4.9$$

$$SG_i = 1.01877$$

Brix after fermentation: 2.4; 2.3; 2.4 (after one week of fermentation)

Average: 2.4

$$SG = 0.9977 + 0.0043 \times {}^{\circ}Brix$$

$$SG = 0.9977 + 0.0043 \times 2.4$$

$$SG_f = 1.00802$$

### Estimating the alcohol content of the cider (ABV)

The alcohol content of beer is measured as Alcohol By Volume (ABV) and corresponds to the calculated number of milliliters of pure ethanol in 100 milliliters of solution. The following equation used to calculate the ABV of a beer.

$$ABV = \frac{1.05\ g}{0.79\ g/mL}\left(\frac{Initial\ SG - Final\ SG}{Final\ SG}\right) x\ 100$$

Where 1.05g represents the amount of $CO_2$ that is produced with every gram of ethanol produced, and 0.79 g/mL represents the density of ethanol.

The amount of sugar present in a liquid measured in Brix units and the Brix value is often used to calculate the ABV of alcoholic beverages. One Brix represents 1g of sugar for 100g of solution.

Using the equation used to calculate ABV in beer, the specific gravity of the cider produced, which was calculated using the Brix value measured, was used to estimate the alcohol content of the cider (ABV).

$$°Brix = 231.61 \times (SG - 0.9977)$$

$$SG = 0.9977 + 0.0043 \times °Brix$$

$$ABV = \frac{1.05}{0.79}\left(\frac{1.01877 - 1.00802}{1.00802}\right) x\ 100$$

$$ABV = 1.42\%$$

This shows that the particular store bought pectinase and amylase was not effective in hydrolysing the polysaccharides present in the pomace. The specific enzymes used in our test were not effective due to the following reasons.

- The enzymes used are meant for clarification purposes i.e. to help remove the haze characteristic of juices, wines and ciders. Therefore, they were the wrong brand type to use for maceration, sugar and juice extraction purposes.

- The enzymes were inactive due to poor storage conditions. They were stored at room temperature prior to use when they should have been stored at temperatures between 2 and 8°C according to the manufacturer's recommendations.

- Temperature and pH was not adjusted to the optimum levels for enzyme activity.

The limitations of the enzymatic pre-treatment process are listed in the section below.

# Discussion

## Limitations of the initially proposed enzymatic pre-treatment process (prototype)

The initially proposed design for the enzymatic pre-treatment of fresh apple pomace was not effective due to the following reasons.

- Enzyme concentrations need were estimated using the quantity of product expected and their typical proportions in the polymers (78% galacturonic acid in Pectin; 100% Maltose/Glucose in Starch)

- Starch present in Apple pomace is insoluble and is therefore not available to the enzymes. A gelatinization process should be included before starch-hydrolysing enzymes are added.

- Pectinase was not used together with cellulase and hemicellulase enzymes. Cell wall material was not fully or effectively hydrolysed.

- The pH and the temperature was not adjusted to optimum values thereby providing the most suitable environment for maximum enzyme activity
- No addition of Calcium ions for α-Amylase stability and effectiveness
- Lack of design for the exclusion of possible enzyme inhibitors – would require expert chemical analysis

Because of these limitations, the pre-treatment process had to be optimized in order to achieve our goal of producing a cider with an alcohol content of at least 3.0%.

## Optimization of enzymatic pre-treatment process

Because of the limitations addressed in the section above, the proposed enzymatic pre-treatment process required re-evaluation, revision and optimization. The objective of the pectinase treatment was disruption of the cell walls while the goal amylase treatment step was to hydrolyze the starch present in the apple pomace.

### Optimization of cell wall components hydrolysis

A major limitation of the pectinase treatment is the presence of other structural polysaccharides in cell walls that interact with pectin. Therefore, the pectinase enzyme alone does not hydrolyze all cell wall components which may have delayed cell wall disruption or affected the efficiency of the process. Additionally, the maximum amount of sugars is achieved when polysaccharides in the pomace including cellulose and hemicellulose which are both present in the cell walls are hydrolyzed into simple sugars. Cellulose is a homopolymer of glucose that consists of β-1,4 glycosidic bonds as opposed to α-1,4 and α-1,6 glycosidic bonds present in starch. On the other hand, hemicellulose is a heteropolymer of pentose and hexoses and sugar acids.[4]

In 1993, Carpita and Gibeuta proposed a structural model for the cell walls of flowering plants that included a pectin matrix. The pectin matrix shields cellulose fibers, which are coated with hemicelluloses. Therefore, pectin obstructs enzymatic hydrolysis of cellulose and hemicelluloses present in the apple cell wall by cellulases and hemicellulases enzymes. (Carpita & Gibeaut 1993) Therefore, pectinases, cellulases and hemicellulases are often used together to enhance juice and sugar extraction and yields by hydrolyzing cell wall components; pectins, cellulose and hemicellulose.

[4] Lecture Six: Cellulose, Hemicellulose and Lignin, Biofuel Feedstocks and Production, Biological and Ecological Engineering Department, Oregon State University http://stl.bee.oregonstate.edu/courses/BFP/Class_Slides_W2011/BFP_Lecture6.pdf

In order to optimize the pectinase treatment step whose main objective is to disrupt the cell walls, Macerozyme® R-10 by Novozymes is used instead of pectinase alone and the optimum pH and temperature of the reaction are used. Macerozyme® is a combination of pectinase (0.5 U/mg), cellulase (0.1 U/mg) and hemicellulase (0.25 U/mg) with the following optimum conditions; pH 3.5 – 7.0 and 40-50ºC. In the new process, a pH of 4.0 and a temperature of 40ºC can be used. The pH can be adjusted using Sodium acetate pH buffer. Apple pomace has a pH of 3 and therefore, a relatively small amount of Sodium acetate will be needed to increase the pH to 4.0.

Since the pectinase treatment focused on the degradation of pectin and the pectinase in Macerozyme® has the highest activity between the three enzymes, the calculations of the amount and concentration of Macerozyme® needed for the process are based on *Rhizipus sp.* pectinase properties.

The pectinase in Macerozyme® is polygalacturonase pectinase from *Rhizopus sp.*, a type of fungi, and it is also known as α-1,4 D- galacturonan glycanohydrolase. At 25ºC and pH 4.0, this enzyme liberates 1.0 µmole of galacturonic acid from polygalacturonic acid per min (Sigma Aldrich n.d.). The enzymes properties at a temperature of 25ºC will be used to estimate the concentration that could be used in the treatment. This may adequately represent the concentration of enzymes required while subjecting the reaction to the enzyme's optimum temperature of 40ºC during the process because the enzyme works faster and more efficiently in optimum conditions.

As mentioned in the initially proposed pectinase treatment described above, it is assumed that pectin is 6.09% of dried apple pomace and 78% of this pectin is composed of polygalacturonic acid. Therefore, 4.75% of dried apple pomace can be assumed to be composed of polygalacturonic acid (molecular weight; 194.139 g/mol). Using the Michaelis-Menten model and the assumption that the maximum reaction rate is the total product expected divided by the reaction time (2 hours), the following calculations were made to estimate the amount and concentration of Macerozyme® enzyme required to treat a 500 Kg batch of fresh apple pomace. A 1:2 ratio of pomace to water mixture is prepared; 1000 L of water is added to 500 Kg of pomace

$$\text{Maximum reaction rate} = V_{max} = \frac{\Delta[P]}{\Delta t} = \frac{4.75\% * \left(20\% * \frac{500\ \text{Kg}}{1000\ \text{L}}\right) * 1000\frac{\text{g}}{\text{Kg}} * 0.001\frac{\text{L}}{\text{mL}}}{2\ \text{hours} * 60\frac{\text{min}}{\text{hour}}}$$

$$\therefore V_{max} = 3.95 \times 10^{-5}\frac{\text{g}}{\text{mL}*\text{min}} = 0.20\frac{\mu\text{mol}}{\text{mL}*\text{min}}$$

The specific activity, $k_{cat}$, of *Rhizopus sp.* pectinase in the Macerozyme® at room temperature and pH 4.0 is 0.5 µmol/min/mg. Therefore;

$$\therefore [E_T] = \frac{V_{max}}{k_{cat}} = \frac{0.20\frac{\mu\text{mol}}{\text{mL} * \text{min}}}{0.5\frac{\mu\text{mol}}{\text{min} * \text{mg}}} = 0.4\frac{\text{mg}}{\text{mL}}$$

Using the assumptions discussed above, the estimated concentration Macerozyme® required to treat 500 Kg of fresh apple pomace in this step is 0.4 mg/mL. A 1.0 L Macerozyme® solution is prepared by dissolving 4.0 grams of Macerozyme® in 1.0 L of water.

The following chart illustrate the optimized cell wall hydrolysis (pectinase) pre-treatment step.

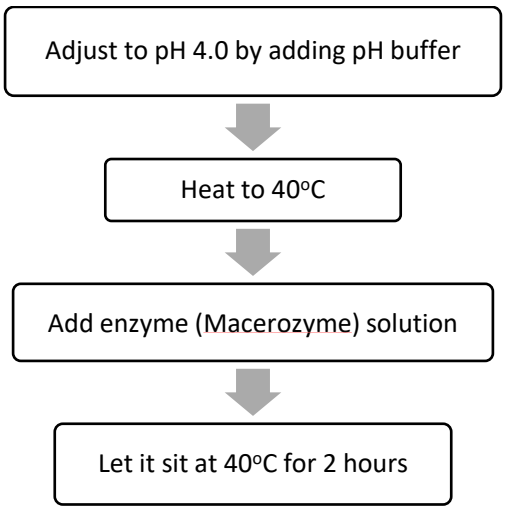


Figure 8: Flow chart for the Optimized pectinase pre-treatment step

Note that the optimized process proposed is a hypothesis as the team has not been able to test how effective this process is in treating apple pomace. It is, however, backed by previous studies in literature and current practices. This is discussed in the limitations and recommendations sections below.

## Optimization of starch hydrolysis

One of the major limitations of starch hydrolysis using enzymes is the availability of the starch molecules to the enzymes. Starch granules are mostly insoluble and therefore, difficult for enzymes to access. For and effective and complete starch saccharification process, the starch is made soluble through a process called gelatinization. Gelatinization involves quickly heating the starch in water to a certain temperature – the gelatinization temperature. Heating causes the starch granules to take up water which makes them swell. Continued heating results in a disruption of the granules and the release of amylose and amylopectin and this results in the dissolution of the starch. Gelatinization temperatures and conditions depend on the source of the starch. (van der Maarel 2009)

In their study of the structures and functional properties of a variety of apple cultivars, Stevenson et al. found that the gelatinization temperatures of the starch from the apples ranged from 64 to 66°C and their gelatinization enthalpies were ranged from 16 to 18 J/g (Stevenson et al. 2006). In another study,

Tirado-Gallegos et al. found that the starches from Golden Delicious Smoothee apples had gelatinization enthalpies between 6.90 and 10.20 J/g (Tirado-Gallegos et al. 2016).

Starch processing in industry involves three steps; gelatinization, liquefaction and saccharification. The gelatinization step involves heating a 30-40% starch slurry to 105$^{O}$C for 5 minutes using methods such as jet-cooking where pressurized steam is injected into the starch slurry. Before the heating process is initiated, the pH is adjusted, calcium and a thermostable α-amylase extracted from *Bacillus licheniformis* or from *Bacillus amyloliquefaciens* bacteria, is added to the slurry. The calcium is for the enzyme's stability. In the liquefaction stage the slurry is flash-cooled at a temperature between 95 and 100$^{o}$C and held at this temperature for 1-2 hours. At the end of liquefaction step, the reaction is either stopped or followed by a saccharification step where the enzymes pullulanase, glucoamylase, β-amylase or α-amylase are added to further degrade the slurry now present in the treated slurry, into maltodextrins, maltose or glucose syrups. (van der Maarel 2009)

By considering both industrial starch processing methods and the gelatinization conditions of starch from apple, the amylase treatment process initially proposed and described in the sections above was optimized by adjusting the temperature and pH to provide optimum conditions for the enzymes and to include a gelatinization, a liquefaction and a saccharification step.

*Gelatinization*. The function of the gelatinization step is to increase the solubility of starch molecules for a time long enough for thermostable α-amylase enzyme molecules to access and hydrolyze the amylose and amylopectin components. Pomace is heated to temperatures 95$^{o}$C for 5 minutes. A temperature of 95$^{o}$C, much higher than the gelatinization temperature of apple starch (Stevenson et al. 2006), is used to increase the probability of complete gelatinization of the whole mass of starch. A thermostable α-amylase enzymes from *B. licheniformis* would be used in this step and it would be added to the pomace-water mixture before the heating process begins.

*Liquefaction.* After 5 minutes at 95$^{o}$C, the mixtures cooled down rapidly to 70$^{o}$C where it is maintained for 1 to 2 hours. This is the liquefaction stage where the *B. licheniformis* enzymes hydrolyse the amylose and amylopectin polysaccharides into dextrins and oligosaccharides.

*Saccharification.* At the end of the liquefaction step, the mixture is cooled down to 50$^{o}$C, the optimum temperature for fungal (*Aspergillus oryzae*) α-amylase enzyme which is added immediately after the cool-down process. The fungal α-amylase hydrolyzes the dextrins and oligosaccharides present in the treated mixture into smaller oligosaccharides and simple sugars.

Note that the optimized process proposed is a hypothesis as the team has not been able to test how effective this process is in treating apple pomace. It is, however, backed by previous studies in literature and current practices. This is discussed in the limitations and recommendations sections below.

The optimized process involves the addition of a thermostable α-amylase enzyme from *B.* licheniformis in a gelatinization step, another α-amylase enzymes from *Aspergillus oryzae*, and a pH buffer to adjust the pH from 4.0 to 6.0. Thermostable α-amylase from *B. licheniformis* is sold as Termamyl®, an enzyme produced by Novozymes Corp, and displays enzymatic activity greater than 500 U/mg. At 20$^{o}$C

and a pH of 6.9, one unit of the enzyme will liberate 1.0 mg of maltose from starch in 3 minutes. Termamyl® thermostable α-amylase has an optimum pH range of 7-9 and displays maximum activity at 90ºC. The enzyme is stable between pH 7 and 10 and between 40 and 60ºC. (Sigma Aldrich product information and specification sheets[5])

The Michaelis-Menten model was used to estimate the amount and concentration of the *B. licheniformis* α-amylase needed to treat 500 Kg of fresh apple pomace in the 5 min gelatinization step and in the liquefaction step. A 1:2 ratio of pomace to water mixture is prepared; 1000 L of water is added to 500 Kg of pomace. As mentioned in the sections above, using results from a study conducted by Singal et al. it is assumed that 17.89% of dried pomace is starch (Singhal K.K. et al. 1991).

$$\text{Maximum reaction rate} = V_{max} = \frac{\Delta[P]}{\Delta t} = \frac{17.89\% * \left(20\% * \frac{500\ \text{Kg}}{1000\ \text{L}}\right) * 1000 \frac{\text{g}}{\text{Kg}} * 0.001 \frac{\text{L}}{\text{mL}}}{5\ \text{min}}$$

$$\therefore V_{max} = 0.03578 \frac{\text{g}}{\text{mL} * \text{min}}$$

For the 1-hour liquefaction stage;

$$\text{Maximum reaction rate} = V_{max} = \frac{\Delta[P]}{\Delta t} = \frac{17.89\% * \left(20\% * \frac{500\ \text{Kg}}{1000\ \text{L}}\right) * 1000 \frac{\text{g}}{\text{Kg}} * 0.001 \frac{\text{L}}{\text{mL}}}{60\ \text{min}}$$

$$\therefore V_{max} = 2.982 \times 10^{-4} \frac{\text{g}}{\text{mL} * \text{min}}$$

It is assumed that the enzyme has a similar specific activity at 20ºC as it does at 95ºC and at 70ºC, the temperature that will be used in the gelatinization step, and that the Termamyl® has an activity of 500 U/mg. Therefore, $k_{cat}$ is assumed to be 486.90 µmol/min/mg (factoring in Maltose and its molecular weight). This step is expected to produce a majority of oligosaccharides with longer chains that maltose which are then broken down in the next step. However, it assumed that calculations made using maltose are representative of the calculations that could be made for the group of oligosaccharides that may be produced.

During gelatinization (95ºC, 5 min), the concentration of *B. licheniformis* α-amylase;

$$[E_T] = \frac{V_{max}}{k_{cat}} = \frac{0.03578\ \frac{\text{g}}{\text{mL} * \text{min}} * \frac{1}{3.423 \times 10^{-4}} \frac{\mu mol}{g}}{486.90 \frac{\mu mol}{mg * \text{min}}} = 2.147 \frac{mg}{mL}$$

[5] Termamyl® α-amylase from *B. licheniformis*
http://www.sigmaaldrich.com/catalog/product/sigma/a3403?lang=en&region=CA

During liquefaction step (70ºC, 1 hour), the concentration of *B. licheniformis* α-amylase;

$$[E_T] = \frac{V_{max}}{k_{cat}} = \frac{2.982 \times 10^{-4} \frac{g}{mL * min} * \frac{1}{3.423 \times 10^{-4}} \frac{\mu mol}{g}}{486.90 \frac{\mu mol}{mg * min}} = 1.789 \times 10^{-3} \frac{mg}{mL}$$

Treating 500 Kg of fresh Apple pomace in the gelatinization and liquefaction step would require a concentration of 2.147 mg/mL of Termamyl® thermostable α-amylase which can be prepared by dissolving 2.147 g of enzyme into 1.0 L of water. Since the enzyme added in the beginning of the gelatinization is not used up in the reaction and deactivated in the process, the enzyme in the gelatinization step continues to acts on the polysaccharides in the liquefaction step and therefore, more enzyme is not added prior to liquefaction.

Two enzymes are used in this process at their optimal conditions; pH 6.0 and 90ºC for *B. licheniformis* α-amylase, and pH 4.5 and 50ºC for *A. oryzae* α-amylase.

*A. oryzae* α-amylase has an activity of 35 Units/mg (Cheirsilp & Umsakul 2008). At a pH of 6.0 and a temperature of 25ºC, one unit corresponds to the amount of enzymes that liberates 1 µmol of maltose per minute. Assuming that this same activity is displayed at the enzyme's optimum conditions of pH 4.5 and 50ºC, it can be said that the enzyme has a specific activity of 35 µmol/min/mg. This is used to estimate the amount and concentration of *A. oryzae* α-amylase required to treat 500 Kg of fresh apple pomace with a reaction time of 3 hours.

$$\text{Maximum reaction rate} = V_{max} = \frac{\Delta[P]}{\Delta t} = \frac{17.89\% * \left(20\% * \frac{500\ \text{Kg}}{1000\ \text{L}}\right) * 1000 \frac{g}{\text{Kg}} * 0.001 \frac{\text{L}}{\text{mL}}}{3\ \text{hours} * 60 \frac{\text{min}}{\text{hour}}}$$

$$\therefore V_{max} = 9.94 \times 10^{-5} \frac{g}{mL * min} = 0.29 \frac{\mu mol}{mL * min}$$

This takes into consideration, the molecular weight of Maltose (342.3 g/mol).

$$\therefore [E_T] = \frac{V_{max}}{k_{cat}} = \frac{0.29 \frac{\mu mol}{mL * min}}{35 \frac{\mu mol}{min * mg}} = 8.29 \times 10^{-3} \frac{mg}{mL}$$

A solution with a concentration of 8.29 mg/L of *A. oryzae* α-amylase is required to treat 500 Kg of apple pomace. The solution is prepared by dissolving 8.29 mg of the enzyme into 1.0 L of water.

## Addition of Calcium ions, $Ca^{2+}$

Calcium ions, $Ca^{2+}$, are required for the stability of the α-amylase enzymes used. *B. licheniformis* α-amylase has a molecular mass of 58.274 kDa and requires three $Ca^{2+}$ ions for every enzyme molecule. *A. oryzae* α-amylase has a molecular mass of 52.490 kDa requires two $Ca^{2+}$ ions for every enzyme molecule. (Duy & Fitter 2005) Calcium chloride ($CaCl_2$) is a permitted non-organic food additive[6] and will be used in this process to provide the enzymes with $Ca^{2+}$ ions. The following table shows the amounts of $CaCl_2$ that would be required the enzymes used. $CaCl_2$ has a molecular mass of 110.98 g/mol.

Table 2: Amount of $CaCl_2$ required for each enzyme addition, to provide $Ca^{2+}$ ions for stability and activity.

| Source and type of α-amylase | Molecular mass of α-amylase (g/mol) | Concentration of enzyme used (g/mL) | Concentration of enzyme used (mol/mL) | No. of $Ca^{2+}$ ions for each molecule | Concentration of $CaCl_2$ required (mg/L) |
|---|---|---|---|---|---|
| *B. licheniformis* | 58274 | $2.147 \times 10^{-3}$ | $3.684 \times 10^{-8}$ | 3 | 12.27 |
| *A. oryzae* | 52490 | $8.29 \times 10^{-6}$ | $1.58 \times 10^{-10}$ | 2 | 0.0351 |

## Adjusting pH

In the optimized enzymatic pre-treatment process, the pH is adjusted three times from pH 3.0 which is the natural pH of the apple pomace, to pH 4.0 (for Macerozyme), to pH 6.0 (for *B. lichniformis* α-amylase) and finally to pH 4.5 (for *A.oryzae* α-amylase). Diluted solutions of pH-adjusting agents are prepared and slowly added into the pomace-water-enzymes mixtures until the desired pH level is achieved. The pH is carefully measured and monitored using a pH meter.

It is noted that pectinase in the Macerozyme hydrolyses pectin's polygalacturonic acid into galacturonic acid molecules which are bound to decrease the pH of the systems. One of the main goals of using a pH buffer will be to prevent such changes in the pH.

For this process, a Sodium acetate and Acetic acid can be used to adjust the pH or both can be used as a buffering system to maintain the pH between 3.7 and 5.6. Both Acetic acid and Sodium acetate are permitted food additive and a permitted food pH adjusting agents (Health Canada)[7]. Other examples of permitted pH-adjusting agents include Ammonium phosphate, Calcium phosphate Calcium carbonate and

[6] Permitted Substances Lists. Public Services and Procurement Canada.
https://www.tpsgc-pwgsc.gc.ca/ongc-cgsb/programme-program/normes-standards/internet/bio-org/permises-permitted-eng.html

[7] List of Permitted pH Adjusting Agents, Acid-Reacting Materials and Water Correcting Agents (Lists of Permitted Food Additives) – Health Canada
http://www.hc-sc.gc.ca/fn-an/securit/addit/list/10-ph-eng.php

Calcium hydroxide. Citrate compounds should be used because citrate ions bind to $Ca^{2+}$ ions and interfere with the binding of $Ca^{2+}$ ions to α-amylase enzyme molecules. Therefore, the use of citrate compounds decreases the stability and activity of α-amylase enzymes.

## Optimized starch hydrolysis enzymatic pre-treatment Summary

The following flow chart provides a summary the optimized starch-hydrolysis process proposed.

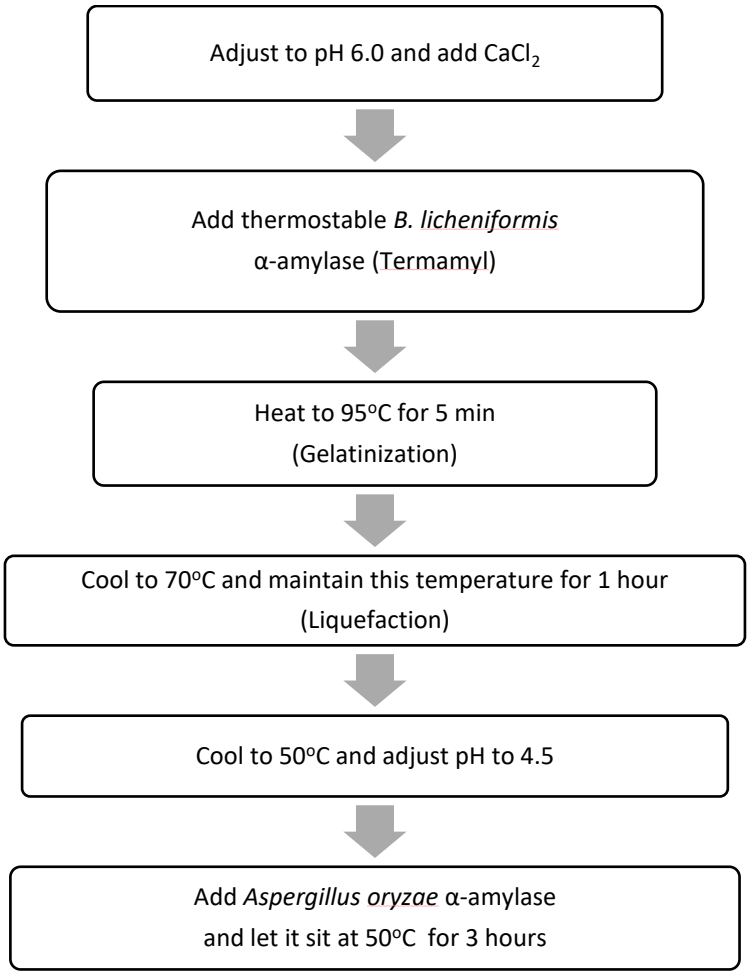


Figure 9: Flow chart of the Optimized Starch hydrolysis process proposed.

## Materials for the optimized pre-treatment process

The masses of enzymes and Calcium chloride used are first dissolved in a litre of water each to prepare their respective solutions.

The materials required to treat 500 Kg of pomace using the optimized enzymatic pre-treatment process.

- 1000 L of water
- 500 Kg of fresh apple pomace
- Dilute solutions of Sodium acetate and Acetic acid buffer
- Solution concentrations prepared by dissolving the respective compounds in water alone
    - 4.0 g/L Macerozyme R-10 by Novozyme Corp.
        - Contains pectinase from *Rhizopus sp.* (0.5 U/mg), cellulase (0.1 U/mg) and hemicellulase (0.25 U/mg)
    - 2.15 g/L of Termamyl® by Novozymes (for brewing), *B. licheniformis* α-amylase, (≥ 500 U/mg)
    - 9.67 mg/L of *A. oryzae* α-amylase (~30 U/mg)
    - 12.27 mg/L of Calcium chloride for Termamyl® treatment
    - 0.035 mg/L of Calcium chloride for *A. oryzae* α-amylase treatment

## Potential results of using the Optimized Enzymatic pre-treatment process

According to a study conducted by Srivastava and Tyagi (2013) on the effect of enzymatic hydrolysis on the juice yield of apple, 1 mg of Pectinase enzyme added to 25 g of fresh apple pulp can result in a 72.3 % Juice Yield. That is 18.075 grams of juice was obtained from the pulp. (Srivastava & Tyagi 2013)

From our lab experiment last semester, we found that the Apple Pomace that we obtained from Quinn farm, a local small-scale apple juice processing facility, has a 80.4% moisture content and the strength tests that we performed on the pomace proved that that close to 20 grams of Apple pomace could still be pressed from the pomace.

It can therefore be assumed that the pectinase treatment can lead to about 70% juice yield when the pectinase-treated fresh apple pomace is pressed. Therefore, using pectinase (in the Macerozyme) may allow 350 Kg of juice to be pressed from 500 Kg of fresh apple pomace. According to the FAO/INFOODS Density Database Version 2.0 (Charrondiere et al. 2012), apple juice has a 1.04 g/mL density[8] thus 350 Kg of apple juice has a 336.5 L volume. Feryal and Aziz (2002), studied the sugar content of apple juices from

[8] FAO/INFOODS Density Database Version 2.0 (2012)

a variety of apples. They found that the apple juices contained 9.30–32.2 g/L of Glucose, 66.10–96.00 g/L of Fructose, 8.5–55.10 g/L of Sucrose, 110.90–164.40 g/L total sugar concentrations and, 11.80–18.60% of total soluble solids (Charrondiere et al. 2012). We can therefore assume that the 336.5 L of apple juice obtained from 500 Kg of pomace can contain close to 55.3 Kg of dissolved sugars; a maximum of 10.8 Kg of glucose, 32.3 Kg of Fructose and 18.5 Kg of Sucrose.

Assuming that the optimized process is successful in extracting the maximum amount of sugars that could be obtained from 500 Kg of fresh apple pomace and that the polysaccharides in this pomace were fully hydrolyzed into their monomers, we could expect the following results.

- 17.89 Kg of Maltose from the complete hydrolysis of starch in 500 Kg of fresh apple pomace assuming that 100 g of dried apple pomace contains 17.89 g of starch (Singhal K.K. et al. 1991).

- 16.67 Kg of Glucose from the complete hydrolysis of cellulose present in 500 Kg of fresh apple pomace assuming that 100 g of dried apple pomace contains 16.67 g of cellulose (Wang & Thomas 1989).

- 55.3 Kg of dissolved sugars in the 336.5 L of apple juice that could be extracted; a maximum of 10.8 Kg of glucose, 32.3 Kg of Fructose and 18.5 Kg of Sucrose.

These values can be used to estimate the amount of ethanol that could be obtained from processing 500 Kg of fresh apple pomace. This will be done using the chemical formulae for the fermentation of the simple sugars into ethanol.

Fermentation of glucose or fructose, ($C_6H_{12}O_6$), into ethanol ($CH_3CH_3OH$);

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_3OH + 2CO_2$$

Fermentation of maltose or sucrose, ($C_{12}H_{22}O_{11}$), into ethanol ($CH_3CH_3OH$);

$$C_{12}H_{22}O_{11} \rightarrow 4CH_3CH_3OH + 4CO_2$$

Maltose + Sucrose (Molecular weight, 342.3 g/mol) = 36.39 Kg = 106.3 mol ==> 425.2 moles of ethanol

Glucose + Fructose (Molecular weight, 180.2 g/mol) = 59.77 Kg = 331.7 mol ==> 663.4 moles of ethanol

Total ethanol (Molecular weight, 46.06 g/mol) = 1,088.6 moles = 50.14 Kg

Ethanol Volume (Density, 789 Kg/m$^3$) = 63.55 L

Water was added to pomace at the start of the process in order to prepare a mixture with a pomace-to-water ration of 1:2. For 500 Kg of fresh apple pomace, 1000 L of water would be added. Therefore the actual volume of the liquid pressed at the end of the enzymatic treatment (in addition to the juice extracted) would be 1336.5 L.

Assuming perfect conditions and complete hydrolysis of the polysaccharides assumed to be present in the pomace, the **Cider ABV** = (63.55/1336.5) = **4.75%**.

This proves that the optimized enzymatic treatment of fresh apple pomace can allow for the production of apple cider with an alcohol content above 3%.

## Limitations of the optimized enzymatic pre-treatment process

As mentioned above, the proposed optimized enzymatic pre-treatment process is more a hypothesis which would be evaluated and confirmed using further chemical analysis using methods and tools such as High-performance liquid chromatography (HPLC) and Differential Scanning Calorimetry. These tools will allow us to determine the products of the enzymatic reactions which will allow us to determine the effectiveness of the enzymes and the conditions used. They will also allow us to modify the process in order to achieve the best conditions. HPLC would also be used to identify potential enzyme inhibitors and potential chemical hazards, such as amygdalin and methanol by-products.

However, the proposed process was designed with reference previous studies in literature and current industrial practices. Therefore, there is a high chance that we will achieve our main objective of extracting the maximum amount juice and sugars from the apple pomace.

Another limitation of the proposed process is a lack of specification on the exact amount of pH adjusting agents to use in order to adjust the pH to optimum condition. This would require a titration experiment involving the use of an accurate pH meter to test the amount of pH adjusting agent and pH buffers to exactly achieve the desired pH during all steps of a full treatment.

## Further recommendations to improve the optimized enzymatic pre-treatment process

In regards to some of the limitations discussed in the previous sections, the following are recommended to further improve the enzymatic pre-treatment process.

- Perform extensive chemical analysis (e.g. HPLC) to determine presence and concentration of;
    - Enzyme inhibitors e.g. $Al^{3+}$ and $Zn^{2+}$ ions
    - Citrate ions which bind to $Ca^{2+}$ ions
    - Harmful by-products e.g. amygdalin and methanol
- Analysis of the apples processed to produce the apple pomace to help determine the effects of factors such as storage (time and conditions), maturity of the fruit and cultivar, on process conditions.

# The Optimized process design

## A summary

Mass Ratio (pomace: water): 1:2

Time = Enzymatic pre-treatment + Fermentation = (2 h + 5 min + 1h + 3h) + (42 h - 2 weeks)

Materials used to process 500 Kg of fresh Apple pomace

| | |
|---|---|
| Enzymatic pre-treatment | |
| Macerozyme R-10 | 4.00 g/L |
| Termamyl® (Novozyme) α-amylase | 2.15 g/L |
| *Aspergillus oryzae* α-amylase | 0.010 g/L |
| Calcium chloride ($CaCl_2$) | 0.012 g/L |
| pH adjusting agents (sodium acetate & acetic acid) | (To be determined via titration) |
| Fermentation | |
| EC 1118 (*S. cerevisiae bayanus)* | 0.4g/L |

The following graph is a summary of the steps used in the optimized process.

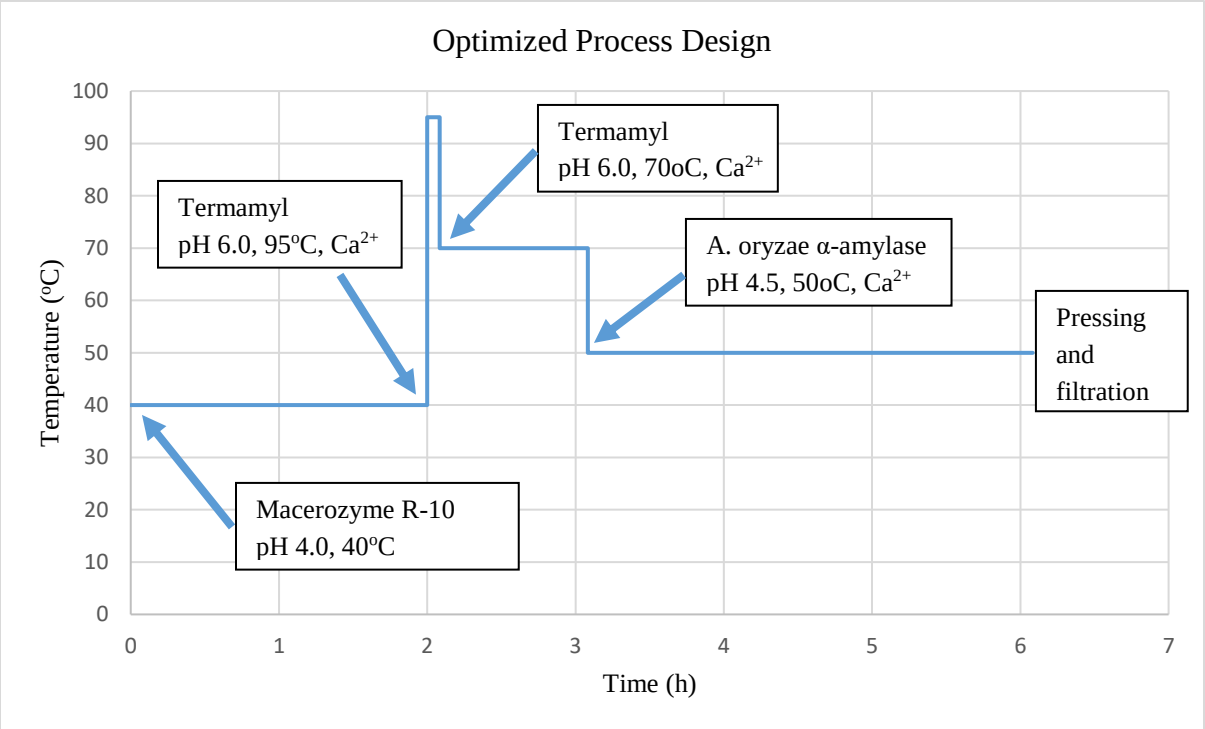


Figure 10: Summary of steps used in the optimized process design

Time (hours.min)

| | |
|---|---|
| T = 0 | Heat to 40°C, add pH-adjusting agent & Macerozyme R-10 |
| T = 2 | Add thermostable *B. licheniformis* & α-amylase (Termamyl)<br>Adjust pH to pH 6.0<br>Heat to 95°C for 5 min (Gelatinization)<br>Cool to 70°C and maintain for 1 hour (Liquefaction) |
| T = 2.05 | |
| T = 3.05 | Cool to 50°C and adjust pH to 4.5<br>Add *Aspergillus oryzae* α-amylase<br>Maintain at 50°C and pH 4.5 for 3 hours |
| T = 6.05 | Liquid is heated to 80°C for 1 minute (Pasteurization)<br>Add EC1118 let ferment |

## Cost analysis: Direct material costs of the optimized enzymatic treatment process

The following table shows the costs of Materials used in the optimized enzymatic pre-treatment process

Table 3: Costs of the materials in the proposed optimized enzymatic treatment process.

| **Material** | **Retail quantities (g)** | **Retail price per package (CAD)** | **Price (CAD per g)** | **Quantity used per 500 Kg batch AP (g)** | **Cost of material per 500 Kg batch AP* (CAD)** |
|---|---|---|---|---|---|
| Macerozyme[9] R-10 | 10 | 320.46 | 32.05 | 4.0 | 128.2 |
| Termamyl® α-amylase[10] | 113.4 (4 oz) | 42.50 | 0.37 | 2.15 | 0.81 |
| *A. oryzae* α-amylase[11] | 10 | 54.50 | 5.45 | 0.010 | 0.055 |
| Calcium chloride[12] (Food grade) | 113.4 (4 oz) | 10 | 0.088 | 0.012 | 0.0011 |

[9] Macerozyme R-10 sold by Gold Biotechnology, USA
https://www.goldbio.com/product/1546/macerozyme-r-10
[10] Termamyl liquid endo-α-amylase
https://homebrewsupplies.ca/product/3144-liquid-endo-alpha-amylase-1qt/
[11] Sigma Aldrich α-Amylase from Aspergillus oryzae
http://www.sigmaaldrich.com/catalog/product/sigma/10065?lang=en&region=CA
[12] Food Grade Calcium chloride, My spice sage website

| | | | | | |
|---|---|---|---|---|---|
| | | | | TOTAL | 129.07 |
| **Other material costs** | | | | | |
| Sodium acetate[13] (Food grade) | 1000.0 | 114.0 | 0.114 | | |
| Acetic acid[14] (Food grade) | 1000.0 | 78.40 | 0.0784 | | |

*AP = fresh Apple pomace

Assuming that 10 g of each pH-adjusting agent or buffer is used in the treatment of 500 Kg of fresh apple pomace, an estimated total CAD 131 is used to purchase the materials required to treat one 500 Kg batch of fresh apple pomace. This would be a variable cost in the production of apple cider from apple pomace using enzymes.

It was estimated that maximum of 1,336.5 L of juice/liquid could be extracted from 500 Kg of fresh apple pomace using the optimized process. Current commercially available cider usually come in bottles that contain 750 mL or 355 mL, sold individually or in 4 packs. The 750 mL bottles would cost the producer CAD 0.80 each, while the 355 mL bottles cost CAD 0.50 each. The 1,336.5 L would produce cider of about the same volume which can fill 892 750-mL bottles and 1880 355-mL bottles. Therefore, other direct variable cost can be calculated

Table 4: Other Direct Material Costs

| Item or Material | Price per unit (CAD) | Price per 500 Kg batch treatment |
|---|---|---|
| Yeast[15] | 1.25 | 72.60 |
| Water[16] | 1.50 for 1000 gal | 0.40 |
| 750 mL bottles[17] | 0.80 | 713.60 |
| 355 mL bottles[18] | 0.50 | 940.00 |
| | TOTAL | 1726.60 |

https://www.myspicesage.com/calcium-chloride-food-grade-p-997.html

[13] Food grade Anhydrous Sodium Acetate – Sigma Aldrich http://www.sigmaaldrich.com/catalog/product/aldrich/w302406?lang=en&region=CA

[14] Food grade Acetic acid – Sigma Aldrich http://www.sigmaaldrich.com/catalog/product/aldrich/w200611?lang=en&region=CA

[15] Yeast from http://www.mainbrew.com/EC1118-Prodview.html

[16] Price of water from Ville de Montreal website: http://ville.montreal.qc.ca/portal/page?_pageid=44,82908&_dad=portal&_schema=PORTAL

[17] The quantity of each type of bottle is based on the assumption that around half of the cider will be sold in 750ml bottles, and the other half in 355ml bottles. 750 ml bottles from https://www.unitedbottles.com/product/belgian-pry-off-750-ml

[18] 355ml bottles from https://www.unitedbottles.com/product/heritage-355-ml-2

Total Direct Material Costs of the Process = CAD 1,857.60 per 500 Kg batch

To estimate the potential selling price of the cider produced, we looked at the prices of cider sold in Quebec and determined the price range of cider with 2-5% ABV. We found that 750 mL bottles of cider are sold at prices CAD 9-12 while a pack of four 355-mL bottles sells for CAD 11-14. The cider produced using the proposed process can be sold at the price on the higher end of the price range. This can be done as the cider is an eco-friendly product produced locally and at a smaller scale which at many times means higher costs incurred per bottle as compared to large scale operations. Therefore, it is assumed that the 750-mL bottle is sold for CAD 12 and the 355-mL bottles is sold for CAD 14.

Assuming that all the bottles made from a 500 Kg batch of Apple pomace are sold, the following sales revenue can be expected.

Sales Revenue = (892 x CAD 12) + (1880/4 x CAD 14) = CAD 17,284.00 per 500 Kg batch processed

The sales revenue less direct material costs is CAD 15,426.40 per 500 Kg batch of pomace processed. This shows the potential profit of using apple pomace to produce apple cider using the proposed treatment process. A fully operated processing facility would also benefit from economies of scale derived from using the same labour and utilities for the cider production as they use for juice production, thereby increasing their gross profit margin

## Downstream processing overview

The enzymatic pre-treatment steps can be carried out in a batch reactor or a water-jacketed tank where the temperature can be regulated and even heating can be achieved. A water-jacketed tank is less costly than a batch reactor and it could therefore be used in small-scale production. An example of a water jacket tank is a 6-gallon (22.7 L) 304-stainless steel model Model 6MH. [19]

At the end of the enzymatic pre-treatment process, the treated pomace is pressed and the liquid is extracted and filtered. This can be done using traditional presses, presses already available in the juice processing facility, or by hand using a cheese cloth and applying force to squeeze out the juices. The liquid is then pasteurized by heating it to 80$^{o}$C for 1 minute. Pasteurizing the liquid will destroy pathogens and inactivate any enzymes present. The liquid is then cooled and then inoculated with the yeast (Saccharomyces cerevisiae bayanus (EC-1118)) and allowed to ferment for 2 weeks, left at a temperature between 15 and 25$^{o}$C. Packaging, or bottling, can take place before or after the start of the fermentation process.

[19] 6MH Water Jacketed
http://www.soapequipment.com/Tanks/6GallonOilTank.htm

# Conclusion

The goal of this project is to design a treatment process that would allow for the production of apple cider with an acceptable alcohol content from apple pomace. Apple cider production involves fermentation of sugars in juice extracted from apple masses by yeast cells, into alcohol and the amount of alcohol produced during fermentation is dependent on the quantity of sugars present. Therefore, the main objective of this project was to extract the maximum amount of sugars and juice from the apple pomace. This led us to study methods of hydrolysing polysaccharides, such as starch, cellulose and hemicellulose, present in the apple pomace and methods of increasing juice yield by breaking down the cell walls. The best method we found of doing so was enzymatic hydrolysis using pectinase, cellulase, hemicellulase and α-amylase, which would hydrolyse pectin, cellulose, hemicellulose and starch, respectively. Pectin, cellulose and hemicellulose are important structural polysaccharides that maintain the structure of the cell wall. Their hydrolysis results in the weakening of the cell walls which allows for an easier extraction of juices and thus significantly higher juice yields. Pectinase, cellulase and hemicellulase are used together and can be found in Macerozyme, a product by Novozymes. Treating apple pomace with pectinase, cellulase, hemicellulase and α-amylase can result in a minimum 70% juice yield and about a 4.75% alcohol content.

After testing and optimization, a process has been designed and proposed for the production of apple cider from apple pomace using enzymes in a pre-treatment step. This process has been summarized in the figure below.

## Process Summary

Mass Ratio (pomace: water): 1:2

Time = Enzymatic pre-treatment + Fermentation = (2 h + 5 min + 1h + 3h) + (42 h - 2 weeks)

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| pH adjusting agents (sodium acetate & acetic acid) | (To be determined via titration) |
| Fermentation | |
| EC 1118 (*S. cerevisiae bayanus)* | 0.4g/L |

The following graph is a summary of the steps used in the optimized process.

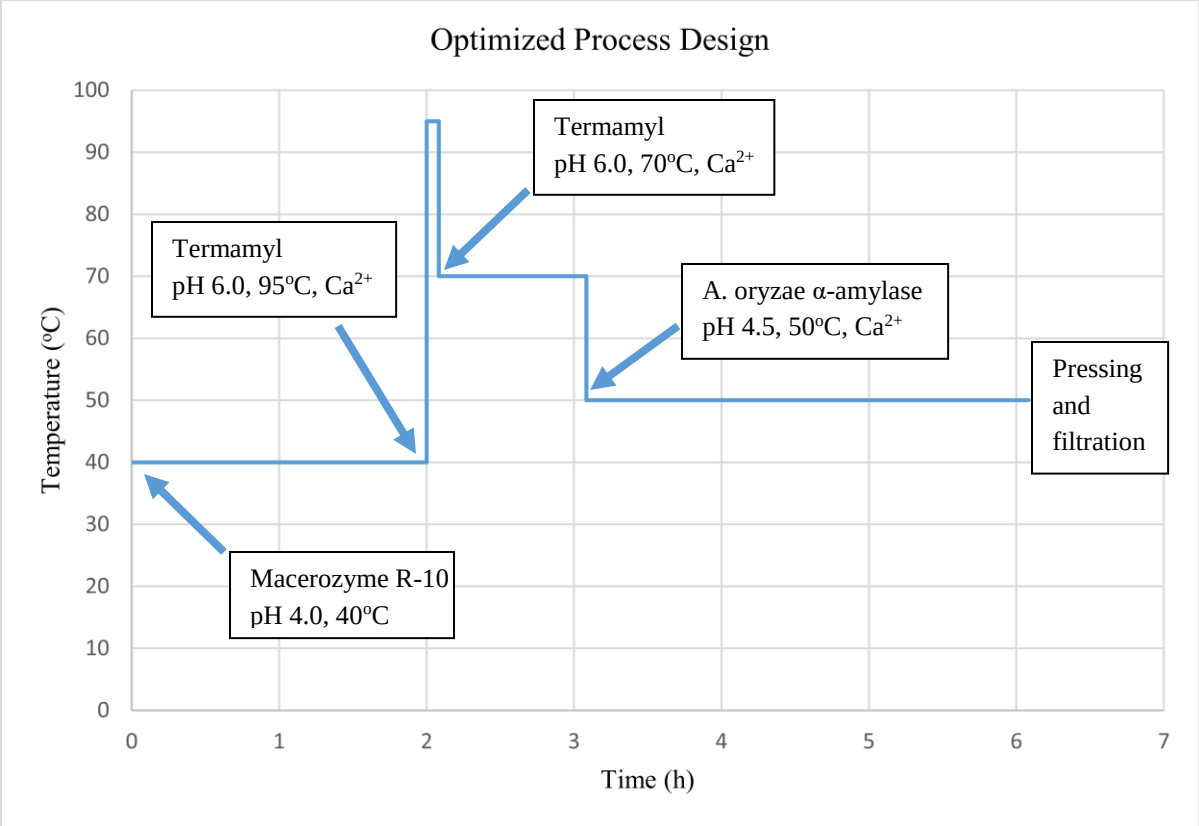


Figure: Graph illustrating processing steps through time

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# Appendix

## Tabulated results of experiments.

Table 5: Results of heat treatment tests performed at 80°C on different water-to-pomace ratios

| | Solution 1 (1:2) (100±1g)/(200±5ml) | | | | Solution 2 (1:4) (100±1g)/(400±13ml) | | | | Solution 3 (1:8) (50±1g)/400±13ml) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| time (min) | | | | **Mean** | | | | **Mean** | | | | **Mean** |
| 0 | 0 | 0 | 0 | **0** | 0 | 0 | 0 | **0** | 0 | 0 | 0 | **0** |
| 15 | 3.6 | 3.5 | 3.7 | **3.6** | 1.5 | 1.4 | 1.2 | **1.4** | 0.7 | 0.7 | 1.0 | **0.8** |
| 30 | 3.8 | 3.4 | 3.8 | **3.7** | 1.9 | 1.5 | 1.8 | **1.7** | 0.8 | 0.7 | 0.6 | **0.7** |
| 45 | 4.1 | 4.3 | 4.4 | **4.3** | 2.2 | 1.9 | 1.9 | **2.0** | 1.2 | 0.7 | 0.6 | **0.8** |
| 60 | 4.5 | 4.0 | 4.1 | **4.2** | 2.1 | 2.2 | 2.0 | **2.1** | 1.1 | 1.2 | 1.0 | **1.1** |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 90 | 4.5 | 4.6 | 4.5 | **4.5** | 2.1 | 2.1 | 2.2 | **2.1** | 1.3 | 1.2 | 1.6 | **1.4** |
| 120 | 4.7 | 4.9 | 4.7 | **4.8** | 2.3 | 2.5 | 2.4 | **2.4** | 1.8 | 1.5 | 1.5 | **1.6** |

Table 6: Results of enzymatic treatment tests performed at room temperature

| Time (min) | Sln 1 0g | Sln 2 0.025g | Sln 3 0.1g | Sln 4 0.4g | Sln 5 2g | Sln 6 4g | Sln 7 4g, 4g | Sln 8 8g | Sln 9 8g, 8g |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0,0 | 0.0 |
| 30 | 2.0 | 2.3 | 2.1 | 2.2 | 2.9 | 2.9 | 3.1 | 3.7 | 3.6 |
| 60 | 2.3 | 2.4 | 2.3 | 2.3 | 2.8 | 2.9 | 3.0 | 4.0 | 4.0 |
| 90 | 2.3 | 2.4 | 2.3 | 2.5 | 2.9 | 3.2 | 3.2 | 4.1 | 3.9 |
| 120 | 2.3 | 2.5 | 2.3 | 2.3 | 3.1 | 3.2 | 3.9 | 4.1 | 5.3 |
| 150 | 2.5 | 2.5 | 2.3 | 2.6 | 3.1 | 3.2 | 4.0 | 4.1 | 5.3 |
| 210 | 2.4 | 2.7 | 2.5 | 2.7 | 3.2 | 3.2 | 4.0 | 4.2 | 5.3 |

*Sln = solution

Table 7: Volume of juice extracted during both experiments

| Table 3. Volume of juice extracted by both series of tests | | | | |
|---|---|---|---|---|
| Heat Treatment | Sln 2 (1:2) | Sln 2 (1:4) | Sln 3 (1:8) | |
| Volume extracted (ml) | 25 | 200 | 250 | |

| Enzymatic Treatment | Sln 1 0g | Sln 2 0.025g | Sln 3 0.1g | Sln 4 0.4g | Sln 5 2g | Sln 6 4g | Sln 7 4g, 4g | Sln 8 8g | Sln 9 8g, 8g |
|---|---|---|---|---|---|---|---|---|---|
| Volume extracted (ml) | 350 | 350 | 350 | 350 | 350 | 350 | 350 | 375 | 375 |

Table 8: Sugar concentration and volum of juice extracted at room temperature from samples of different water-to-pomace ratios

| Ratio (mass pomace/mass water) | | Sugar concentration plateau (120min-210) average | | Juice extracted plateau (120min-210) average | |
|---|---|---|---|---|---|
| 0.50 | ±0.02 | 0.049 | ±0.003 | 50 | ±5 |
| 0.25 | ±0.01 | 0.024 | ±0.001 | 350 | ±13 |
| 0.13 | ±0.01 | 0.015 | ±0.002 | 800 | ±13 |