Enzyme Treatment of Wheat Bran to Release Antioxidants and Combination Processing to Further Enhance this Release.

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Abstract

Whole grains have long been associated with good health due to the complex carbohydrates, vitamins, and other nutrients such as antioxidants, found within them. It is this last class that has the most untapped nutritional power when looking at whole grain products. This is because a majority of the antioxidants found are in the insoluble bound form and are not bioavailable to the consumer. Treating whole grains with multiple processes, including physical, chemical, and biological, can release some of these nutrients into a free or soluble bound form that is much more bioavailable, making the whole grain products much more beneficial to health.

Treating wheat bran with different individual and combinations of enzymes over varying lengths of time and at different concentrations was done in a 3x4 factorial to show which combination led to the highest release of the indicator phenolic compound- ferulic acid. The information from enzyme optimization was then combined with multiple pre-processing steps including a NaOH soaking period, high shear mixing, and high pressure homogenization to provide the greatest increase in ferulic acid release, as well as the largest increase in viscosity, which is a positive driver for functionality in whole grain products.

Results of the enzyme optimization study showed the highest release was almost always found at the highest concentration and time combination- 1.0% and 48 hours. Three individual enzymes showed high release- Xylanase BX-AN, S and Pentopan. The testing done on combinations of enzymes did not show a significantly larger release than any individual enzyme and so a synergistic effect among enzymes was not found.

When testing the enzyme with the pre-processing steps there was a significant increase in the release of ferulic acid. The NaOH soaking treatment proved extremely effective in aiding in release of ferulic acid. The high pressure homogenization treatment provided a dramatic increase in viscosity. Overall, the combination of treatments gave the highest release of ferulic acid and the largest increase in viscosity.

A preliminary animal study was done that showed the use of treated wheat bran gave an increase in intestinal contents viscosity, which leads to cholesterol lowering. A longer 12 week study by another graduate student is planned to confirm these findings.

Table of Contents

List of Tables:

List of Figures:

1 Literature Review

1.1 Whole Grains and Health

The food guide pyramid has a suggested intake of 6-11 servings of grains, with a minimum of 3 being whole grains. This recommendation is seldom met, with less than 10% of Americans reaching that goal. This is unusual considering the general knowledge about whole grains being a healthier option due to the fiber and nutrients remaining in the whole grain as compared to white flour products. However, people continue to choose white flour or other refined grain products due to the accepted appearance, texture, and taste they've grown accustomed to (Marquart et al. 2007).

There is increasing evidence that consumption of whole grains has many health benefits including the reduced risk of many chronic diseases. These include cancer (Jacobs et al. 1998), coronary heart disease (Steffen et al. 2003), diabetes (Fung et al. 2002), and obesity (Koh-Banerjee et al. 2004). Because some of these findings are based upon clinical studies, the US Food and Drug Administration allow for certain health claims to be made on certain whole grain products.

The whole grains council states that to qualify as a whole grain product, it must contain all the parts of the grain (bran, germ and endosperm) (Whole Grains Council 2004). The wheat berry, or kernel, is roughly 80% starchy endosperm, 15% bran, and 5% germ. Within the bran and germ lies the overwhelming majority of phytochemicals present in the kernel. These phytochemicals are the key to the health benefits of whole grains because they provide more than just nutrition. A small molecular weight subset of phytochemicals is antioxidants, which include carotenoids, tocopherols, lignans, and phenolic acids. These antioxidants provide protection to the body by protecting important molecules, such as DNA, from oxidative damage through various mechanisms (Yu 2008).

Although all of these beneficial compounds are present in whole grains, they are not always available to the body when food is ingested, or during the digestion of the food. Approximately 75% of the phenolics are found in an insoluble bound form,

and they avoid stomach or intestinal digestion/absorption to reach the colon intact (Yu 2008). This leads to two very important classifications when discussing antioxidants, bioavailability and bioaccessibility. Bioavailability is the degree and rate at which a substance is absorbed into a living system, or is made available at the site of physiological activity. Bioaccessibility is the amount of an ingested substance that is available for absorption in the gut after digestion (Hedrén et al. 2002). So this means that not only do these important nutrients have to be freely available in sufficient quantities to the body, but they must be in a form that allows them to be absorbed by the body.

Clearly multiple factors affect the overall health benefits of whole grains, including the processing the grain undergoes, and the food matrix it is presented in. Because there is such a wealth of nutrients that are unavailable to the body, work is being done to release these nutrients and make them both bioaccessible and bioavailable. The following pages discuss important aspects of whole grains, and sheds insight on to why this work is being undertaken, and what is essential to its success.

1.2 Wheat Kernel Structure

As mentioned above the wheat kernel can be separated into the endosperm, bran, and germ. The most crucial fraction to health, the bran, is approximately 15% of the kernel's weight. It is made up of many layers that protect the bran from the harmful effects of multiple sources- including digestion from animals, insects, bacteria, severe weather, etc… The endosperm, which is 80% of the kernel's weight, is mainly starch and some protein, but has almost no antioxidants present (less than 0.5% of total). The germ, which is 5% of the weight, is removed because its fat content can lead to lipid oxidation and rancidity. **Figure 1.1** indicates the three main components, and the additional layers of the bran.

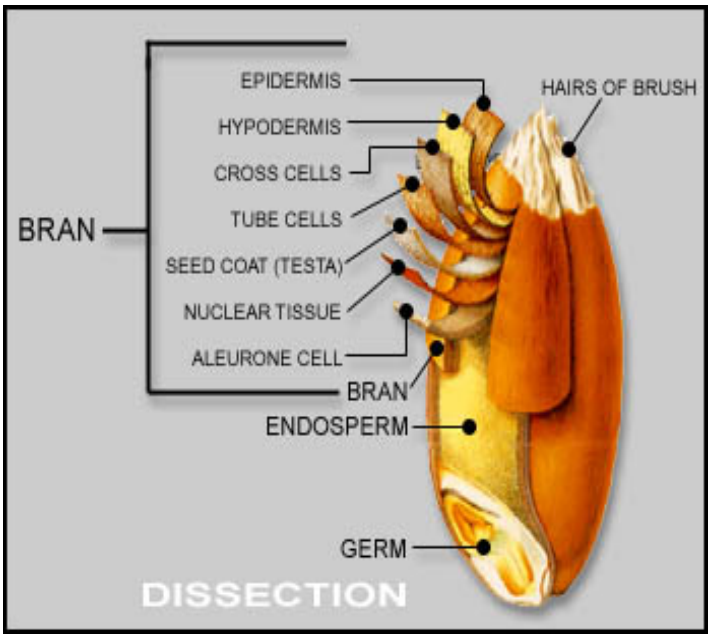

Figure 1.1: Wheat Kernel Cross-section (The New Zealand Institute for Crop & Food Research Limited 2002)

1.2.1 Bran Layer Identification

It is important to provide details on specific layers of the bran because the majority of phenolics are found in certain ones. In **Figure 1.2** the bran layers have been separated into **four** groups through tissue dissection at natural boundaries by Parker et al. (2005): Beeswing bran (**BWB**) consisting of the outer pericarp layers including brush hairs, **Cross cells**, **Testa + NE** (consisting of the pigmented testa and the nuclear epidermis (NE, hyaline layer)), and the single layer of **aleurone** cells. The aleurone layer contains the majority of phenolics as seen in **Figure 1.3**, they are evident because phenolics fluoresce (an important trait crucial to its measurement) and the brightest sections signify the highest concentration of phenolics.

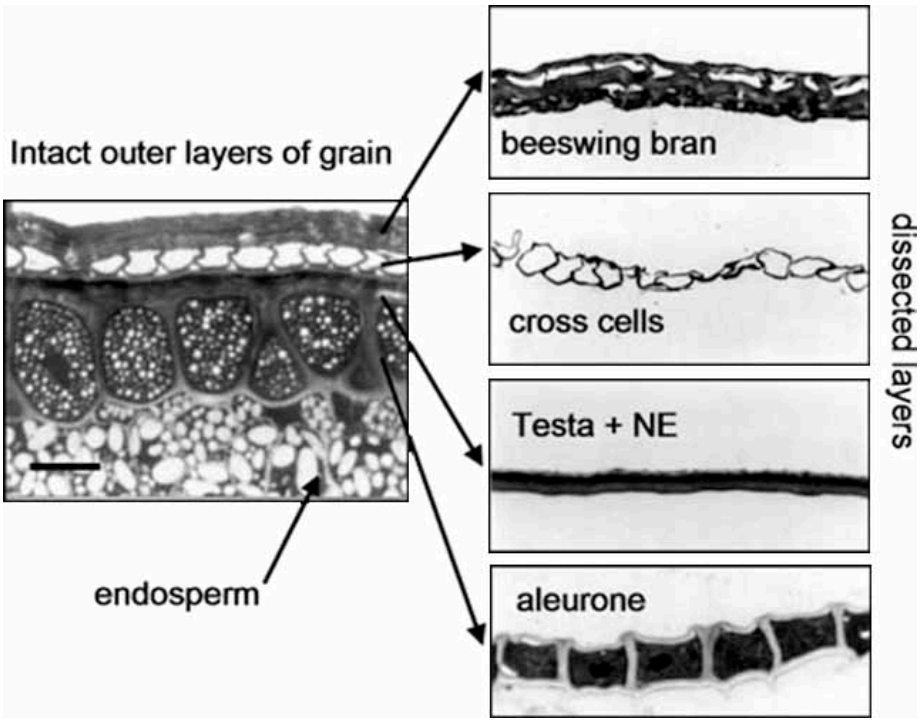

Figure 1.2: Separation of bran layers in wheat bran (Wheat Antioxidants, Yu 2008)

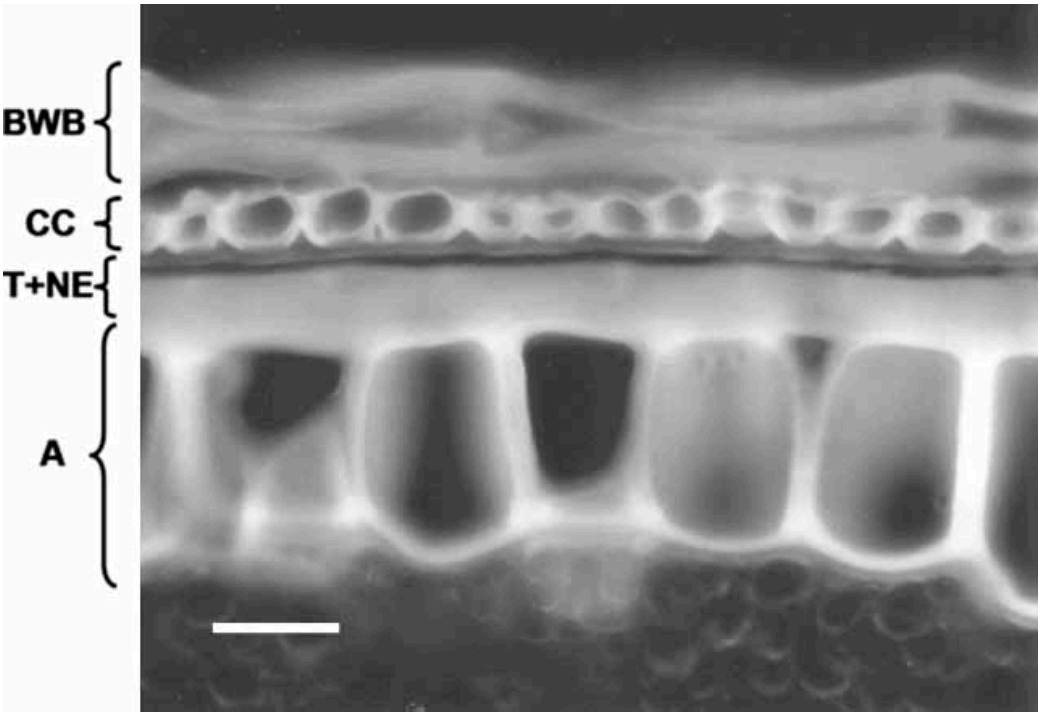

Figure 1.3: Fluorescence micrograph of bran layers (Wheat Antioxidants, Yu 2008)

1.2.2 Arabinoxylan Structure

Wheat bran is mainly composed of cell wall polysaccharides, among which xylans represent 40% of the dry matter (Thiago and Kellaway 1982), as well as β-glucans, xyloglucans and cellulose. Arabinoxylans are the main non-starch polysaccharide of cell walls of wheat. They consist of a linear backbone of (1→4)-linked β-D-xylopyranosyl units, substituted with monomeric α-L-arabinofuranosyl units by 1, 2- and/or 1,3- α-glycosidic linkages. **Figure 1.4** below shows a small section of the arabinoxylan backbone.

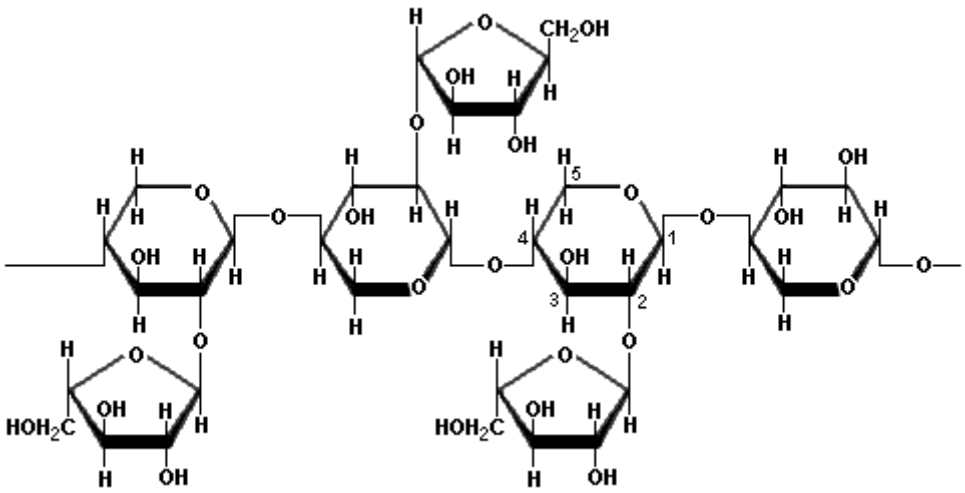

Figure 1.4: Section of Arabinoxylan showing substituents (Carbohydrate Polymers, Schooneveld-Bergmans 1998)

Arabinoxylans are either water-extractable (WE-AX) or water-unexctractable (WU-AX). These two types have very different physiochemical properties; WE-AX can give a very viscous solution while WU-AX has a large water-holding capacity (Meuser et al 1986). Because we are looking for a final product that exhibits both of these properties it is important to have both in the product. WU-AX can be partially solubilized if treated with alkaline. Differences in the water extractability of these polysaccharides has been thought to be attributed to multiple factors including the variations in the substitution patterns and resulting physical entanglement, covalent

ester bonding between carboxyl groups of uronic acids and the hydroxyl groups of arabinoxylan, as well as diferulic acid bridges between adjacent arabinoxylan chains (Izydorczyk & Biliaderis 1995) . The spatial arrangement of the substituents as well as degree of branching and ferulic acid content can also attribute to the visco-elastic properties of gels formed by wheat bran.

A unique feature of arabinoxylans is the presence of ferulic acid, the main phenolic acid found in plants and is usually reported in its *trans*-isomeric form for wheat bran. Ferulic acid is associated to arabinoxylans covalently through an ester linkage, this ester bond occurs through the acetylation of an acidic group with the primary hydroxyl at the C5 position of α-L-arabinofuranosyl residues (Ishii 1997). The diferulates in wheat bran can also be found in the 5,5' and 8,5' derivatives (Garcia et al. 1997). These ferulates can form either ester or ether linkages to attach to the arabinoxylan backbone and provide a convenient mechanism to cross-link the polysaccharides; this changes the overall structure and some of the mechanical properties (Grabber et al. 2000). These cross-linkings' also interfere with the attachment of ruminant bacteria to plant cell walls thus making the graminaceous matter less susceptible to digestion (Izydorczyk & Biliaderis 1995). This results in significantly limited digestion of complex plant materials by ruminants and therefore minimal breakdown when humans ingest the plant material. Breaking down the polysaccharide cross-linking through physical, chemical and/or biological processes frees the phenolics esterified to the arabinoxylan backbone. Those processes are crucial to making the phenolics and other antioxidants available to the body during digestion.

1.3 Wheat Antioxidants

There are a multitude of phytonutrients found in wheat but the most important groups are phenolic acids, carotenoids, tocopherols, lignans, inulin, and β-glucans. These important compounds are mainly found in the bran and germ (Fulcher and Rooney-Duke 2002). There has been research on all of these compounds but

phenolics have been the focus of hundreds of studies due to the high concentration in wheat. The bound phenolics constitute up to 90% of the total phenolics in wheat, and can be separated into two groups- hydroxycinnamic acids and hydroxybenzoic acids, based on differences in their chemical structures (Yu 2008). These two groups contain ferulic, vanillic, *p*-coumaric, protocatechuic, syringic, *p*-hydroxybenzoic, caffeic, gentistic, and cholorogenic (Yu 2008). All of the structures of these phenolics can be found in Appendix A.

Ferulic acid is present in the highest concentration and therefore has been used as a benchmark for quantifying the release of antioxidants. This allows the researcher to compare the efficiency of different processing techniques used to liberate the phenolics. Also present in the bran and germ are amino acids, high levels of B vitamins (thiamin, niacin, pantothenic acid and riboflavin), and some minerals (calcium, iron, sodium, magnesium, and phosphorus). All of these phytonutrients contribute to the health benefits of whole grains. Numerous mechanisms have been presented to show the anti-carcinogenic effect (Wattenberg 1985) and other health benefits such as lowering cholesterol (Yu 2008) of whole grains. However, the availability of these nutrients is a crucial factor when determining how effective the nutrients will be at promoting health benefits.

1.3.1 Antioxidant Availability

As mentioned earlier, most grain products made from wheat are mainly refined white flour products containing only the endosperm. Liyana et al. (2006) showed that through pearling (stripping away the bran layer) the antioxidant capacity of the bran was greatly reduced, ending in refined flour that consisted mainly of endosperm and had minimal antioxidant activity. This is because the bran and aleurone layers of wheat that are being removed contain the highest concentrations of phenolics. A majority of these phenolics are found in the insoluble bound form and their amount is greatly underestimated in literature. This is because these bound phenolics are esterified to the cell wall polymers and cannot be absorbed in the

gastrointestinal tract. However, after entering the colon, the microbial enzymes found there, such as xylanase and esterase, solubilize and release feruloylated oligosaccharides or free ferulic acid (Kroon et al. 1997)

Of the many factors that affect food nutrient availability for absorption in the gut, the following are the most important: processing conditions, chemical state of the phytonutrient, release from the food matrix, possible interactions with other food components, presence of suppressors or cofactors, formation of stable compounds, and the food microstructure (Parada and Aguilera 2007). The effect of the bran microstructure was illustrated best by Adam et al. (2002) when they compared the absorption of ferulic acid in rats when perfused with either free ferulic acid or in a cereal product. They found that 56.1% of the ferulic acid was absorbed into the bloodstream and 49.9% was distributed to peripheral tissues as conjugate forms when fed the free ferulic acid. When fed just the cereal the concentration of absorbed ferulic acid was 83.5% lower, and more than 38% of the ingested ferulic acid from bran was excreted in the fecal matter (Adam et al. 2002). This shows the importance of presenting these anti-oxidants in a form that is compatible with our biological systems.

1.4 Measurement of Phenolics

It has been shown that wheat may provide a significant amount of dietary phenolic acids, with one gram of grains providing as much as 0.5 milligrams of total phenolic acids (Moore et al. 2005). However, the phenolic contents in wheat have been underestimated in literature because such a large amount (~90%) are bound and are not able to be directly extracted without specific treatments. There have been many methods developed to quantify the phenolics liberated from wheat bran, two of which were used in this research.

Previous methodology only used various aqueous solutions of methanol, ethanol, and acetone to extract soluble phenolics for HPLC measurement (Liyana et al.

2006). This methodology ignored phenolics found in forms besides free-, esterified, and insoluble bound. It is important to use different methodologies depending on which form of phenolics you want to quantify. There are numerous methodologies used to quantify free phenolics, besides Liyana et al.'s, Yuan et al. (2005)used a spectrometry method taking advantage of the phenolics ability to fluoresce. The concentrations of feruloyl oligosaccharides were determined at 285 and 325 nm for both free ferulic acid and esterified ferulic acid using a UV-1000 UV/VIS spectrophotometer (Yuan et al. 2005).

This researcher used the spectrophotometric methodology of quantifying phenolics. This method was chosen over the more precise HPLC methods because the goal was not to find the exact amounts of ferulic acid released, but instead a more general estimation. This is because the enzyme processing will be combined with pre-processing steps to discover which combination of treatments works synergistically to yield the highest overall release of phenolics, as well as effect positive changes in the viscosity of the wheat bran. Therefore, a less rigid method that was quick was preferred. This allowed the research to move forward faster with the enzyme optimization as well as the overall processing optimization.

1.5 Processing Technologies

Since people have harvested wheat they have processed it on some level, the most rudimentary being grinding or milling it to make flour for all kinds of products. But as technology improved, new processing techniques arrived bringing more possibilities for extracting all of the nutrients locked in the wheat kernels. The most basic improvements in processing included not only more thorough physical processing, but chemical and heat treatments as well. These treatments have allowed researchers to alter the structure and properties of wheat bran making a larger portion of the phytonutrients more available.

1.5.1 Physical Processing

Physical processing has progressed past simple milling and includes both wet and dry processing where the focus is to reduce particle size as much as possible. This decrease in particle size breaks open the structure and can release nutrients by physically breaking the bonds holding them to the carbohydrate backbone. This increases the number of sites on the milled bran that enzymes can act upon once in the gastrointestinal tract. Nystrom et al. (2007) reported that a fine particle size is crucial to releasing plant sterols. Only cryogenic grinding with liquid nitrogen yielded a high release of plant sterols when compared to milling with a centrifugal mill (Nystrom et al. 2007). Wet processing has also been shown to be effective at reducing particle size. One such form is high pressure homogenization (HPH), it has been shown to be extremely effective in reducing particle size and also prompting changes in the functional properties of the wheat bran. Chau et al.(2006) reported that HPH alone produced dramatic increases in water holding capacity, swelling capacity, oil-holding capacity, and fiber solubility.

The goal of processing and reducing the particle size is to increase the bioavailability of the phytonutrients present in the wheat bran. Yu and Kies(1993) showed better apparent bioavailability was achieved with finely ground, dry-milled maize bran versus the other test brans (finely ground, wet-milled maize bran; coarsely ground, wet-milled maize bran; finely ground, dry-milled corn bran; and coarsely ground, dry-milled maize bran). The increase in bioavailability may be accredited to damaged cells and an increase in the surface area of the finely milled particles (Yu and Kies 1993). However, there is still a large amount of nutrients remaining in the finely ground particles and further treatments are needed to release them.

1.5.2 Chemical

Chemical treatment of wheat bran often entails either an alkaline or acid hydrolysis. The acid or base breaks the ester linkages binding the phenolics to the arabinoxylan backbone, releasing a large portion of the insoluble bound fraction. Kim

et al. (2006) treated wheat bran samples with 2 M NaOH and 6M HCl (repeated with acid first, followed by alkaline) to compare the release to traditional methanol extractions. They found that the quantity of bound phenolic contents was considerably higher than the methanol extracted phenolic contents. This signifies that the major phenolic acids in wheat bran are not extractable by methanol. Although both acid and alkali were successful in releasing bound phenolics, the alkali treatment released a higher amount of free ferulic acid. This was further illustrated when the alkaline hydrolysable fraction had greater antioxidant activities than the acid hydrolysable fraction (Kim et al. 2006).

It has been shown that the free and soluble extract can provide some antioxidant activity, but the insoluble bound fraction contains the most activity in wheat bran. In a study looking at the importance of insoluble bound phenolics to the antioxidant properties of wheat, Liyana-Pathirana and Shahidi (2006) confirmed the findings on the alkaline treatment of wheat bran and the value of the insoluble bound fraction. They tested the antioxidant activity of the hydrolyzed bound fraction and found that the total antioxidant capacity was 89.6% of the total, an overwhelming majority.

Although the bound fraction contributes the highest antioxidant activity, there is an increase in the release of free and soluble conjugate phenolics through chemical treatments as well. Ohta et al. (1997) showed that both free and the soluble conjugate phenolics are absorbed and bioavailable when administered to rats and in an LDL oxidation system. Both types of phenolics are important to have in solution when considering the health benefits given by these anti-oxidants.

1.5.3 Thermal

Thermal treatments can vary from traditional commercial processes, such as baking, retort, or extrusion, to industrial processes like steam explosion, which is currently used in the paper industry. With wheat bran, thermal processes are often a pretreatment or intermediary step, before more effective treatments. However it is

important not to expose the wheat bran to an extremely high temperature for too long. Such actions may result in lowering antioxidant activity because the heat may accelerate oxidation and other degrading reactions affecting the antioxidants. This was shown when wheat bran underwent heat stress treatments at 100˚ and 140˚C. The results showed a dramatic decrease in the scavenging capability of the antioxidants and a decrease in the total phenolic content (Cheng et al. 2006).

Extrusion processes can modify the phenolic content, arabinoxylan release and also affect the soluble and insoluble dietary fiber. Figueroa et al. (2004) tested high energy extrusion and high shear mixing combined with an enzyme treatment to solubilize arabinoxylans. It was found that the extrusion process gave the best solubilization rate and that the high shear mixing treatment ultimately did not improve the arabinoxylan solubilization as expected (Figueroa et al. 2004). Soluble and insoluble dietary fibers are important because they lead to an increase in bile acid binding, which leads to lower cholesterol. Kahlon et al. (2006a) researched the effects of different cereal grains on in vitro bile acid binding. They concluded that only high viscosity beta glucan soluble fibers in oats and barley contributed to a significant increase in bile acid binding. There are many factors that affect bile acid binding but viscosity is a functional property that is important when evaluating processing technologies (Kahlon et al 2006a). If results are to be validated then in vitro studies must be taken to the next level and human or animal studies must be done to prove the results. Kahlon et al.(2006b) fed hamsters extruded wheat bran, and although the variability left a majority of their results not significant, comparing the results between animals reduced the variable significance for blood lipids and cholesterol; and the total liver lipid and total liver cholesterol were considerably lower (23-35% and 28-31% respectively) (Kahlon et al. 2006b). This shows the positive effect extruded wheat bran can have on biological systems.

Another method is hydrothermal processing which often appears as steam or flash explosion; this has been a process in use for years in the paper and potato industries. It involves heating a material with high pressure steam and then releasing

the pressure in an instant. The moisture in the sample flashes to steam and physically breaks open the fibers of the material. This process can be extremely beneficial when used as a pretreatment step before chemical or biological processing. Because the structure has been opened up and is porous there is more surface area for reagents to react.

Schooneveld-Bergmans et al. (1998) researched the effects of reaction time (5, 10, and 15 min.) and temperature (180˚C, 190˚C, and 200˚C) of destarched wheat bran on the water extractable glucuronoarabinoxylans and the ferulic acid content of the bran. They found that they were able to free 19-28% of the glucuronoarabinoxylans present in the wheat bran. The esterified ferulic acid bound to the arabinoxylans is not lost during the process but remains bound. However, they were able to extract up to 6 times the quantity of ferulic acid released in untreated bran (Schooneveld-Bergmans et al. 1998). These findings show the great potential this form of treatment can provide, especially when used in combination with more site specific treatments, such as biological processing.

1.5.4 Biological Processing

Biological processing uses enzymes to break down the arabinoxylan backbone and cleave the ester linkages to liberate both free phenolics and soluble conjugated phenolics. When focusing on phenolics there are many enzymes to be considered but the two most common are xylanase and ferulic acid esterase(FAE). FAE is expensive and not commercially available so it is not often used. Therefore a lot of work focuses on the use of xylanases and commercially made xylanase enzyme “cocktails” like Celluclast® from Novozyme®.

The term xylanase refers to enzymes that are capable of cleaving the 1,4-beta-D-xylopyranosyl linkages of the l,4-beta-D-xylans, namely arabinoxylan, arabinoglucuronoxylan, arabino-4-O-methy-D-glucuronoxylan and glucuronoxylan. Diverse forms of these enzymes exist, displaying varying mechanisms of action, substrate specificities, hydrolytic activities (yields, rates and products), folds and

physicochemical characteristics. Families 10 (EC 3.2.1.8) and 11 (EC 3.2.1.32) (http://www.cazy.org/Glycoside-Hydrolases.html) of the glycoside hydrolases are the most commonly employed when choosing xylanases. These families are mainly endo-xylanases and focus on xylanases that are either endo-1,4-β-xylanase or endo-1,3-β-xylanase, respectively.

Family 10 xylanases have higher molecular weights and more complex structures than Family 11. They exhibit greater catalytic versatility (lower substrate selectivity) and cleave the decorated regions of the arabinoxylan and are less hampered by the presence of 4-*O*-methyl-D-glucuronorate, acetate, and α-L-arabinofuranosyl substituents on the xylan backbone (Biely et al 1997). Recent research has shown that Family 10 enzymes have the capacity to cleave an arabinose residue at the -2 subsite, the -1 and +1, as well as the +2 and +3 subsites if unsubstituted (Fujimoto et al 2004). Because they can degrade the more substituted solubilized regions on the backbone they would produce a higher relative yield of FA-substituted products as compare to xylanases from Family 11. However, high levels of arabinose decoration along the arabinoxylan backbone can obstruct the action of the enzyme. The reaction for Family 10 xylanases is the endohydrolysis of (1→4)-β-D-xylosidic linkages in xylans. Another form of xylanase in Family 10 is exo-xylanase, the most common is xylan 1,4-β-xylosidase (EC 3.2.1.37). The reaction for this is the hydrolysis of (1→4)-β-D-xylans, to remove successive D-xylose residues from the non-reducing termini.

Family 11 xylanases preferentially cleave in unsubstituted regions of the arabinoxylan backbone and degrade more arabinoxylan from the insoluble fraction. The reaction for Family 11 is random hydrolysis of (1→3)-β-D-glycosidic linkages in (1→3)-β-D-xylans.

Arabinoxylans are best degraded by finding synergistic combinations of enzymes, however when you want to release specific compounds your selectivity needs to be sharper. For example you can degrade arabinoglucuronoxylans by using a combination of exo-glycosidases such as alpha-L-arabinosidases, alpha-D-

glucuronidases, beta-D-xylosidases, and the exo-and endo-acting xylanases. However, you would be degrading the structure far more than needed and would be solubilizing unfavorable components. Conversely, using both Family 10 and 11 enzymes might provide a synergistic effect for degrading arabinoxylans by producing certain specific arabinoxylosaccharide products and increasing the amount of ferulic acid associated xylans.

Because of this reasoning xylanases were the focus of this research as they are especially important because their effect on wheat bran is two-fold. First, the increase in extractable arabinoxylans produces changes in the physical characteristics of the bran (Trogh et al. 2004/11). Second, releasing insoluble bound phenolics increases the bioavailability and antioxidant activity of the bran (Napolitano et al. 2006).

1.5.4.1 Traditional Enzymes Employed

Various enzymes have been used to release phenolics, solubilize carbohydrates and dietary fiber, and in turn alter the physical characteristics of wheat bran to improve baking qualities. As previously mentioned a vast majority of experiments performed for this task have been xylanases. A lot of this work involved either individual enzymes or in combinations, many of which are pre-made commercial cocktails. Maes et al. (2004) used two endo-xylanases, one produced by *Bacillis subtilis* and another by *Aspergillus aculeatus,* to solubilize water-unextractable arabinoxylans. They showed the first was useful to convert insoluble fiber to soluble fiber (Maes et al 2004). Sorenson et al. (2002) found a synergistic effect between the commercial enzymes Ultraflo L and Celluclast 1.5L to hydrolyze arabinoxylan for improved utilization of wheat hemicelluloses in the ethanol fermentation industry. The samples were treated with a 10 wt% enzyme /substrate ratio at 50˚C at a pH 5 for 48 hours (Sorenson et al. 2003). Szwajgier and Targonski also used 5 commercial enzyme preparations (Celluclast®, Viscozyme®, Shearzyme®, Cereflo®, and Ultraflo®) to treat brewer's spent grain in order to release feruloylated arabinoxylans and free ferulic acid. They found that Viscozyme® and Shearzyme® resulted in effective release

of both ferulolyated oligosaccharides and free ferulic acid due to the ferulic acid esterase activity found only in these enzymes (Szwajgier and Targonski 2006).

Yuan et al.(2006) also used a xylanase produced by *Bacillus subtilis* to produce feruloyl oligosaccharides from wheat bran insoluble dietary fiber. The focus was to find the best possible combination of reaction temperature, pH, time, enzyme concentration, and substrate concentration for maximum oligosaccharide production. The optimum reaction conditions they found were 42˚C, pH 5.2, 35h, 4.8g/l enzyme concentration, and a 120g/l substrate concentration (Yuan et al. 2006). These feruloylated oligosaccharides were then used with rat erythrocytes to investigate the antioxidant activity and effects. They found that they efficiently protect the normal rat erythrocytes against hemolysis induced by free radicals and showed strong free radical scavenging activity (Yuan et al. 2005).

The use of ferulic acid esterase (FAE) has been proven to be very effective in releasing ferulic acid from wheat bran. Faulds and Williamson (1995) used an FAE from *Aspergillus niger* and a xylanase from *Trichoderma viride* to release a staggering 95% of the total ferulic acid. They showed that FAE could release ferulic acid on its own but the release was 24-fold higher in the presence of xylanase. Overall, they showed that the FAE and the xylanase work together to break down feruloylated plant cell-wall polysaccharides to give a high yield of ferulic acid (Faulds and Williamson 1995). A similar result was shown by McKinnon and Christensen (2005) using the same FAE and xylanase in addition to cellulase, endo-glucanase and β-glucanase. They increased the release of ferulic acid in order to improve the nutritional value of oat hulls by degrading the dry matter and lowering the digestion inhibition that occurs from cross-linking of polysaccharides (McKinnon and Christensen 2005). Because of the high cost of FAE it was not used for this research. Instead, the focus was on the xylanases that are available in larger quantities and are much less expensive.

1.5.4.2. Enzymes and Ingredient Functionality

All of these processes are used to release phenolics and increase the bioavailability of antioxidants. However it is important to consider other aspects besides antioxidant release; it is critical to retain ingredient functionality properties so product quality doesn't suffer. Trogh et al.(2004/11) utilized a xylanase enzyme during bread making to convert water-unextractable arabinoxylan to soluble arabinoxylan giving the bread an increased loaf volume. They showed that as the xylanase levels were increased the amount of arabinoxylan solubilized increased while decreasing the average molecular weight. This is vital because at low levels of enzymes the viscosity of bread dough was increased while at a higher level the viscosity was lowered due to the molecular weight reduction (Trogh et al. 2004/11).

1.5.5 Combined Processing

All of these individual processes are effective at releasing phenolics and increasing the bioavailability of antioxidants. When you combine one, two, three, or even four processes you can dramatically increase bioavailability and alter functional properties to be advantageous to your end product. It was shown that enzyme treated maize bran released 30% of the total ferulic acid, but with steam explosion pretreatment that number increased to 80% solubilized ferulic acid (Saulnier et al. 2001). Another combined treatment was done by Figueroa et al. combining an extrusion pretreatment with enzymatic solubilization to release a maximum amount of arabinoxylans (Figueroa et al. 2004). Nystrom et al showed that a combination of physical processing resulting in small particle size and enzymatic processing resulted in optimal sterol availability (Nystrom et al. 2007). Combining physical, chemical, thermal, and biological treatments is considered an effective way to release the maximum amount of phenolics and increase bioavailability. Using this approach an optimized process can be developed to release phenolics, increase bioavailability, and retain or improve functional properties to yield a wheat bran ingredient that can be used to produce new products or replace existing ones. This is the goal of the

following research- to combine multiple processes in the best combination to produce a unique wheat ingredient. This ingredient will not only have an enhanced phenolic profile, but these anti-oxidants in addition to the physiological changes in the wheat will provide a value-added product that delivers much more to the ever health conscious consumer.

2 Individual and Combination Enzyme Optimization

2.1 Introduction

It is widely known that whole grains should be included in a healthy diet because of the fiber and nutrients found in whole grain products. There are many health benefits that come from eating whole grains including reducing risk of many chronic diseases including cancer, coronary heart disease, obesity, etc… However, whole grains are still not the first choice when it comes to consumers selecting products- refined flour products are dominant in the market. With the wide range of products available it is becoming important to offer consumers value-added items. This means the products need to taste good, and provide above and beyond the basic nutrients. New technologies and ingredients in foods are answering the call for value-added products and that is what this study strived to offer.

Whole wheat products have a wealth of nutrients but unfortunately many of the beneficial compounds are never made available to the body. Wheat bran is the most beneficial fraction of whole wheat, it contains almost all of the phytonutrients found in wheat and is also the main source of fiber. However, many of these phytonutrients, such as phenolic acids, are covalently bonded to the arabinoxylan structure that makes up the bran. These bonds do not allow the nutrients to be freely available to the gastrointestinal system so they cannot be absorbed by the body and give all the health benefits the nutrients can provide. Different processing technologies can break those bonds and liberate the nutrients so they become bioavailable and bioaccessible.

One of these processes is using enzymes to degrade the arabinoxylan structure and release the phenolics. Popular enzymes used to free phenolics include xylanases, ferulic acid esterase, cellulases and others. Although ferulic acid esterase can directly break the ester bonds holding ferulic acid to the polysaccharide structure, it is extremely expensive and not commercially available. Xylanases are a great option because they break the bond between the xylopyranosyl backbone and the α-L-

arabinofuranosyl substituents on which the phenolics are esterified. This frees the insoluble bound phenolics and can also increase the free and soluble-conjugate phenolics fractions as well.

The objective of this study was to optimize an enzyme treatment of wheat bran that will increase the bioavailability and bioaccessibility of antioxidants in the wheat bran that are otherwise unavailable to the consumer during digestion. This optimization involves testing various xylanases and other enzymes at different concentrations and different lengths of time to find the combination of those factors that gives the highest release of phenolics. The enzymes that perform the best on an individual basis will then be used in 2- or 3- enzyme combinations to see if a synergistic effect can be found for a more effective release of phenolics. The phenolic acid ferulic acid is used as the baseline for judging release because it is present in the highest amount in wheat bran (~60% of all phenolics). Also, it is easy to measure using a spectrophotometer, which was the preferred method for this research.

2.2 Materials & Methods

2.2.1 Materials

2.2.1.1 Enzyme selection

In this study it was important to test multiple xylanase enzymes from different companies. This is because they may contain small amounts of other enzymes and therefore illicit different side reactions. Also, some cellulases and hemicellulases will be used as negative controls because they should not give a significant release of ferulic acid. This is because they work primarily on the cellulose backbone, and not on the xylan chains. A table of all the enzymes used can be found in **Table 2.1** on the following page. As mentioned above, the enzymes showing the highest release will be chosen for the combination testing.

Table 2.1: Table of all the enzymes tested, including their main activities, bacterial source and optimal pH and temperature ranges.

Enzyme	Provider	Main Activity	Side activities	Source	Optimal pH of main activity	Optimal temp. (°C)
Xylanase S	Enzyme Development Corp.	Xylanase	β-1,3 glucanase, galactomannase, and cellulase	*Trichoderma longibrachiatum*	4.0 - 7.0 (5.0)	45-55°C (55°C)
Xylanase AN 900	Enzyme Development Corp.	Hemicellulolytic preparation	Cellulase, some mannase	*Aspergillus niger*	4.5-6.0	30-45°C
Xylanase BX-AN	Enzyme Development Corp.	Combination of 2 xylanases	Some mannase, and β-glucanase	*Trichoderma longibrachiatum, A. niger*	4.0-5.5	40-60°C
Xylanase N	Sigma-Aldrich	Xylanase	N/A	*Thermomyces lanuginosas*	4.0-5.5	45-60°C
Grindamyl Powerbake 900	Danisco	Xylanase	N/A	*Bacillus subtilis*	4.0-5.0	40-55°C
Grindamyl Powerbake 950	Danisco	Xylanase	Some cellulase	*Bacillus subtilis*	4.0-5.1	40-60°C
Pentopan Mono BG	Novozyme	Xylanase	N/A	*Thermomyces lanuginosas*	4.0-6.0	45-55°C
Cellulase	Enzyme Development Corp.	Cellulase	N/A	*Aspergillus niger*	3.0-5.5	45-60°C
Hemi-cellulase	Enzyme Development Corp.	Beta-mannase	Cellulose and hemi-cellulase	*Aspergillus niger*	3.0-6.0	50-60°C

2.2.1.2 Wheat Bran selection

For this study King Wheat Bran (from King Wheat Milling Co.) was used for all enzyme testing. King wheat bran is a light soft white wheat bran.

2.2.1.3 Sodium acetate buffer

A sodium acetate buffer solution was used comprising of sodium acetate (Fisher), acetic acid (Fisher), and deionized water.

2.2.2 Methods

2.2.2.1 Wheat bran Preliminary Testing

There was preliminary testing of the wheat bran that included milling, moisture content, and particle size distribution.

1. Milling for Particle Size Reduction

   The King Wheat Bran was milled using a rotor beater milling technology (Retsch GmbH, Model SR 300, Haan, Germany) in order to reduce particle size to 0.5 mm and 0.2 mm. The 0.5 mm size was used for all testing because the smaller size did not increase in release of phenolics, and it also reduced processing to make the entire system faster and cheaper when considering a scale up to industry.

2. Moisture Content of duplicate samples was measured by AACC Moisture method 44-15A.

3. Particle Size Distribution

   Particle size was measured using a Laser diffraction particle size analyzer (Beckman Coulter, LS-13-320, Brea, California).

2.2.2.2 Sodium Acetate Buffer

To make the buffer, 0.6805g of sodium acetate was dissolved in 1000 mL of deionized water, and then 0.30025 mL of acetic acid was added while under stirring to achieve the desired pH of 5.23. These quantities were quadrupled to make a large stock of buffer used to prepare enzyme solutions as well as the samples.

2.2.2.3 Enzyme Solution Preparation

During the study, enzyme solutions were prepared at three different concentrations 0.1, 0.3, and 1.0%. The concentrations were calculated based

on the amount of wheat bran in the sample. For 4 g wheat bran samples the enzyme solutions were made as follows:

For Individual Enzymes:

0.1% = 4g x 0.001 = 0.004 x 12 (# samples in set) = 0.0048g enzyme powder dissolved in 48 mL buffer while being stirred (4 mL aliquot for each sample)

0.3% = 4g x 0.003 = 0.012 x 12 = 0.144g enzyme powder/ 48 mL buffer

1.0% = 4g x 0.1 = 0.04 x 12 = 0.48g enzyme powder/ 48 mL buffer

For Combination of Enzymes:

The concentrations are prepared in the same way for each level, and the total amount of enzyme solution to be added is split amongst the enzyme solutions. So if 4 mL of 0.3% enzyme solution needs to be added and you're mixing Xylanase S and BX-AN, you add 2 mL each of 0.3% S and 0.3% BX-AN for a total of 4 mL.

2.2.2.4 Sample Preparation

For Wheat Bran

1. 1 lb of King Wheat bran is milled to a 0.5 mm particle size
2. 4.408g (4g dry matter based on 10.211% moisture content) was weighed into a 50 mL centrifuge tube.
3. Samples were then autoclaved (Consolidated Steromaster MIC II Autoclave, Boston, Massachusetts) for 30 min at 15 psi and 121°C to kill any residual bacteria in the wheat bran.
4. After samples cooled to room temperature 36 mL of the sodium acetate buffer is added, followed by 4 mL of the appropriate enzyme solution under a sterile hood to prevent contamination.
5. Samples are placed on the Innova Incubator Shaker (Enfield, Connecticut) at 50°C and 220 rpm.

6. Samples are removed after the appropriate length of time, either 12, 24, 36, or 48 hours.
7. Immediately after removal from the incubator shaker the samples are centrifuged at 680xg for 20 minutes.
8. After centrifugation the supernatant is poured off into a correspondingly labeled 50 mL centrifuge tube and placed in the -80°C freezer until solid.
9. Tube caps are removed and the sample is covered with parafilm and perforated, and placed back into the freezer to ensure the sample is frozen.
10. Frozen samples are freeze dried (Virtis Freezemobile 12EL, Warminster, Pennsylvania) using an Edwards Ultra Grade 19 Vacuum pump VWR(Sanborn, New York) until samples were completely dry.

For UV spectrometry measurement

1. Freeze dried samples are reconstituted with 10 mL deionized water in their 50 mL centrifuge tubes.
2. A 0.3 mL aliquot of the reconstituted sample is pipette into a 2.0 mL centrifuge tube containing 1.2 mL of deionized water to give a 1.5 mL sample.
3. Sample cap is closed and then the sample is vortexed for 10 seconds to thoroughly mix the sample.
4. Steps 2 & 3 are repeated two more times to give a total of 3 dilutions.
5. The final 1.5 mL diluted sample is poured into a cuvette and placed in a Spectronic Genesys 5 spectrophotometer (Thermo Fisher Scientific, Madison, Wisconsin) that has been zeroed with deionized water.
6. The absorbance at 280 and 325 nm is then read and recorded for each sample within one hour of prepping samples.

2.2.2.5 Statistical Analysis

The data was analyzed using R version 2.8.1 and was in the form of an Analysis of Variance (ANOVA). An example of the output data is shown below:

```
> p<-lm(ffa~frep+fconc+ftime+fconc*ftime)
> anova(p)
Analysis of Variance Table
Response: ffa
```

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
freps	2	563	281.5	0.5752	0.57085
fconc	2	17099.2	8549.6	17.4672	2.868e-05 ***
ftime	3	5526.8	1842.3	3.7639	0.02544 *
fconc:ftime	6	26006.3	4334.4	8.8553	5.670e-05 ***
Residuals	22	10768.3	489.5		

```
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

The data from the ANOVA was used for a least significance difference test (LSD) which results in a means separation test. An example of the output data is shown below for concentration:

conc

t(0.025,12)=2.56 TINV(0.025,12)=2.560033

LSD= $\text{sqrt}(2MS_e/n) = \text{sqrt}(2*489.5/12) = 9.03$

2.56*9.03 = **23.1 = LSD**

This value is then used when comparing the means of the different treatments to see if they are significantly different or not. The data tables for all of the enzymes show the LSD value and whether or not the different treatments (time and concentration) had an effect.

2.3 Results and Analysis

2.3.1 Wheat Bran Preliminary Testing Results

2.3.1.1 Milling for Particle Size Reduction

The goal of reducing particle size prior to enzyme processing is to find the optimal range of size reduction that yields the best results when combined with further testing. This is done to keep overall costs at a minimum when considering the processing in combination. Multiple dry and wet milling processes were tested but the Retsch mill was found to be the most efficient. The wheat bran was able to be ground to 0.5 mm with just a single pass through the 0.5 mm screen size on the mill. Milling through a smaller size (0.12 mm) clogs the mill and requires time and energy to clean it out and continue milling. The 0.5mm particle size serves numerous functions in the research. Reducing the particle size through milling physically breaks apart the bran wall, thereby producing fractures in the cell wall structure creating more sites on the arabinoxylan backbone for the enzymes to act on, which can aid in increasing the overall release. Also, a small particle size is necessary for the combination processing.

2.3.1.2 Moisture Content

The moisture content was measured for the wheat bran so the actual dry matter amount could be used when calculating the amount of ferulic acid released from the bran. The average moisture content was found to be 10.2%, with a standard deviation of 0.01%, this means the bran is 89.8% dry matter.

When weighing out samples to achieve 4g bran dry matter, 4.408g of bran was weighed to account for the amount of water in the bran.

2.3.1.3 Particle Size Distribution

The average particle size was taken after milling to determine the particle size distribution. Having a starting particle size of less than 0.5 mm is important for the

second half of this research, when the enzyme optimization is combined with chemical and high pressure homogenization treatments. A particle size of less than 0.5 mm is required to not plug the reaction chamber in the homogenizer. The size distribution table below shows that a majority of the particles are less than 0.335 mm, within the range necessary for high pressure homogenization. **Table 2.2** shows the particle size distribution range for wheat milled through the 0.5 mm screen

Table 2.2: Particle size distribution for wheat bran milled through 0.5 mm screen

Fraction (overs)	Opening (mm)	Bran, 0.5mm %
30	**>0.6**	0.07%
45	**0.335-0.6**	11.27%
60	**0.25-0.335**	26.24%
80	**0.18-0.25**	26.42%
100	**0.15-0.18**	10.32%
120	**0.125-0.15**	19.16%
140	**0.106-0.125**	2.21%
pan	**<0.106**	4.31%
Total		100.00%

2.3.2 Enzyme Optimization Results

The focus of this research was to find the best enzyme(s) to release phenolic acids from wheat bran. Because the spotlight is on the enzymes and their relative release compared to one another, quantitative analysis of the samples was not necessary. Qualitative data gives a good estimate of how effective the enzymes were at releasing the phenolics, without needing exact quantitative data. As mentioned previously, the enzymes were tested individually and then in combination based on these results.

2.3.2.1 Standard Curve & Raw Data Analysis

The absorbance values taken for the samples were crucial for this research because the amount of free ferulic acid released can be calculated from them. This was done by running a standard curve of ferulic acid and using the equation from the trend line to calculate the amount from the absorbancies of unknowns. The standard curve was generated by making five known concentration of pure ferulic acid and measuring them at 325 nm on a spectrophotometer in triplicate, and then plotting the averages at each concentration. **Figure 2.1** below shows the standard curve for ferulic acid generated from those concentrations, and the resulting trend line and equation is shown on the graph as well.

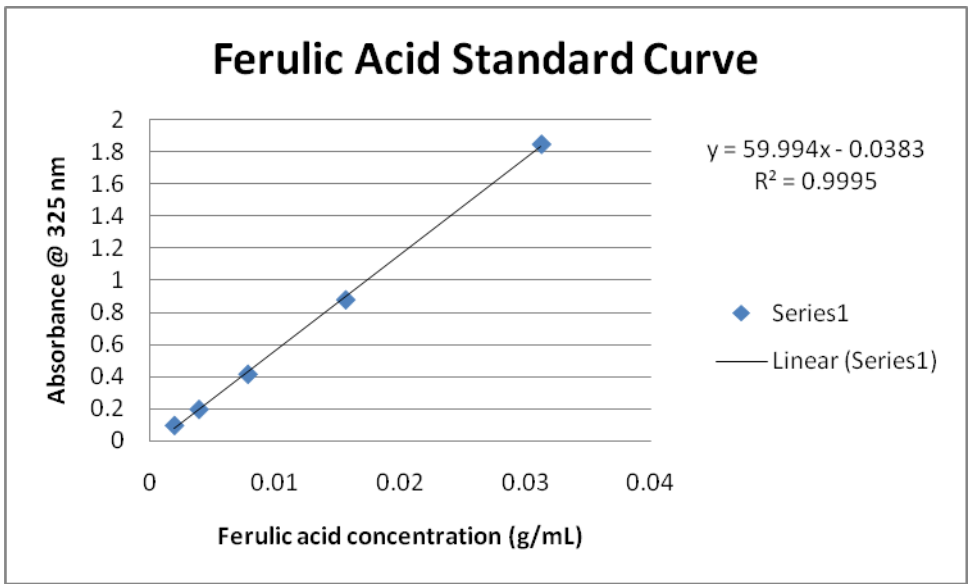

Figure 2.1: Ferulic acid standard curve created from averages of triplicate measurements for each point.

From the equation **y = 59.994x - 0.0383** (Equation 2.1), the amount of free ferulic acid, (x), is calculated, an example is shown below:

$$y=mx+b$$
$$x=(y-b)/m$$

m	b
59.99	0.0383

Example:
1. Solve for x using y=0.922 as the absorbance:

$$x = (0.922 - 0.0383)/ 59.99 = 0.0147 = \text{concentration (g/mL)}$$

2. Calculate bran concentration from dilutions:

We begin with 4.0g of bran as opposed to the measured amount of bran after the freeze drying step because the amounts were so small as not give accurate numbers. It was attempted to weigh every single 50 mL cylinder before and after, but the variation between cylinders, as well as the accuracy of the scale did not allow for consistent measurements. Due to these difficulties, all samples were based off the original 4.0g of bran used, and the same calculation was applied throughout as to keep all calculations consistent. This allows the data to be compared across all experiments within this research.

So, we start with 4.0g bran d.m., and after freeze drying, the material undergoes three 4:1 dilutions.

$$4.0/4/4/4 = \quad 0.0625$$

3. Calculate free ferulic acid per gram (f.a./g) using the example from step 1 and the bran concentration calculated in step 2:

$$0.0147/ 0.0625 = 0.2352 \text{ f.a./g}$$

This is multiplied by 1000 to give free f.a. milli eq./g:

$$x1000 = 0.2352(1000) = 235.2 \text{ free f.a. milli eq. /g}$$

***The milli eq./g is what is reported and graphed for all samples.**

The absorbance values collected for the samples were used for calculating the actual amount of released ferulic acid. A graph showing the values at each concentration and time (including the three replicates) is constructed for each enzyme. An example of a full set of data can be found in **Figure 2.2** below. It is an excellent example of the general trend found throughout this research that as both

the time and concentration increase, so will the release of phenolics, with the highest time (48 hours) and concentration (1.0%) showing the largest release. Full sets of the raw data and the finished graphs for all of the enzymes can be found in Appendix B.

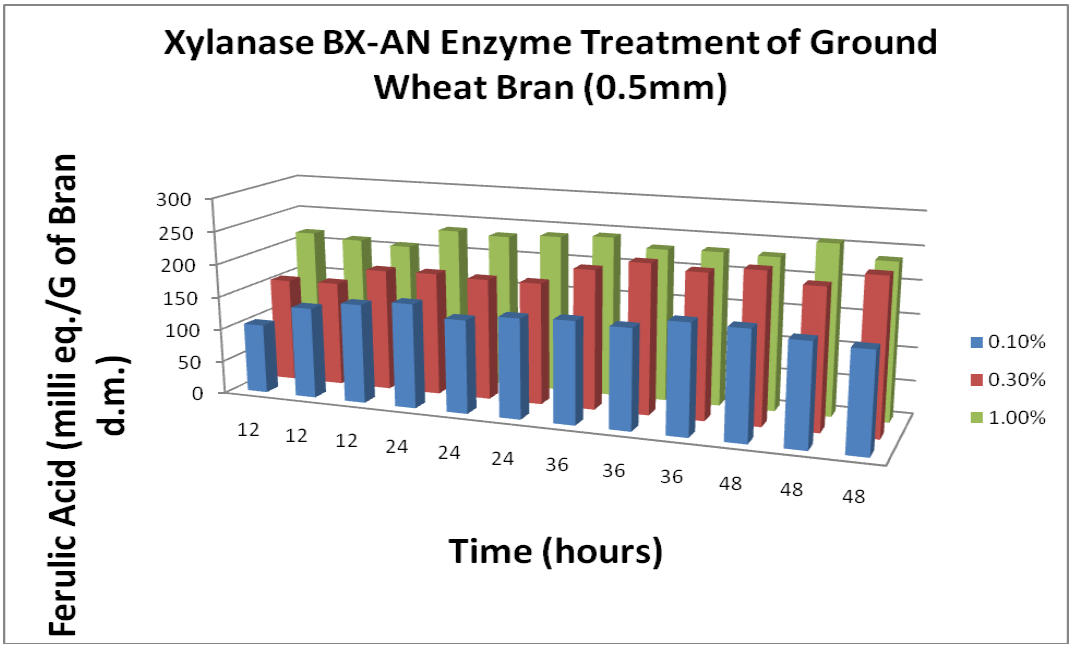

Figure 2.2: Example of a full 36 sample set for Xylanase BX-AN showing three concentrations and three times (samples done in triplicate).

Data was analyzed using R version 2.8.1 and was in the form of an ANOVA. This was performed on all the individual enzymes to see if time, enzyme concentration, or both were significant within the individual enzyme testing. Time and concentration were the dependent variables, and the amount of free ferulic acid was the response variable. Afterwards, a Mean Separation test was run comparing the samples to each other to see which was the most effective.

The four combination tests done after individual optimization were not analyzed. This is because the samples may have undergone degradation during freeze drying because they were subjected to two freeze-thaw cycles due to equipment malfunction. The resulting data is not consistent with past findings leading the

researcher to disregard it in the analysis and so it was not considered. **Table 2.3** below lists all the individual and combinations of enzymes tested in this study.

Table 2.3: List of all individual and combinations of enzymes tested in the study.

List of Enzyme(s) Tested
Individual
Xylanase AN 900
Xylanase BX-AN
Xylanase S Concentrate
Xylanase N
Pentopan Mono BG
Grindamyl Powerbake 900
Grindamyl Powerbake 950
Cellulase
Hemicellulase
Combination
BX-AN & S
BX-AN & Pentopan
Pentopan & S
BX-AN, S, & Pentopan

Table 2.4 shows the enzymes, concentrations and time combinations that gave the largest release of ferulic acid. With the first few sets of samples 0.3% was the highest concentration tested, but it was later switched to 1.0% for the remaining data. Therefore, if the sample has the concentration 0.3% listed as the highest concentration, it is because the sample was not tested at the 1.0% concentration. However, this did not pose a problem for comparing the enzymes because all were able to be compared at 0.3%, and the first few sets limited at 0.3% had the lowest release when compared to the remaining samples.

The xylanases S and BX-AN had the highest release for the individual enzymes at 248 and 259 F.A. milli eq./g respectively. While Pentopan also showed good release compared to the others with 204 F.A. milli eq./g . The table also includes the time at which all of these samples had the highest release which was 48 hours for all enzymes.

This would infer that the longest time is always going to be the most effective, however, the values in the table reflect the highest individual release value. The statistical analysis will show that both 24 and 36 hours also gave high releases, and in some cases were not significantly different than the 48 hours.

Table 2.4 also includes the control sample (with no enzyme) and the cellulase and hemicellulase samples. These were used as negative controls because they act differently on the arabinoxylan backbone than xylanases, they hydrolyze the 1,4-beta-D-glycosidic linkages in cellulose, which mainly liberates sugars, and not phenolic compounds. The values for those samples did not show any significant release of phenolics and illustrates that using enzymes from the xylanase class are crucial in releasing these phenolic acids.

Table 2.4: Maximum release of ferulic acid for each enzyme and the respective variables involved. To be able to compare all samples the 0.3% concentration and respective FA milli eq values are given, they are in parentheses when 1.0% was the highest concentration.

Enzyme	Conc. (%)	Time (hrs)	Ferulic acid milli eq./g wheat bran
Grindamyl Powerbake 900	0.3	48	102.19
Xylanase S Concentrate	1.0 (0.3)	48	248.21 (204.3)
Xylanase AN-900	0.3	48	116.49
Xylanase BX-AN	1.0 (0.3)	48	259.95 (210.34)
Pentopan	1.0 (0.3)	48	204 (132)
Grindamyl Powerbake 950	1.0 (0.3)	48	126 (76)
Xylanase N	1.0 (0.3)	48	130 (104)
Cellulase	0.3	48	68.6
Hemi-cellulase	1.0 (0.3)	48	21 (15)
Control	N/A	48	57.7

2.3.2.2 Statistical Analysis of Individual Enzymes

ANOVA's

An Analysis of Variance was performed on all of the individual enzymatic sets of data, focusing on whether time or concentration was significant. As mentioned before the amount of free f.a., the time, and enzyme concentration were the three variables used in the statistical analysis. The results varied widely among the enzymes and **Table 2.5** below shows the resulting significance of those three variables.

When looking at the table it can be seen that for xylanases N, S, and BX-AN, the concentration was significant with a *p*-value of ≤ 0.001. However, for xylanase AN-900, Grindamyl Powerbake 900, and the hemicellulase, the concentration was not shown to be significant. For the first three (N, S, & BX-AN) and hemicellulase, time was found to be significant. For xylanase N, the *p*-value was very low (0.001), but for S, BX-AN, and hemicellulase the significance value was at the highest level that is still significant (0.05). In preliminary testing, AN-900 and Grindamyl Powerbake 900 did not show strong release and time replicates were not done, therefore an ANOVA was not performed for their time variable.

Table 2.5: ANOVA for the individual enzymes showing the significance whether time and/or concentration affected the release of ferulic acid.

Enzyme	Time Significance	Conc. Significance
Xylanase N	$p \leq 0.001$	$p \leq 0.001$
Xylanase S	$p \leq 0.05$	$p \leq 0.001$
Xylanase BX-AN	$p \leq 0.05$	$p \leq 0.001$
Xylanase AN-900	N/A	Not significantly Different
Hemi-cellulase	$p \leq 0.05$	Not significantly Different
Grindamyl Powerbake 900	N/A	Not significantly Different

A mean separation test was performed after the ANOVA to see the differences from the time and concentration variables within the individual enzymes. Below are these concentration and time tables for each individual enzyme. When reading the tables, the 'Difference' column indicates whether the differences within the treatment variable (i.e. for time- 12, 24, 36, 48 hrs) are significantly different from each other. For example, if the letters (a, b, c, & d) are different for each individual time then one can assume they are all significantly different. However, if there are overlaps among the letters (i.e ab, bc, bcd etc…) then the times are not significantly different from each other. Through these tables conclusions can be surmised about which variable, time or concentration, is more important, and furthermore which can be manipulated to give optimal phenolic release.

For Xylanase BX-AN:

In Tables 2.6a & b if the same letters are present at the same treatments it means they are not significantly different from each other, if the letters are different they are significantly different.

Table 2.6a: Mean separation test for the three concentrations calculated from 3 replicates, degree of difference is $p \leq 0.001$.

Concentration	Mean (FFA milli eq)	Difference
1.00%	230.5	a
0.30%	195.1	b
0.10%	148.5	c

LSD = 23.6

Table 2.6b: Mean separation test for the four times calculated from 3 replicates, degree of difference is $p \leq 0.05$.

Time	Mean (FFA milli eq)	Difference
12h	173.03	a
24h	188.14	ab
36h	203.5	bc
48h	200.9	bc

LSD = 27.28

From **Table 2.6a** it can be seen that all three concentration levels are significantly different from each other with the highest concentration, 1.0%, having an extremely high mean value of 230.5 compared to the lowest concentrations' mean value of 148.5. **Table 2.6b** shows that time is not as important a factor as concentration. The key observation here is that that the 24 and 48 hour treatments are not significantly different, leading to the possibility of using a reduced time treatment (24 h). This would lower the overall costs and processing time for this type of treatment. However, it is important to note that while the three highest times (24, 36 and 48) were not significantly different from each other, the 36 hour treatment did have the highest mean value. This means that if time was not a limiting factor in the overall process you could do a longer treatment, yet the slight increase in yield may not be enough to warrant it, especially since it was not significantly different from the 24 and 48 hour treatments.

For Xylanase S:

In Tables 2.7a & b if the same letters are present at the same treatments it means they are not significantly different from each other, if the letters are different they are significantly different.

Table 2.7a: Mean separation test for the three concentrations calculated from 3 replicates, degree of difference is $p \leq 0.001$.

Concentration	Mean (FFA milli eq)	Difference
1.0%	153.9	b
0.3%	180.3	a
0.1%	126.9	c
LSD = 23.1		

Table 2.7b: Mean separation test for the four times calculated from 3 replicates, degree of difference is $p \leq 0.05$.

Time	Mean (FFA milli eq)	Difference
12h	138.9	bc
24h	156.8	ab
36h	146.9	ab
48h	172.1	a

LSD = 26.4

Once again all the concentrations are significantly different for xylanase S as shown in **Table 2.7a.** However, the difference for S is that its 0.3% concentration actually had the highest mean value. This is a departure from the general trend that the highest concentration will always yield the largest release of phenolics. The data from **Table 2.7b** exhibits a similar trend as BX-AN, with no significant difference between the 24 and 48 hour treatments. This reinforces the belief that time may not be as important when optimizing the final treatment procedure for the wheat bran.

For Xylanase N:

In Tables 2.8a & b if the same letters are present at the same treatments it means they are not significantly different from each other, if the letters are different they are significantly different.

Table 2.8a: Mean separation test for the three concentrations from 3 replicates, degree of difference is $p \leq 0.001$.

Concentration	Mean (FFA milli eq)	Difference
1.0%	112.2	a
0.3%	81.8	b
0.1%	57.8	c

LSD = 8.52

Table 2.8b: Mean separation test for the four times from 3 replicates, degree of difference is $p \leq 0.001$.

Time	Mean (FFA milli eq)	Difference
12h	64.3	c
24h	90.5	ab
36h	84.0	b
48h	96.0	a

LSD = 9.73

Xylanase N demonstrated similar trends as BX-AN for both concentration and time. **Table 2.8a** shows that all the concentration treatments were significantly different form each other with the 1.0% concentration having the highest mean value. The similarity to BX-AN continues when viewing the time treatments in **Table 2.8b.** The 24 and 48 hour times are not significantly different, but the 36 hour treatment is significantly different from the 48 hour treatment. This might be because xylanase behavior can be seen as a function of substrate solubility. The behavior towards insoluble and soluble substrates can be referred to as substrate selectivity of the enzyme, defined as the ratio of enzyme activity towards insoluble substrate over enzyme activity towards soluble substrate (Moers et al 2003). The enzymes ability to act on the substrate over time may vary- the enzyme can become inactivated, or there is an inability of the enzyme to attack the remaining polysaccharide through steric hindrance. However, these can be overcome as the breakdown of the arabinoxylan backbone continues and more subsites are exposed for the enzymes. This could be part of the reason for the variations in the release at 24, 36, and 48 hours.

An established trend seen with all the enzymes so far is that it is necessary to treat for longer than 12 hours. All the enzymes have confirmed this, showing a significant difference between the 12 hour time treatment and all the other time treatments.

For Hemicellulase:

In Table 2.9 if the same letters are present at the same treatments it means they are not significantly different from each other, if the letters are different they are significantly different.

Table 2.9: Mean separation test for the four times from 3 replicates, degree of difference is $p \leq 0.05$.

Time	Mean (FFA milli eq)	Difference
12h	12.0	bc
24h	16.1	ab
36h	10.5	c
48h	18.0	a

LSD = 5.12

The concentration was not found to be significantly different when the ANOVA was executed so a mean separation test was not performed. However, there was a significant difference among times, again the 24 and 48 hour times were not significantly different, but the 48 was different from the 12 hour treatment. It is not surprising that the concentrations were not significantly different because this enzyme was used as a negative control. There should not be a large release from hemicellulase because it does not react at the same sites that liberate phenolics from the arabinoxyan backbone as the xylanases. This is why the concentration did not have an effect on the release, and subsequently these treatments were not significantly different from one another. However, the longer the enzyme is allowed to react the more degradation of the cell wall structure will occur, and therefore more phenolics may be cleaved in the resulting side reactions.

For **Xylanase AN 900** and **Grindamyl Powerbake 900** a mean separation test was not performed for two reasons. First, there were no significant differences found for the concentrations, and second, there were no time replicates so you wouldn't be able to perform an ANOVA to get results for the time treatments. These two enzymes

had similar levels of ferulic acid release to each other, and were not as efficient at liberating phenolics as xylanases S, BX-AN, and N, and so did not move past preliminary testing into the larger enzyme combination designs. However, AN 900 has possible side reactions that can occur from its hemicellulolytic preparation; due to this the AN 900 xylanase was used in a combination in the second half of the research.

Comparison of the Enzymes against One Another

An ANOVA and mean separation test comparing the three top enzymes- S, BX-AN, and N, and the negative control hemicellulase- was done to see which enzyme performed the best when compared to the others. The overall averages of each data set were used as the points for the analysis. The data showed there was a significant difference when comparing enzyme vs. enzyme, and also when comparing the enzyme: concentration matrices. The enzymes were shown to be significantly different with a *p*-value of 0.001, while the enzyme: concentration matrix showed a significant difference with a *p*-value of 0.05. **Table 2.10** on the next page shows the mean separation test for the four enzymes compared against each other.

For Table 2.10 if the same letters are present at the same treatments it means they are not significantly different from each other, if the letters are different they are significantly different.

Table 2.10: Mean separation test comparing the individual enzymes against one another.

Enzyme	Mean (FFA milli eq)	Difference
BX-AN	212.9	a
S	167.0	b
N	97.5	c
Hemi-cellulase	14.2	d
LSD = 5.5		

Table 2.10 shows that all the enzymes treatments were significantly different from one another. It clearly demonstrates that xylanases are extremely effective at liberating phenolics, although not all xylanases are created equal. The xylanases were all significantly different with the largest difference in effectiveness showing when comparing against the negative control, hemicellulase. Xylanase N, the lowest performing of the xylanases, showed an impressive 7x the amount of ferulic acid released when compared to the hemicellulase. What is even more astounding is that the clear winner in release- BX-AN- had an extraordinary 15x the amount of ferulic acid released than hemicellulase. The hemicellulase had the lowest release of the three controls. Whereas cellulase and the no enzyme control were similar, as illustrated in **Table 2.11** below.

Table 2.11: Ferulic acid released for the three controls.

Enzyme	F.A. released (FFA milli eq)
Cellulase	68.6
Hemi-cellulase	21.0
Control (no enzyme)	57.7

It is important to note that all of the xylanases were significantly different from the hemicellulase and the control. This shows that they are doing a successful job at releasing bound ferulic acid and making it available in the soluble portion of the sample. The top three enzymes, BX-AN, S, and N were all significantly different from each other, with BX-AN being the best individual enzyme. It is interesting to note that the 24 and 48 hour samples were not significantly different for some enzymes. As previously mentioned, the inactivation of the enzyme over time or its inability to attack the remaining arabinoxylan through steric hindrance might be a factor in why extending the time treatment is ineffective. Also the enzymic removal of arabinose substituents may also lead to the intermolecular aggregation through non-covalent

interactions between less substituted chains and *increase* the insolubility of arabinoxylan (Kormelink et al 1991). That effect may be overcome with the longer exposure of the backbone to the enzymes but it may account for why there is variation of release over time.

The data shows that the length of time the reaction is allowed to occur may be the more arbitrary factor when deciding between time and concentration. The highest concentration (1.0%) was generally the best, even though the middle concentration of 0.3% did an excellent job at releasing as well.

Pentopan Data

In the original screening Pentopan showed promise with the 1.0%/48h sample releasing ≥200 f.a. milli q./g of wheat bran, which was close to xylanases S and BX-AN samples. Because of this initial good result additional tests were run with those three enzymes, combining them with one another in pairs, and then all three together, at the 0.3% concentration level. The middle concentration was chosen because when combining enzymes the hope is for a synergistic effect between them, allowing you to use less enzyme overall. Having a lower usage level for the enzyme is a good way to reduce process costs, especially when you can lower the most expensive part- the enzyme cost.

However, when the first 25 of the 48 samples of the set were being dried the freeze dryer had difficulties and the samples were allowed to thaw and remained unfrozen for over 2 days sitting in the chamber at room temperature. Those environmental conditions allow for degradation of the phenolic compounds and the resulting data can be inaccurate or skewed. The following figure shows the complete set of data, including data that was compromised in addition to the samples that were unaffected.

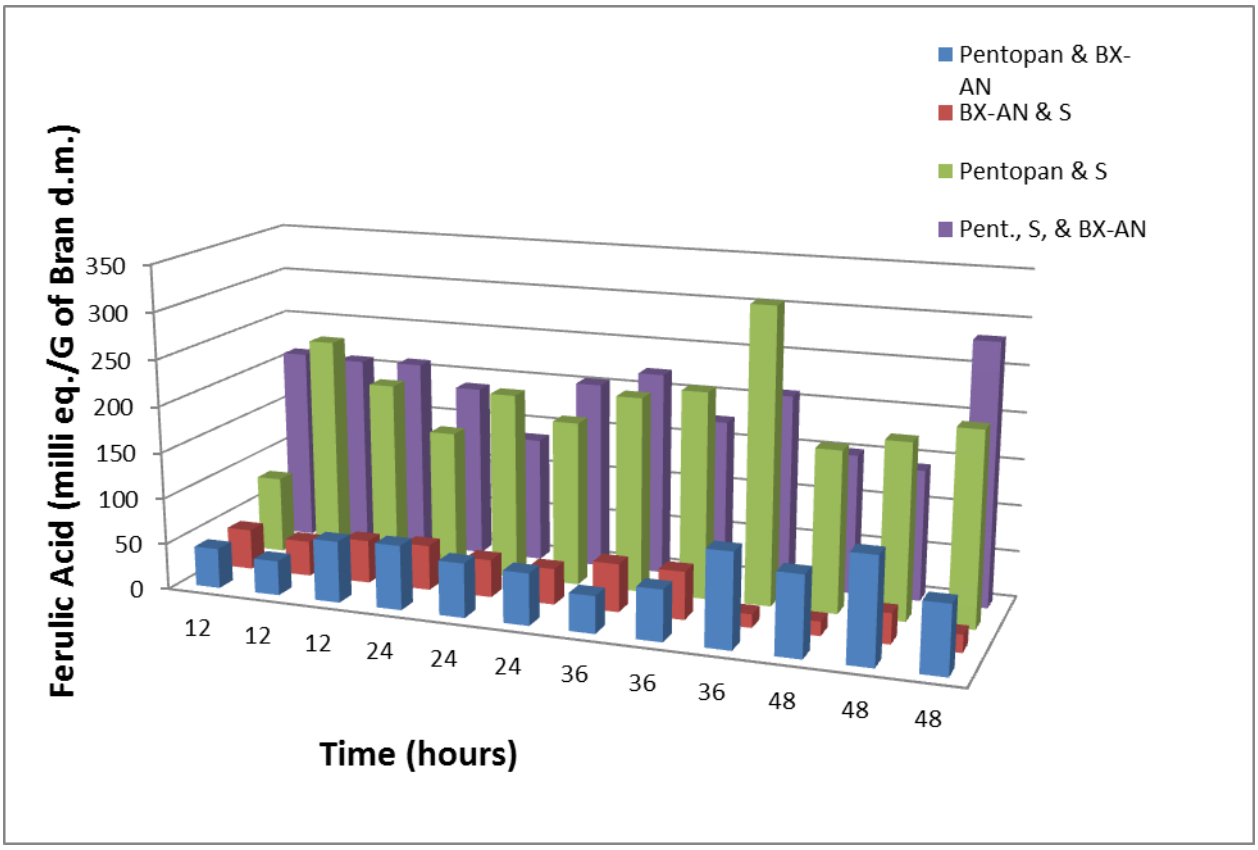

Figure 2.3: Combined enzyme treatment of Ground Wheat Bran (0.5mm) using Pentopan, Xylanase S, & BX-AN at 0.3% enzyme concentration.

It can be seen that the first two combinations (the two sets in the front half of the graph), Pentopan & BX-AN and BX-AN & S, are extremely low in comparison to the second two. Those front sets were the samples that were degraded and it is quite obvious because many of the samples barely reach 50 f.a. milli eq./g. However, when viewing the second half of the samples, the Pentopan & S and the combination of all three enzymes (samples 26-48), it can be seen that their values are, on average, much closer to the expected values. Whether the differences are due to human error during the addition of the enzymes to the samples or other degradation that may have taken place is unknown. But because these samples were kept together during their drying stage it can be assumed that a comparison of treatments can be made. **Table 2.12** below shows the averages for each time treatment.

Table 2.12: Average f.a. milli eq./g released for each time interval for the combination enzyme treatments

Enzyme Treatment	Time	Avg. f.a. (FA milli eq/g)
Pentopan & S	12	227.28
	24	178.16
	36	250.87
	48	190.69
Pentopan, S, & BX-AN	12	209.98
	24	175.75
	36	201.27
	48	191.57

From **Figure 2.2** and the averages in **Table 2.12** it is clear that there was not good consistency among the replicates, and that the different times did not follow the established increasing pattern, and instead seemed completely arbitrary. This is especially clear because the 12 hour samples in both the P&S and P, S, & BX-AN treatments showed a higher release than almost all of the other times. It seems that the P & S samples were more efficient at releasing ferulic acid than all three enzymes together because those averages are all equivalent or higher than the three enzymes together, as seen in **Table 2.12**. The combinations of enzymes had some release values similar to the individual enzymes (mid-200's), but the individuals still were higher. This may infer that using combinations would not be prudent when an individual enzyme would do the same job, perhaps more effectively. Beaugrand et al (2004) also found no synergistic effect when combining endo-xylanases to enhance the solubilization of arabinoxylan. A reason why the enzymes may not have worked together to release an increased amount of ferulic acid is that they are all endo-xylanases of the same family, and so the enzymes are competing to cleave the same bonding sites on the arabinoxylan backbone. As mentioned earlier family 11 and family 10 xylanases preferentially cleave different areas on the arabinoxylan

backbone- at the linear unsubstituted region or the “decorated” regions, respectively. And so using xylanases from different families would possibly yield better results. However, because the enzymes came from different sources they have different side activities, and the side reactions from them may influence how and where phenolics are liberated. This could alter the overall fractions of soluble, soluble-conjugate, and insoluble bound phenolics released. Because this possibility exists the Pentopan & S, and the Pentopan & BX-AN combinations were used in the combined processing portion of this research to see what could be discovered.

2.3.3. Conclusions

From the statistical analysis it is clear that xylanase BX-AN was the most efficient at liberating phenolics, and for that specific enzyme the 1.0% concentration was the most effective. However, xylanase S and Pentopan also showed effective results, and S showed promise that the 0.3% concentration could be just as effective if not more so than the 1.0%. When comparing the different concentrations overall concentration was shown to be significant. With the highest concentration, 1.0%, giving the best release overall as compared to the 0.3 or 0.1% levels. As mentioned before, the data shows that concentration effectiveness does vary from enzyme to enzyme.

When determining why certain enzymes were more effective than others in releasing phenolics there are a few attributes we must consider. Aside from all the enzymes being endo-xylanases, the enzymes vary in their bacterial source, side activities, optimal temperature and pH range, as well as different activities. Xylanase BX-AN and S both had β-glucanase as a side activity. Xylanase S had β-1,3 glucanase, this allows for the hydrolysis of (1→3)-β-D-glucosidic linkages in (1→3)-β-D-glucans. BX-AN did not specify the type of glucanase but it would be expected to be either 1,3 or 1,4. Having this enzyme in solution is important because it targets the arabinoxylan backbone breaking the polysaccharide chains into smaller pieces, thereby providing

more subsites for the target enzymes to act on. This is a probable cause for the excellent release given by Xylanases BX-AN and S.

When looking at the different time treatments 48 hours did give the highest release, but it was not statistically different from the 24 or 36 hour times. This leads to the conclusion that time may be the more arbitrary variable and can be lowered to save process time and costs.

When combining enzymes no firm conclusions can be made because the data set is incomplete. However, the two combination treatments that weren't affected by degradation (Pentopan & S, and Pentopan, S, & BX-AN), showed release similar to the individual enzymes. The possible side reactions that occur from combining enzymes could yield different profiles of the phenolic fractions released, giving a potentially more well-rounded overall phenolic profile. Ferulic acid esterase has been shown to be extremely effective at releasing phenolics but it is very expensive. Faulds et al combined FAE with xylanases and found the combination together to be more effective than either individual treatment. So, if FAE is combined in the right proportion to xylanases it could yield a much higher release at possible cost parity. Exploring the use of FAE and/or xylanases that have different side reactions or specificities such as exo-xylanases would be other directions to further this research to obtain the most efficient combination of enzymes at the lowest cost.

Another important part of this research that is addressed in the following combined process portion of the research is functionality of the final product. Increases in certain properties such as viscosity and water-holding capacity are crucial in delivering a product with added health benefits. Because there was minimal increase in viscosity in these enzyme-treated samples it was not considered an important characterization test to perform. However, in the combined processing section there was a large increase in viscosity and it was important to maintain that functionality benefit throughout the process so the topic is attended to there.

3 Combination Processing Optimization

3.1 Introduction

The first study showed that the use of individual and combinations of enzymes is effective in releasing bound phenolics and increasing the overall presence of soluble phenolics in solution. However, other processing methods discussed in the literature including physical and chemical, have been shown to be extremely successful for phenolic release as well. Therefore a natural progression was to find the best combination of treatments to maximize the release of phenolics, and increase the functionality of the wheat bran. The combined processing treatment of biological, physical, and chemical methods proved to be the most effective.

Another graduate student in the group optimized the other individual processing treatments. The chemical treatment was finalized to use an alkaline solution of 0.1 N NaOH for a 24 hour soaking period, after which it was neutralized with 6 N HCl. The second step, the physical processing, consists of two parts. In the first part the sample is high shear mixed at 5,000 rpm for 5 minutes. In the second part a 2% solution of the wheat bran is run through a high pressure homogenizer three times at 22,000 psi. This was then followed by various enzymatic treatments to pinpoint which one is the most effective in combination with the other processing aids. The flow chart below (**Figure 3.1**) shows the processing scheme used for this study. A diagram of the homogenizer used in the combined processing can be found in Appendix A. The ferulic acid was measured in the same way as in the first study to determine which treatment had the highest release.

A concentration level of 0.3% was chosen for all the enzymes, this was done for multiple reasons. First, it is already understood that the highest concentration is the most effective with only an enzymatic treatment, but when used in combination with other pre-processing treatments the lower concentration may be just as effective. This is because the other treatments are doing some of the work as far as particle reduction and are opening up the structure so the enzyme has more sites to

interact with making the enzyme more effective. Also, an advantage of perfecting a system with a lower concentration makes the entire process cheaper as enzymes can be an expensive part of the process.

The next step in evaluating the processed wheat bran for health benefits would be an animal study. Animal feeding studies evaluate the intestinal supernatant viscosity before and after feeding to see if the processed bran can lead to a lower in serum cholesterol. Dietary fiber was shown by others to lower serum cholesterol in hypercholesterolemic men and women after 6 weeks of consumption (Andreasen et al. 2001). Therefore, it is possible that if there is a difference in ex vivo viscosity using this wheat bran it may lead to a decrease in the level of cholesterol.

An animal feeding study was executed by students in Dan Gallaher's lab at the University of Minnesota. However, because the data was not performed by the author it will not be included here.

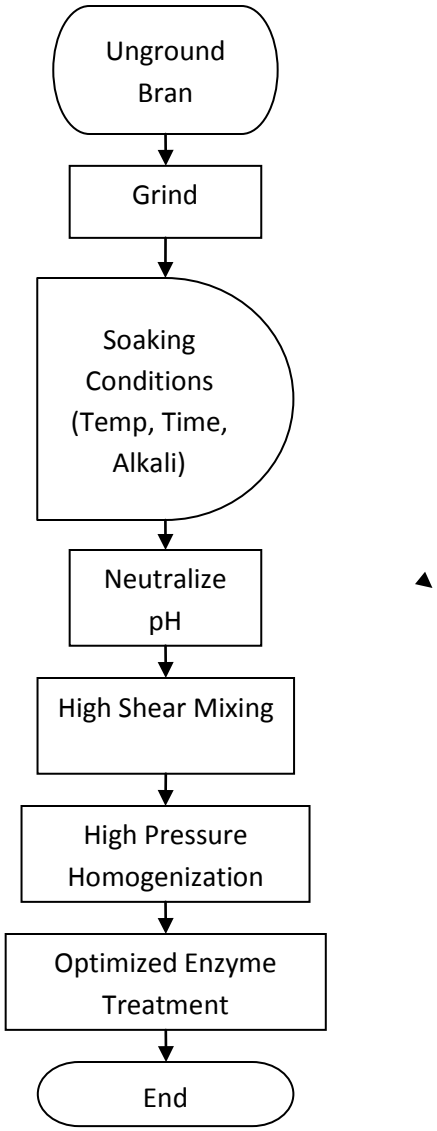

Figure 3.1: Bran processing flowchart showing the combination of physical, chemical, and biological processing steps.

3.2 Materials & Methods

3.2.1 Materials:

3.2.1.1 Enzyme Selection

After reviewing the most effective enzymes from the first study multiple individual and combinations of enzymes were chosen to test with the combined processing. The following individual enzymes were tested at 0.3%: Pentopan, Xylanase S, Xylanase BX-AN, and AN 100. The following combination of enzymes were also tested at a total concentration of 0.3%: Pentopan & BX-AN, Pentopan & Xylanase S, Xylanase S and AN 100, and Xylanase S and Xylanase BX-AN. For the enzyme combinations the total mL of the enzyme added was the same as the individual enzymes, as in the method discussed earlier (2.2.2.3).

3.2.1.2 Wheat Bran selection

The same bran as in the previous study was used, King Wheat Bran (from King Wheat Milling Co., Lowell, Michigan). King wheat bran is a light soft white wheat bran.

3.2.1.3 Sodium acetate buffer

A sodium acetate buffer solution was used comprising of sodium acetate (Fisher Scientific, Waltham, Massachusetts), acetic acid (Fisher Scientific, Waltham, Massachusetts), and deionized water.

3.2.1.4 NaOH solution

A NaOH solution was used comprising of NaOH(dry) and deionized water.

3.2.1.5 HCL solution

An HCl solution was used comprising of 37% HCl (liquid) and deionized water.

3.2.2 Methods

3.2.2.1 Sodium Acetate Buffer

To make 1 Liter of buffer 0.6805g of sodium acetate was dissolved in 1000 mL of deionized water, and then 0.30025 mL of acetic acid was added while under stirring to bring the pH down to the desired 5.23. These quantities were quadrupled to make a 4L stock of buffer used to prepare the enzyme solutions as well as the samples.

3.2.2.2 NaOH Solution

To make a 2 L stock solution of 0.1 N NaOH, 8 g of dry NaOH was dissolved into 2 L of deionized H_2O.

3.2.2.3 HCl Solution

To make a 1 L stock solution of HCl 86.2 mL of 37% liquid HCl was added to a 1 L volumetric flask, deionized H_2O added to make 1 L.

3.2.2.4 Enzyme Solutions

During the second study enzyme solution were prepared at a single concentration of 0.3%. The concentrations were calculated based on the amount of wheat bran in the sample. For 4 g wheat bran samples the enzyme solutions were made as follows:

0.3% = 4g x 0.003 = 0.012 x 12 = 0.144g enzyme powder/ 48 mL buffer

For the half and half enzyme solutions a 0.3% solution was made of each and then half the quantities added were taken of each for the addition.

3.2.2.5 Sample Preparation

1. 1 kg of King Wheat bran is milled using a rotor beater milling technology (Retsch GmbH, Model SR 300, Haan, Germany) to a 0.5 mm particle size.
2. Approximately 260 mL of 5% bran solutions were prepared by mixing 12.5g wheat bran with 250 mL of 0.1N NaOH.
3. Samples were then held at 60°C for 24 hours to allow the bran to soak in the alkali conditions.

4. After 24 hours the samples were neutralized using enough 6N HCl to reach a pH of 7. The bran solution was diluted down to a 2% by adding enough water to bring the sample to 600 mL.
5. Samples were then mixed with a high shear mixer(Carter model XMUC752BF; T25 Basic SI Homogenizer, IKA Works; or Greerco, Homogenizer 1L, Colloid Mill W200V, North Andover, MA) at 24,000 rpm for 5 minutes, a viscosity measurement was taken post-shear mixing.
6. The samples were then high pressure homogenized (M-110Y Laboratory Microfluidizer® Processor (Newton, MA) at 23,000 psi through the 100 dispersion chamber twice, the viscosity was taken post-HPH.
7. The sample was portioned off as 36 mL aliquots into 50 mL centrifuge tubes and 4 mL of the appropriate enzyme solution was added.
8. The samples were then placed on the Innova Incubator Shaker(Enfield, Connecticut) at 50°C and 220 rpm.
9. Samples are removed after the appropriate length of time, either 24 or 48 hours.
10. Immediately after removal from the incubator shaker the samples are centrifuged at 680 x g for 20 minutes.
11. After centrifugation the supernatant is poured off into a correspondingly labeled 50 mL centrifuge tube and placed in the -80°C freezer until solid.
12. Sample caps removed and the sample is covered with parafilm and then perforated, and then placed back in the freezer to ensure the sample is frozen.
13. Frozen samples are freeze dried (Virtis Freezemobile 12EL, Warminster, Pennsylvania) using an Edwards Ultra Grade 19 Vacuum pump VWR(Sanborn, New York) until samples were completely dry.

3.2.2.6 UV spectrometry measurement

1. Freeze dried samples are reconstituted with 10 mL deionized water in their 50 mL centrifuge tubes.

2. A 0.3 µL aliquot of the reconstituted sample is pipette into a 2.0 mL centrifuge tube containing 1.2 mL of deionized water to give a 1.5 mL sample.
3. Sample cap is closed and then the sample is vortexed for 10 seconds to thoroughly mix the sample.
4. Steps 2 & 3 are repeated two more times to give a total of 3 dilutions.
5. The final 1.5 mL diluted sample is poured into a cuvette and placed in a Spectronic Genesys 5 spectrophotometer that has been zeroed with deionized water.
6. The absorbance at 280 and 325 nm is then read and recorded for each sample within one hour of prepping samples.

3.2.2.7 Viscosity measurement

Viscosity was measured on a Rapid Visco Analyzer (RVA, Newport Scientific, Jessup, Maryland).

i. As-is samples from intermediate processing steps at 2% dry matter were stirred at 160 rpm at 37°C until viscosity stabilized (usually after 2-3 minutes) and a 3 minute average viscosity was taken.

ii. Dried samples were used at 6% dry matter suspensions, stirred at 160 rpm 37°C, and a three minute average viscosity was taken in the stable area from minutes 5 to 8.

3.3 Results and Analysis

The combination of NaOH, physical processing, and enzymes proved to be extremely effective for releasing phenolics from wheat bran. The combined processes showed the highest release when compared to either the pre-processing conditions or enzyme treatments alone. This shows that both are needed to achieve the best

possible release. Tests were run with the enzymes showing that BX-AN had the highest individual release, closely followed by S, and also AN 900 which showed promise early on. Also, a set using Pentopan combined with BX-AN and S was done, however equipment problems were encountered during the drying phase rendering the data unacceptable for analysis, however, it is included in Appendix B.

3.3.1 Combined Enzyme Results

An ANOVA was run on the data from the combined processing in the same fashion as the enzyme optimization. The figures showing the ferulic acid release as well the statistical analysis tables are included for each set.

The first experiment used Xylanase S and AN 100. The enzymes were added after the bran underwent all of the processing conditions (0.1N NaOH at 60°C for 24 hours, high shear mixing, and high pressure homogenization (HPH) at 22,000 psi). The optimally processed bran was then treated with enzymes for either 24 or 48 hours at 50°C. The same set was done without the NaOH pretreatment to see the overall effectiveness of NaOH and how it compared when used with the enzymes. **Figure 3.2** on page 54 shows a graph of the ferulic acid release from these two treatments at only the 48 hour time. **Table 3.1a** below shows the mean separation data comparing the enzymes to one another.

For Tables 3.1a & b if the same letters are present at the same treatments it means they are not significantly different from each other, if the letters are different they are significantly different.

Table 3.1a: Mean separation data comparing the enzyme treatments to one another.

Enzyme	Mean (FFA milli eq)	Difference
AN 100	299	a
S	332	b
½ & ½	334	b
LSD = 6.6		

When comparing the individual enzymes to the mixed enzyme treatment it was shown that AN 100 was significantly different from ½ S & ½ AN, and had a lower release. This continues the trend of Xylanase S being extremely effective at releasing ferulic acid.

When considering just the NaOH pretreatment **Table 3.1b** below shows that using NaOH is an extremely effective solo treatment for releasing phenolics. It is significantly different from the bran not treated with NaOH with a large difference between the means.

Table 3.1b: Mean separation test comparing samples processed with enzymes but treated with and without NaOH.

Treatment	Mean (FFA milli eq)	Difference
No NaOH	243	a
NaOH	400	b
LSD = 5.3		

In **Figure 3.2** below the differences between samples treated with and without NaOH can easily be seen, as well as differences among the enzyme treatments. The samples were run for 24 or 48 hours, the 48 hour samples showed a larger increase in release. An important observation is that the combination of chemical pretreatment followed by enzyme treatment resulted in an increase in ferulic acid release in every case. And after running the ANOVA on the data it was shown that both the NaOH treatments and the enzyme treatments were significantly different from the controls.

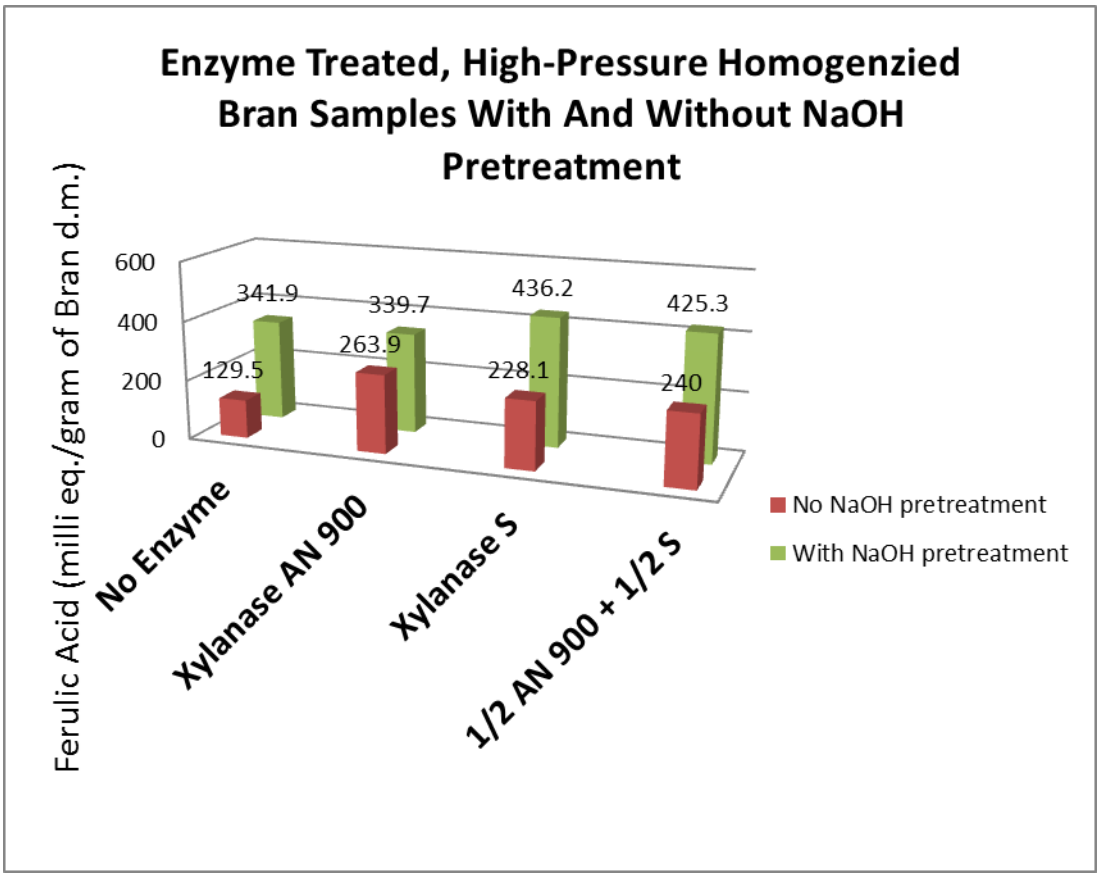

Figure 3.2: Phenolic release for HPH + enzyme treated (Xylanase S and AN900) bran samples with and without NaOH averaged from 2 replicates.

The second set of combined samples used Xylanase S and BX-AN. These two were chosen because both BX-AN and S had both shown good release individually as well as in combination with each other. In **Table 3.2** below the mean separation data shows that all three treatments were significantly different with BX-AN showing the best release followed by S and then the ½ and ½ mixture. A point of difference of these samples compared to the previous set was running some of the samples without the use of HPH as part of the combination testing. This was done to illustrate the importance of each step in the combination processing and to see if any of the additional processing steps showed a large effect when combined with the enzyme treatment or if certain steps rendered others unnecessary.

For Table 3.2 if the same letters are present at the same treatments it means they are not significantly different from each other, if the letters are different they are significantly different.

Table 3.2: Mean separation test comparing the enzyme treatments to one another.

Treatment	Mean (FFA milli eq)	Difference
S	325	b
BX-AN	362	c
½ & ½	312	a
LSD=10.36		

Figure 3.3 shows the amount of ferulic acid released by Xylanase S, BX-AN, and the ½ and ½ mixture used in combination with the optimized pre-processed and HPH treated bran. Besides the enzyme combinations, controls were done using NaOH and HPH without enzyme, NaOH without HPH, and a final without NaOH. Another important note was that all the treatments (with enzyme, NaOH, and HPH) were significantly different than the controls (No NaOH, no HPH, or no enzyme). The samples treated with both NaOH and enzymes had the highest release, however samples with just NaOH treatment had a release similar to the enzyme only treatments. A vital step with combined processing appears to be the use of NaOH because samples processed without NaOH or enzymes showed a poor release. This stresses the importance of using both enzymes and NaOH to get the largest release of bound phenolics from wheat bran.

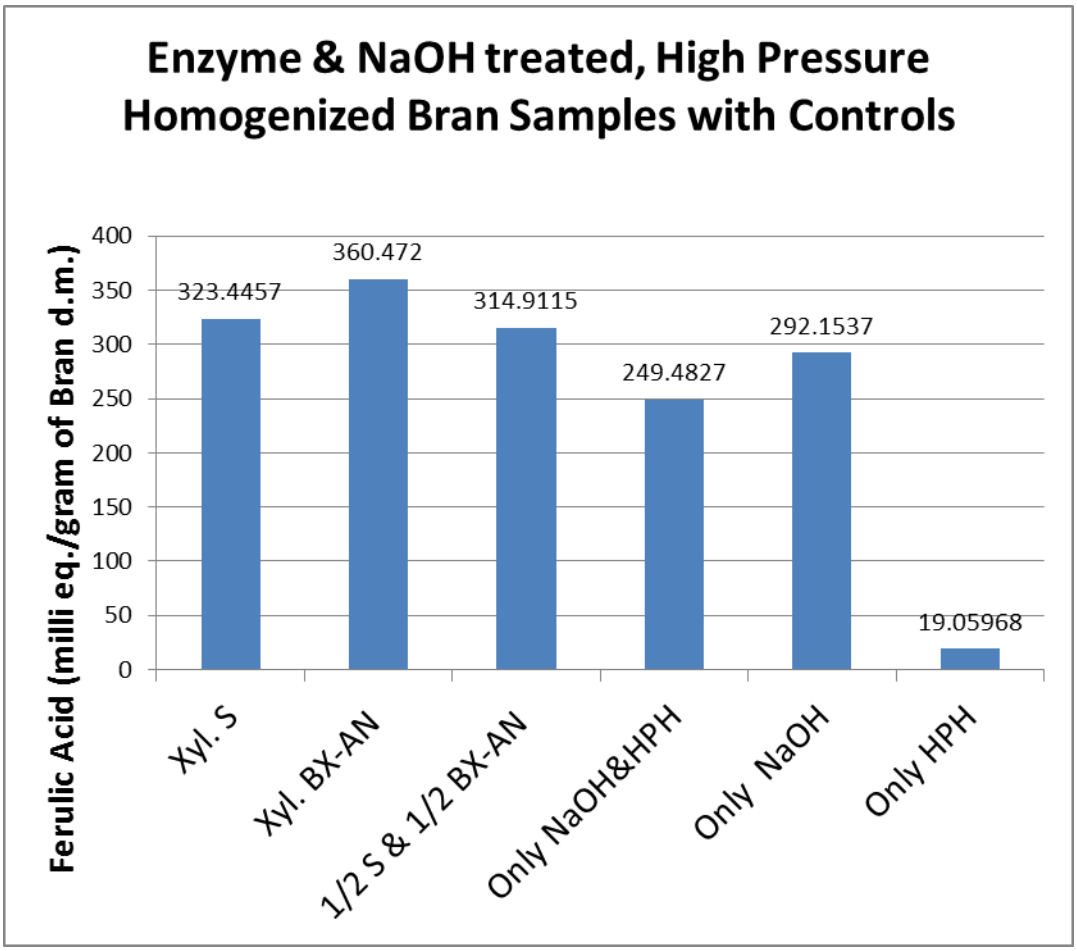

Figure 3.3: Phenolic release for NaOH +HPH + enzyme combined treatment bran samples using Xylanase S and BX-AN, and also various controls.

3.3.2 Viscosity data

In **Figure 3.4** below, viscosity data is presented for all the samples from **Figure 3.3**. The viscosities were taken after high shear mixing, after HPH, and after the enzyme treatment. From the graph it is clear that high pressure homogenization is the critical factor affecting viscosity, with an approximate doubling of viscosity after HPH. It is also clear that the use of NaOH is also very important as the sample that was not treated with NaOH had a very low release equivalent to the sample that lacked the HPH treatment. It is also worthy to note that the viscosities didn't drop significantly

after the enzyme treatment due to the specificity of the enzyme, and therefore functional properties gained from HPH and NaOH(increased viscosity and water holding capacity) are retained in the final product.

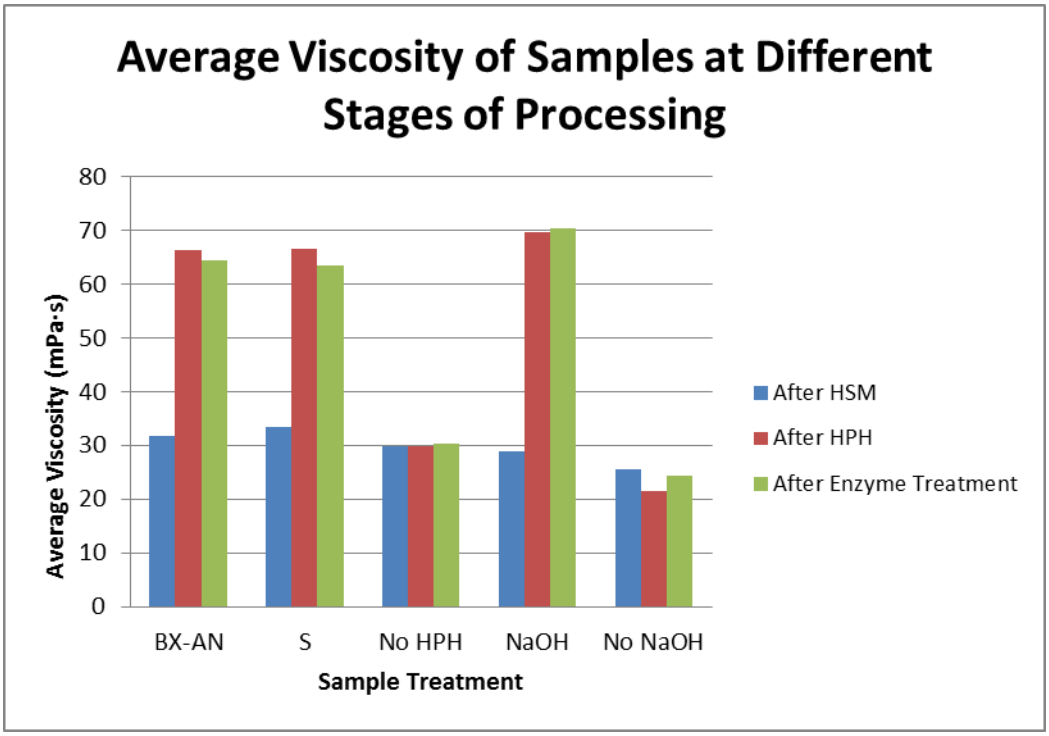

Figure 3.4: Viscosity data for the second set of combined treatments given in mPa·s.

3.4 Conclusions

Overall the combination of enzyme treatments with the pre-processing steps showed the highest release for phenolics. One of the important benefits to the combination processing besides the high release of phenolics is maintaining the functionality of the wheat bran. This was achieved for viscosity, and it can be assumed to continue with the water-holding capacity as well. This is because the high pressure homogenization is expanding and opening up the bran structure allowing for those increases.

Again, Xylanase BX-AN showed the best release individually, while it also did well in combination with Xylanase S. Further testing using Pentopan in combination with Xylanases S and BX-AN would be needed to complete the combination testing for a full set of data to compensate for the set that was lost. As of now a synergistic effect has not been found among the enzymes. The possible use of ferulic acid esterase or other exo-xylanases as mentioned it the individual enzyme treatments may contribute to achieving this synergistic effect.

It was clear that the use of a NaOH pre-treatment is imperative for increasing the release of phenolics. This is because the NaOH is able to break ester bonds between phenolic acids and bran polysaccharides thereby breaking down the wheat bran and opening up the structure. This exposes more sites for the enzymes to react with increasing their effectiveness. However, the NaOH treatment was extremely effective on its own without enzymes, releasing only a slightly lower amount of phenolics. This could be seen by some as a reason not to use the enzyme treatment because the two treatments were not significantly different, and proceeding with the enzyme treatment is both time consuming and an added expense. However, because the two treatments release phenolics in different ways the profiles released by each could be drastically different, giving a much more appealing phenolic profile that has multiple kinds of phenolics and conjugates. Having a balanced and varied antioxidant profile would contribute to the perceived health benefits of the wheat bran ingredient.

When considering the use of HPH it does not seem to have an effect on the overall release of phenolics. However, it does have a pronounced effect on the viscosity of the bran solution as well as the water holding capacity. This is critical when looking at the implications in the human biological use of the bran for health benefits, including cholesterol lowering. Because of this HPH is considered a valued step in the combination processing. However, the high shear mixing did not seem to have any effect on the viscosity of the bran solution. Because of this that step may be eliminated from the process to save time and money. In summary, all three

treatments- chemical, physical, and biological- have their place in releasing phenolics or improving the viscosity and are important to obtain the best overall wheat bran product.

4 Future Research

As mentioned, In the future it may be pertinent to test the use of ferulic acid esterase in combination with some of the enzymes that have shown the highest release. However, the expense of FAE can be off-putting for scale-up in the industry due to its high cost.

With regards to the optimization of the combined processing, preliminary work was being done on the use of steam explosion, which has been shown to thoroughly break open the wheat bran structure, allowing for more subsite access for the enzymes. This used in addition too, or in place of, high pressure homogenization may be an extremely effective method for release of phenolics and increasing the wheat bran's water holding capacity.

Also, work using HPLC to show the difference in profiles of the phenolics released from the enzymes and from the NaOH treatment would be of value. The NaOH does an excellent job of releasing phenolics on its own, begging the question is the use of enzymes necessary? If testing can be done to show that these two treatments release different phenolics, and that both are of value to the body, then a process using both would be worthwhile for the added-value benefits it would give in the final wheat bran product.

And finally, when looking at the actual nutritional value and functionality of using this wheat bran as an ingredient it is necessary to do longer term feeding studies. There are future feeding studies that are longer (~12 week length) to confirm cholesterol reduction, increased bioavailability of antioxidants, and additional physiological attributes. These studies would be executed by the students in Dr. Gallaher's lab.

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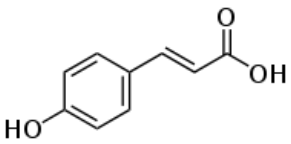
6 Appendices

Appendix A:

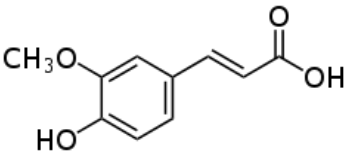
Phenolic Acid Structures

Equipment used

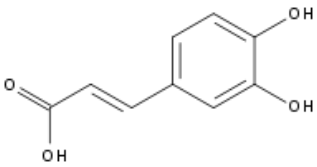
Phenolic Acid Structures



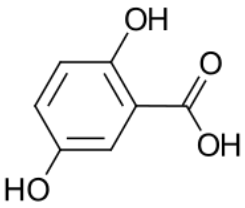
p-Coumaric acid



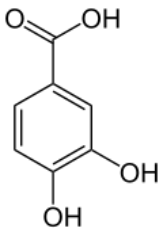
Ferulic acid



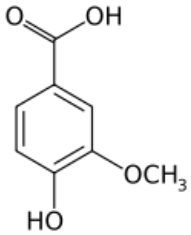
Caffeic acid



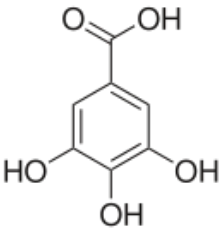
Gentistic acid



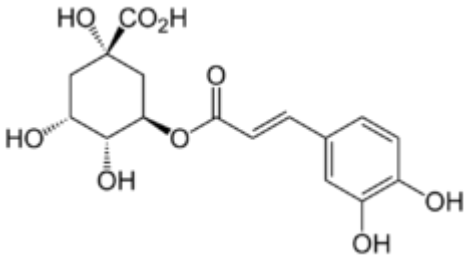
Protocatechuic acid



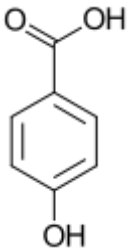
Vanillic acid



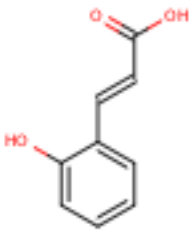
Gallic acid



Chlorogenic acid



p-Hydroxybenzoic acid



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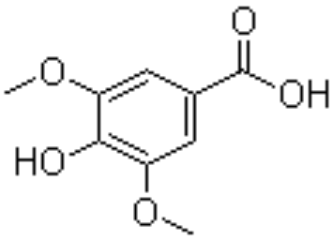


Syringic acid

Equipment Used:

Innova Incubator Shaker

Microfuidizer

Appendix B: Raw Data & Individual Graphs for all the enzyme treatments.

Raw Data:

Standard Curve
Xylanase AN 900
Grindamyl Powerbake 900
Xylanase BX-AN
Xylanase S
Xylanase N
Hemicellulase
Pentopan
Grindamyl Powerbake 950
Pentopan combined with Xylanase S and BX-AN
Combined Process with Xylanase S and AN-100
Combined Process with Xylanase S and BX-AN
Combined Process with Xylanase S, BX-AN, and Pentopan

Individual graphs for all enzyme treatments

Standard Curve
Xylanase AN 900
Grindamyl Powerbake 900
Xylanase BX-AN
Xylanase S
Xylanase N
Hemicellulase
Pentopan
Grindamyl Powerbake 950
Pentopan combined with Xylanase S and BX-AN
Combined Process with Xylanase S and AN-900
Combined Process with Xylanase S and BX-AN
Combined Process with Xylanase S, BX-AN, and Pentopan

Raw Data

Standard Curve

	Absorbance @ 325			
Concentration	Rep 1	Rep 2	Rep 3	Avg
0.03125	1.846	1.844	1.854	**1.848**
0.015625	0.91	0.84	0.89	**0.88**
0.0078125	0.419	0.416	0.416	**0.417**
0.00390625	0.199	0.201	0.194	**0.198**
0.001953125	0.095	0.099	0.1	**0.098**

Averages used in the graph.

Xylanse AN 900

Sample	Absorbance @ 325	time	x	Sample Wt	Dilution	Diluted Extracted	free f.a.	f.a milli
				g d.m.		Bran g.	eq./g	eq./g
1	0.275	12	0.00392	4.5	0.0156	0.0703125	0.0558	55.8095
2	0.312	24	0.00449	4.5	0.0156	0.0703125	0.0638	63.8288
3	0.424	36	0.00619	4.5	0.0156	0.0703125	0.0881	88.1032
4	0.454	48	0.00665	4.5	0.0156	0.0703125	0.0946	94.6053
5	0.69	12	0.01025	4.5				
6	0.329	24	0.00475	4.5	0.0156	0.0703125	0.0675	67.5133
7	0.516	36	0.0076	4.5	0.0156	0.0703125	0.108	108.043
8	0.555	48	0.00819	4.5	0.0156	0.0703125	0.1165	116.496
9	0.177	12	0.00243	4.5	0.0156	0.0703125	0.0346	34.5694
10	0.209	24	0.00292	4.5	0.0156	0.0703125	0.0415	41.505
11	0.34	36	0.00491	4.5	0.0156	0.0703125	0.0699	69.8974
12	0.54	48	0.00796	4.5	0.0156	0.0703125	0.1132	113.245

Grindamyl Powerbake 900

Sample	Absorbance @ 325 time		x	Sample Wt g d.m.	Dilution	Diluted Extracted Bran g.	free f.a. eq./g	f.a milli eq./g
1	0.262	12	0.00373	4.5	0.0156	0.0703125	0.053	52.992
2		24	-0.0003	4.5	0.0156	0.0703125	-0.0038	-3.79288
3	0.319	36	0.00459	4.5	0.0156	0.0703125	0.0653	65.3459
4	0.473	48	0.00694	4.5	0.0156	0.0703125	0.0987	98.7233
5	0.284	12	0.00406	4.5	0.0156	0.0703125	0.0578	57.7602
6	0.33	24	0.00476	4.5	0.0156	0.0703125	0.0677	67.73
7	0.396	36	0.00577	4.5	0.0156	0.0703125	0.082	82.0346
8	0.489	48	0.00719	4.5	0.0156	0.0703125	0.1022	102.191
9	0.179	12	0.00246	4.5	0.0156	0.0703125	0.035	35.0029
10	0.259	24	0.00368	4.5	0.0156	0.0703125	0.0523	52.3418
11	0.334	36	0.00482	4.5	0.0156	0.0703125	0.0686	68.597
12	0.365	48	0.0053	4.5	0.0156	0.0703125	0.0753	75.3158

Xylanase BX-AN

Sample	Absorbance @ 325	time	x	Sample Wt	Dilution	Diluted Extracted	free f.a.	f.a milli
				g d.m.		Bran g.	eq./g	eq./g
1	0.433	12	0.006579	4	0.015625	0.0625	0.1052639	105.26386
2	0.555	12	0.0086125	4	0.015625	0.0625	0.1378004	137.80045
3	0.601	12	0.0093793	4	0.015625	0.0625	0.1500683	150.06834
4	0.631	24	0.0098793	4	0.015625	0.0625	0.1580691	158.06914
5	0.566	24	0.0087959	4	0.015625	0.0625	0.1407341	140.73407
6	0.603	24	0.0094126	4	0.015625	0.0625	0.1506017	150.60173
7	0.615	36	0.0096126	4	0.015625	0.0625	0.153802	153.80205
8	0.604	36	0.0094293	4	0.015625	0.0625	0.1508684	150.86842
9	0.66	36	0.0103627	4	0.015625	0.0625	0.1658032	165.80325
10	0.654	48	0.0102627	4	0.015625	0.0625	0.1642031	164.20309
11	0.617	48	0.009646	4	0.015625	0.0625	0.1543354	154.33543
12	0.601	48	0.0093793	4	0.015625	0.0625	0.1500683	150.06834
13	0.628	12	0.0098293	4	0.015625	0.0625	0.1572691	157.26906
14	0.634	12	0.0099293	4	0.015625	0.0625	0.1588692	158.86922
15	0.731	12	0.0115462	4	0.015625	0.0625	0.1847385	184.73847
16	0.736	24	0.0116295	4	0.015625	0.0625	0.1860719	186.07194
17	0.726	24	0.0114628	4	0.015625	0.0625	0.183405	183.40501
18	0.728	24	0.0114961	4	0.015625	0.0625	0.1839384	183.93839
19	0.827	36	0.0131463	4	0.015625	0.0625	0.210341	210.34103
20	0.886	36	0.0141297	4	0.015625	0.0625	0.2260759	226.07594
21	0.858	36	0.013663	4	0.015625	0.0625	0.2186085	218.60853
22	0.892	48	0.0142298	4	0.015625	0.0625	0.2276761	227.6761
23	0.83	48	0.0131963	4	0.015625	0.0625	0.2111411	211.14111
24	0.91	48	0.0145298	4	0.015625	0.0625	0.2324766	232.47658
25	0.855	12	0.013613	4	0.015625	0.0625	0.2178084	217.80845
26	0.832	12	0.0132297	4	0.015625	0.0625	0.2116745	211.6745
27	0.815	12	0.0129463	4	0.015625	0.0625	0.2071407	207.14071
28	0.926	24	0.0147965	4	0.015625	0.0625	0.2367437	236.74367
29	0.913	24	0.0145798	4	0.015625	0.0625	0.2332767	233.27666
30	0.933	24	0.0149132	4	0.015625	0.0625	0.2386105	238.61053
31	0.95	36	0.0151965	4	0.015625	0.0625	0.2431443	243.14431
32	0.902	36	0.0143964	4	0.015625	0.0625	0.230343	230.34303
33	0.909	36	0.0145131	4	0.015625	0.0625	0.2322099	232.20989
34	0.903	48	0.0144131	4	0.015625	0.0625	0.2306097	230.60973
35	0.998	48	0.0159966	4	0.015625	0.0625	0.2559456	255.94559
36	0.922	48	0.0147298	4	0.015625	0.0625	0.2356769	235.6769

Xyalanse S

Sample	Absorbance @ 325	time	x	Sample Wt	Dilution	Diluted Extracted bran (g)	Free f.a. eq./g	f.a milli eq./g
1	0.367	12	0.0055	4	0.01563	0.0625	0.0876621	87.6621
2	0.33	12	0.0049	4	0.01563	0.0625	0.0777944	77.794446
3	0.361	12	0.0054	4	0.01563	0.0625	0.0860619	86.06194
4	0.415	24	0.0063	4	0.01563	0.0625	0.1004634	100.46338
5	0.401	24	0.006	4	0.01563	0.0625	0.0967297	96.729673
6	0.426	24	0.0065	4	0.01563	0.0625	0.103397	103.39701
7	0.51	36	0.0079	4	0.01563	0.0625	0.1257992	125.79925
8	0.521	36	0.008	4	0.01563	0.0625	0.1287329	128.73287
9	0.535	36	0.0083	4	0.01563	0.0625	0.1324666	132.46658
10	0.744	48	0.0118	4	0.01563	0.0625	0.1882055	188.20549
11	0.706	48	0.0111	4	0.01563	0.0625	0.1780711	178.07114
12	0.853	48	0.0136	4	0.01563	0.0625	0.2172751	217.27506
13	0.686	12	0.0108	4	0.01563	0.0625	0.1727373	172.73727
14	0.675	12	0.0106	4	0.01563	0.0625	0.1698036	169.80365
15	0.669	12	0.0105	4	0.01563	0.0625	0.1682035	168.20349
16	0.733	24	0.0116	4	0.01563	0.0625	0.1852719	185.27186
17	0.736	24	0.0116	4	0.01563	0.0625	0.1860719	186.07194
18	0.72	24	0.0114	4	0.01563	0.0625	0.1818048	181.80485
19	0.604	36	0.0094	4	0.01563	0.0625	0.1508684	150.86842
20	0.65	36	0.0102	4	0.01563	0.0625	0.1631363	163.13631
21	0.692	36	0.0109	4	0.01563	0.0625	0.1743374	174.33743
22	0.676	48	0.0106	4	0.01563	0.0625	0.1700703	170.07034
23	0.807	48	0.0128	4	0.01563	0.0625	0.2050072	205.00717
24	0.623	48	0.0097	4	0.01563	0.0625	0.1559356	155.93559
25	0.611	12	0.0095	4	0.01563	0.0625	0.1527353	152.73527
26	0.653	12	0.0102	4	0.01563	0.0625	0.1639364	163.93639
27	0.68	12	0.0107	4	0.01563	0.0625	0.1711371	171.13711
28	0.732	24	0.0116	4	0.01563	0.0625	0.1850052	185.00517
29	0.714	24	0.0113	4	0.01563	0.0625	0.1802047	180.20469
30	0.759	24	0.012	4	0.01563	0.0625	0.1922059	192.20589
31	0.59	36	0.0092	4	0.01563	0.0625	0.1471347	147.13471
32	0.602	36	0.0094	4	0.01563	0.0625	0.150335	150.33503
33	0.598	36	0.0093	4	0.01563	0.0625	0.1492683	149.26826
34	0.835	48	0.0133	4	0.01563	0.0625	0.2124746	212.47458
35	0.806	48	0.0128	4	0.01563	0.0625	0.2047405	204.74047
36	0.703	48	0.0111	4	0.01563	0.0625	0.1772711	177.27106

Xylanase N

Sample	Absorbance @ 325	time	x	Sample Wt g. dry matter	Dilution	Diluted extracted g.	free f.a. eq./g	f.a. milli eq./g
1	0.209	12	0.00285	4	0.01563	0.0625	0.0455246	45.524552
2	0.195	12	0.00261	4	0.01563	0.0625	0.0417908	41.790846
3	0.185	12	0.00245	4	0.01563	0.0625	0.0391239	39.123912
4	0.239	24	0.00335	4	0.01563	0.0625	0.0535254	53.525353
5	0.253	24	0.00358	4	0.01563	0.0625	0.0572591	57.259059
6	0.274	24	0.00393	4	0.01563	0.0625	0.0628596	62.859619
7	0.256	36	0.00363	4	0.01563	0.0625	0.0580591	58.059139
8	0.228	36	0.00316	4	0.01563	0.0625	0.0505917	50.591726
9	0.27	36	0.00386	4	0.01563	0.0625	0.0617928	61.792846
10	0.298	48	0.00433	4	0.01563	0.0625	0.0692603	69.260259
11	0.304	48	0.00443	4	0.01563	0.0625	0.0708604	70.860419
12	0.348	48	0.00516	4	0.01563	0.0625	0.0825949	82.594926
13	0.252	12	0.00356	4	0.01563	0.0625	0.0569924	56.992366
14	0.237	12	0.00331	4	0.01563	0.0625	0.052992	52.991966
15	0.213	12	0.00291	4	0.01563	0.0625	0.0465913	46.591326
16	0.358	24	0.00533	4	0.01563	0.0625	0.0852619	85.26186
17	0.362	24	0.0054	4	0.01563	0.0625	0.0863286	86.328633
18	0.337	24	0.00498	4	0.01563	0.0625	0.0796613	79.661299
19	0.357	36	0.00531	4	0.01563	0.0625	0.0849952	84.995166
20	0.351	36	0.00521	4	0.01563	0.0625	0.083395	83.395006
21	0.39	36	0.00586	4	0.01563	0.0625	0.093796	93.796046
22	0.428	48	0.0065	4	0.01563	0.0625	0.1039304	103.93039
23	0.413	48	0.00625	4	0.01563	0.0625	0.09993	99.929993
24	0.443	48	0.00675	4	0.01563	0.0625	0.1079308	107.93079
25	0.367	12	0.00548	4	0.01563	0.0625	0.0876621	87.6621
26	0.403	12	0.00608	4	0.01563	0.0625	0.0972631	97.26306
27	0.455	12	0.00695	4	0.01563	0.0625	0.1111311	111.13111
28	0.5	24	0.0077	4	0.01563	0.0625	0.1231323	123.13231
29	0.542	24	0.0084	4	0.01563	0.0625	0.1343334	134.33343
30	0.537	24	0.00831	4	0.01563	0.0625	0.133	132.99997
31	0.432	36	0.00656	4	0.01563	0.0625	0.1049972	104.99717
32	0.437	36	0.00665	4	0.01563	0.0625	0.1063306	106.33063
33	0.472	36	0.00723	4	0.01563	0.0625	0.1156649	115.6649
34	0.254	48	0.0036	4	0.01563	0.0625	0.0575258	57.525753
35	0.524	48	0.0081	4	0.01563	0.0625	0.129533	129.53295
36	0.383	48	0.00575	4	0.01563	0.0625	0.0919292	91.929193

Hemicellulase

Sample	Absorbance @ 325	time	x	Sample Wt g d.m.	Dilution	Diluted extracted g.	free f.a. eq./g	free f.a. milli eq./g
1	0.102	12	0.0010618	4	0.01563	0.0625	0.016988	16.98837
2	0.018	12	-0.0003384	-4	0.01563	-0.0625	0.005414	5.413875
3	0.089	12	0.0008451	4	0.01563	0.0625	0.013521	13.52135
4	0.106	24	0.0011284	4	0.01563	0.0625	0.018055	18.05514
5	0.103	24	0.0010784	4	0.01563	0.0625	0.017255	17.25506
6	0.1	24	0.0010284	4	0.01563	0.0625	0.016455	16.45498
7	0.114	36	0.0012618	4	0.01563	0.0625	0.020189	20.18869
8	0.083	36	0.0007451	4	0.01563	0.0625	0.011921	11.92119
9	0.078	36	0.0006617	4	0.01563	0.0625	0.010588	10.58773
10	0.091	48	0.0008784	4	0.01563	0.0625	0.014055	14.05474
11	0.1	48	0.0010284	4	0.01563	0.0625	0.016455	16.45498
12	0.117	48	0.0013118	4	0.01563	0.0625	0.020989	20.98877
13	0.086	12	0.0007951	4	0.01563	0.0625	0.012721	12.72127
14	0.092	12	0.0008951	4	0.01563	0.0625	0.014321	14.32143
15	0.083	12	0.0007451	4	0.01563	0.0625	0.011921	11.92119
16	0.093	24	0.0009118	4	0.01563	0.0625	0.014588	14.58813
17	0.101	24	0.0010451	4	0.01563	0.0625	0.016722	16.72167
18	0.09	24	0.0008618	4	0.01563	0.0625	0.013788	13.78805
19	0.074	36	0.0005951	4	0.01563	0.0625	0.009521	9.520952
20	0.071	36	0.0005451	4	0.01563	0.0625	0.008721	8.720872
21	0.046	36	0.0001283	4	0.01563	0.0625	0.002054	2.053539
22	0.107	48	0.0011451	4	0.01563	0.0625	0.018322	18.32183
23	0.128	48	0.0014951	4	0.01563	0.0625	0.023922	23.92239
24	0.092	48	0.0008951	4	0.01563	0.0625	0.014321	14.32143

Pentopan

Sample	Absorbance @ 325	time	x	Sample Wt g d.m.	Dilution	Diluted extracted g.	free f.a. eq./g	f.a. milli eq./g
1	0.217	12	0.002979	4	0.015625	0.0625	0.047658	47.658099
2	0.23	12	0.003195	4	0.015625	0.0625	0.051125	51.125113
3	0.256	12	0.003629	4	0.015625	0.0625	0.058059	58.059139
4	0.262	24	0.003729	4	0.015625	0.0625	0.059659	59.659299
5	0.273	24	0.003912	4	0.015625	0.0625	0.062593	62.592926
6	0.25	24	0.003529	4	0.015625	0.0625	0.056459	56.458979
7	0.259	36	0.003679	4	0.015625	0.0625	0.058859	58.859219
8	0.267	36	0.003812	4	0.015625	0.0625	0.060993	60.992766
9	0.266	36	0.003795	4	0.015625	0.0625	0.060726	60.726073
10	0.325	48	0.004779	4	0.015625	0.0625	0.076461	76.460979
11	0.35	48	0.005196	4	0.015625	0.0625	0.083128	83.128313
12	0.321	48	0.004712	4	0.015625	0.0625	0.075394	75.394206
13	0.342	12	0.005062	4	0.015625	0.0625	0.080995	80.994766
14	0.364	12	0.005429	4	0.015625	0.0625	0.086862	86.86202
15	0.37	12	0.005529	4	0.015625	0.0625	0.088462	88.46218
16	0.471	24	0.007212	4	0.015625	0.0625	0.115398	115.39821
17	0.418	24	0.006329	4	0.015625	0.0625	0.101263	101.26346
18	0.443	24	0.006746	4	0.015625	0.0625	0.107931	107.93079
19	0.41	36	0.006196	4	0.015625	0.0625	0.09913	99.129913
20	0.444	36	0.006762	4	0.015625	0.0625	0.108197	108.19749
21	0.403	36	0.006079	4	0.015625	0.0625	0.097263	97.26306
22	0.536	48	0.008296	4	0.015625	0.0625	0.132733	132.73327
23	0.416	48	0.006296	4	0.015625	0.0625	0.10073	100.73007
24	0.443	48	0.006746	4	0.015625	0.0625	0.107931	107.93079
25	0.62	12	0.009696	4	0.015625	0.0625	0.155136	155.13551
26	0.62	12	0.009696	4	0.015625	0.0625	0.155136	155.13551
27	0.612	**12**	**0.009563**	4	0.015625	0.0625	0.153002	153.00197
28	0.725	24	0.011446	4	0.015625	0.0625	0.183138	183.13831
29	0.719	24	0.011346	4	0.015625	0.0625	0.181538	181.53815
30	0.728	24	0.011496	4	0.015625	0.0625	0.183938	183.93839
31	0.797	36	0.012646	4	0.015625	0.0625	0.20234	202.34023
32	0.741	36	0.011713	4	0.015625	0.0625	0.187405	187.40541
33	0.68	36	0.010696	4	0.015625	0.0625	0.171137	171.13711
34	0.806	48	0.012796	4	0.015625	0.0625	0.20474	204.74047
35	0.724	48	0.011429	4	0.015625	0.0625	0.182872	182.87162
36	0.799	48	0.01268	4	0.015625	0.0625	0.202874	202.87362

Grindamyl Powerbake 950

Sample	Absorbance @ 325	time	x	Sample Wt g d.m.	Dilution	Diluted extracted g.	free f.a. eq./g	f.a. milli eq./g
1	0.13	12	0.0015285	4	0.015625	0.0625	0.0244558	24.455779
2	0.194	12	0.0025953	4	0.015625	0.0625	0.0415242	41.524152
3	0.22	12	0.0030286	4	0.015625	0.0625	0.0484582	48.458179
4	0.234	24	0.003262	4	0.015625	0.0625	0.0521919	52.191886
5	0.203	24	0.0027453	4	0.015625	0.0625	0.0439244	43.924392
6	0.215	24	0.0029453	4	0.015625	0.0625	0.0471247	47.124712
7	0.298	36	0.0043288	4	0.015625	0.0625	0.0692603	69.260259
8	0.274	36	0.0039287	4	0.015625	0.0625	0.0628596	62.859619
9	0.228	36	0.003162	4	0.015625	0.0625	0.0505917	50.591726
10	0.214	48	0.0029286	4	0.015625	0.0625	0.046858	46.858019
11	0.275	48	0.0039454	4	0.015625	0.0625	0.0631263	63.126313
12	0.226	48	0.0031286	4	0.015625	0.0625	0.0500583	50.058339
13	0.3	12	0.0043621	4	0.015625	0.0625	0.0697936	69.793646
14	0.292	12	0.0042288	4	0.015625	0.0625	0.0676601	67.660099
15	0.279	12	0.0040121	4	0.015625	0.0625	0.0641931	64.193086
16	0.344	24	0.0050955	4	0.015625	0.0625	0.0815282	81.528153
17	0.344	24	0.0050955	4	0.015625	0.0625	0.0815282	81.528153
18	0.364	24	0.0054289	4	0.015625	0.0625	0.086862	86.86202
19	0.325	36	0.0047788	4	0.015625	0.0625	0.076461	76.460979
20	0.297	36	0.0043121	4	0.015625	0.0625	0.0689936	68.993566
21	0.304	36	0.0044288	4	0.015625	0.0625	0.0708604	70.860419
22	0.36	48	0.0053622	4	0.015625	0.0625	0.0857952	85.795246
23	0.357	48	0.0053122	4	0.015625	0.0625	0.0849952	84.995166
24	0.372	48	0.0055622	4	0.015625	0.0625	0.0889956	88.995566
25	0.415	12	0.006279	4	0.015625	0.0625	0.1004634	100.46338
26	0.444	12	0.0067623	4	0.015625	0.0625	0.1081975	108.19749
27	0.44	12	0.0066957	4	0.015625	0.0625	0.1071307	107.13071
28	0.483	24	0.0074124	4	0.015625	0.0625	0.1185985	118.59853
29	0.438	24	0.0066623	4	0.015625	0.0625	0.1065973	106.59733
30	0.467	24	0.0071457	4	0.015625	0.0625	0.1143314	114.33143
31	0.469	36	0.0071791	4	0.015625	0.0625	0.1148648	114.86482
32	0.455	36	0.0069457	4	0.015625	0.0625	0.1111311	111.13111
33	0.462	36	0.0070624	4	0.015625	0.0625	0.112998	112.99797
34	0.502	48	0.0077291	4	0.015625	0.0625	0.1236657	123.6657
35	0.519	48	0.0080125	4	0.015625	0.0625	0.1281995	128.19949
36	0.382	48	0.0057289	4	0.015625	0.0625	0.0916625	91.6625

Pentopan combined with Xylanase S and BX-AN

Sample-Enzyme	Absorbance @ 325	time	x	Sample Wt g d. m.	Dilution	Diluted extracted g.	free f.a. eq./g	free f.a. milli eq./g
1-B & P	0.201	12	0.00271	4	0.015625	0.0625	0.043391	43.391006
2-B & P	0.179	12	0.00235	4	0.015625	0.0625	0.0375238	37.523752
3-B & P	0.288	12	0.00416	4	0.015625	0.0625	0.0665933	66.593326
4-B & P	0.3	24	0.00436	4	0.015625	0.0625	0.0697936	69.793646
5-B & P	0.258	24	0.00366	4	0.015625	0.0625	0.0585925	58.592526
6-B & P	0.248	24	0.0035	4	0.015625	0.0625	0.0559256	55.925593
7-B & P	0.189	36	0.00251	4	0.015625	0.0625	0.0401907	40.190686
8-B & P	0.245	36	0.00345	4	0.015625	0.0625	0.0551255	55.125513
9-B & P	0.421	36	0.00638	4	0.015625	0.0625	0.1020635	102.06354
10-B & P	0.364	48	0.00543	4	0.015625	0.0625	0.086862	86.86202
11-B & P	0.466	48	0.00713	4	0.015625	0.0625	0.1140647	114.06474
12-B & P	0.315	48	0.00461	4	0.015625	0.0625	0.073794	73.794046
13-B & S	0.203	12	0.00275	4	0.015625	0.0625	0.0439244	43.924392
14-B & S	0.182	12	0.0024	4	0.015625	0.0625	0.0383238	38.323832
15-B & S	0.213	12	0.00291	4	0.015625	0.0625	0.0465913	46.591326
16-B & S	0.221	24	0.00305	4	0.015625	0.0625	0.0487249	48.724872
17-B & S	0.191	24	0.00255	4	0.015625	0.0625	0.0407241	40.724072
18-B & S	0.184	24	0.00243	4	0.015625	0.0625	0.0388572	38.857219
19-B & S	0.233	36	0.00325	4	0.015625	0.0625	0.0519252	51.925193
20-B & S	0.232	36	0.00323	4	0.015625	0.0625	0.0516585	51.658499
21-B & S	0.093	36	0.00091	4	0.015625	0.0625	0.0145881	14.588125
22-B & S	0.096	48	0.00096	4	0.015625	0.0625	0.0153882	15.388205
23-B & S	0.161	48	0.00205	4	0.015625	0.0625	0.0327233	32.723272
24-B & S	0.11	48	0.0012	4	0.015625	0.0625	0.0191219	19.121912
25-S & P	0.351	12	0.00521	4	0.015625	0.0625	0.083395	83.395006
26-S & P	0.954	12	0.01526	4	0.015625	0.0625	0.2442111	244.21109
27-S & P	0.793	**12**	**0.01258**	4	0.015625	0.0625	0.2012735	201.27346
28-S & P	0.616	24	0.00963	4	0.015625	0.0625	0.1540687	154.06874
29-S & P	0.797	24	0.01265	4	0.015625	0.0625	0.2023402	202.34023
30-S & P	0.706	24	0.01113	4	0.015625	0.0625	0.1780711	178.07114
31-S & P	0.83	36	0.0132	4	0.015625	0.0625	0.2111411	211.14111
32-S & P	0.875	36	0.01395	4	0.015625	0.0625	0.2231423	223.14231
33-S & P	1.232	36	0.0199	4	0.015625	0.0625	0.3183518	318.35184
34-S & P	0.692	48	0.0109	4	0.015625	0.0625	0.1743374	174.33743
35-S & P	0.748	48	0.01183	4	0.015625	0.0625	0.1892723	189.27226
36-S & P	0.82	48	0.01303	4	0.015625	0.0625	0.2084742	208.47418
37-B, S, P	0.831	12	0.01321	4	0.015625	0.0625	0.2114078	211.40781

38-B, S, P	0.82	12	0.01303	4	0.015625	0.0625	0.2084742	208.47418
39-B, S, P	0.826	12	0.01313	4	0.015625	0.0625	0.2100743	210.07434
40-B, S, P	0.742	24	0.01173	4	0.015625	0.0625	0.1876721	187.6721
41-B, S, P	0.547	24	0.00848	4	0.015625	0.0625	0.1356669	135.6669
42-B, S, P	0.803	24	0.01275	4	0.015625	0.0625	0.2039404	203.94039
43-B, S, P	0.866	36	0.0138	4	0.015625	0.0625	0.2207421	220.74207
44-B, S, P	0.692	36	0.0109	4	0.015625	0.0625	0.1743374	174.33743
45-B, S, P	0.821	36	0.01305	4	0.015625	0.0625	0.2087409	208.74087
46-B, S, P	0.605	48	0.00945	4	0.015625	0.0625	0.1511351	151.13511
47-B, S, P	0.568	48	0.00883	4	0.015625	0.0625	0.1412675	141.26746
48-B, S, P	1.097	48	0.01765	4	0.015625	0.0625	0.2823482	282.34823

Combined Process with Xylanase S and AN-900

Sample	Absorbance @ 325	x	Sample Wt	Dilution	Diluted Extracted	free f.a.	f.a milli
	time		g d.m.		Bran g.	eq./g	eq./g
1	0.333	0.0048	0.9	0.0156	0.0140625	0.3419	341.901
2	0.28	0.004	0.9	0.0156	0.0140625	0.2845	284.466
3	0.331	0.0048	0.9	0.0156	0.0140625	0.3397	339.734
4	0.379	0.0055	0.9	0.0156	0.0140625	0.3918	391.75
5	0.42	0.0061	0.9	0.0156	0.0140625	0.4362	436.181
6	0.371	0.0054	0.9	0.0156	0.0140625	0.3831	383.081
7	0.41	0.006	0.9	0.0156	0.0140625	0.4253	425.345
8	0.352	0.0051	0.9	0.0156	0.0140625	0.3625	362.491
9	0.137	0.0018	0.9	0.0156	0.0140625	0.1295	129.5
10	0.098	0.0012	0.9	0.0156	0.0140625	0.0872	87.2363
11	0.261	0.0037	0.9	0.0156	0.0140625	0.2639	263.876
12	0.28	0.004	0.9	0.0156	0.0140625	0.2845	284.466
13	0.228	0.0032	0.9	0.0156	0.0140625	0.2281	228.115
14	0.206	0.0029	0.9	0.0156	0.0140625	0.2043	204.274
15	0.239	0.0034	0.9	0.0156	0.0140625	0.24	240.035
16	0.201	0.0028	0.9	0.0156	0.0140625	0.1989	198.855
17	0.035	0.0003	0.9	0.0156	0.0140625	0.019	18.9644
18	0.205	0.0029	0.9	0.0156	0.0140625	0.2032	203.19

Combined Process with Xylanase S and BX-AN

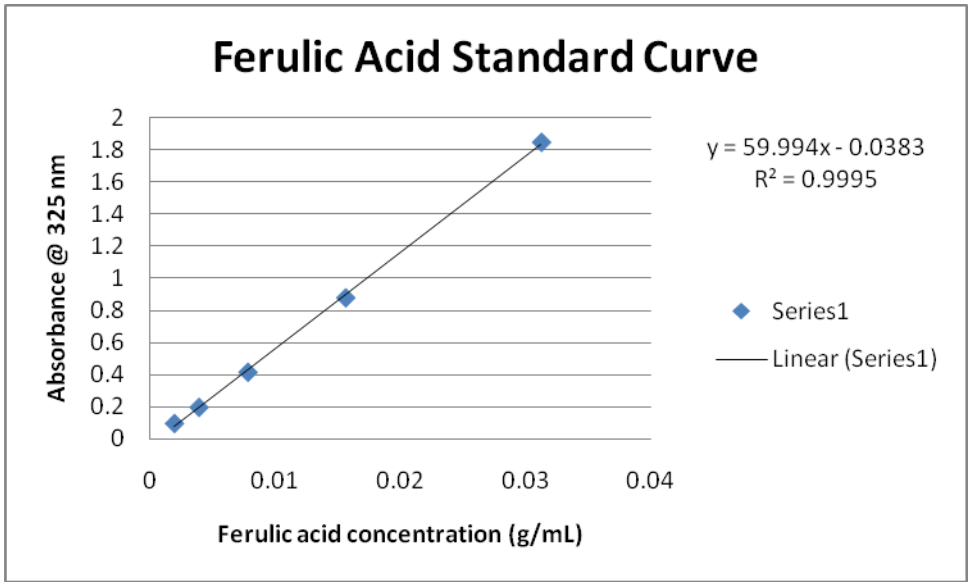
Sample	Absorbance @ 325	time	x	Sample Wt g d.m.	Dilution	Diluted extracted g.	free f.a. eq./g	free f.a. milli eq./g
1	0.16	12	0.002029	0.375	0.01563	0.0058594	0.346204	346.20351
2	0.128	12	0.001495	0.375	0.01563	0.0058594	0.255172	255.17218
3	0.138	12	0.001662	0.375	0.01563	0.0058594	0.283619	283.61947
4	0.138	24	0.001662	0.375	0.01563	0.0058594	0.283619	283.61947
5	0.152	24	0.001895	0.375	0.01563	0.0058594	0.323446	323.44568
6	0.126	24	0.001462	0.375	0.01563	0.0058594	0.249483	249.48273
7	0.125	36	0.001445	0.375	0.01563	0.0058594	0.246638	246.638
8	0.139	36	0.001679	0.375	0.01563	0.0058594	0.286464	286.4642
9	0.174	36	0.002262	0.375	0.01563	0.0058594	0.38603	386.02971
10	0.149	48	0.001845	0.375	0.01563	0.0058594	0.314911	314.91149
11	0.142	48	0.001729	0.375	0.01563	0.0058594	0.294998	294.99839
12	0.165	48	0.002112	0.375	0.01563	0.0058594	0.360427	360.42715
13	0	12	-0.00064	0.375	0.01563	0.0058594	-0.10895	-108.95312
14	0.138	12	0.001662	0.375	0.01563	0.0058594	0.283619	283.61947
15	0.287	12	0.004145	0.375	0.01563	0.0058594	0.707484	707.48408
16	0.123	24	0.001412	0.375	0.01563	0.0058594	0.240949	240.94854
17	0.104	24	0.001095	0.375	0.01563	0.0058594	0.186899	186.89869
18	0.124	24	0.001428	0.375	0.01563	0.0058594	0.243793	243.79327
19	0.149	36	0.001845	0.375	0.01563	0.0058594	0.314911	314.91149
20	0	36	-0.00064	0.375	0.01563	0.0058594	-0.10895	-108.95312
21	0.154	36	0.001929	0.375	0.01563	0.0058594	0.329135	329.13514
22	0.125	48	0.001445	0.375	0.01563	0.0058594	0.246638	246.638
23	0.164	48	0.002095	0.375	0.01563	0.0058594	0.357582	357.58242
24	0.126	48	0.001462	0.375	0.01563	0.0058594	0.249483	249.48273
25	0.141	12	0.001712	0.375	0.01563	0.0058594	0.292154	292.15366
26	0.045	12	0.000112	0.375	0.01563	0.0058594	0.01906	19.059684

Combined Process with Xylanase S, BX-AN, and Pentopan

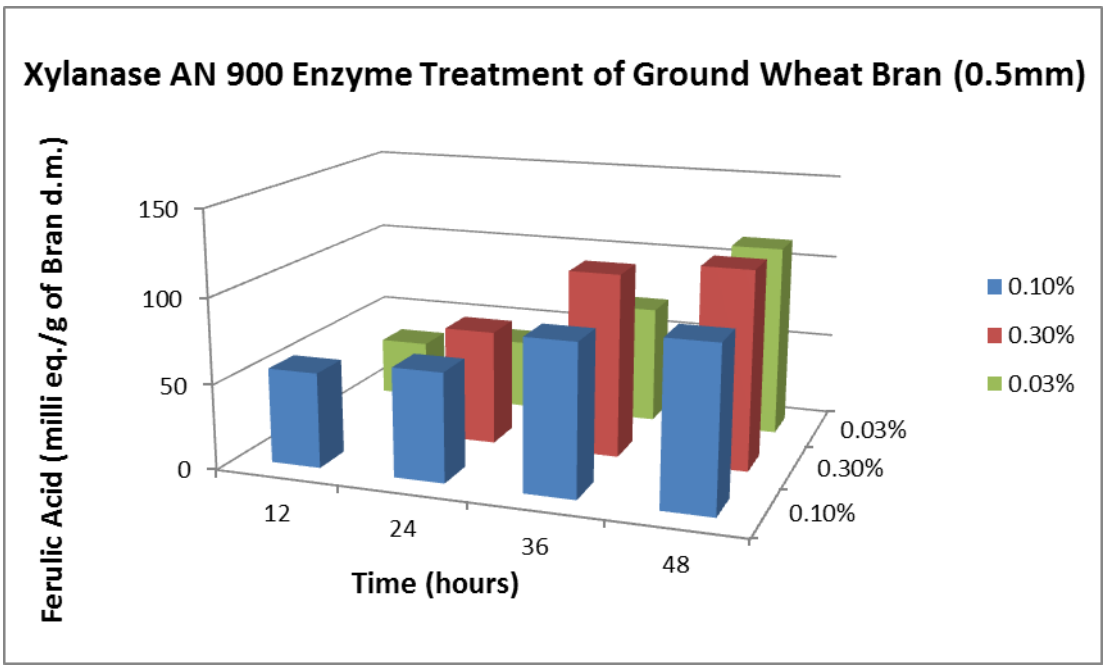
Sample-Enzyme	Absorbance @ 325	time	x	Sample Wt g d. m.	Dilution	Diluted extracted g.	free f.a. eq./g	free f.a. milli eq./g
1-P & B	0.825	12	0.01311	4	0.015625	0.0625	0.20981	209.80765
2-P & B	0.832	12	0.01323	4	0.015625	0.0625	0.21167	211.6745
3-P & B	0.817	12	0.01298	4	0.015625	0.0625	0.20767	207.6741
4-P & B	0.79	24	0.01253	4	0.015625	0.0625	0.20047	200.47338
5-P & S	0.84	24	0.01336	4	0.015625	0.0625	0.21381	213.80805
6-P & S	812	24	13.534	4	0.015625	0.0625	216.545	216544.77
7-P & S	0.418	36	0.00633	4	0.015625	0.0625	0.10126	101.26346
8-P & S	0.395	36	0.00595	4	0.015625	0.0625	0.09513	95.129513
9-P	0.807	36	0.01281	4	0.015625	0.0625	0.20501	205.00717
10-P	0.805	48	0.01278	4	0.015625	0.0625	0.20447	204.47378
11-P	0.77	48	0.0122	4	0.015625	0.0625	0.19514	195.13951
12-P	0.856	48	0.01363	4	0.015625	0.0625	0.21808	218.07514
13-P & B	0.649	12	0.01018	4	0.015625	0.0625	0.16287	162.86962
14-P & B	0.806	12	0.0128	4	0.015625	0.0625	0.20474	204.74047
15-P & B	0.9	12	0.01436	4	0.015625	0.0625	0.22981	229.80965
16-P & B	0.751	24	0.01188	4	0.015625	0.0625	0.19007	190.07234
17-P & S	0.79	24	0.01253	4	0.015625	0.0625	0.20047	200.47338
18-P & S	0.823	24	0.01308	4	0.015625	0.0625	0.20927	209.27426
19-P & S	0.814	36	0.01293	4	0.015625	0.0625	0.20687	206.87402
20-P & S	0.885	36	0.01411	4	0.015625	0.0625	0.22581	225.80925
21-P	0.633	36	0.00991	4	0.015625	0.0625	0.1586	158.60253
22-P	0.782	48	0.0124	4	0.015625	0.0625	0.19834	198.33983
23-P	0.679	48	0.01068	4	0.015625	0.0625	0.17087	170.87042
24-P	0.781	48	0.01238	4	0.015625	0.0625	0.19807	198.07314
25-cntrl	0.785	12	0.01245	4	0.015625	0.0625	0.19914	199.13991

Individual graphs

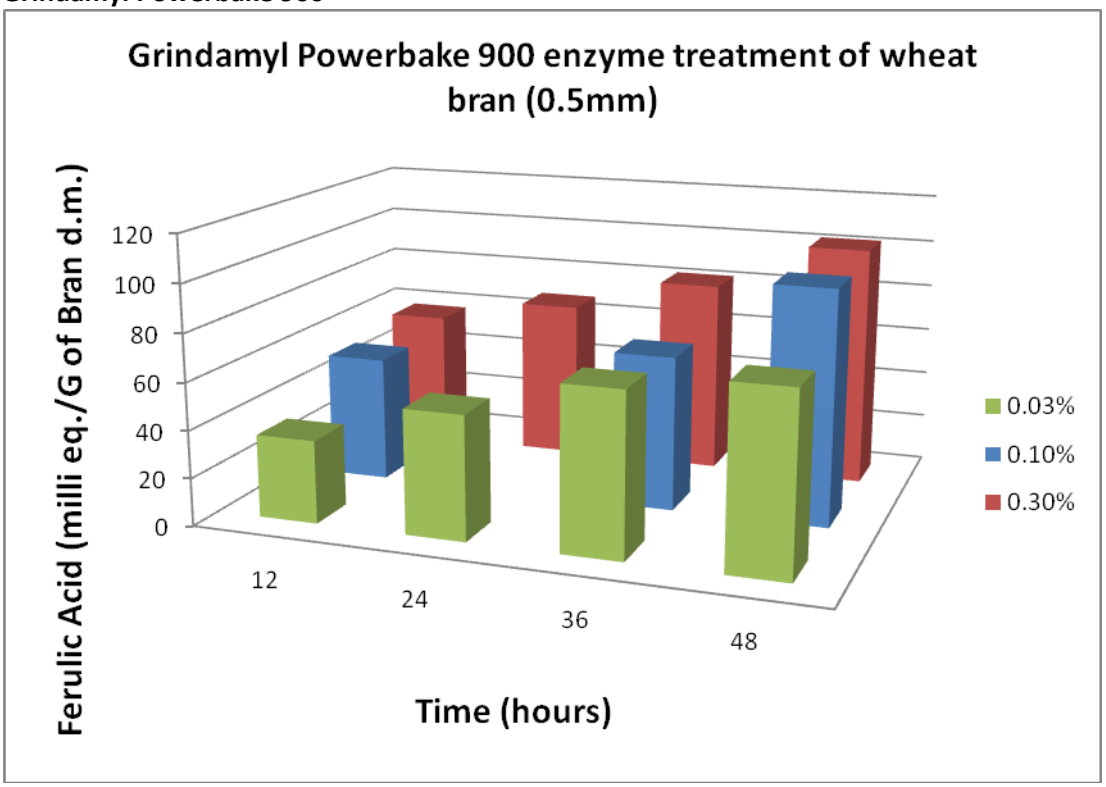
Standard Curve



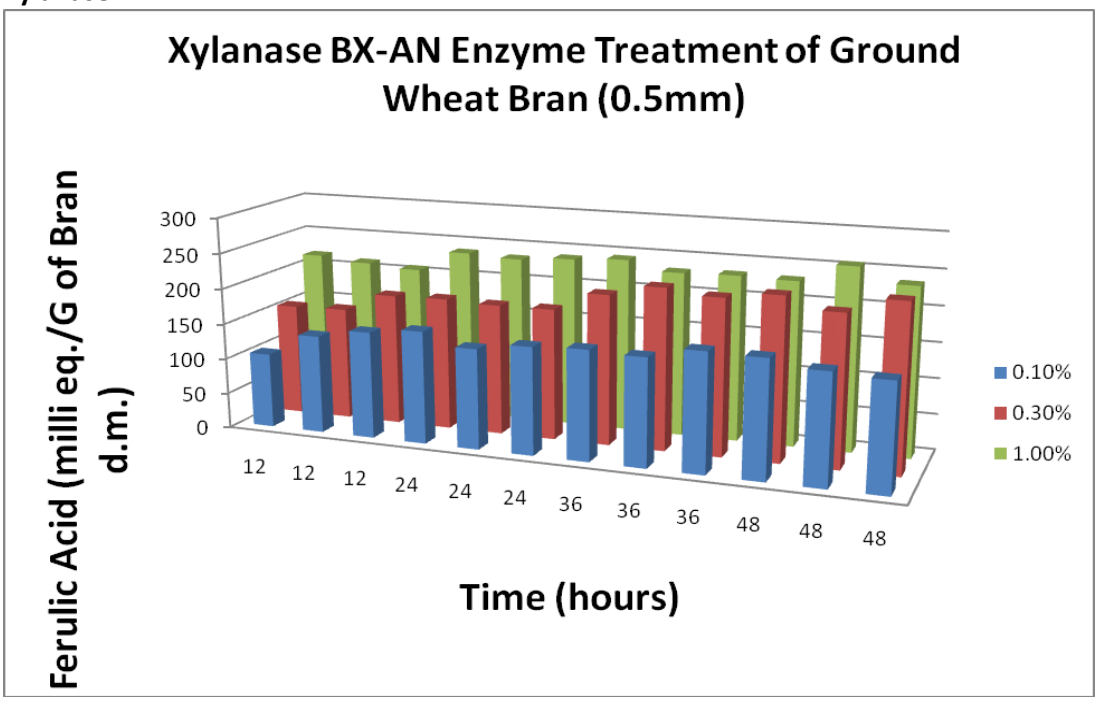
Xylanase AN 900



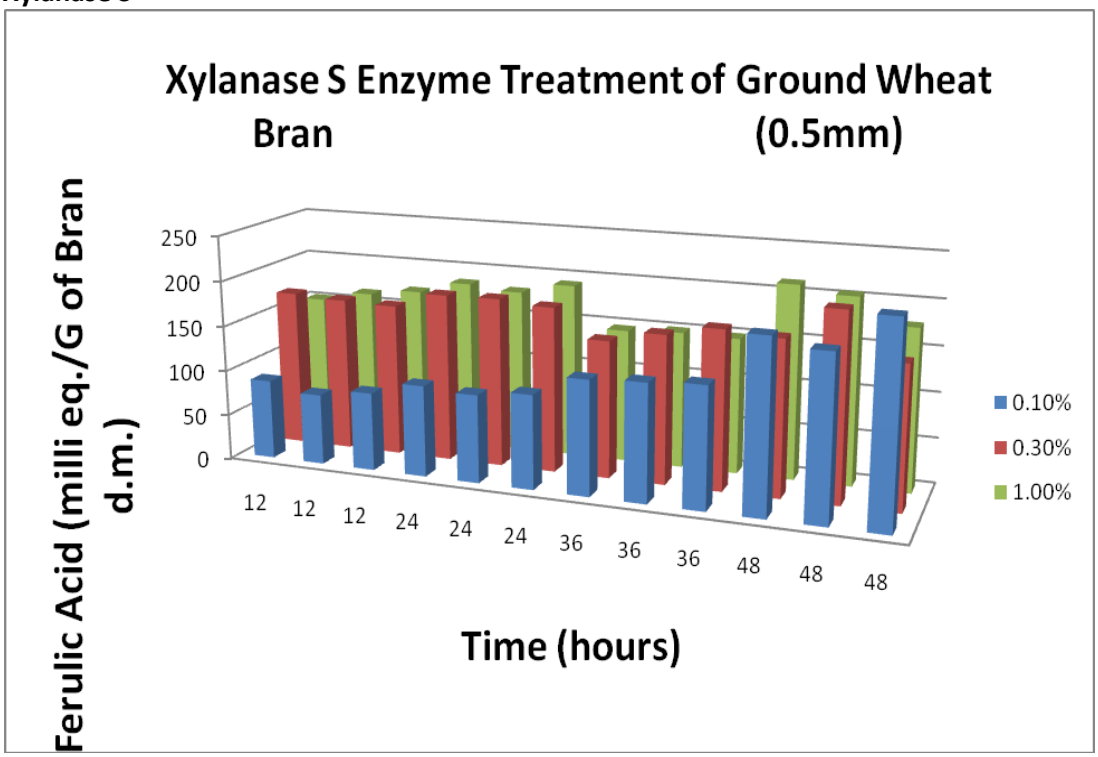
Grindamyl Powerbake 900



Xylanase BX-AN



Xylanase S



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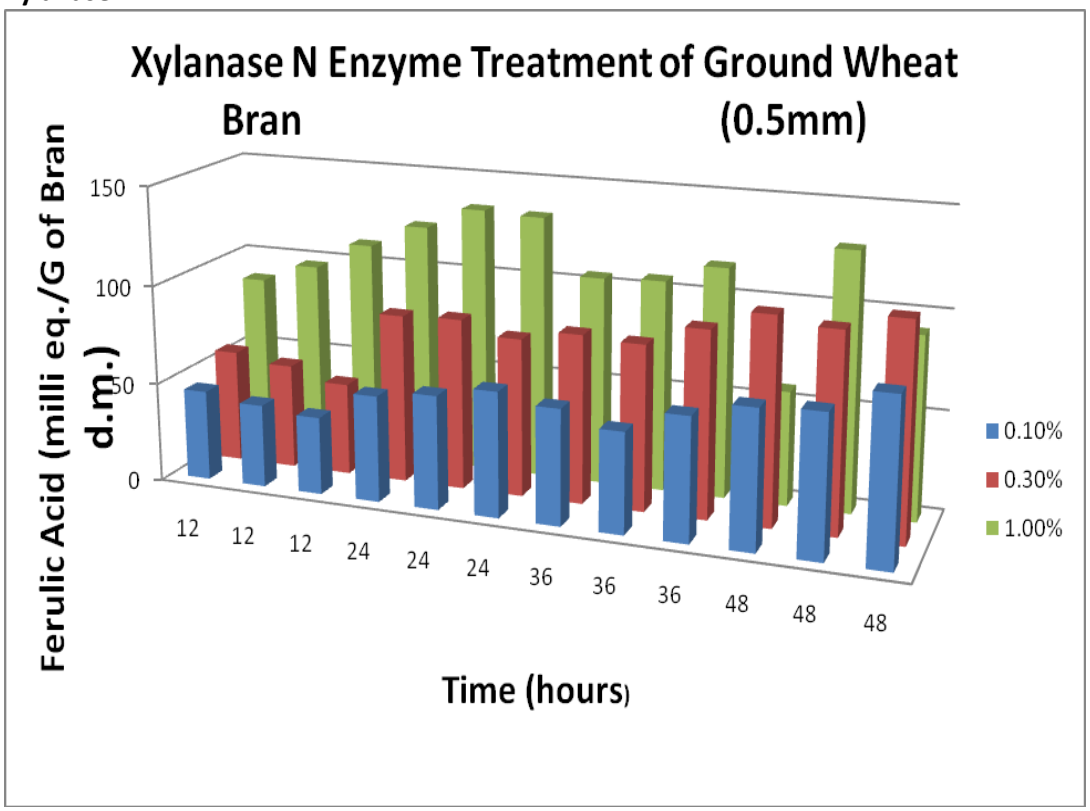
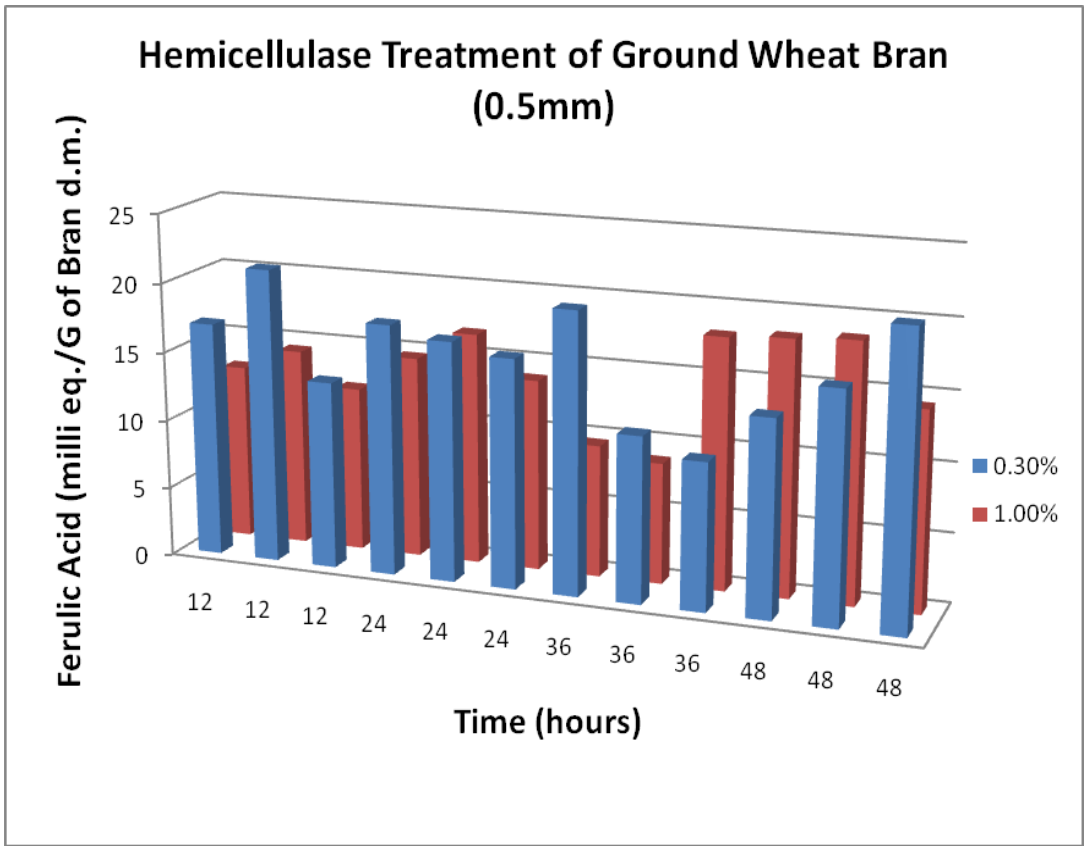
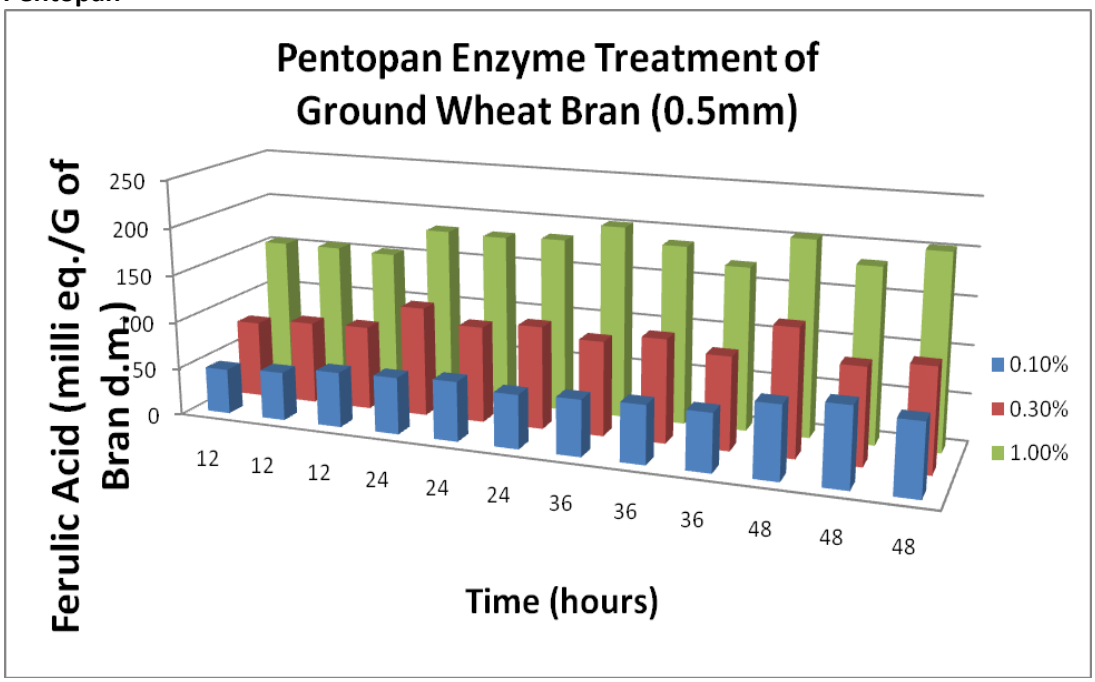


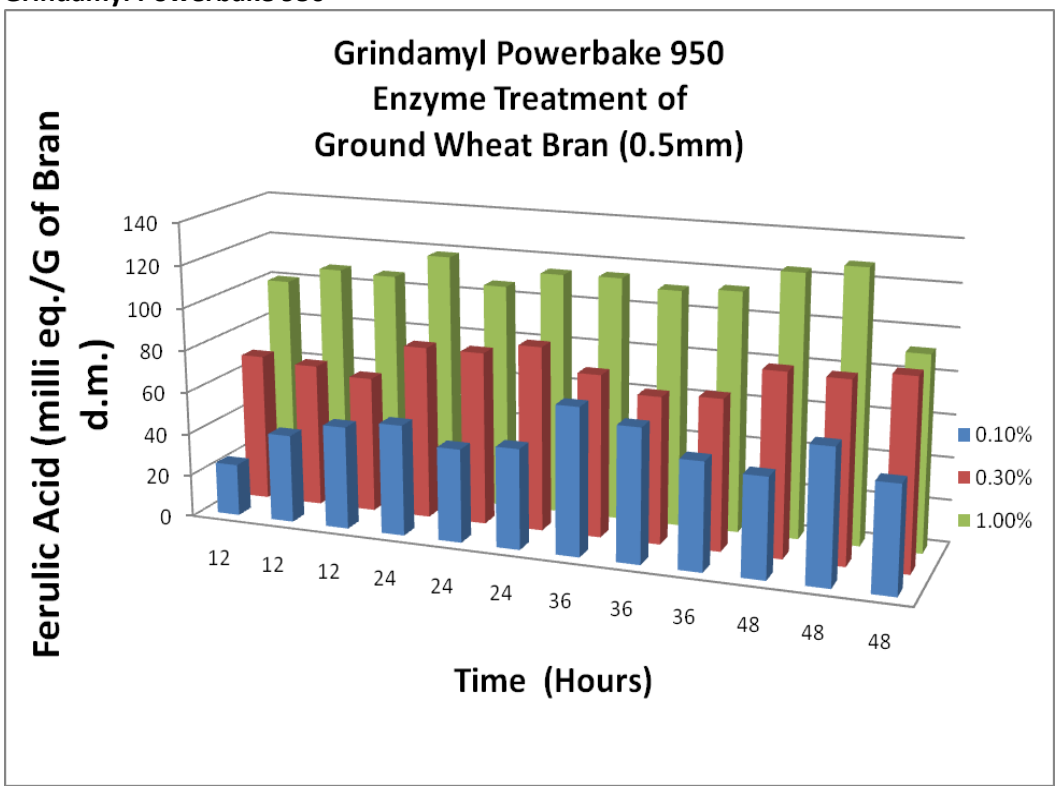
Hemicellulase



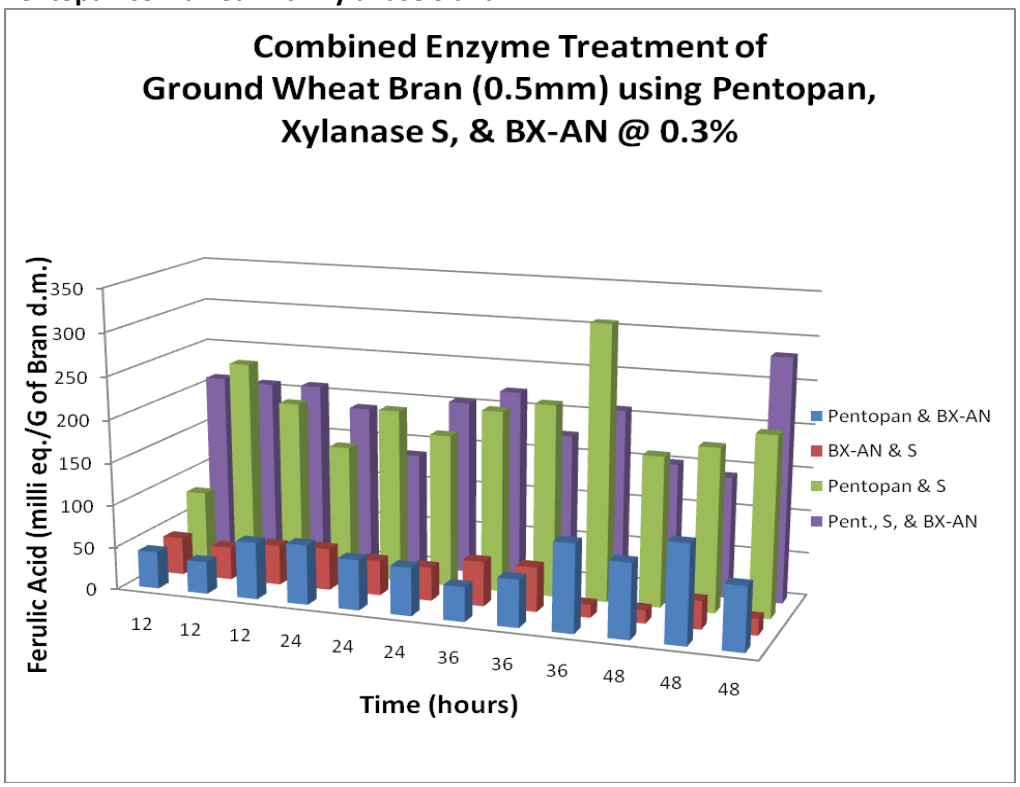
Pentopan



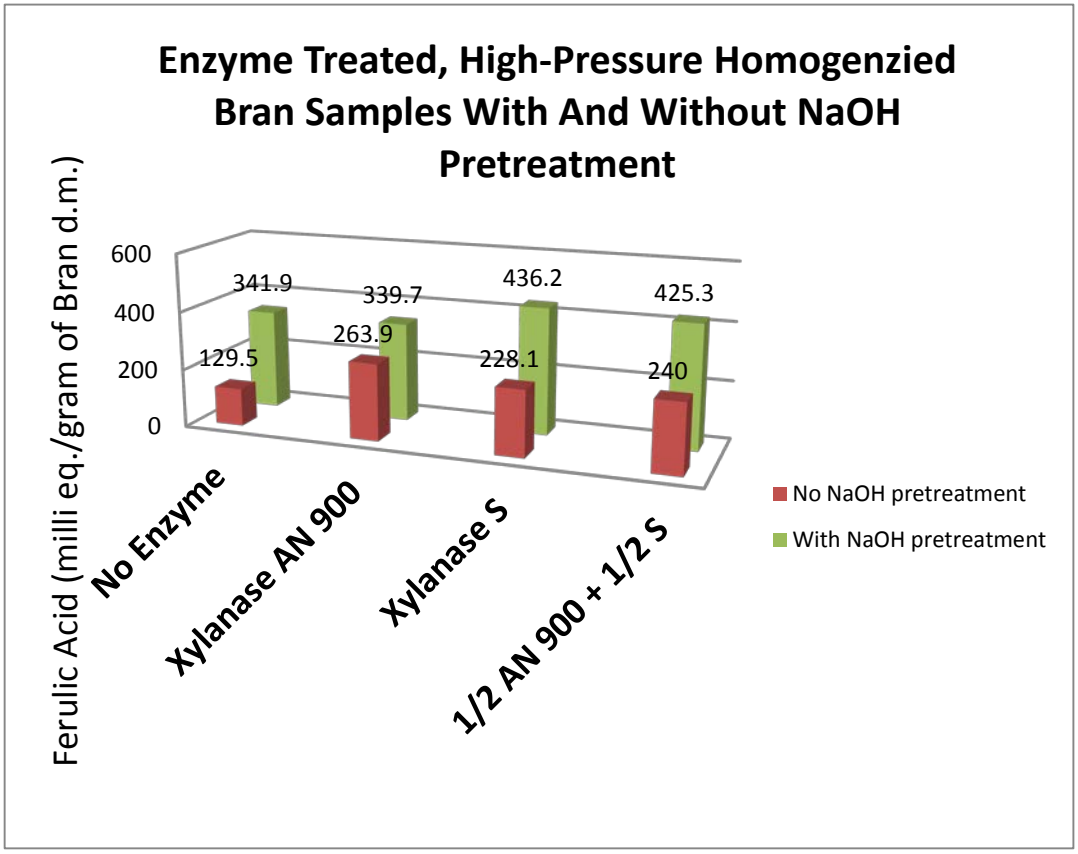
Grindamyl Powerbake 950



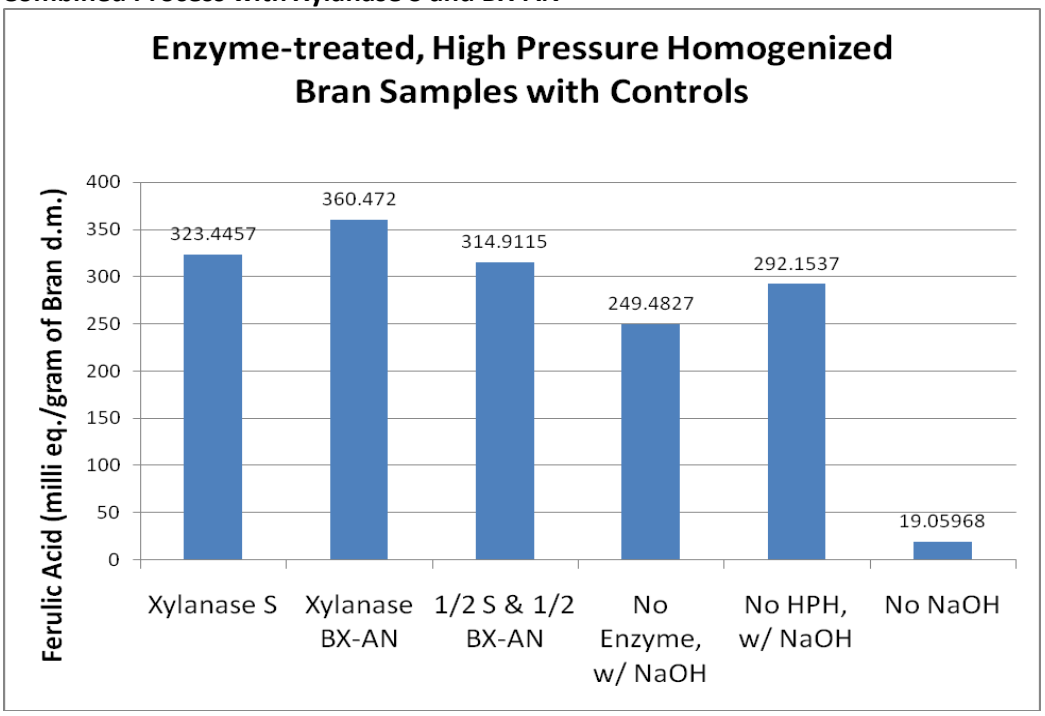
Pentopan combined with Xylanase S and BX-AN



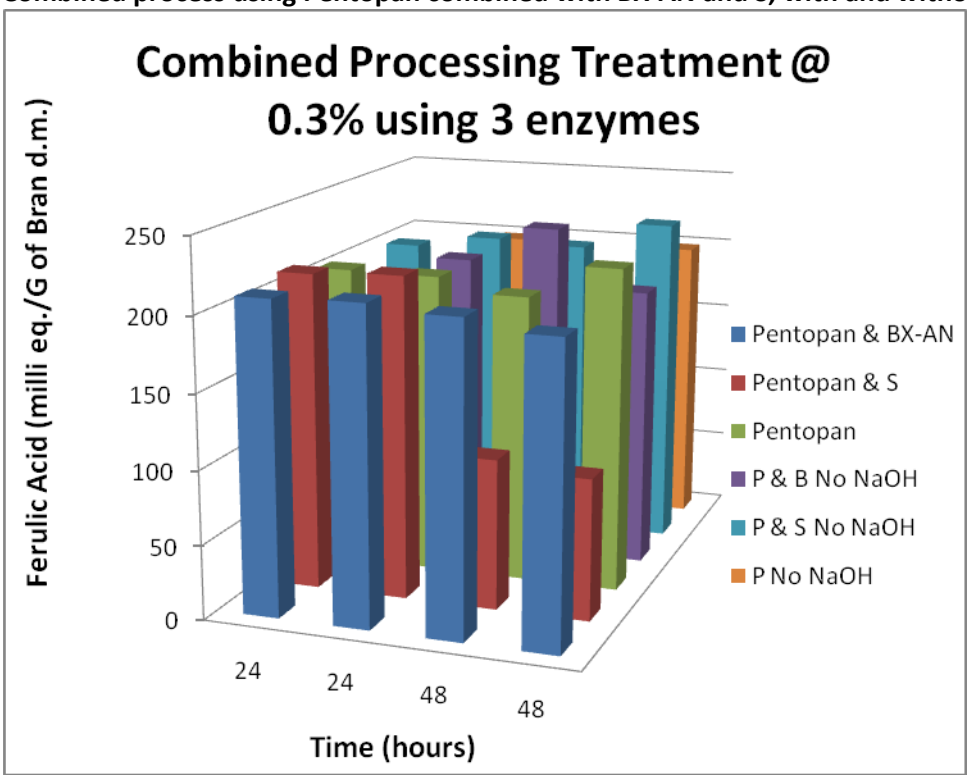
Combined Process with Xylanase S and AN-900



Combined Process with Xylanase S and BX-AN



Combined process using Pentopan combined with BX-AN and S, with and without NaOH.



Appendix C: Viscosity Data