

**EVALUATION OF XYLANASE AND LACCASE ENZYMES WITH MICROWAVE
TREATMENT, TO BREAK DOWN COTTON SEED COAT FRAGMENTS.**

by

RENUKA DHANDAPANI

(Under the Direction of Ian R. Hardin)

ABSTRACT

Enzymatic applications have various advantages that make this an ideal process to be adapted in the textile industry. But limitation such as failure in removing seed coat fragments (SCF's) from cotton greige fabric during bio-scouring prevents the overall acceptance of enzymatic applications. Cotton seed coat fragments are recalcitrant by nature and are found embedded in the fabric surface. SCF's were subjected to four groups of treatments including conventional sodium hydroxide at three different concentrations, enzymatic treatments including xylanase and laccase enzymes, 2.5 min and 5 min microwave treatments and a combination of microwave and enzymatic treatments. The effectiveness of the treatment on SCF's were evaluated by measuring the weight loss obtained for the SCF's, analyzing the sugar content present in the supernatant separated from SCF's using GC-MS and measuring any change in the chemical structure of SCF using FTIR, and Raman spectroscopy (SERS). The overall conclusion to be drawn from this research is that enzymes alone cannot be used to effectively break down the SCF's due to their complex structure. By combining microwave pretreatment with enzymatic treatments gave results that were comparable to conventional treatments.

INDEX WORDS: *Xylanase, Laccase, Microwave, Seed coat fragments, GC-MS, FTIR, SERS*

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CHAPTER 1

INTRODUCTION

An enzyme is a protein that acts as a catalyst in chemical reactions, increasing the rate of the reaction by lowering the activation energy of the reaction. Similar to other catalysts, enzymes are neither consumed in reactions nor do they alter the equilibrium of the reactions. The well known advantages of an enzyme include its specific action that results in a high overall efficiency, requiring mild treatment conditions to be active and easily be deactivated by changing either the pH or the temperature of the bath. These characteristic features of an enzyme are highly desired in an industrial environment as the use of enzymes will increase the product quality, lower the manufacturing cost, reduce the energy consumption and decrease the waste generated. In addition to these advantages, since an enzyme is a catalyst relatively low concentration of the enzyme is sufficient to carry out a reaction (Illanes 2008).

Enzymes are often used to replace harmful chemicals or processes that cause environmental safety issues. For example, enzymes are used:

- To replace strong acids, alkalis and oxidizing agents used in fabric desizing process.
- To replace pumice stones for 'stone washing' of jeans.
- Can be used instead of chlorine bleach to remove stains from the fabrics and save energy as the clothes can be washed at lower temperatures to get effective results.
- To provide a safer working environment for the workers during the production processes

With advanced research in biotechnology and enzymology, different functions of enzymes are being studied and comprehended. Due to the selective nature of this natural substance application to different textile wet processing stages are being researched.

Cotton fibers contain non-cellulosic materials in addition to the primary cellulose polymer. Non-cellulosic material creates a physical hydrophobic barrier that protects the fiber from damage during its growth. However, in textile wet processing these non-cellulosic materials cause hindrance to application of treatments and hence are removed using conventional methods. The various pretreatment stages through which a greige cotton fabric is passed include singeing, desizing, scouring, bleaching, mercerizing and depilling. The purpose of applying these pretreatments is to remove the impurities present in the fabric and to improve its surface properties resulting in a fabric appropriate for dyeing.

Desizing is a wet treatment process where starch present in the greige fabric is removed by hydrolyzing starch into soluble material and thus improving the wettability of the fabric. Desizing is performed using enzymes such as α -amylase and its derivatives to assist in the breakdown of starch. The next stage is scouring, which is done to remove natural waxes, pectins and other non-cellulosic components present in the fabric. Scouring is done by a conventional method using sodium hydroxide (NaOH); the treatment is done at a high temperature followed by extensive rinsing. Though sodium hydroxide is less expensive and alkaline scouring is effective, the scouring process is considered inefficient because of the large quantities of water and energy required to carry out this process, and extra chemicals needed to neutralize the waste water (Agrawal, Nierstrasz et al. 2007). Due to this negative impact of sodium hydroxide on the consumer and the environment, much research has been done on replacing the alkaline scouring process by an enzymatic process. Enzymes such as cellulases, pectinases, lipases and proteases

were investigated by researchers as possible replacements for alkaline scouring (Hartzell-Lawson and Hsieh 1998; Buchert and Pere 2000; Aly, Moustafa et al. 2004; Kim, Choe et al. 2006). Of these enzymes, two potential candidates for enzymatic scouring were identified as cellulases and pectinases (Li and Hardin 1997; Csiszar, Szakacs et al. 1998; Calafell and Garriga 2004).

Treatment by an alkaline pectinase was found to be effective in destabilizing the pectin present in the primary cell wall of cotton fibers which, when followed by washing, resulted in removal of various impurities present in the fiber. While cellulase enzyme is effective in attacking impurities, it also degrades the cellulose in the fiber along with the impurities, thus reducing the strength of the fabric (Agrawal, Nierstrasz et al. 2007). Pectinase is also the preferred enzyme for bioscouring because pectin is one of the main components present on the surface of cotton that glues and stabilizes the outer layer on cotton that must be removed for successive treatments (Schnitzhofer, Kandelbauer et al. 2006). The purpose of scouring is to increase the absorbency of the fabric to achieve uniformity in dyeing. One limitation of bioscouring is that it does not remove seed coat fragments (SCF's) present in the fabric as does the conventional alkaline scouring process.

Bleaching is the next pretreatment stage. Fabrics are treated with hydrogen peroxide to remove natural coloring material present in the fiber and thus improve the whiteness of the sample before being dyed. The drawback of peroxide bleaching is that after treatment an excess of peroxide present on the treated fabric must be removed by washing. This consumes large quantities of water. Since the late 1990's, catalase enzymes have been effectively used to remove the excess peroxide present in effluent by decomposing peroxide into oxygen and water (Cavaco-Paulo and Gubitiz 2003). In depilling or biofinishing process of the cotton fabric,

cellulase enzyme is used to remove fibrils and fuzz fibers from the surface of the fabric thus giving it a smooth finish (Ramos, Pinto et al. 2007).

The significance of enzymes in different sectors of textile wet processing has been acknowledged but their application in textile industry has yet to be widely accepted. One of the main reasons preventing the overall acceptance of enzymatic treatments is the failure in removal of SCF's from the fabric by an enzymatic treatment. Seed coat fragments are portions of mature or immature cotton seeds that are either entangled in the fibers or embedded in the fabric. They are mostly black or dark brown in color. Their presence can result in major imperfections in the yarn structure and, in turn, the fabric structure that affects the fabric appearance and quality. For example, non-removal of SCF's can result in formation of dark specks in the fabric upon dyeing. Seed coat fragments are conventionally removed by alkaline scouring of greige desized fabric followed by bleaching. Removal of SCF's from the fabric is comparatively difficult with respect to other impurities present in the fabric. This is due to their intricate entanglement with the fibers and their complex chemical structure. Also, a mature cotton seed has a high degree of micro-hardness similar to annealed aluminum and hence is difficult to break. As a result, compared to other impurities, longer reaction times and higher chemical concentrations are required to facilitate SCF removal. This in turn can affect the cellulose in the fabric, resulting in a decrease in fabric strength (Losonczy, Csiszar et al. 2005; Csiszar, Losonczy et al. 2006).

The complex chemical structure that contributes to the recalcitrance of seed coat is more precisely due to presence of lignin in different layers of a cotton seed. The lignin structure forms a protective layer that restricts enzyme access to the hydrolyzable compounds of cotton seed. Therefore, for an effective enzymatic treatment this protective layer has to be modified by a pre

treatment so that it enhances the enzyme accessibility by removing lignin and increasing the porosity of the complex structure.

CHAPTER 2

LITERATURE REVIEW

I. General Background

Cotton is the most widely used natural fiber, with world consumption estimated to be around 114.6 million bales of cotton for the year 2010/11 (Cotton 2012). It is a staple fiber that grows naturally in the form of fluffy balls of fibers that encapsulate the seeds of cotton plants of the *Gossypium* genus.

A. Chemical Composition of a Cotton Fiber

Table 1: Composition of cotton fibers (McCall and Jurgens 1951; Tripp, Moore et al. 1951; Li and Hardin 1997)

Constituent	Composition (% dry weight)	
	Typical %	Range%
Cellulose	95.0	88.0-96.0
Protein	1.3	1.1-1.9
Pectic substances	0.9	0.7-1.2
Ash	1.2	0.7-1.6
Wax	0.6	0.4-1.0
Total sugars	0.3	0.1-1.0
Organic acids	0.8	0.5-1.0
Pigment	Trace	-
Others	1.4	-

The chemical composition of cotton fibers is given in Table 1 above. The major component of cotton is cellulose, which accounts for 95 percent of the fiber. After the desizing and scouring treatments of cotton the cellulose content increases to 99 percent.

B. Generation of Impurities in Cotton

The main plant contaminants found in the harvested cotton fibers are leaves, stem fragments, and seed coat fragments (SCF's). The seed coat fragments occur when cotton lint is separated from its seeds by passing the harvested cotton through the ginning process. During this process the various forces applied to separate the fibers from the seed result in seed fragments that remain attached to the fibers. During spinning these fragments become tightly embedded in the yarn and eventually end up on the surface of a woven fabric (Krifa, Gourlot et al. 2008). These fragments usually appear as black or dark brown colored material with fibers or linters attached to their surfaces. SCF's differ in chemical composition, color and morphology when compared to other impurities present in the fabric. They are difficult to eliminate during the cleaning and fiber spinning processes because of fibers attached to the surface of the seed coat. In addition, the cleaning operation carried out after ginning generates further fragmentation in SCF's, resulting in an increase in the fragments found on the fibers. Therefore, after carding, the number of SCF's usually increases while the size of the fragments decreases. Intensifying the cleaning process would result in fiber length reduction that reduces the overall quality of the fibers by increasing the proportion of short fibers in the bulk (Krifa, Frydrych et al. 2002).

After weaving, the fabric is passed through a desizing process to remove the starch that was applied to the warp yarns before weaving. Since the early 1950's, enzymatic desizing using α -amylases has been successfully used in textile industries to remove the starch from fabrics. More recently, thermostable α -amylases produced by *Bacillus subtilis* applicable at a range of

temperatures have been used in desizing. After desizing, the fabric is subjected to conventional scouring using caustic soda (NaOH) before dyeing and finishing of the fabric. The main purpose of scouring is to increase the hydrophilicity of the cotton fabric. This is obtained by removing any non-cellulosic impurities such as waxes, fats and pectin present in the fiber, along with foreign substances such as dirt, lint, and SCF's attached to the fabric. The success of subsequent treatment processes applied to the cotton fabric depends on the efficiency of the scouring process. Conventional alkaline scouring was found to be effective on all types of non-cellulosic impurities and resulted in major removal of impurities (Agrawal 2005).

Scouring involves the use of substantial amounts of sodium hydroxide at high treatment temperatures (90 °C-100 °C). Because of the use of NaOH, the treated fabric must be subjected to extensive washing, which in turn results in increased water consumption. Also, due to the use of high concentrations of NaOH, additional chemicals are required to neutralize the alkalinity of the wastewater. Therefore, though conventional scouring is effective in removal of foreign material from cotton fabric, it is characterized by the use of high temperatures, significant concentrations of chemicals and an alkaline pH in the liquor bath. This is one of the main reasons that there is the desire to shift wet textile treatments toward sustainable processes based on biocatalysts. Other factors that support this include less demand on fresh water usage, less pollution and the potential to meet environmental restrictions (Tzanov, Calafell et al. 2001; Nierstrasz and Warmoeskerken 2003; Agrawal, Nierstrasz et al. 2007).

Much research has been done to study the impact of environmentally friendly enzymatic processes (Li and Hardin 1997; Buschle-Diller, El Mogahzy et al. 1998; Csiszar, Losonczi et al. 2001; Tzanov, Calafell et al. 2001; Yachmenev, Bertoniere et al. 2001; Lenting and Zwier 2002). Enzymatic processes have been developed to degrade and remove undesired components from

cotton fibers using different enzymes such as pectinases, cellulases and lipases. These enzymatic treatments can successfully remove hydrophobic material from the surface of cotton fiber and thus improve the wet processing of yarns and fabrics. However, so far no effective enzymatic treatment has been developed to successfully remove seed coat fragments from fabric.

C. Cotton Seed Coat Fragments

Seed coat fragments are portions of cotton seed envelopes along with the lint attached to them and are present both within and on the surface of a cotton fabric (Figure 1). As seen in Figure 2, the quality of a dyed fabric is reduced when SCF's appear as dark specks after dyeing of the fabric. Although some SCFs originate in the cotton field before reaching the ginning mill, the quantity of SCF's is usually increased greatly in the ginning. Boykin determined experimentally that 32 percent (by weight) of SCF's created in the ginning process originated from seeds damaged either in the field or during ginning, while 15 percent (by weight) resulted from immature seeds. Damaged seeds usually increased from 5 percent to 12 percent as the fibers passed through the gin and therefore were a major source of SCF's created in the gin stand (Boykin 2008). Figure 3 represents a scanning electron microscope (SEM) pictograph of a seed coat fragment entangled on a fabric surface.

Along with processing factors, genetic factors also influence the formation of SCF's in textiles. A cotton seed is covered by a layer of palisade cells that gives the seed added strength and rigidity (Vizia, Jadhav et al. 1995). The entire outer surface of a seed coat is coated with a waxy layer that extends to fibers and provides the additional strength to the fiber attachment with the epidermis (Vigil, Anthony et al. 1996).

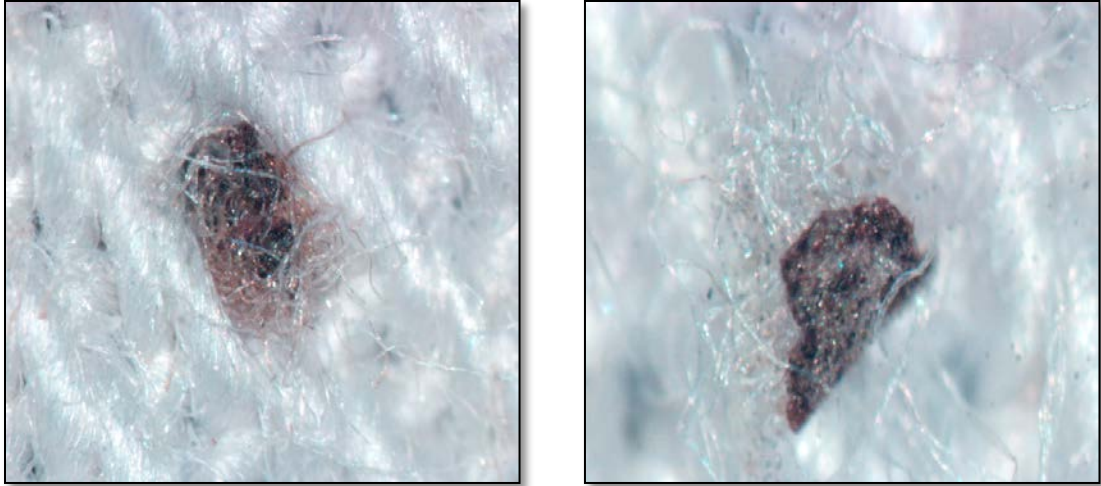


Figure 1: Seed Coat Fragments as seen on the Surface of a Fabric

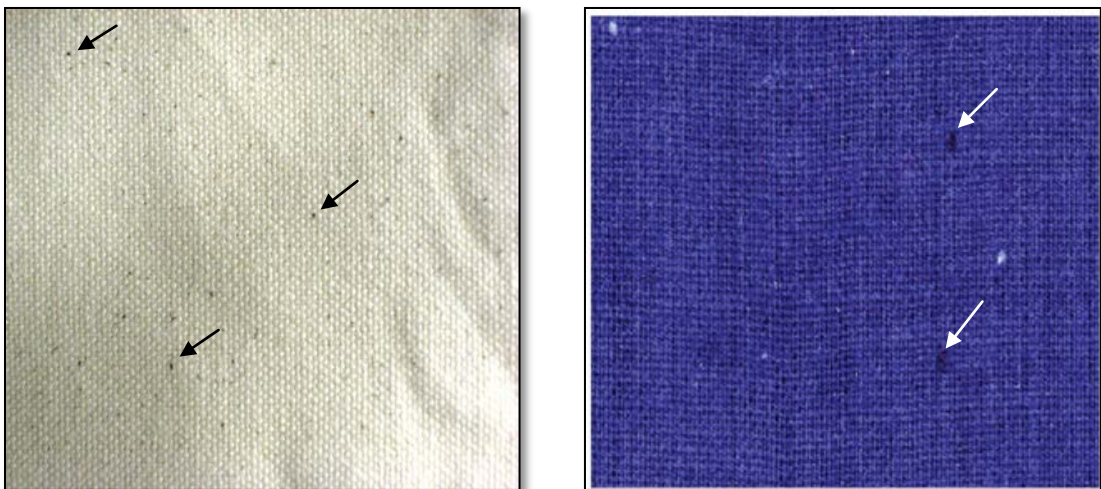


Figure 2: Seed Coat Fragments as seen on a Greige and Colored Fabric (USDA)

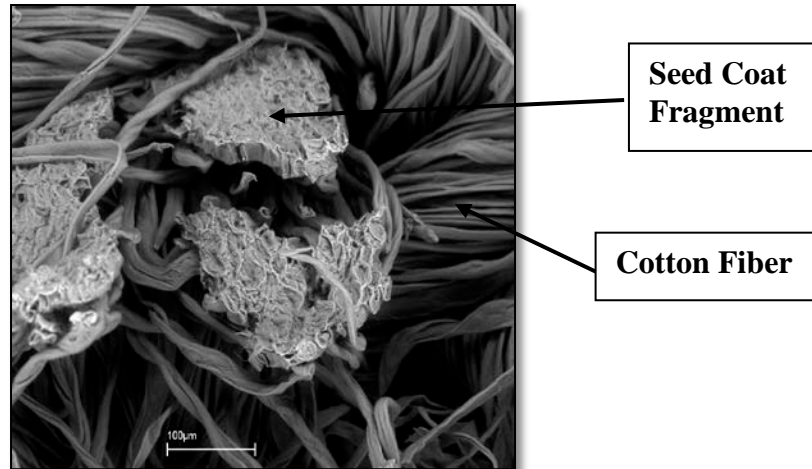


Figure 3: SEM of a Seed Coat Fragment as seen on a Greige Fabric

The Vigil study determined that the protective layer did not extend to the chalazal end of the seed where cells are small and spongy with intercellular spaces present between them. Also, fiber density is considerably larger towards the chalazal end compared to other areas on the seed coat. Due to a high fiber density, a greater force is applied at the chalazal region during ginning and more fibers are caught in the gin roller and easily pulled out of the seed (Vizia, Jadhav et al. 1995).

The cotton fibers attached to a seed coat have three sections, with the fiber foot being the part of the fiber that is embedded deep within the outer surface of a seed coat, the fiber shank being the part of the fiber near the surface of outer layer of the seed coat, while the fiber elbow is the junction of the fiber and the seed coat, visible on the surface of the seed coat (Himmelsbach, Akin et al. 2003). The extent of difficulty in removal of fibers from the seed coat depends on the strength of attachment of individual fibers, which in turn depends on the thickness of primary cell wall material present at the fiber elbow on the seed coat surface (Vigil, Anthony et al. 1996).

D. Composition of Seed Coat Fragments

A seed coat fragment is primarily a combination of cellulose and lignin. The composition of SCF's is summarized in the Table 2 given below:

Table 2: Composition of Cotton Seed Coat Fragments (Dollear and Markley 1948; Reicher and Csiszar 1992)

Component	Seed Coat %
Cellulose	43-48
Lignin	22-26
Pentosan	5-10
Wax	5-7
Protein	2-4
Calcium	3.7
Ash	2.6-2.8
Magnesium	0.7

According to Vigil and Yan, the outer epidermal layer of the seed coat consists of wax and cutin along with cellulose. The cuticle forms a water impermeable barrier that protects the plant by preventing penetration of any kind of liquids or attacks by parasites and plant pathogens. Figure 4 provides a cross sectional representation of a seed coat fragment. The palisade layer of the seed coat contains polysaccharides that have components of hemicelluloses and pectin, along with lignin (Vigil, Anthony et al. 1996; Yan, Hua et al. 2009). The seed coat fragments found on the cotton fabric are mainly constituted of this layer. The inner and outer pigment layers have similar structural and chemical composition and are composed mainly of lignin (Figure 5). The basic repeat structure in lignin is shown in Figure 6. Lignin is made of phenylpropane units

called syringyl and guaiacyl units (Figure 7). Lignin content in the colorless layer varies from strong to moderate, depending on the position of the layer in the cotton seed coat (Rokhin, L.V.Kanitskaya et al. 1994; Himmelsbach, Akin et al. 2003; Yan, Hua et al. 2009).

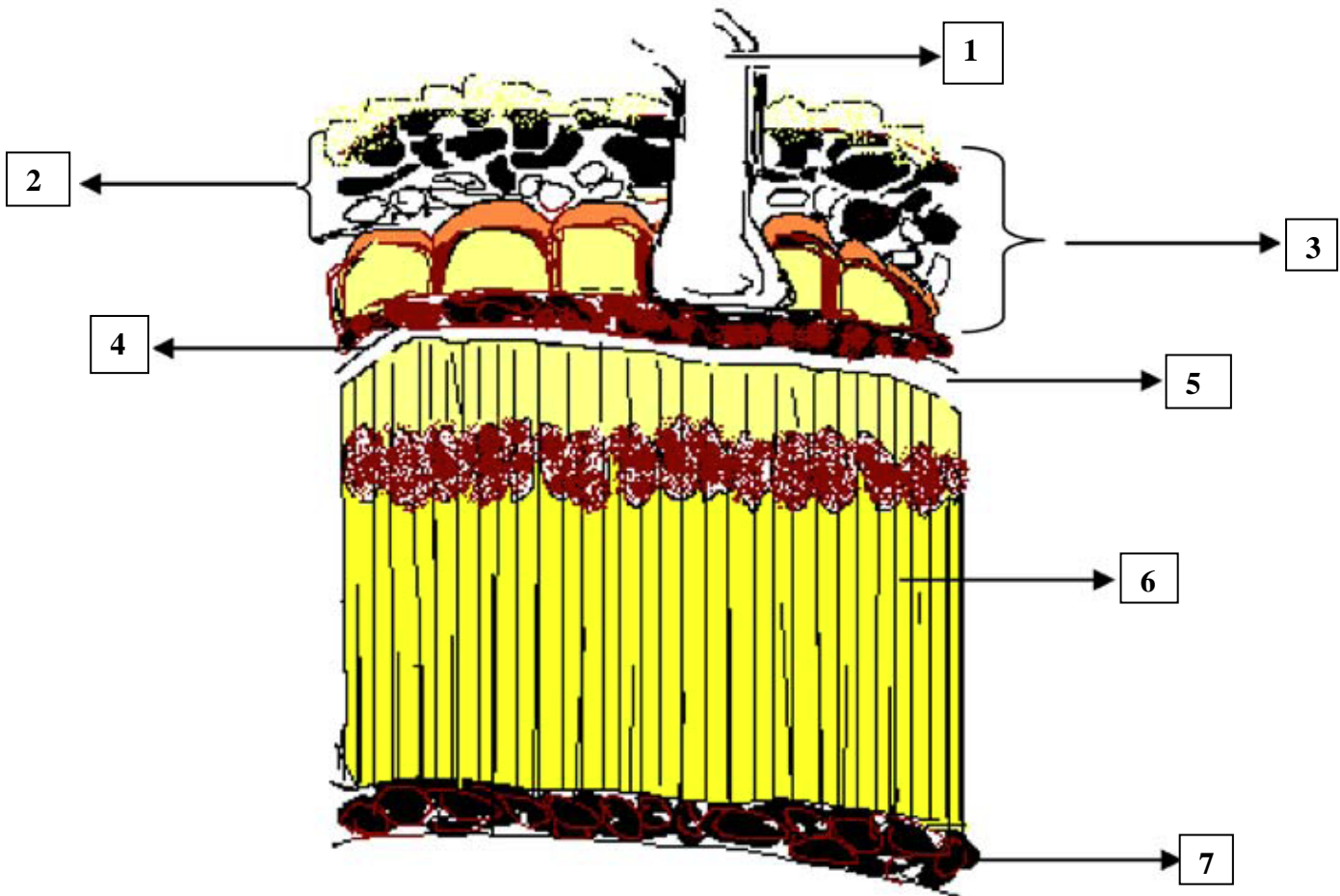


Figure 4: Cross Section View of a Seed Coat Fragment (Yan, Hua et al. 2009)

- 1: Cotton fiber
- 2: Cuticle
- 3: Outer epidermis layer
- 4: Outer pigment layer
- 5: Colorless layer
- 6: Palisade layer
- 7: Inner pigment layer

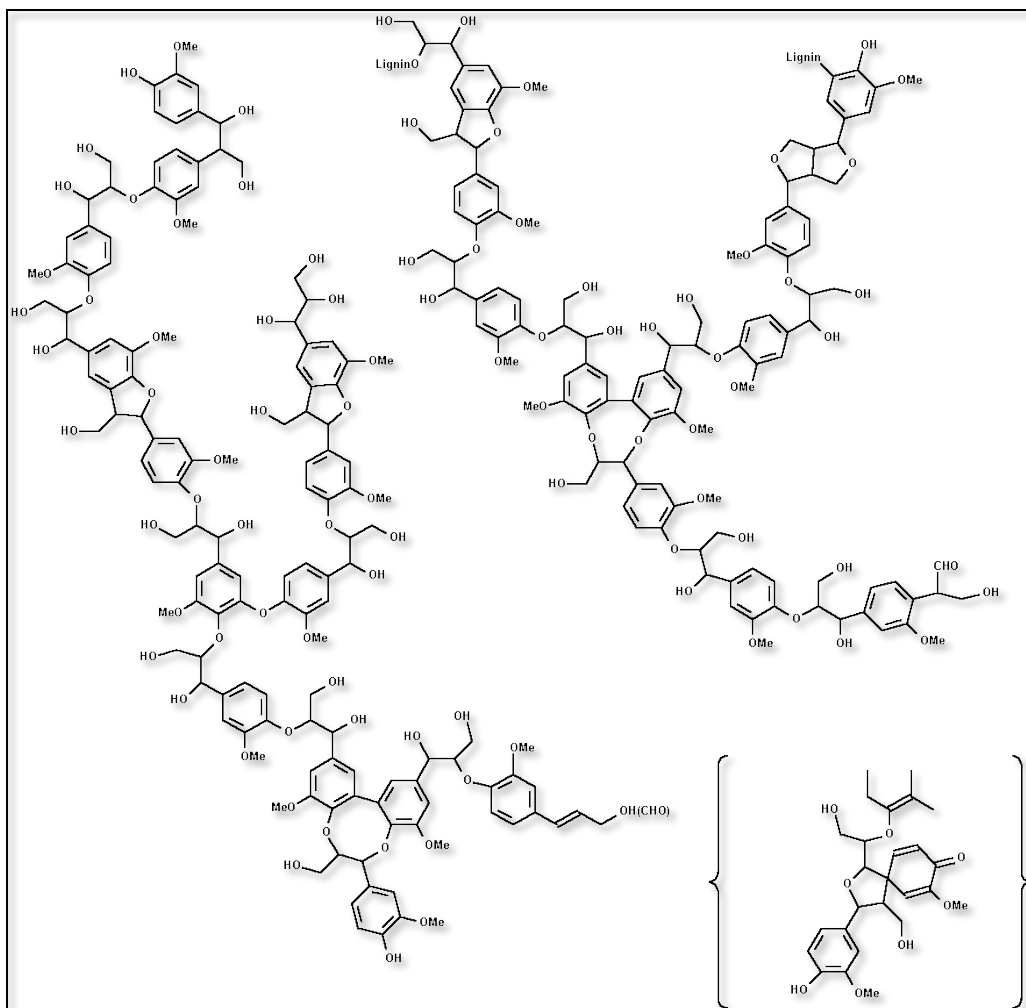


Figure 5: Irregular, Branched Phenolic Structure of Lignin (Webmaster 2011)

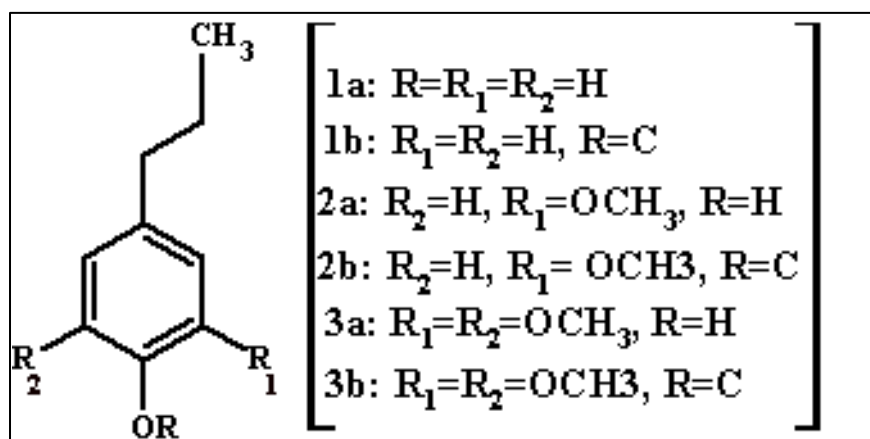


Figure 6: Basic Structure of Lignin (Lundquist and Parkas 2011)

From Figure 6 lignin can be divided into two main types of units namely:

- Phenolic units containing 1a, 2a, and 3a structures in the above figure
- Non phenolic units containing 1b, 2b and 3 b structures in the above figure

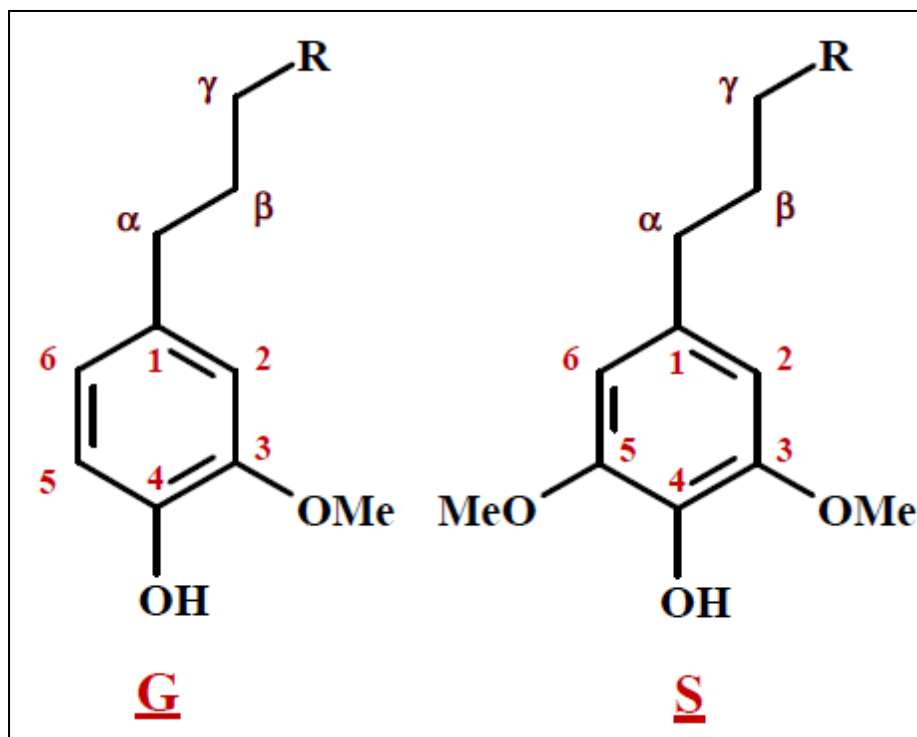


Figure 7: Chemical Structures of Guaiacyl (G) and Syringyl (S) Units in Lignin (Wikberg and Liisa Maunu 2004)

The outer epidermis layer changes its color and composition as the seed grows and matures. During their growth the seeds are initially white in color, and change to a dark brown color upon maturation of the seed. The seeds are impervious to liquids and this impermeable nature of seeds becomes prominent only upon maturity of the seed with oxidation and coloration of the phenolic compounds present in the seed coat. Therefore, oxidized phenolics are considered to play an important role in causing the cotton seed to become impermeable to liquids (John M

1982; Halloin, Lee et al. 1991). Seed hardness is also a factor that is directly related to impermeability in the seed coat. An increase in seed hardness results in an increase in the impermeability. Paiziev and Krakhmalev (2006) observed that during maturation of the seed coat a tremendous increase in the micro-hardness (from 45 kgmm^{-2} to 80 kgmm^{-2}) of seeds occurred. The external integument of the seed coat was determined to have the same hardness as annealed aluminum, while the palisade layer had a micro-hardness similar to that of annealed copper metal. Due to this hardness in the SCF's, cotton fabric requires additional treatments to successfully remove SCF's from the fabric. Alkaline scouring using sodium hydroxide is the conventional method used for treating the fabric for removal of SCF's, but due to its negative impact on the environment alternate sources using enzymes are being investigated.

II. Overview on Enzymes

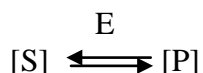
A. Enzyme Introduction

Enzymes are proteins produced by living organisms that function as catalysts in specific biochemical reactions. The fundamental actions of enzymes are to increase the rate of any chemical reaction without themselves being consumed in the reaction or being altered by the reaction. The increase in the rate of the reaction is done without altering the chemical equilibrium between the reactants and the products produced. Enzymes are highly specific with respect to the type of substrate they bind to and are vital for cellular metabolism due to their regional specificity. The enzyme forms a perfect fit with the substrate by changing the shape of its active site to fit with the substrate. The enzyme forms a combination of hydrogen, ionic bonds, hydrophobic and Van der Waals interactions type of bonds with a substrate (Jenkins 2003).

In a chemical reaction, substrate [S] is converted to product [P] where the substrate and the product are maintained in a chemical equilibrium.



By introducing an enzyme in the reaction, the conversion of [S] to [P] is accelerated but the equilibrium between [S] and [P] remains unaltered because the enzyme accelerates both the forward and reverse reactions equally.



The role of an enzyme in the chemical reaction is depicted in the energy diagram (Figure 8) of a catalyzed vs. uncatalyzed reaction. In the reaction, the substrate is first converted to a higher energy compound called the transition state before forming products. The rate of the reaction depends on the activation energy (E_a), which is the amount of energy the substrate has to overcome to reach the transition state. An enzyme reduces this activation energy, thereby increasing the rate of reaction and favoring formation of the transition state.

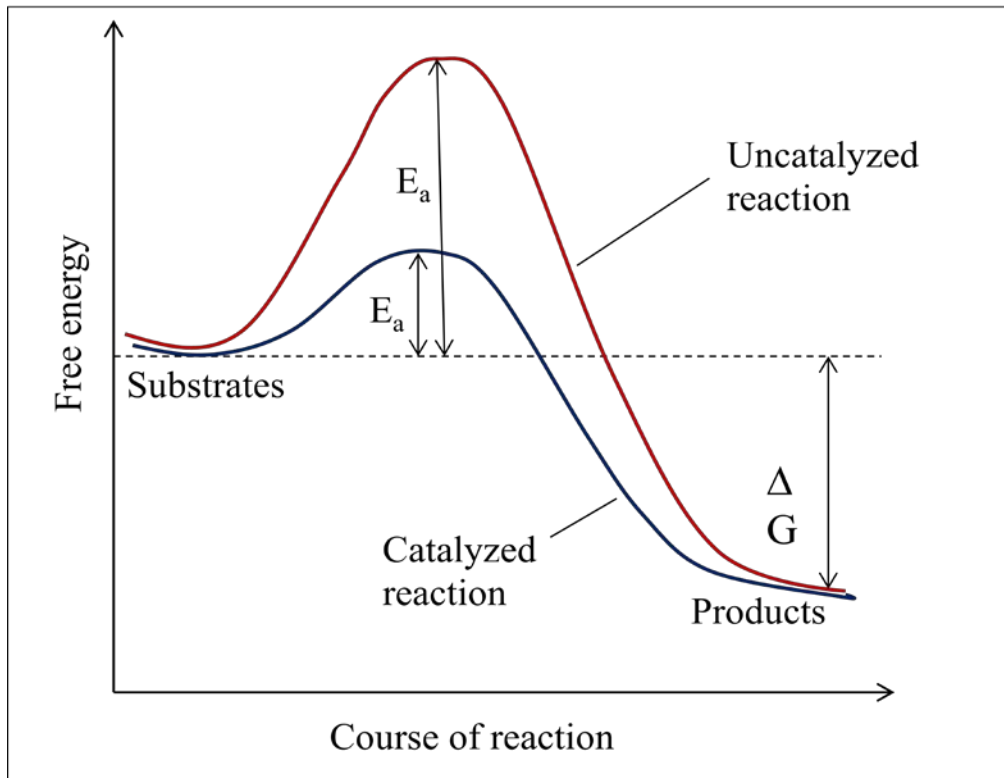


Figure 8: Energy Diagrams for Catalyzed and Uncatalyzed Reactions

In the catalytic reaction the substrates bind to the active site on the enzyme to form an enzyme-substrate complex (ES). While bound to the active site the substrate is converted to the product and is released from the enzyme.



Substrates bind only to specific active sites on the enzyme. Active sites are made of amino acid groups and are present on the surface of an enzyme where the substrate initially binds through non-covalent interactions. At this stage after binding multiple mechanisms such as altering substrate conformation occurs to accelerate the conversion of substrate to products. As seen in Figure 9, an enzyme can be described as a platform upon which two substrates properly

align together to result in the transition state where they interact with each other (Cooper and Hausman 2009).

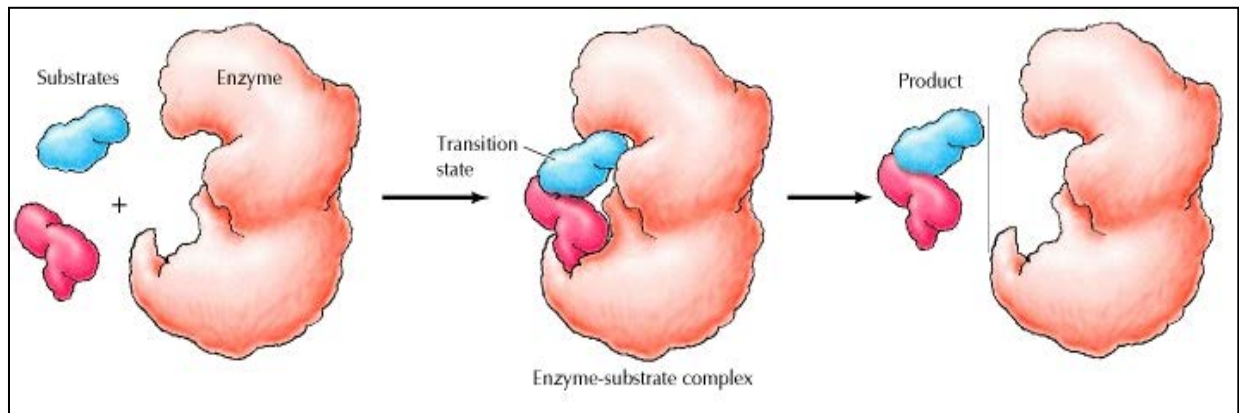


Figure 9: Enzymatic Catalysis of a Reaction Between Two Substrates (Cooper and Hausman 2009)

Lock and key and induced fit (Figure 10) are the two main models used to define the enzyme-substrate interaction. In the former model, the substrate fits exactly to the active site. In the latter model both the enzyme and the substrate are modified by the substrate binding.

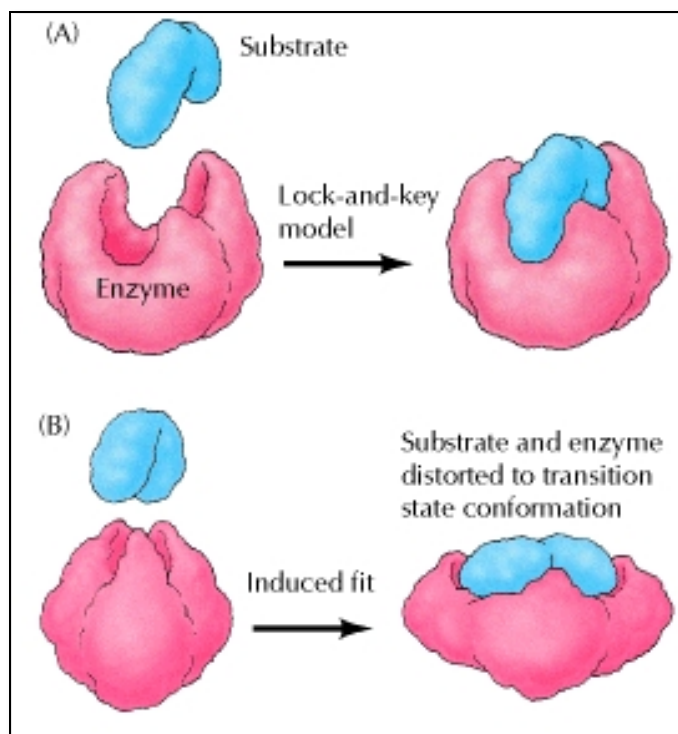


Figure 10: Enzyme Substrate Interaction Models (Cooper and Hausman 2009)

Advances in biotechnology and enzymology have helped in the development of various enzymatic applications in textile wet processing. Enzymes are considered to provide promising solutions for the problems related to use of sodium hydroxide because of their specificity in action, ability to work under mild conditions and their high efficiency output. Enzymes, produced from fully biodegradable, renewable resources, in turn result in the production of biomass which is either transferred to agricultural land as soil conditioners or fertilizers, thus making the enzymes compatible with the environment (Schäfer, Borchert et al. 2007).

B. Different Types of Enzymes Used in Textile Industry

The use of enzymes in textile processing started in 1857 when starch-treated (sized) cloth was soaked in a water bath containing barley for several hours. During this soaking period, barley germinates and produces enzymes such as α -amylases and proteases. It is α -amylase enzyme that breaks down the starch present on the fabric into simple sugars, thus removing the

starch (desizing) from the fabric. After this initial use as desizing agent, other enzymes were introduced for various applications in textiles. In 1912, bacterial and animal amylases were produced for enzymatic desizing. In the 1980's, cellulases were produced for depilling fabrics made from cellulosic fibers and for producing stone washed effects in denim fabrics (Cavaco-Paulo and Gübitz 2003). In the 1990's, catalase enzymes were introduced to break down excess hydrogen peroxide that remains on the fabric after bleaching, thereby reducing the overall water consumption required for washing the fabric. The possibilities of replacing alkaline scouring with enzymatic scouring for removal of cotton impurities and improving wettability of cotton using enzymes such as cellulases, pectinases, lipases, hemicellulases and proteases were investigated by several researchers (Li and Hardin 1997; Hartzell-Lawson and Hsieh 1998; Li and Hardin 1998; Buchert and Pere 2000; Aly, Moustafa et al. 2004; Kim, Choe et al. 2006). Of these enzymes, cellulases and pectinases were identified as the two potential candidates for enzymatic scouring (Csiszar, Szakacs et al. 1998; Li and Hardin 1998; Calafell and Garriga 2004). The source for these enzymes used in textile processing varied depending on the type of enzyme. For example, commercial α -amylase is mainly obtained from bacteria such as *Bacillus subtilis*, while cellulase is extracted from fungi such as *Trichoderma reesei* and *Humicola insolens* (Cavaco-Paulo and Gübitz 2003). Purified pectinase is obtained from fungi such as *Aspergillus niger*.

Waxes and pectins present on the greige cotton fabric impede the wetting and wicking properties of the fabric and consequently reduce the effectiveness of any wet treatment applied to the fabric. Therefore, for an effective enzymatic treatment the surface waxes must be solubilized to increase the enzyme access to the hydrolyzable parts in the substrate. The wax content can be classified as saponifiable, consisting of fatty acids such as hexadecanoic acid and n-tetracosanic

acid, and non-saponifiable, consisting of high molecular weight monohydric alcohols such as gossypol ($C_{30}H_{30}O_8$), montanyl ($C_{28}H_{57}OH$) and ceryl ($C_{28}H_{53}OH$) (Bailey, Biely et al. 1992; Agrawal, Nierstrasz et al. 2007). As a pre-treatment, two minutes boiling in water was found to enhance the enzyme accessibility to pectin. This occurred because waxes are on the surface of raw cotton fibers. Boiling water melts and removes some of these waxes, resulting in an increase in wettability of the cotton fabric while reducing overall enzyme concentration, temperature and time required (Goldstein 1991; Bailey, Biely et al. 1992; Hartzell-Lawson and Hsieh 1998). By using solvents such as n-hexane in the pre-treatment process a higher amount of wax extraction was made possible that resulted in an increase in the hydrophilic properties of the fabric. Results similar to hexane extraction were obtained using cutinase enzyme that required considerably lower scouring temperature and reduced the treatment time to produce similar results (Agrawal 2005; Agrawal, Nierstrasz et al. 2007). Lipase enzyme was also found to hydrolyze cotton wax resulting in a scoured fabric with lower amount of fatty acid and wax content present on the surface (Sae-be, Sangwatanaroj et al. 2007). Different enzymes have varying efficiencies in removal of wax from cotton fabrics.

An enzyme alone is usually not effective enough to sufficiently remove all impurities from the cotton fiber. Pectinase treatment of an n-hexane extracted fabric resulted in 1.65 times higher pectin hydrolysis than the pectinase treatment of the greige fabric without wax extraction (Agrawal, Nierstrasz et al. 2007). Pectinase treatment of fabric alone resulted in little improvement in fabric wettability, while use of cellulase resulted in significant loss in fiber strength (Hartzell-Lawson and Hsieh 1998; Agrawal, Nierstrasz et al. 2007). Enzymes are therefore used in combinations in order to utilize the synergistic advantage obtained from combining different types of enzymes. Significant degradation of the surface fibrils, SCF's,

water-extractable material and other natural impurities were reported in a biotreatment containing commercial cellulases and xylanases of amylase desized cotton fabric (Zamora 2012).

A combination of lipase and protease followed by a cellulase treatment was found to give better results than a single-enzyme pectinase treatment. In another combination, hydrophilicity was improved when pectinase and lipase were used along with a cellulase treatment (Goldstein 1991; Buchert and Pere 2000; Traore and Buschle-Diller 2000; Degani, Gepstein et al. 2002; Losonczi 2004; Sae-be, Sangwatanaroj et al. 2007). Combinations of pectinases and cellulases were also studied with good results (Hartzell-Lawson and Hsieh 1998). However, in general, the use of cellulases is not preferred since cellulases readily degrade cellulose chains in the fibers and thus reduce the tensile strength of the fabric (Goldstein 1991).

Pectinases are the preferred enzymes for bioscouring because pectin is one of the main components present on the surface of cotton that connects the other components and stabilizes the outer layer of the structure (Goldstein 1991; Valle, Onos et al. 2006). Cotton can be bioscoured using either an acidic pectinase or an alkaline pectinase (Calafell, Klug-Santner et al. 2005). To achieve optimal enzyme activity using an acidic pectinase the use of a non-ionic surfactant and pH control are key parameters to be considered (Calafell and Garriga 2004). Alkaline pectinase (Bioprep 3000L) has the advantage of providing similar results to that obtained using acidic pectinase (Pect 062L) but at lower enzyme concentrations (Tzanov, Calafell et al. 2001). Also, bleaching after scouring of cotton fabric is done using hydrogen peroxide at an alkaline pH of 10.5-12. Therefore, in this case use of alkaline pectinase results in an easy transfer of fabric from one treatment bath to another with less washing involved.

Xylanases are enzymes that hydrolyse xylan, a main heteropolymer of hemicellulose. Xylan is a complex polysaccharide that consists mainly of D-xylose as the monomeric unit along

with traces of L-arabinose. Due to its complex nature, it is difficult to achieve a complete breakdown of xylan. This is done conventionally by alkaline treatment which causes removal of acetyl groups and thus results in breakdown of cross linkages. The xylanase enzyme is effective in cleaving the xylan backbone into smaller oligosaccharides and causes effective removal of hemicellulosic impurities (Pastor, Gallardo et al. 2007; Dhiman, Sharma et al. 2008). Xylanase was used as pre-treatment to degrade the xylan constituent in the lignocellulose structure of SCF's followed by alkaline extraction. The results showed release of significant amounts of reducing sugars in the treatment bath, indicating the effectiveness of the enzyme in breaking down xylan (Csiszar, Losonczy et al. 2006). Some of the parameters that increased the efficiency of xylanase in biotreatment of cotton fabric were agitation and the addition of a chelating agent such as ethylenediamine-tetra-acetic acid (EDTA) to the treatment bath (Losonczy, Csiszar et al. 2005; Zamora 2012).

The purpose of scouring is to achieve uniformity upon dyeing, and therefore scouring should increase the absorbency of the fabric. Some of the parameters that could be added to optimize enzymatic scouring, resulting in an increase in fabric absorbency, include addition of a chelating agent and surfactants. A chelating agent such as EDTA enhanced the direct enzymatic action on all impurities present on cotton except wax by removing calcium ions present in the structure thus opening up the structure for enzyme action (Csiszar, Losonczy et al. 2001; Kim, Choe et al. 2006). Surfactants, especially non-ionics, improved the emulsifying ability of the enzyme and thus the absorbency of the fabric treated with this combination. Non-ionic surfactants are preferred because anionic surfactants are poor emulsifiers and they strongly affect scouring by deactivating the enzyme, thus impeding its scouring action and reducing absorbency of the fabric (Sawada and Ueda 2001; Kim, Choe et al. 2006). When scouring was carried out

with agitation an increase in enzyme efficiency resulted. The results obtained from agitation in the bath were found to be independent of treatment time and enzyme concentration used (Zamora 2012).

After scouring, the fabric is bleached to obtain a high whiteness index, especially for white and light shade fabrics. About 70 percent of cotton fabrics are bleached with hydrogen peroxide. Enzymatic bleaching using glucose oxidase with glucose, starch or cellulose as a substrate was found to produce *in situ* peroxide which resulted in the desired fabric whiteness (Schäfer, Borchert et al. 2007). According to Schäfer, for large scale production the process had to be made more cost effective to produce larger quantities of peroxide to improve the bleaching power. A new experimental concept was developed that combined two oxidoreductases in the bleaching process (Opwis, Knittel et al. 2008). In this experiment, the starting enzyme, glucose oxidase, was used on a glucose substrate to generate hydrogen peroxide *in situ*. The second enzyme used was peroxidase, which instantly consumed the freshly made hydrogen peroxide as a substrate and oxidized color impurities present on cotton, resulting in a higher degree of whiteness in the fabric.

Conventional bleaching results in deposition of residual peroxide in the fabric, which needs to be removed before dyeing and finishing of the fabric. Usually, this is done by repeated washing with water or chemical reducing agents. These are now being slowly replaced by the use of catalase enzymes that decompose hydrogen peroxide to oxygen and water. Enzymatic degradation of hydrogen peroxide with catalase enzyme results in reduced water consumption during the washing of the bleached cotton fabric. This, in turn, improves the quality of the dyed fabrics (Tzanov, Costa et al. 2001; Heikinheimo 2002). In a study involving the use of catalase enzymes, a significant process savings of a 24 percent reduction in energy consumption, a 50

percent reduction in water consumption, and a 33 percent reduction in processing time was found possible (Schäfer, Borchert et al. 2007).

C. Specific Enzymes Related to this Research

1. Enzymes for Degradation of Hemicellulose

The chemical composition of an SCF consists of hemicellulose (5%-10%), lignin (22%-26%), and wax (5%-7%), in addition to cellulose (43%-48%) (Dollear and Markley 1948; Reicher and Csiszar 1992). Hemicelluloses are carbohydrate polymers associated with cellulose present in the palisade layer of the SCF. The hemicellulose forms an interphase between lignin and pectin by covalently linking lignin phenolic residues to pectins. Figure 11 is an example of hemicellulose consisting of pentosan, a polysaccharide having xylose and arabinose units found in bran of grasses. The xylan acts as a backbone with L-arabinofuranose attached randomly to xylose units through $\alpha(1\rightarrow2)$ and $\alpha(1\rightarrow3)$ linkages throughout the chain (Goldstein 1991; Zamora 2012).

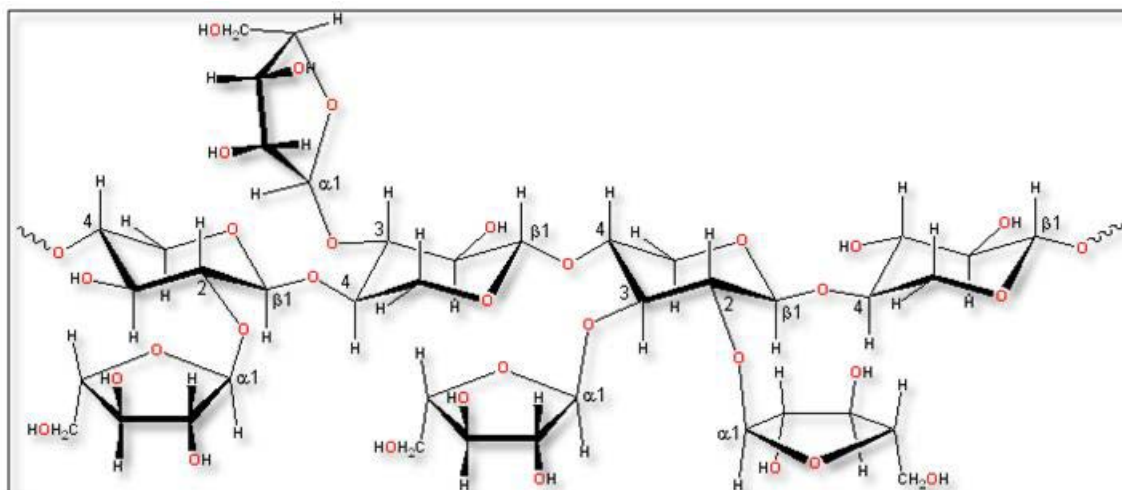


Figure 11: Arabinoxylan, a Polysaccharide with Linked $\beta(1\rightarrow4)$ Xylose Backbone Having Randomly Linked Arabinose Residues at $\alpha(1\rightarrow2)$ and $\alpha(1\rightarrow3)$ Linkages (Sadosky 2008; Chaplin 2012)

Hemicelluloses add rigidity to the cellulose-hemicellulose-lignin complex and are insoluble in water. However, they dissolve in strong alkali and can be readily hydrolyzed with an acid. Since hemicellulose is a part of SCF composition and has an insoluble structure, it requires specific treatments that hydrolyze hemicellulose and solubilize it to help in breakdown of SCFs.

Xylanases (EC 3.2.1.8) are the enzymes that can catalyze the hydrolysis of xylan, the major backbone of the pentosan structure, into D-xylose monomer. Use of xylanase enzymes at the industrial level has increased over the past couple of decades. Xylanase enzymes are being used in pulp bleaching, waste paper treatment and textile bio processing. Xylanases are produced by both fungal species (*Trichoderma harzianum*) and bacterial species (*Bacillus circulans*) (Dhiman, Sharma et al. 2008). The use of alkaline bacterial xylanases in bio-processing is more advantageous than fungal enzymes because the pH of the treatment for the next wet processing stage (bleaching) is done in an alkaline environment.

Pulpzyme[®] HC (Novozymes), which contains an alkaline, thermostable xylanase enzyme produced from genetically modified *Bacillus* microorganism, was the enzyme used to decompose hemicellulose in SCF's. Csiszar, in her earlier work, determined that the addition of Pulpzyme HC[™] in the presence of a chelating agent such as ethylenediaminetetraacetic acid (EDTA) with agitation accelerated the degradation of SCF's (Csiszar, Losonczi et al. 2001; Csiszar, Urbanszki et al. 2001; Csiszar, Losonczi et al. 2006). She used Pulpzyme HC as a pre-treatment followed by alkaline scouring and concluded that pretreatment opened the substrate structure and accelerated the degradation of the substrate in subsequent alkaline extraction (Csiszar, Losonczi et al. 2006).

2. *Enzymes for Degradation of Lignin*

After cellulose, lignin is the major component present in an SCF. In a cotton seed lignin is present in the palisade layer along with hemicellulose and pectin and is also found in the outer and inner pigment layers of the seed (Yan, Hua et al. 2009). Lignins are three dimensional polymers of phenylpropane units with different linkages between monomers forming a complex structure that imparts structural support, impermeability, and barrier properties against enzymatic degradation of the substrate. It acts as a shield, preventing digestible parts of the substrate from hydrolyzing, thus limiting the rate and extent of enzymatic hydrolysis (Goldstein 1991). Lignin is recalcitrant and is highly difficult to degrade. A chemical recipe used for lignin degradation includes 80% (w/w) acetic acid with 0.92% (w/w) nitric acid as a catalyst at 120 °C for 20 minutes which results in 92% degradation of the original lignin. With an increase of nitric acid concentration to 8.5% (w/w), 98% degradation of original lignin was achieved (Sun, Sun et al. 2004).

Lignin in wood is degraded by *Basidiomycetes*, also known as white rot fungi. The fungi decompose lignin in wood to gain access to the cellulose and hemicellulose embedded in the lignin matrix. A multi enzyme system is used by the fungus to transform and degrade the lignin barrier; this enzyme system can be divided into three groups (Hammel 1997). The first group includes cellulase, hemicellulase, laccase and different peroxidase enzymes that directly attack wood constituents or their primary degradation products. The second group comprises of aryl alcohol oxidase and glyoxal oxidase enzymes that do not directly attack wood components but rather assist with the first group by providing hydrogen peroxide (H_2O_2) for the peroxidases. The third enzyme group includes feedback type enzymes such as glucose oxidase and cellobiose dehydrogenase which play a key role in biotransformation of high molecular mass wood

constituents. Further, under group one, two classes of extracellular oxidative enzyme peroxidases and laccases catalyze cleavage of carbon-carbon or carbon-oxygen bonds in the complex lignin polymer, thus producing ligninolysis. Lignin peroxidase (LiP) and manganese peroxidase (MnP) require hydrogen peroxide (H_2O_2) generated by other enzymes to be active against lignin while laccase (Lac) requires oxygen (O_2) to be active (Leonowicz, Cho et al. 2001).

Laccases (EC 1.10.3.2) are glycoproteins that are a family of 'blue copper' oxidases containing multiple copper atoms which are reduced as the substrate is oxidized (Figure 12). The enzyme's active site contains four specifically structured copper ions. The type 1 (T_1) copper imparts the characteristic light blue color to the enzyme while both type 2 and type 3 copper ions are 'non-blue'. The active site's copper ions (Figure 13) are linked to histidine residues. Laccase enzyme stores electrons from individual oxidation reactions and in turn reduces molecular oxygen to water, thus returning to its native form. Laccase can only oxidize phenolic units to phenolic radicals due to the random polymer nature of lignin, low redox potential of the enzyme and its high molecular weight. Thus a mediator, a small and low molecular weight compound with a higher redox potential than laccase, is used to oxidize the non-phenolic part of lignin. The enzyme oxidizes the mediator into a strong oxidizing intermediate mediator that acts as an electron shuttle and diffuses away from the enzymatic pocket, and in turn oxidizes that part of substrate that the enzyme cannot directly act on (Hammel 1997; Leonowicz, Cho et al. 2001; Couto and Toca-Herrera 2006; Riva 2006; Kunamneni, Ballesteros et al. 2007). HBT (1-hydroxybenzotriazole) and ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] (Figure 14) are used as mediators with laccase.

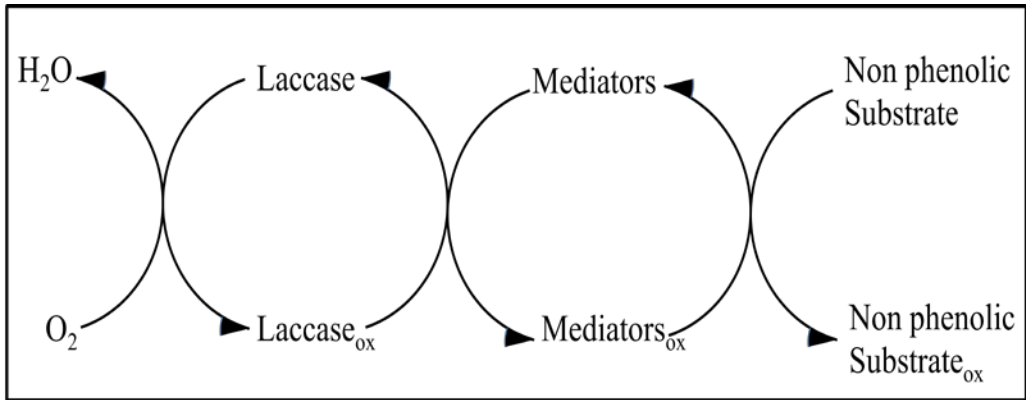
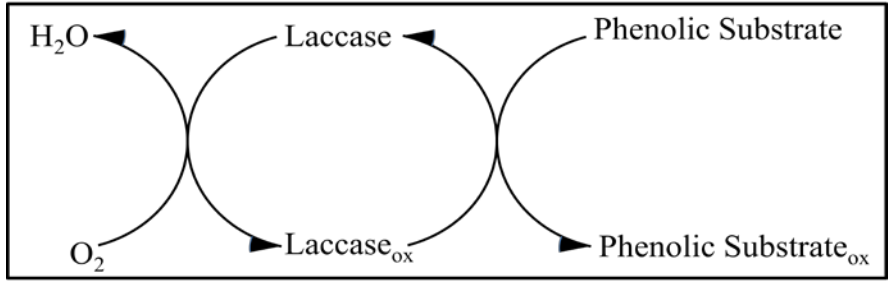


Figure 12: A Comparison of the Catalytic Cycles of Laccase With and Without Mediators (Wells, Teria et al. 2006; Gochev and Krastanov 2007)

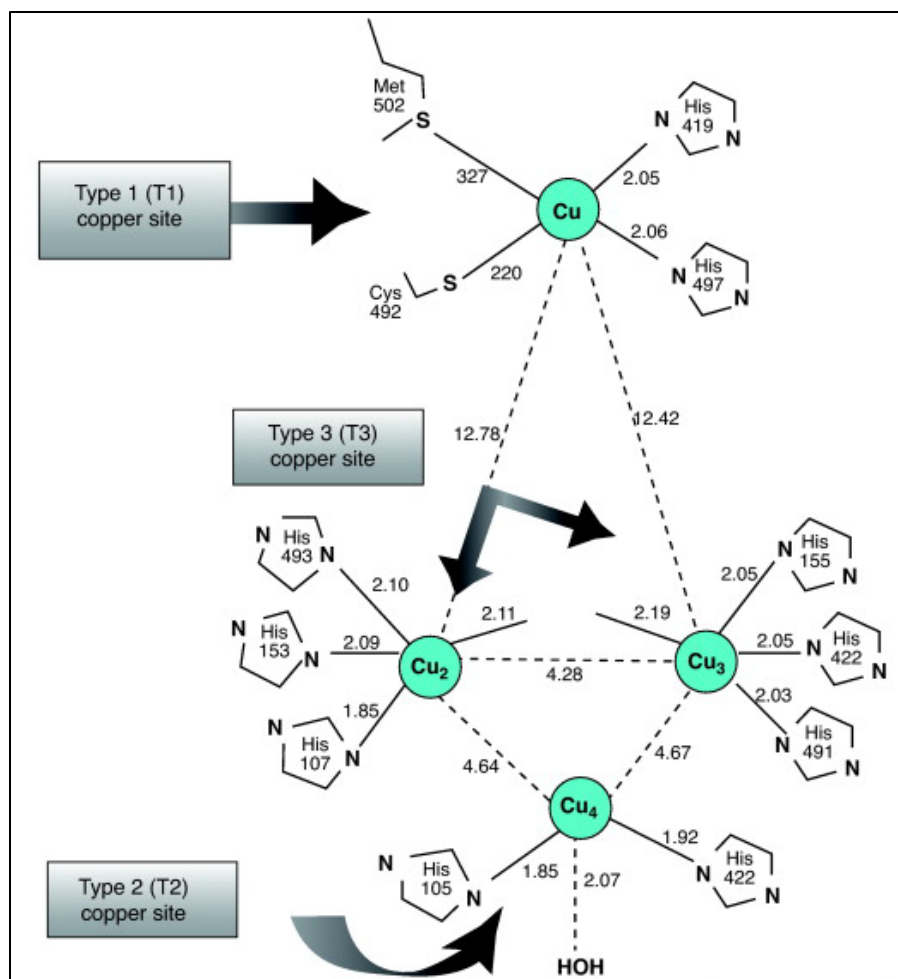


Figure 13: Schematic Representation of Active Sites for Laccase Enzymes (Santhanam, Vivanco et al. 2011)

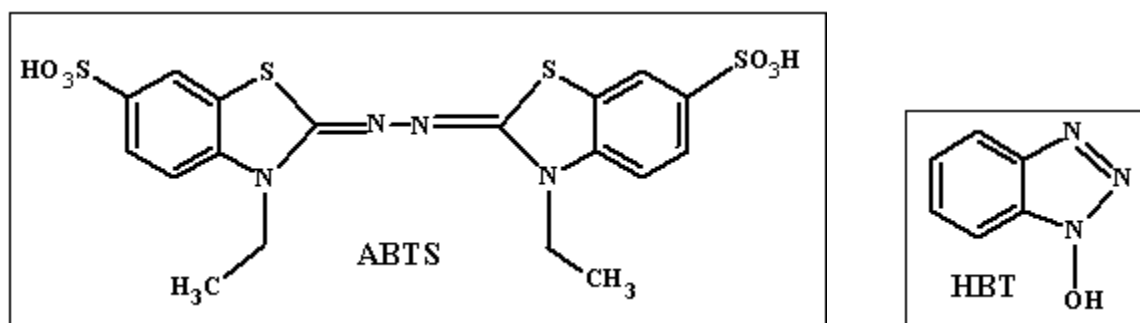


Figure 14: Common Mediators used with Laccase Enzymes (Wells, Teria et al. 2006)

III. Conclusions

From the literature review the main conclusions to be drawn are that on the cotton fabric enzymes play a significant in removing hydrophobic impurities. Compared to conventional treatments used in textile wet processing, various enzymes used have a positive impact on the environment as they result in fewer by-products and are easy to dispose. Though enzymes are effective on major impurities present on cotton fabric, they have less impact on cotton seed coat fragments due the latter's chemical composition. The recalcitrant nature of seed coat fragments is due to presence of lignin, a complex polyphenolic structure that is difficult to break down. Conventional scouring using sodium hydroxide is effective in breaking down the seed coat fragments, but due to its negative impact on the environment a more eco-friendly substitute is required.

For seed coat fragment impurities, enzymes can be an effective alternate treatment to conventional method provided the lignin structure can be modified by a pretreatment to open the substrate for the enzyme action. Also, enzymes specifically attacking the lignin structure can be used to breakdown the recalcitrant compound.

IV. Research Objectives

The overall objective of this research was to investigate the feasibility of designing an effective treatment incorporating enzymes to aid the complete breakdown of seed coat fragments which are present as impurities in cotton greige fabric. The specific research objectives were-

- Analyze the effect of conventional sodium hydroxide treatment on cotton seed coat fragments.
- Screen enzymes that were effective in hydrolyzing the hemicellulose and lignin, two of the major constituents of the seed coat fragments.

- Determine if the components used in the enzymatic treatment could boost the enzyme action on seed coat fragments.
- Investigate pre-treatments that increased the effectiveness of enzymes and reduced the sodium hydroxide concentration needed to break down and remove seed coat fragments.

CHAPTER 3

MATERIALS AND METHODS

I. Research Design

A systematic diagram on the next page gives an outline of the experimental structure followed in this research. The flow chart starts with the preparation of raw material (seed coat fragments), followed by investigation of different treatments and finally the multiple analytical approaches used for each treatment. Each section of the flow chart is described in detail in the following pages.

SCF Preparation

1. Conventional NaOH Treatment

- i. 0.5%, 2%, & 4% NaOH
- ii. Weight Loss
- iii. Sugar Analysis by GC-MS
- iv. Lignin Analysis
 - . Klason Lignin
 - . py-MBMS
 - . py-GC/MS
 - . SERS

2. Enzymatic Treatment Only

- i. Xylanase & Laccase Enzymes
- ii. Enzyme Assays
- iii. Weight Loss
- iv. Sugar Analysis by GC-MS
- v. Lignin Analysis
 - . Klason Lignin
 - . py-MBMS
 - . py-GC/MS
 - . SERS

3. Microwave Treatment Only

- i. 2.5 min & 5 min treatment times
- ii. Weight Loss
- iii. Sugar Analysis by GC-MS
- iv. FTIR

4. Combined Microwave and Enzyme Treatments

- i. Xylanase enzyme
- ii. Laccase enzyme
- iii. Weight Loss
- iv. Sugar Analysis by GC-MS
- v. FTIR
- vi. Lignin Analysis
 - . Klason Lignin
 - . py-MBMS
 - . py-GC/MS
 - . SERS

II. Materials

A. Seed Coat Fragment Preparation Sequence

The preparation of cotton seed coat fragment samples (SCF's) is represented pictorially in Figure 15. The cotton seeds were manually plucked from a bale of cotton and the kernels separated from seed coats by splitting the seeds in half. The seed coat fragments were then generated by grinding the split seed coats using a Wiley Mill with a 10 mesh sieve. The ground SCF's were then conditioned for 24 hours at 65% relative humidity and 70 °F before using in the experiments. This method of SCF production was selected to replicate the SCF's found in a greige cotton fabric.

Scanning electron microscopy was used to study the structure of SCF's. The samples were prepared by mounting them on an aluminum stub, with double-sided sticky tape. The samples were coated with a 90 Å layer of high purity silver using a vacuum evaporator prior to analysis.

B. Sodium Hydroxide (NaOH) Solutions

Sodium hydroxide solutions of required concentrations were prepared using a 50 percent (w/v) NaOH-Baker reagent. Three different concentrations of 0.5%, 2%, and 4% were prepared by adding 1ml, 4ml and 8ml respectively of 50 percent NaOH solution to water in a 100 ml volumetric flask. Fresh NaOH solutions were prepared daily and any leftover solution was discarded in the waste container at the end of the day.

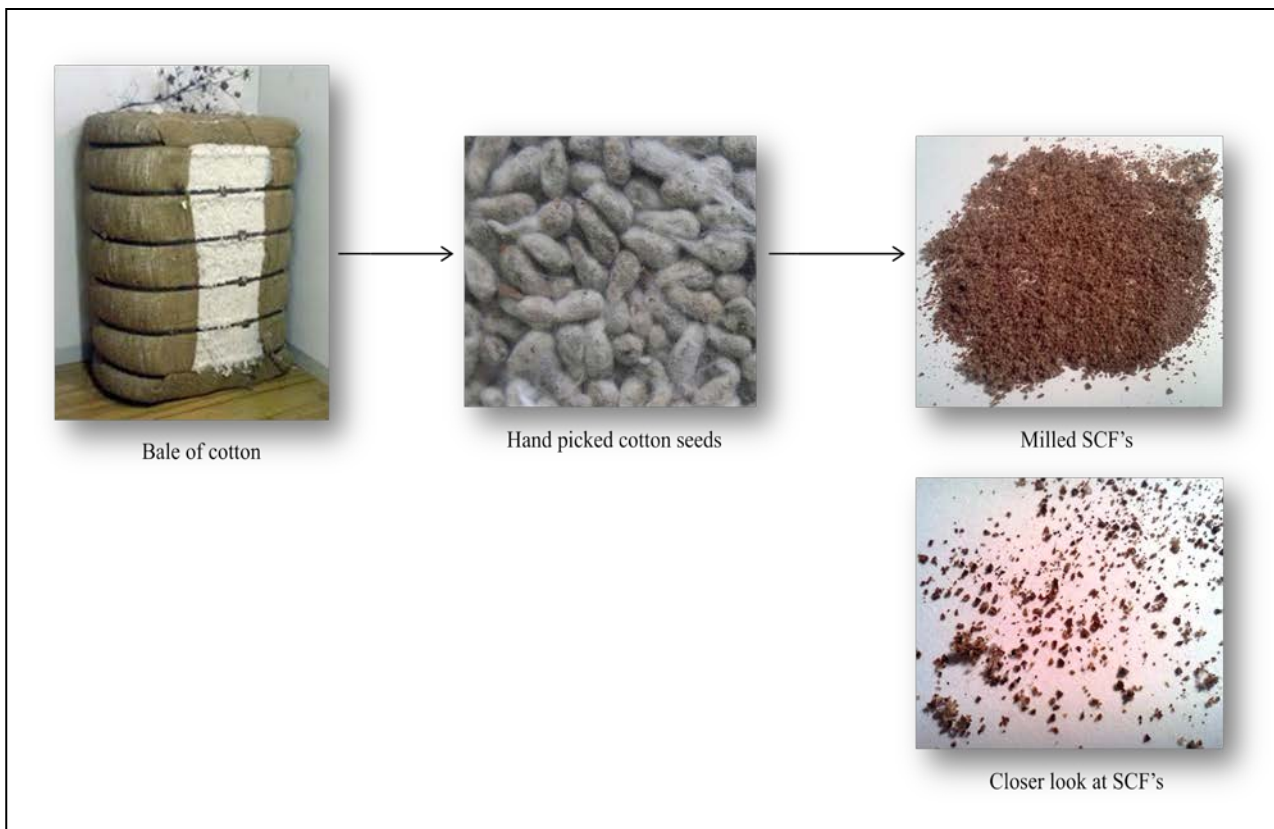


Figure 15: Seed Coat Fragments Preparation Sequence

C. Buffer Solutions

Tris Buffer: 5mM tris(hydroxymethyl)aminomethane solution (tris) was prepared by adding 6.055 g of tris in 1L of water. The pH of the buffer was adjusted to pH 8-8.5 using concentrated HCl.

Britton's Robinson Buffer: Britton Robinson's Universal pH buffer was prepared by adding 0.1M acetic acid, 0.1M boric acid and 0.1M phosphoric acid in 1L of water to form 0.1M Britton Robinson Universal buffer. The pH of the buffer was adjusted between pH3 to pH8 by adding 0.2M sodium hydroxide solution to the buffer.

Boric Acid-Borax Buffer: Initially 0.2M boric acid and 0.05M borax (sodium borate) solutions were prepared by adding 12.4g of boric acid and 9.07g of borax to 1L of water, respectively. The

boric acid-borax buffer was prepared by adding 250ml of the 0.2M boric acid solution and 41.5ml of the 0.05M borax solution to a 1L volumetric flask and adding water to make up the remaining volume in the flask. The pH of the final solution was in the range of pH 8.

D. Enzymes

1. Pulpzyme[®] HC (Xylanase Enzyme)

Pulpzyme HC is an alkaline, thermostable xylanase enzyme solution obtained from Novozymes[®]. This enzyme is found to be active in alkaline conditions of pH 6.5-9.5 and at temperatures of 40 °C-65 °C. This enzyme is produced by submerged fermentation of a genetically modified *Bacillus* microorganism. The enzyme's three dimensional structure as seen in Figure 16, is able to catalyze the hydrolysis of xylan substrates as it contains endo-1,4-beta-D-xylanase activity (E.C.3.2.1.8) only and has no cellulase activity.



Figure 16: Docked Structure of Endo-1,4-Beta-Xylanase Enzyme, PDB ID: 1H4H (Sabini, Sulzenbacher et al. 1999)

2. Laccase Enzyme

White rot fungi produce extracellular lignin-degrading enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac). Laccase enzymes require only molecular oxygen as an oxidant in the lignin-degrading process converting oxygen to water, whereas the peroxidases require hydrogen peroxide as an oxidant. This was the main reason laccase enzyme was selected from the group of possible enzymes to treat the lignin present in the SCF's. The laccase enzyme from *Trametes versicolor* (Figure 17) was obtained from Sigma-Aldrich (38429 Sigma). Laccase enzyme catalyzes the oxidation of phenol containing compounds. In the presence of a mediator, it catalyzes the oxidation of non-phenolic compounds present in lignin. Figure 17 represents the three dimensional structure of an oxidized laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) enzyme where the copper ions present in the enzyme are clearly seen as black balls attached in the figure which is absent in xylanase enzyme in Figure 16.

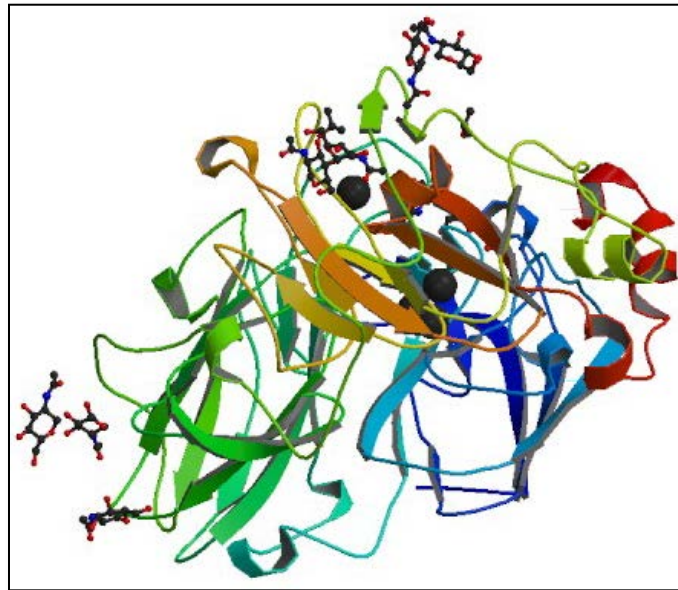


Figure 17: Three-Dimensional Structure of Laccase from *Trametes versicolor* in its Oxidized Form, PDB ID: 1GYC (Piontek, Antorini et al. 2002)

E. Enzyme Assays

1. Xylanase Assay

The xylanase enzyme, Pulpzyme HC (1000 AXU/g, 1.15 g/ml density), is a bacterial enzyme that was obtained in solution form from Novozymes[®]. The activity for this enzyme was determined using the dinitrosalicylic acid (DNS) assay method as adapted by Bailey (Bailey, Biely et al. 1992). The assay was performed as follows:

The substrate for this assay, birchwood xylan (1%) was added to the tris buffer (pH8) at 60 °C and boiled under constant stirring, cooled to room temperature and stored at 4 °C. The enzyme stock solution was prepared by adding 0.1ml of Pulpzyme HC enzyme to 10ml of buffer. Four different dilutions were prepared by adding 0.2ml, 0.15ml, 0.10ml, and 0.05ml of the stock solution to 50ml of buffer.

A standard xylose solution was prepared by adding 0.15g of xylose (0.01M) in 100 ml of buffer. From the standard sugar stock solution five different dilutions were prepared by adding 1:1, 1:1.5, 1:2, 1:2.5, and 1:3 ratios of xylose solution to buffer to obtain 10 µmol/ml, 6.67 µmol/ml, 5 µmol/ml, 4 µmol/ml and 3.33 µmol/ml concentration xylose standards. During the experiment, 3 ml of DNS solution was added to each of the xylose standard solutions.

The DNS solution was prepared by adding 2.5g sodium hydroxide, 2.5g DNS, 48g potassium sodium tartarate (Rochelle salt), 0.5g phenol, and 0.125g sodium sulphite to 250ml of distilled water under constant stirring. An amount of the substrate solution (1.8ml) was added to 15ml test tubes followed by 200µl of the enzyme solution at different concentrations. The test tubes were incubated at 50 °C for 10 min.

At the end of the incubation period, 3 ml of the prepared DNS solution was added to each test tube and then boiled for 10 min, followed by cooling in cold water. For a blank, the same

procedure was followed except that the enzyme was left out of the mixture. DNS (3,5-Dinitrosalicylic acid) reacts with the reducing sugar molecules present in solution to form 3-amino-5-nitrosalicylic acid (Figure 18). Depending on the concentration of reducing sugars present in solution, the intensity of color formed varies and can be measured at 540 nm using a UV/Vis spectrophotometer.

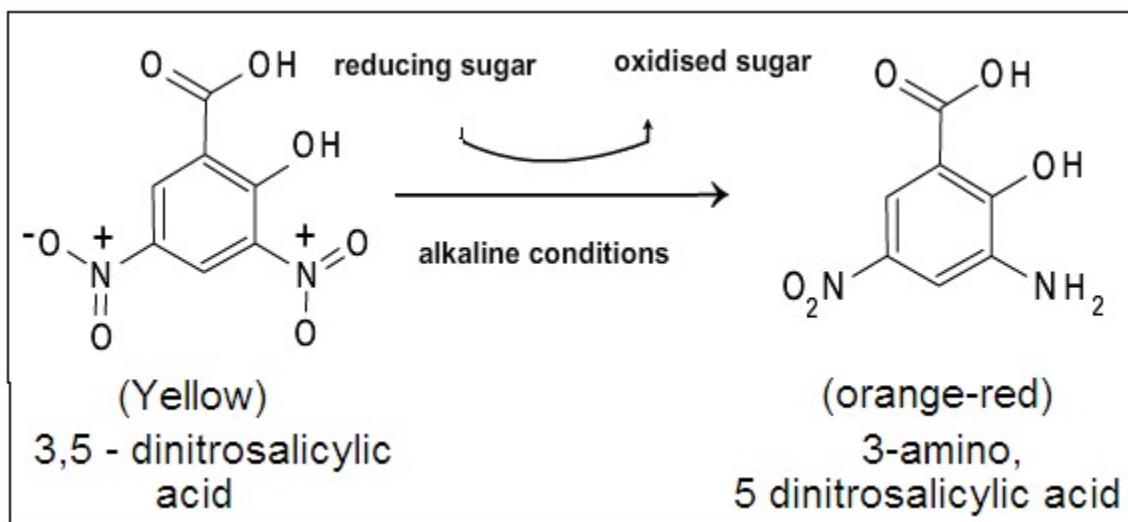


Figure 18: DNS reduced to 3-amino-5-nitrosalicylic acid results in color change (Goud 2011)

A standard line was plotted using the five xylose standard points with absorbance on the ordinate axis and xylose concentration converted to nkat/ml on the abscissa. Katal (kat) is a derived SI unit of catalytic activity of enzymes and is equal to mole per second. Enzyme unit (IU) is an off-system and is equal to $1 \mu\text{mol}\cdot\text{min}^{-1} \approx 16.67 \text{ nkat}$ (Dybkaer 2001). The enzyme activity was obtained by plotting the absorbance obtained for the enzyme on the standard line and finding the corresponding concentration in nkat/ml units.

2. *Laccase Assay*

The specific activity of the laccase used was assayed by monitoring color change of the substrate in solution using a UV-Vis spectrophotometer to record the increase in absorbance values from oxidation of different substrates at room temperature.

The assay was performed using four different substrates in a range of pH buffers to determine the perfect pH range and substrate to be used in the study. The different substrates selected for this study were 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), guaiacol, syringaldazine and vanillin. The Britton Robinson pH buffer was used in this assay for these substrates in the pH range of pH3 to pH8. The assay mixture for the test cuvette consisted of 2.2 ml of Britton buffer, 0.3 ml of substrate solution and 0.5 ml of laccase 1mg/ml solution. The assay mixture for the blank cuvette consisted of 2.2 ml of Britton buffer, 0.3 ml of substrate solution and 0.5 ml of deionized water. After adding the substrate solution to the buffer and the enzyme solution, the mixture was immediately mixed by inverting the cuvette and the increase in the absorbance of the oxidized product of the substrate was recorded for 10 minutes. The increase in absorbance values for the product was recorded for ABTS at 420 nm, guaiacol at 465 nm, syringaldazine at 525 nm and vanillin at 308 nm.

III. Treatments

A. Conventional Sodium Hydroxide Treatment

For the conventional alkaline treatments, a heating mantle was used to boil 0.5 grams of ground cotton seed coat fragments at reflux for 60 min. The treatment bath contained 0.5% (v/v) NaOH, 2% (v/v) NaOH and 4% (v/v) NaOH concentrated solutions respectively along with 0.1% (v/v) nonionic surfactant. After treatments, the SCF's were separated from the supernatant liquid using vacuum suction. The supernatant liquid was saved for further analysis, while the SCF's

were thoroughly washed, dried and conditioned at 65% relative humidity and 70 °F for 24 hours before the treated SCF's were weighed.

B. Enzymatic Treatment only

1. Xylanase Enzyme

In the xylanase enzyme treatment of seed coat fragments, boric acid-borax buffer at pH 8 was used in a shaking water bath with the agitation set at 180 rpm at 60°C for 90 min. Materials to liquor ratio of 1: 40 and enzyme concentrations of 1% and 2% on weight of substrate (ows) were used on one half gram of SCF's. Different variables such as enzyme concentration, and the addition of ethylenediamine-tetra-acetic acid (EDTA), nonionic surfactant (Triton X 100) and vanillin were considered, and their effect on the SCF's was studied. In earlier studies, Csiszar determined that a 0.5 mmol of EDTA increased the weight loss obtained for Pulpzyme HC enzyme. The chelating agent sequestered the calcium ions present in the substrate, thus opening the structure and improving the degradation of the substrate (Csiszar, Losonczi et al. 2001).

Nonionic surfactant of concentration 0.1% (v/v) was used in this study to increase the enzyme action by preventing unproductive surface adsorption of enzyme to lignin, and also increase the accessibility of the substrate (Csiszar, Losonczi et al. 2001; Kristensen, Borjesson et al. 2007). According to Kaya, addition of 0.2% (v/v) vanillin increased the enzymatic hydrolysis of xylan as the enzyme has an affinity towards soluble lignin products (Kaya, Heitmann et al. 2000). Also, the possible synergistic effect in boosting the enzyme action on SCF's was analyzed. Vanillin was added to the solutions by dissolving vanillin powder in a few drops of ethanol and then adding the solution to the buffer. After treatment, the SCF's were separated from the supernatant liquid using vacuum suction. The separated supernatant liquid was saved for further analysis, while the SCF's were washed, air dried and conditioned at 65% relative

humidity and 70 °F for 24 hours before they were weighed. For the control samples, the seed coat fragments were treated in the buffer solution without enzyme or the other components under the same conditions of the xylanase treatment.

2. *Laccase Enzyme*

The laccase treatment was conducted in a 150 mL Erlenmeyer flask containing 0.5 grams of SCF's in the Britton Robinson's buffer. The various parameters studied included mediators (ABTS and guaiacol), laccase enzyme concentrations, nonionic surfactant, and treatment time. ABTS is one of the commonly used mediators in laccase mediator systems (LMS) and hence used in this study (Bourbonnais and Paice 1996; Archibald, Bourbonnais et al. 1997; Solis-Oba, Almendariz et al. 2008). The experiment was carried out at pH 6.5, found to be optimal for the enzyme-ABTS substrate combination from the enzyme assay work. Enzyme treatments were performed according to a 2³ factorial design by opting for low, medium and high enzyme concentrations of 2U/g, 20U/g and 40U/g. The other factors were ABTS (mediator) concentrations of 0.5%, 2.5% and 5% in the time ranges of 1 hr, 4 hr, and 6 hr (Garcia, Camarero et al. 2003; Valls, Vidal et al. 2010). For guaiacol, although pH 4 was found to be optimal for the enzyme-substrate combination from the laccase assay, pH 5 was actually used. The reason for this was that pH 4 was highly acidic and strongly affected the fabric's tensile strength (the effect of different pH's on desized cotton fabric was studied separately). The buffer solution initially contained 0.05% of a nonionic surfactant, Triton X100 (C₁₄H₂₂O(C₂H₄O)_n). However, this surfactant caused a problem as it produced a high amount of frothing when oxygen was bubbled in the solution. To overcome this problem, a different surfactant, Tween 80 (C₆₄H₁₂₄O₂₆), was tried. A low foaming nonionic surfactant, Merspol A (ethylene oxide condensate), of 0.01% concentration was finally used. The whole system was immersed in a shaking water bath (150

rpm) at room temperature with constant bubbling of oxygen through the sample (Camarero, Ibarra et al. 2007; Gutierrez, Rencoret et al. 2007). A material to liquor ratio of 1:100 was used to facilitate proper immersion of sample in the buffer solution. For the control samples, the seed coat fragments were treated in the buffer solution without enzyme or the other components under the same treatment conditions as for the laccase treatment.

C. Microwave Treatment only

One of the early works done using radiation to assist in enzymatic hydrolysis was in 1978 where irradiation was used as a pretreatment to induce decomposition in plant biomass. The source of irradiation used were high energy electron beam accelerator and gamma rays (Kumakura and Kaetsu 1982; Kumakura, Kojima et al. 1982). The insoluble components, such as lignin and hemicellulose in rice straw, wheat straw and sawdust were dissolved by using a pretreatment by irradiation followed by the cellulose breakdown to glucose using cellulase enzyme (Kumakura and Kaetsu 1978). Since the enzyme's accessibility to the cellulosic material is restricted by the structure of the substrate, irradiation treatment was used to indirectly accelerate the enzymatic hydrolysis reaction. In more recent times less energetic microwaves (as seen in Figure 19) have been used as the source of radiation (Zhu, Wu et al. 2005; Keshwani, Cheng et al. 2007; Hu and Wen 2008).

Microwave irradiation is electromagnetic energy with a frequency range of 300 to 300,000 MHz and a wavelength of 1 mm to 30 cm. Two main mechanisms that result in heating using microwave are dipole rotation and ionic conduction. A rapidly changing electric field causes constant rotation motion as the polar molecules try to align with the field. The presence of free ions in the electric field generates ionic motion as the ions align with the rapidly changing

electric field. As the molecules move they generate heat as a byproduct and as the temperature increases transfer of energy increases (Sanchez 2008).

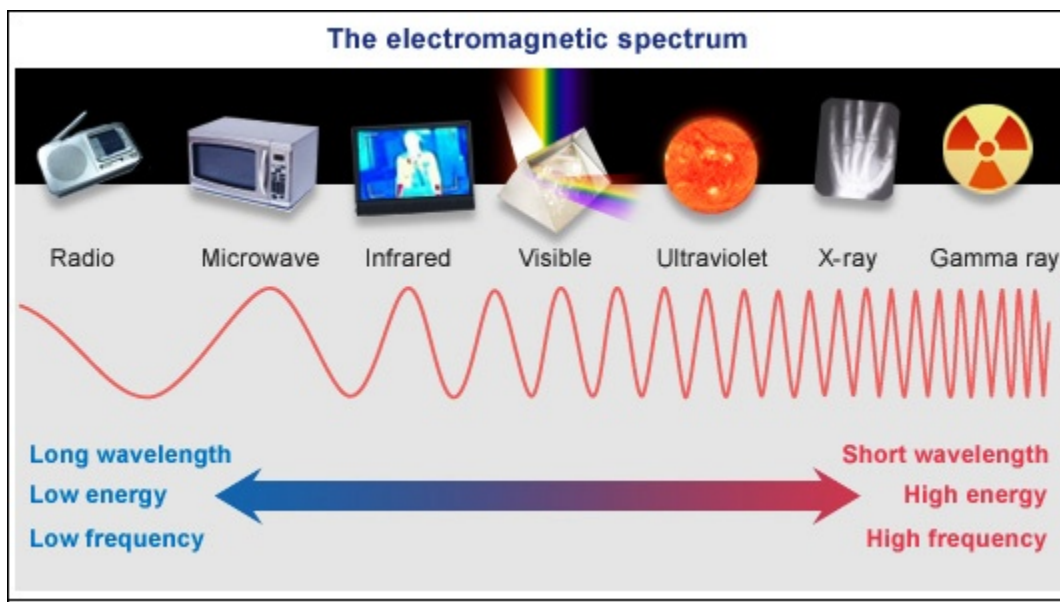


Figure 19: The Electromagnetic Spectrum Depicting the Hierarchy of Different Types of Electromagnetic Radiation with Respect to their Wavelength (Hobbs 2010).

Microwave heating's major advantage over conventional heating (conductive heating) is reduction in reaction time. In conductive heating, the heating source is located outside and the heat is driven to reach the solvent and the reactants after passing through the walls of the vessel (Figure 20). This slows down the transfer of energy to the core of the material, thus reducing the efficiency of the process.

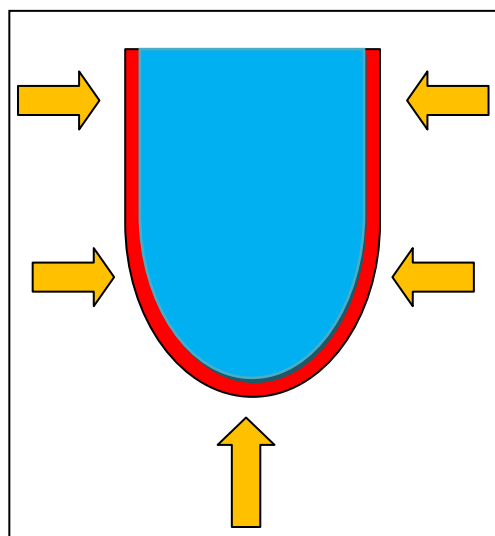


Figure 20: Conductive Heating Using an External Source, Heat Reaches the Reactants-Solvent Mixture Through the Vessel Walls (Hayes 2002; Sanchez 2008).

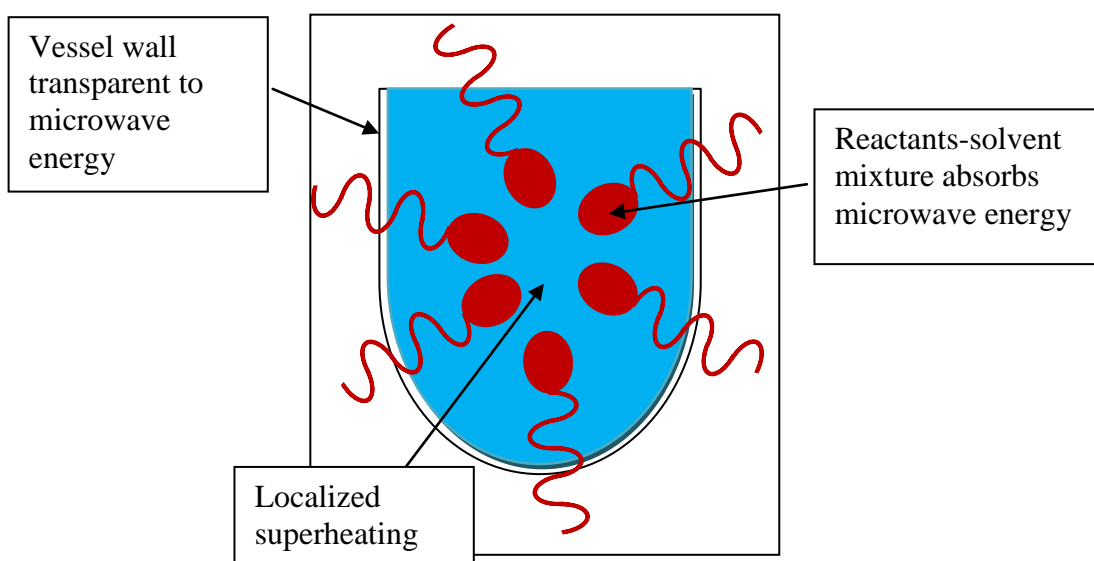


Figure 21: Microwave Heating Where the Waves Directly React With the Molecules Present in the Reaction Medium (Hayes 2002; Sanchez 2008).

In microwave heating, microwaves react with the polar molecules or ions (Figure 21) present in the solution and produce a rapid increase in temperature. Therefore, the heat loss in the

energy transfer is at a minimum. One of the studies done on the impact of microwave synthesis on drug discovery compared the temperature difference for a dielectric heating microwave and heating using an oil-bath by convection currents. They carried out both reactions for the same period of time in the same reaction vessel under constant stirring action (Kappe and Dallinger 2006).

A regular domestic microwave model, Emerson 1000W with a power output frequency of 2450 MHz was used to conduct experiment on SCF's in this study. Initially, a temperature profile was constructed using 20 ml of pure water, and 0.5% and 4% sodium hydroxide solutions in the microwave oven at 30% power for 5 min. For pure water alone, 10% and 50% power for 5 min and 30% power for 7 min were also performed to study the effect of microwave radiation on water temperature with changes in power and time. Statistical software (SAS 9.1) was used to determine if any significant difference existed between each temperature profile. Parameters included time levels of 2.5 min and 5 min and NaOH concentrations of 0.5%, 1%, 2%, and 4% (v/v). Water treated samples at two different time levels were used as a control. One half gram of SCF's and 20 ml of NaOH solutions were placed in a 400 ml beaker and irradiated at the conditions described above.

D. Combined Microwave and Enzyme Treatments

1. Xylanase Enzyme

Following the construction of temperature profiles for the microwave, the amount of power to be used and the time cycle were determined. SCF's were microwave treated at 2.5 min and 5 min respectively in 0.5%, 1%, 2%, and 4% (v/v) sodium hydroxide. The SCF's were separated from the supernatant liquid, thoroughly washed, and then subjected to enzymatic treatment in a shaking water bath. The parameters were set as follows: agitation at 180 rpm,

treatment temperature at 60 °C, treatment time for 90 minutes, pH of 8.0, and a material to liquor ratio of 1: 40 based on the information obtained for the enzyme. In the treatment liquor, a 1% (w/v) xylanase enzyme was used along with 0.5 mmol of EDTA, 0.1% (v/v) nonionic surfactant and 0.2% (w/v) vanillin. At the end of 90 min, the SCF's were separated from the supernatant liquid by vacuum filtering, washed, air dried and conditioned for 24 hours before being weighed. The supernatant liquid was saved for further analysis.

2. *Laccase Enzyme*

One gram of SCF's were treated with 40 U/g of laccase enzyme in presence of 5% (v/v) ABTS mediator and 0.05% (v/v) surfactant in a pH 6.5 Universal buffer for 0.5, 1 and 2 hours. The enzymatic treatment was followed by separating the SCF's from the supernatant liquid and subjecting the SCF's to 0.5% (v/v) and 1% (v/v) sodium hydroxide treatment in a microwave for 5 min. According to Jeon et.al (2008), a cocktail of mediators was able to enhance the laccase enzyme action in lignin biodegradation. A 1:1 molar ratio of ABTS and vanillin adding up to 2 mM concentration was shown to have good effect on biodegradation by multiple sequential electron transfers occurring between laccase and its mediators (Jeon, Murugesan et al. 2008). Therefore, in this experiment, a cocktail mediator mixture of (0.97 mM and 1 mM) ABTS, and (0.97 mM and 1 mM) vanillin was used following the sample process as outlined above.

IV. **Analysis of Treated Seed Coat Fragments**

A. **Seed Coat Fragments Weight Loss**

The final weight of each specimen was recorded after conditioning the sample for 24 hours under standard conditions. The weight loss for the SCF's was calculated by the following formula

$$\frac{W_1 - W_2}{W_1} \times 100$$

Where: W_1 and W_2 are the weights of the SCF's measured before and after treatments.

Samples were treated in replicates of three and their average values were reported as the weight loss percent for the SCF's at different treatment conditions.

B. Analysis of Sugar Removed Using Gas Chromatography-Mass Spectrometry (GC-MS)

After each enzymatic treatment the supernatant liquid was separated from SCF's using a vacuum suction filter. The liquid sample thus separated was passed through a Dowex[®] ion exchange resin. The column was prepared by loading 20 grams of Dowex resin in a glass column and washing the column in sequence with 4M HCl, water, ethanol and water. The supernatant liquid samples were then passed through the column to remove salts present in the sample and thus prevent these salts from hindering the next process.

After desalting, the liquid samples were dried to powder form using a FreeZone[®] 4.5 Liter freeze dry system. An internal standard (myo-inositol) of 250 μ l was then added to the sample to be analyzed so that the amount of sugar released in the solutions could be quantified and compared. The dried samples were next hydrolyzed using methanolic-HCl to release all types of monosaccharides present by hydrolysis under a single set of treatment conditions with minimal destruction of sample. This release was achieved when the glycosidic bonds (Figure 22) connecting the two monosaccharides in a polysaccharide were cleaved to form methyl glycosides. The samples treated with methanolic HCl were stored in tightly closed vials in an oven at 80 °C for over 16 hours to allow the hydrolysis reaction to take place.

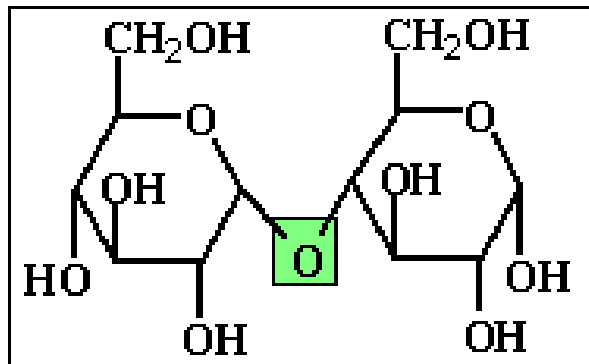


Figure 22: $\alpha(1\rightarrow4)$ Glycosidic Linkage Between Two Monosaccharides (King 2012)

Subsequently the samples were dried over nitrogen gas at 60 °C. The separated neutral monosaccharides and uronic acids were derivatized using Tri-Sil Reagent into alditol acetates and per O-trimethylsilyl ethers. During analysis, the monosaccharides tend to interact with each other and with the GC column. Thus, derivatization is an important step required to prevent interaction that results in poor peak resolution. TMS derivatives form particular fragmentation patterns and mass ions that are easily chromatographed and recognized with GC-MS. The samples were resolved on a 30 meter ZB-1 column (0.25 x 0.25, id.: Phenomenex) in a Shimadzu QP5000 GC-MS. The initial temperature of the gas chromatograph was set at 90 °C, then raised to 200 °C at 2°/min and finally increased to 260 °C for 10°/min. Silyl derivatives were obtained by replacing the active hydrogen from acids, alcohols, thiols, amines, amides and enolizable ketones and aldehydes with trimethylsilyl groups (Molnar-Perl and Katona 2000).

Three replications were repeated for each enzymatic treatment. From the quantitative results of sugars present in the liquid we can determine the effectiveness of the enzyme used on SCFs by calculating the concentration of a particular sugar removed from the substrate by the enzyme.

C. FTIR Spectroscopy

FTIR absorption spectroscopy is an important analytical technique used to identify unknown compounds. Mid- infrared spectroscopy occurs in the range of 4000- 400 cm^{-1} region of the electromagnetic spectrum (Figure 19). IR spectrum is the result of energy absorbed by a molecule exhibiting dipole moment in vibrational motion. Therefore, the energy at which a peak is observed in an absorption spectrum corresponds to the frequency of vibration of the molecule. Vibration can either be in stretching mode or deformation mode. In stretching mode the bond length of the molecule changes, while in deformation mode, the bond angle of the molecule changes due to scissoring, wagging, rocking and twisting actions (Stuart 2004; Lammers 2008). It is difficult to analyze the spectra directly because each component has several vibrational modes and bands that overlap in the spectra. Therefore, due to the interfering bands it is difficult to determine the wavelength and absorbance of individual bands. By differentiating the IR spectra we can separate the overlapping bands and resolve the absorption spectra to reveal useful information. As seen in Figure 23, first derivative curve intercepts the wavelength axis at a point equivalent to the maximum (λ_{max}) of the original zero-order curve while the second derivative curve is obtained as an inverted main peak enclosed by two smaller satellite peaks. As the derivative order increases it helps in resolving the overlapping bands and producing fine spectral detail. Also the amplitudes of the peaks and troughs produced by the derivatives are proportional to the concentration of the substance being analyzed. Second order derivative curve gives an increased resolution in comparison to the normal and first derivative spectra (Bridge, Fell et al. 1987). Processing of the derivatized FTIR data along with multivariate analysis helps in easy processing of the data obtained from FTIR spectra. PCA breaks down the data to its basic difference and can be used to detect sample patterns and groupings of similar compounds. The

analysis will result in reducing a data set containing many variables to a smaller number of variables called principal components (PC's). The initial components can be plotted and most of the variations in the data set can be determined. In multivariate analysis (refer to Appendix), principal component analysis (PCA) and Hierarchical cluster analysis (HCA) were performed using R statistics and Origin software.

FTIR spectra of ball milled seed coat fragments were recorded with the FTIR spectrometer (Nicolet 6700) at a resolution of 4 cm^{-1} in the range of $4000\text{-}400\text{ cm}^{-1}$. For the FTIR sample 3 mg of oven dried SCF's was mixed with 300 mg of KBr and crushed in a mortar under a heat lamp until a uniform mixture was obtained. After grinding, one third of the weighed sample was transferred into a pellet holder, covered with a stainless steel disk and placed under a hydraulic press to form thin pellets. After recording the background in FTIR instrument the pellet was mounted in the holder and 128 scans of the sample was recorded as absorbance values. The FTIR spectra were baseline corrected, normalized and an average of three readings was taken as the mean spectra for further analysis. Since the finger print region contained most of the spectral information, only the region between $1900\text{-}800\text{ cm}^{-1}$ was considered for the multivariate analysis techniques. Origin software was used to obtain the 1st order derivative (Savitzky-Golay Smoothing of 2nd order polynomial and 15 points) and 2nd order derivative (Savitzky-Golay Smoothing of 2nd order polynomial and 20 points) for the data sets (Rana, Muller et al. 2008; Chen, Ferrari et al. 2010; Bahng, Donohoe et al. 2011).

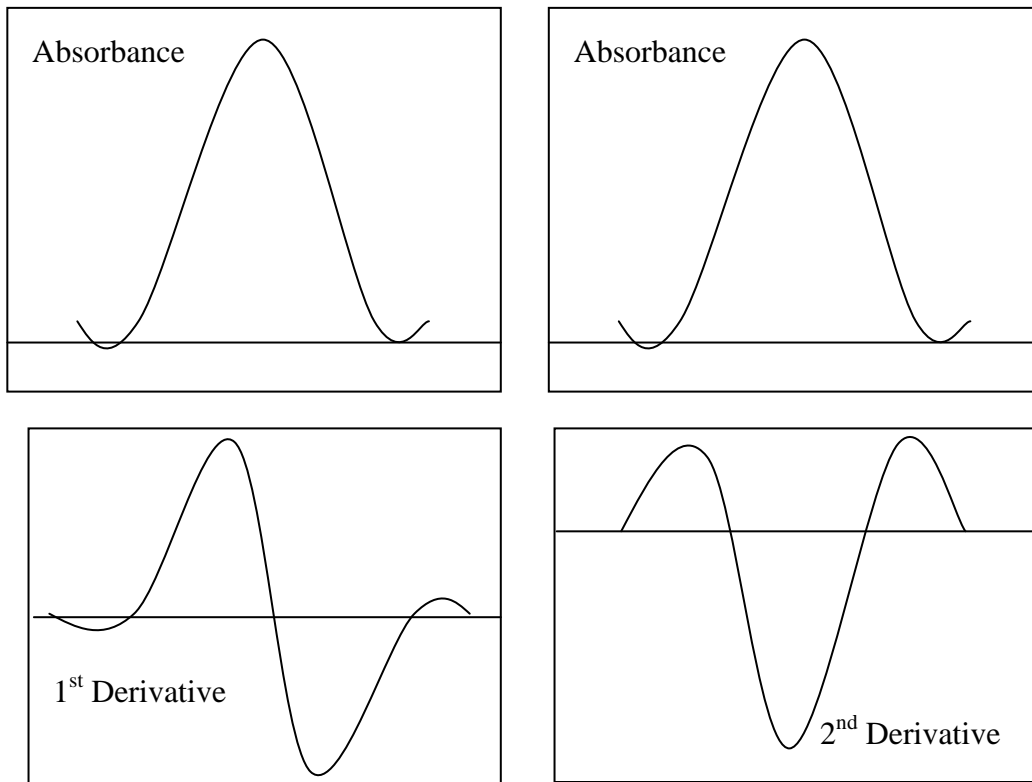


Figure 23: Absorbance and Derivative Spectras (Owen 1995)

D. Analysis of Lignin Removed

1. Klason Treatment

The Klason method of lignin analysis was performed following National Renewable Energy Laboratory (NREL) procedure. The whole experiment can be divided into three sub classes. First, the total solids in biomass were determined following LAP-001: Standard method for determination of total solids in biomass. Next the recalcitrant insoluble lignin in the biomass was determined following Procedure A: Summative Analysis from LAP-003: Determination of acid-insoluble lignin in biomass. Finally, the soluble lignin in the biomass was determined following LAP-004: Determination of acid soluble lignin in biomass.

The seed coat fragments that were separated from the supernatant solution were washed, dried, conditioned for 24 hours and the weight loss recorded before the sample was ball milled to convert SCF's to a finer powder. The ball milled samples were then conditioned in a vacuum oven at 45 °C overnight before using in the Klason experiment. The crucibles were heat-treated at 575 °C in a muffle furnace for three hours to achieve a constant weight. The heat-treated crucibles were then stored in a desiccator to allow the crucibles to cool to room temperature before weighing to the nearest ±0.1 mg.

A 72% (v/v) sulfuric acid (H₂SO₄) solution was prepared by adding 66.5 ml of concentrated sulfuric acid to 30 ml of water placed in an ice bath. For total solids determination, 0.2 g of SCF's were weighed and transferred to a pre weighed aluminum weigh boat. The sample was oven dried at 105 °C for more than 8 hours before the weight of the sample was measured.

The percentage of the total solids was determined by the following formula:

$$\frac{\textit{Weight of dried sample plus boat} - \textit{weight of boat}}{\textit{Weight of sample as received}} \times 100$$

Two replications were done for each sample and the average of total solids was reported as T_{final} in the calculations of acid insoluble lignin.

In the acid insoluble lignin procedure, 0.3 g of SCF's (2 replications) was weighed and placed in a 50 ml beaker. The initial weight of the sample was recorded as W₁. To the weighed sample 3 ml of 72% (v/v) sulfuric acid was added and the sample was mixed using a glass stirring rod for two minutes until the sample was thoroughly wetted. Acid was added to each sample at two minute intervals and left under a hood with stirring done every 15 minutes. The hydrolysis of the SCF's was allowed to take place for two hours before the hydrolyzate was transferred to a glass autoclave bottle. The concentrated acid was diluted to 4% (v/v) concentration by washing the beaker with 84 ml of water and transferring all the residual solids

into the bottle. The autoclave was set to liquid vent cycle at a temperature of 121 °C and the samples were autoclaved for one hour. After the autoclave cycle the samples were left out in the room temperature to cool before opening the lid of the glass bottle.

The samples were vacuum filtered using the stored, heat-treated and pre-weighed crucible. Hot deionized water was used to wash the particles clinging to the glass bottle. A 20 minute time span was allotted to collect the vacuum filtered samples after washing. The filtrate collected from each sample was weighed and 25 ml of the filtrate was saved for acid-soluble lignin analysis. The filtrate was stored in a refrigerator and used within six hours of hydrolysis. The crucibles and its contents were dried at 105 °C for 2 hours and then left in a desiccator to cool to room temperature. The weight of the sample along with the crucible was recorded as W_2 . The crucible was then placed in a muffle furnace and heat-treated at 575 °C for 3 hours and then cooled to room temperature by placing in a desiccator. The cooled crucible was weighed and the weight recorded as W_3 . The percentage of acid insoluble lignin was calculated using the formula given below:

$$\frac{W_2 - W_3}{W_1 \times \frac{T_{final}}{100}} \times 100$$

Where:

W_1 = initial sample weight

W_2 = weight of crucible, acid-insoluble lignin and acid insoluble ash,

W_3 = weight of crucible and acid insoluble ash

T_{final} = % total solids content of the prepared sample used in lignin analysis, on a 105 °C dry weight basis (as determined by LAP-001)

Acid-soluble lignin determination was done using UV/Vis spectrophotometer where the absorbance of the saved filtrate sample was measured at 205 nm. For reference a blank of 4%

sulfuric acid was used. Both the sample and the blank were diluted until the absorbance reading fell between 0.2 and 0.7. The acid-soluble lignin for the SCF's was estimated by using the following equation:

$$\frac{\frac{A}{b \times a} \times df \times V \times \frac{L}{1000 \text{ mL}}}{\frac{W \times T_{final}}{100}} \times 100$$

Where:

A= absorbance at 205 nm

df= dilution factor

b= cell path length, 1 cm

a= absorptivity equal to 110 L/g-cm

V= weight of the filtrate obtained from LAP 003 Klason lignin protocol expressed in mL

W= initial biomass sample weight in grams

T_{final}= % total solids content of biomass determined during LaP-003 analysis

2. *Pyrolysis Molecular Beam Mass Spectrometry (py-MBMS)*

Seed coat fragments in the range of 4 mg were weighed in 80 µl stainless steel sample cups of a commercially available auto sampler of double shot pyrolyzer (PY-2020iD, Frontier Ltd). The sample was then pyrolyzed at 500 °C with helium (carrier gas) at an interface temperature of 350 °C and a total pyrolysis time of 2 minutes. The residues of the pyrolysis were then analyzed using a custom built supersonic molecular beam mass spectrometer (Extrel Model MAX-1000). An in-house standard such as NIST 8491 standards having a lignin content of 24.2 % was included in each set of samples.

Mass spectral data from m/z 30-450 were acquired on a Merlin Automation Data System version 3.3. Multivariate analysis was performed using Unscrambler software version 10.1

(CAMO). The intensities of the lignin peaks (m/z 120, 124, 137, 138, 150, 152, 154, 164, 167, 168, 178, 180, 181, 182, 194, 208 and 210) were summed and averaged in order to estimate the lignin contents in the sample. Syringyl to Guaiacol (S/G) ratios were also determined by summing syringyl peaks m/z at 154, 167, 168, 182, 194, 208 and 210 and dividing by the sum of the guaiacol peaks m/z 124, 137, 138, 150, 164 and 178 (Evans and Milne 1987). The lignin values thus generated and calculated were compared with the results obtained from Klason lignin analysis.

2. Pyrolysis Gas Chromatography Mass Spectrometry (Py/GC-MS)

The SCF's samples were weighed (2mg) into 80 μ l stainless steel sample cups of a commercially available auto sampler of double shot pyrolyzer (PY-2020iD, Frontier Ltd). The samples were then pyrolyzed at 500 °C with helium as the carrier gas and an interface temperature of 280 °C. Each pyrolysis reaction was completed in 1.2 min with a total pyrolysis time of 2 min. The residues were carried into an Ultra Alloy-5 Capillary Column (30mx.25mm) with 5% diphenyl 95% dimethylpolysiloxane as the stationary phase. The temperature was held at 50°C for 2 min to trap and focus the volatile compounds. The temperature was then programmed to a final temperature of 280°C at 5°C/min, and held for 5 min. The total run time was 53 min per sample. The compounds were detected using Agilent Technologies 6890N GC and 5975B Mass Spec Detector.

The resulting lignin peaks were compared with the lignin library obtained from Taiwan Forestry Research Institute. The total area of 'S' and 'G' lignin peaks were calculated and the S/G ratio were determined.

3. *Surface Enhanced Raman Spectroscopy (SERS)*

Monochromatic light incident on a molecule causes scattering of photons usually of same energy (elastic) called Raleigh scattering. A very small fraction of the photons (1×10^{-7}) is inelastically scattered (having a different frequency than that of the incident photons). This difference in wavelength of the inelastic photon can easily be translated into chemical and structural information of the molecule. Raman scattered photons can either have higher energy (anti-Stokes radiation) or lower energy (Stokes radiation) than that of the incident photon. The observed shift of the photons is a direct measurement of the vibrational energy that is specific with respect to the chemical bonds in the molecule. Only those molecules having symmetrical stretching and bending with a constant change in the polarizability ' α ' of the molecule during the vibration are Raman active.

A typical Raman spectrum is a plot of Raman scattering intensity vs Raman shift in wave numbers. The Raman scattering signal is very weak compared to Raleigh scattering. In biological samples the broad fluorescence background signals of the sample masks the weak Raman spectrum and makes it invisible. Surface enhanced Raman spectroscopy (SERS) was developed in 1974 as a method to greatly enhance the weak Raman signal. Using localized surface plasmons of metals such as silver, gold or copper, the Raman signal was enhanced 10^6 times. To obtain a highly enhanced signal, the target molecules should be kept in immediate contact with the surface of SERS active substrate. The substrate should be present in nano scaled structure as it offers more surface area for the target molecules to be in good contact with the active substrate (Sur 2010).

Silver nanorod arrays prepared in another lab were used as the active substrate for this experiment. The arrays were individual 1in by 3in substrate with 40 diameter wells of 3mm

formed by polydimethylsiloxane. Samples for the experiment were prepared by diluting the sample to a very low dilution with water. 1 ml of liquid sample was mixed with 1 ml of ethanol and using a pipette, 1 μ l of the sample was transferred onto the array. The substrate was allowed to air-dry overnight before spectra was collected. Spectral signals were collected in 10 sec acquisitions with Bruker optics on a Senterra dispersive Raman microscope using the 785 nm laser at a 25mW power level, a 10 x objective and a 10 sec acquisition with a resolution of 3 to 5 wavenumbers using OPUS 6.5 software were also used.

Spectra were first averaged using the GRAMS32/AI spectral software package to assess signal-to-noise ratio spectral quality and baseline corrected using a concave rubber band algorithm which performed 10 iterations on 64 points to aid in a preliminary evaluation/comparison and peak assignment. Spectra were pre-processed for chemometric analysis by normalizing to unit vector length with R-Statistics. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were used to explore clustering of similar spectra using R statistics.

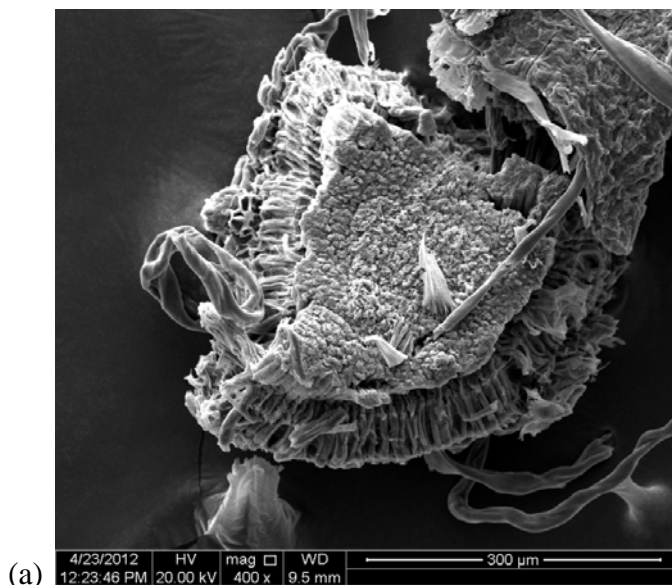
CHAPTER 4

RESULTS AND DISCUSSION

I. General

A. Scanning Electron Microscope (SEM)

A scanning electron microscope was used to capture the surface topography of the seed coat fragments. A high magnification of 400 X to 800 X was used in obtaining the Figure 24. The samples for SEM were prepared by mounting them on an aluminum stub, with a double-sided sticky tape. The samples were coated with a 90 Å layer of high purity silver under a vacuum evaporator prior to analysis. Figure 24 (b) represents a clear cross sectional view of a seed coat fragment that can be related to the cross sectional diagram in Figure 4.



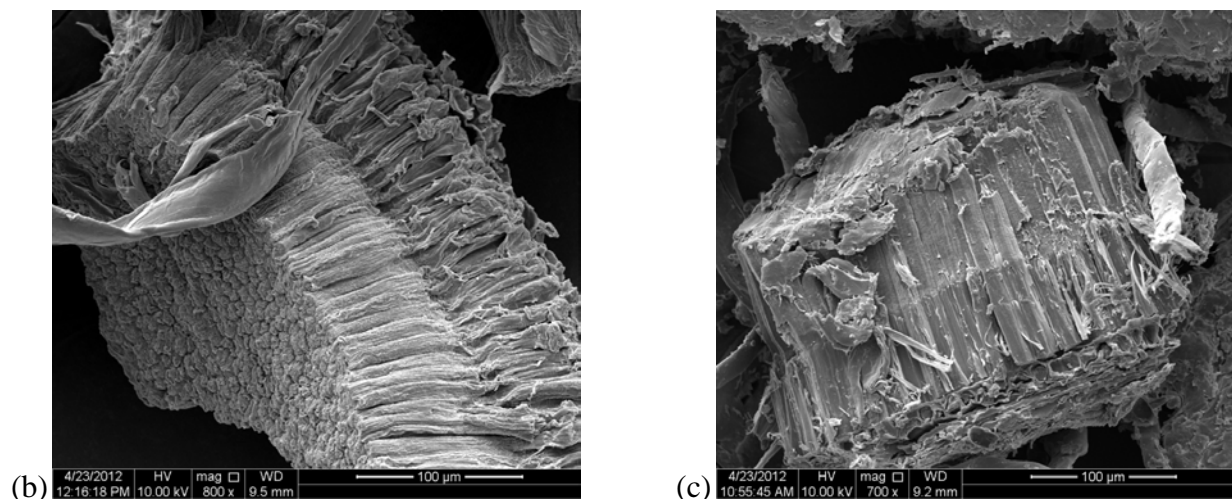


Figure 24: Scanning electron micrographs of untreated seed coat fragments

B. Enzyme Assays

1. Xylanase Assay

Standard solutions of xylose sugar at five different concentrations (10 μ mol/ml, 6.67 μ mol/ml, 5 μ mol/ml, 4 μ mol/ml, 3.33 μ mol/ml) were prepared. DNS reagent added to the solution is reduced by xylose (a reducing sugar) to 3-amino-5-nitrosalicylic acid (an orange-red complex) that absorbs strongly at 540 nm. The intensity of the color measured depends on the amount of reducing sugar in solution. The standard line was constructed using five points with absorbance on the ordinate and xylose concentrations converted to nkat ml⁻¹ units on the abscissa. Table 3 gives the absorbance values recorded for the DNS product with respect to concentrations of xylose in solution. The xylose sugar standard constructed using Table 3 is shown in Figure 25.

The xylose sugar concentration (μ mol/ml) unit was converted to SI units (nkat/ml) of reducing sugar (xylose) using the following formula:

$$\frac{10 \frac{\mu\text{mol}}{\text{ml}}}{600 \text{ s}} = 0.167 \mu\text{mol ml}^{-1}\text{s}^{-1} = 16.7 \text{ nkat ml}^{-1}$$

Table 3: Absorbance obtained for DNS product converted to xylose concentrations

Xylose units (nkat/ml)	Absorbance (A)
16.7	2.328
11.11	1.450
8.33	0.967
6.67	0.713
5.56	0.549

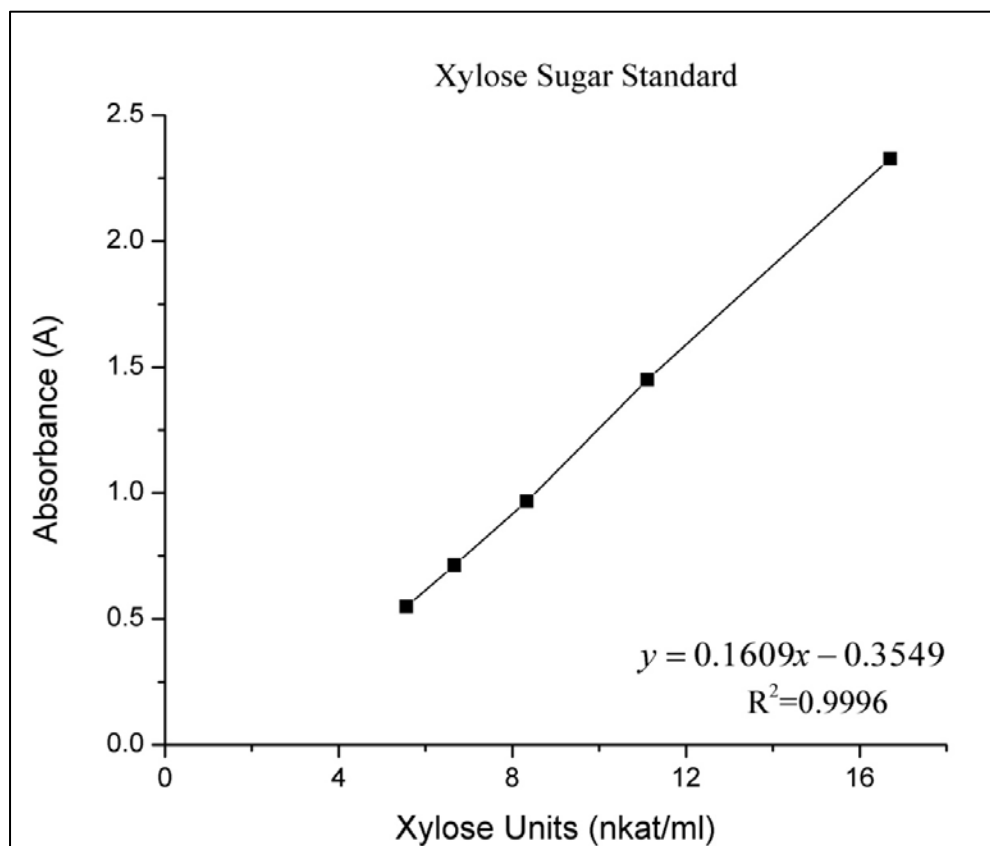


Figure 25: Xylose sugar standard curve

Table 4: Xylanase enzyme activity calculated by measuring the absorbance for DNS product reduced by the xylose obtained by hydrolysis of birchwood xylan

Sample No.	Absorbance (A) measured	Enzyme Activity (nkat/ml)
1.	0.9747	15888
2.	1.283	15937
3.	0.9423	16019
4.	0.8250	16247
5.	1.3799	16839
6.	1.530	16946
7.	1.438	17545
8.	1.189	17864
9.	0.6581	18862
10.	0.7816	19132
11.	0.6830	19311
12.	1.922	19356
13.	0.900	20658
Avg.		17739
Std. Dev.		1585

A stock solution containing 0.1ml of enzyme in 10ml of buffer was further diluted by adding 0.2ml, 0.15ml, 0.10ml and 0.05ml of the stock solution to 50ml buffer. The final dilution factors calculated for the enzyme solutions were 25,000, 33,333, 50,000, and 100,000

respectively. The enzyme activity obtained in nkat ml^{-1} units using the xylose standard curve is given in Table 4.

The enzyme activity for the xylanase enzyme was determined using the following formula:

Enzyme activity = $17,739 \pm 1585 \text{ nkat/ml}$ (1IU = 16.7 nkat) (Bailey, Biely et al. 1992)

Therefore, for Pulpzyme HC, a xylanase enzyme activity of $1062 \pm 95 \text{ IU/ml}$ was obtained in international (IU) units using the xylose standard curve.

2. *Laccase Assay*

The activity assay for the laccase enzyme was done using four different substrates in pH's ranging from 3 to 8. The absorbance values of the products formed for each substrate were plotted against time in minutes. As the concentration of the product formed increased, the absorbance values increased with time.

ABTS, a non-phenolic substrate, was oxidized to the cation radical ABTS^+ , as seen in Figure 26. The absorbance of the cation radical was measured at 420 nm because the radical has a maximum absorbance value at this wavelength. Figure 27 gives the graphical representation of the average absorbance values obtained for three replications of the ABTS substrate measured at different pH values.

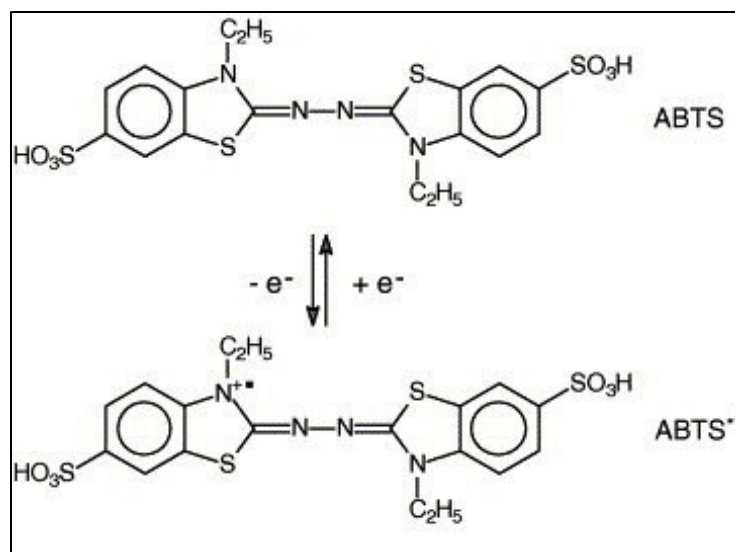


Figure 26: ABTS oxidized to ABTS cation (Solís-Oba, Ugalde-Saldívar et al. 2005)

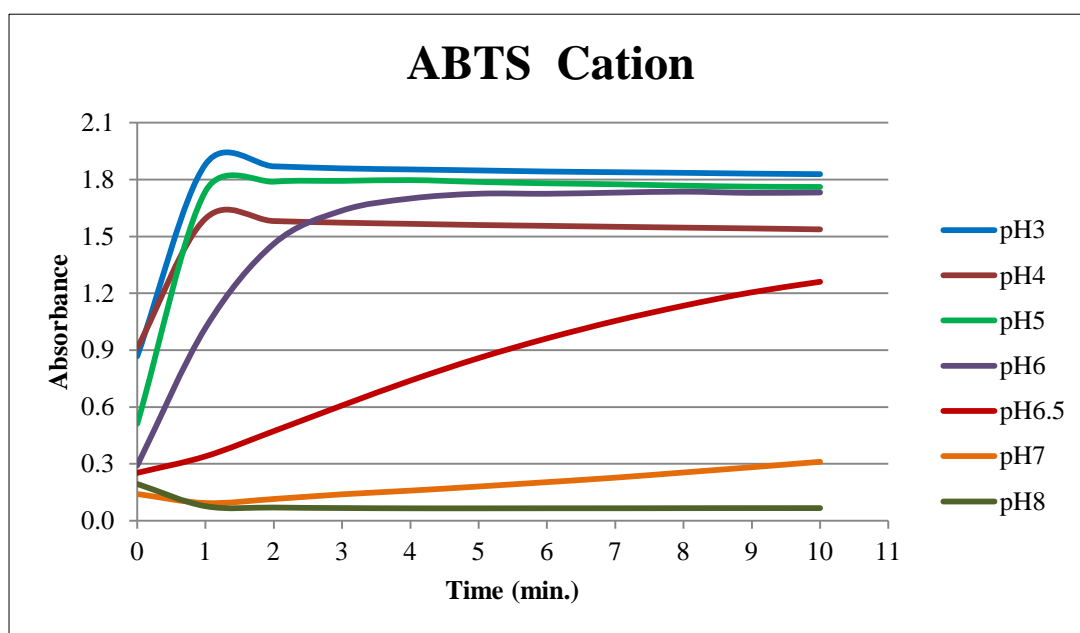


Figure 27: Absorbance values measured at 420 nm for ABTS cation obtained by oxidizing substrate using laccase enzyme.

Syringaldazine, a phenolic substrate, was oxidized to a quinone compound (Figure 28) in the presence of laccase enzyme. The quinone product has a maximum absorbance value at 525 nm wavelength. Hence, the absorbance was measured at this wavelength. Figure 29 gives the

graphical representation of the average absorbance values obtained for three replications of the syringaldazine oxidation measured at different pH values.

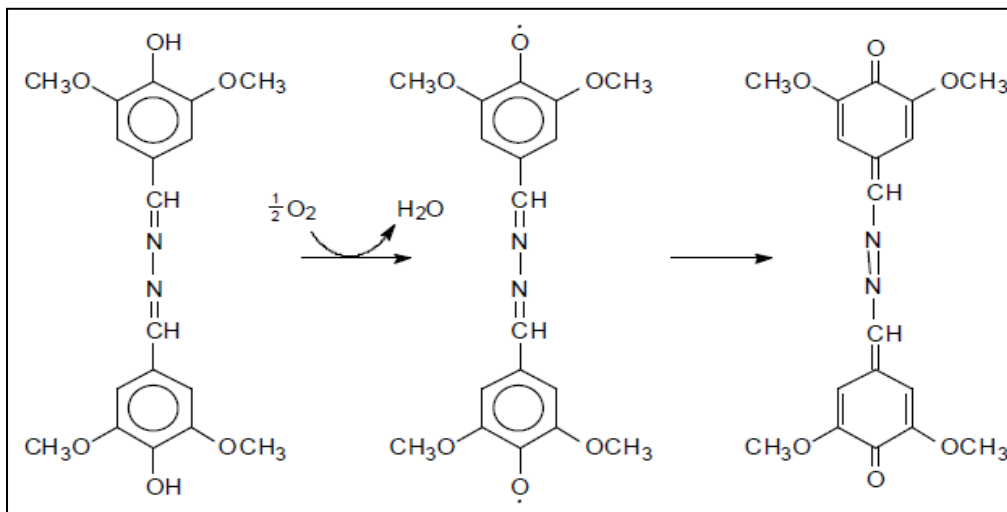


Figure 28: Syringaldazine oxidized to quinone product (Sanchez-Amat and Solano 1997)

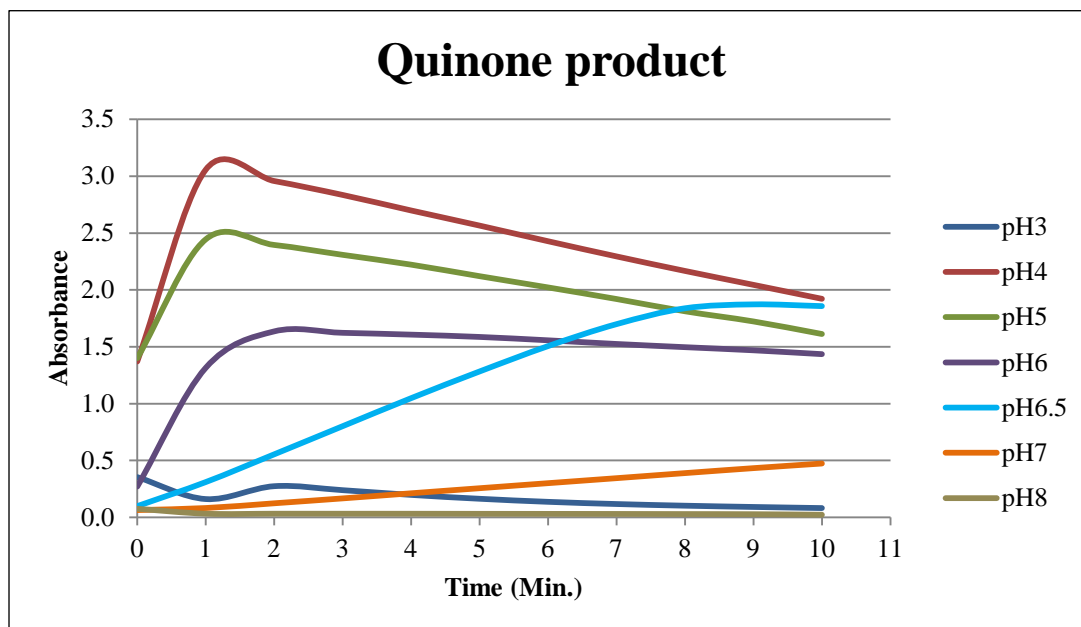


Figure 29: Absorbance values measured at 525 nm for quinone product obtained by oxidizing syringaldazine substrate using laccase enzyme.

Guaiacol is a phenolic compound that is easily oxidized in the presence of laccase enzyme to its radical form (Figure 30). The absorbance vs time in minutes at different pHs for the product of the laccase enzyme activity on the guaiacol substrate is given in Figure 31.

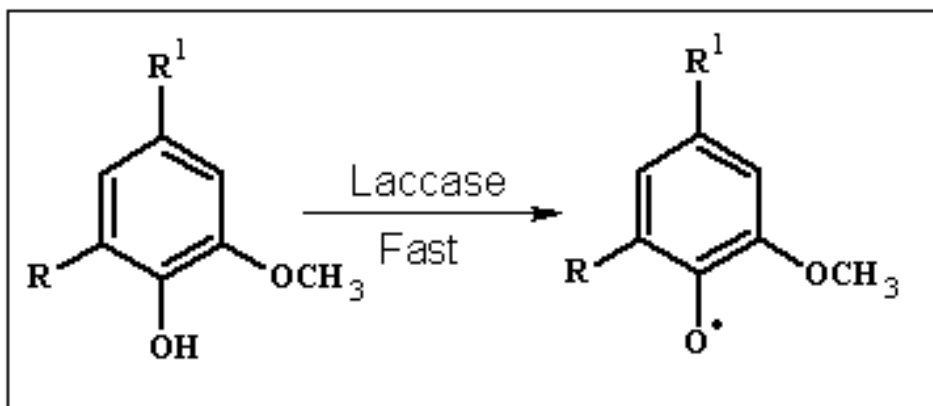


Figure 30: Guaiacol oxidized to its radical by laccase enzyme (Shleev, Persson et al. 2006)

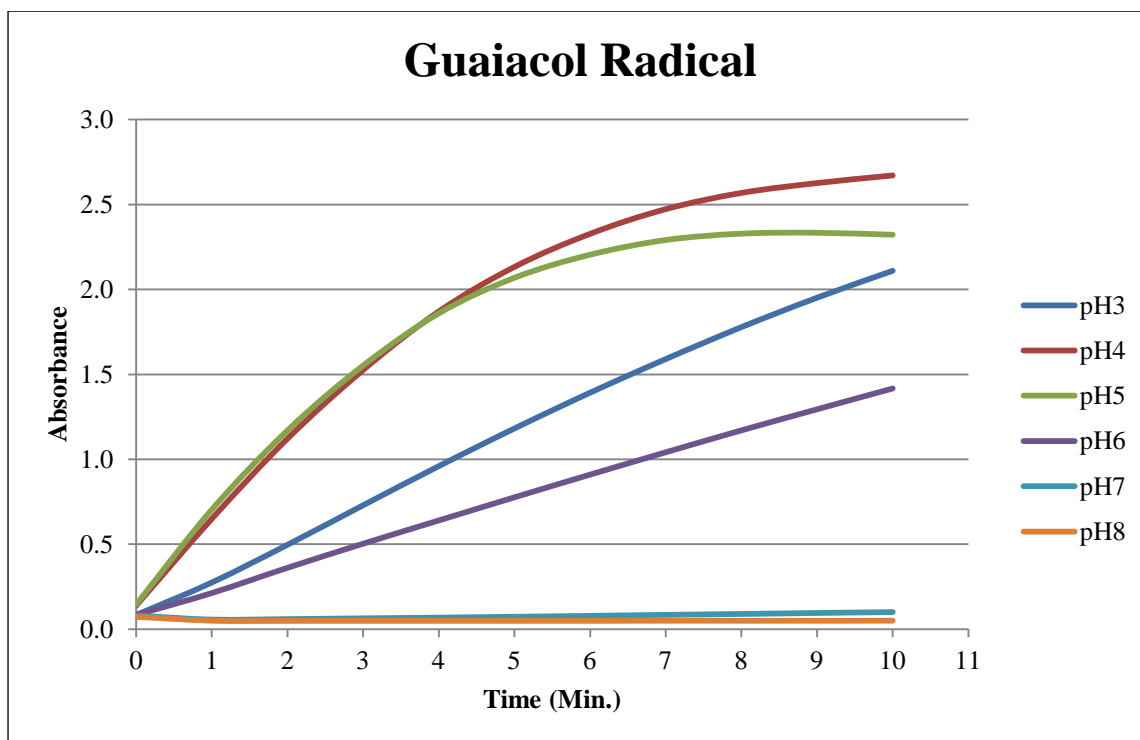


Figure 31: Absorbance values measured at 465 nm for guaiacol radical obtained by oxidizing the guaiacol substrate by laccase enzyme.

Vanillin is a phenolic aldehyde substrate that is oxidized by laccase enzyme. The absorbance values recorded at 308 nm for vanillin substrate at pH ranging from pH 3-8 are given in Figure 32.

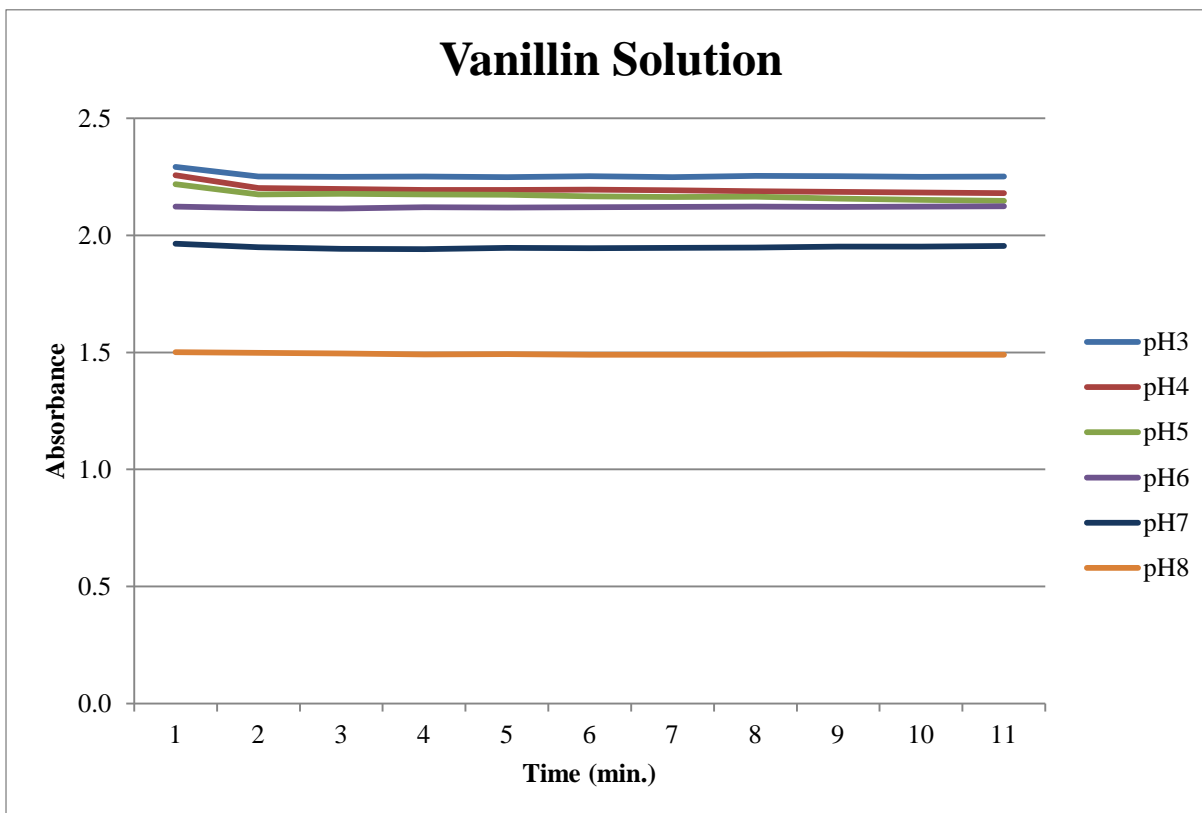


Figure 32: Absorbance values measured for vanillin solution at 308 nm after vanillin substrate was oxidized by laccase enzyme.

The laccase enzyme activity was calculated using the following equation:

$$\text{Activity} \left(\frac{U}{mL} \right) = (\text{average slope}) \times (1000) \times 2.0$$

One unit is defined as a change of 0.001 absorbance units in one second. Since the amount of enzyme solution used was 0.5ml instead of 1ml, the activity was multiplied by 2.0 in the above equation.

The enzyme activities obtained for different substrates at various pH ranges are given in Table 5. (blanks indicate that the activity obtained for the enzyme at that pH was negligible)

Table 5: Laccase enzyme activity given in U/ml for different substrates measured at pH's 3-8. The blank spaces indicate negligible activity obtained.

Substrate	pH3	pH4	pH5	pH6	pH6.5	pH7	pH8
ABTS (U/mL)			0.6	2.2	4		
Syringaldazine (U/mL)					7.6	1.4	
Guaiacol (U/mL)	7.4	10.2	8.8	4.6		0.2	
Vanillin(U/mL)							

The activity table, shows that the laccase enzyme for substrates ABTS and syringaldazine was more active at pH 6.5 compared to the other pH values. For guaiacol the laccase enzyme was highly active at pH 4. For vanillin, laccase enzyme was not active at any of the pH's. Therefore, the seed coat fragments were treated with laccase enzyme and the substrates as a mediator at their active pH.

II. Treatments

A. Conventional Sodium Hydroxide Treatment

Seed coat fragments were treated at concentrations of 0.5% (v/v) NaOH, 2% (v/v) NaOH, and 4% (v/v) NaOH, respectively, along with 0.1% (v/v) surfactant for 60 min. The average weight loss for three replications of each treatment is represented in the Figure 33.

The weight loss obtained for the treated SCF's increased with an increase in the concentration of sodium hydroxide. One of the reasons for this could be that with an increase in

sodium hydroxide concentration a corresponding increase in dissolution of non-degraded polysaccharides, peeling-off reactions and decomposition of polysaccharides occurred (Mirahmadi, Kabir et al. 2010).

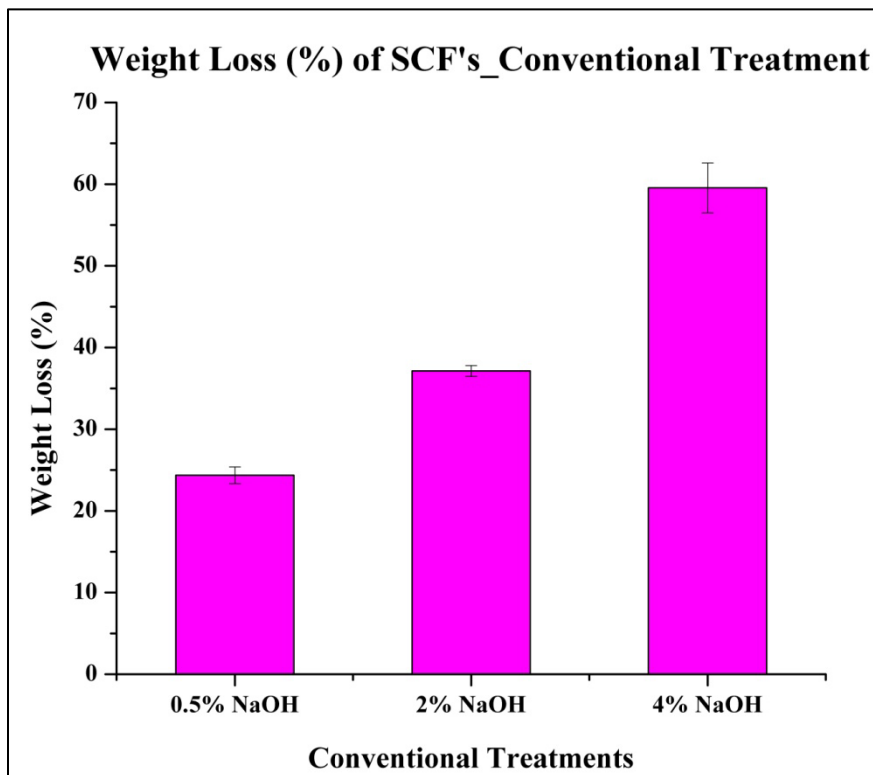


Figure 33: Weight loss for SCF's after sodium hydroxide treatment.

B. Enzymatic Treatment only

1. Xylanase Enzyme

In the initial part of the experiment, the effects of different variables such as enzyme concentration, presence of chelating agent (EDTA), surfactant and vanillin substrate on SCF's were studied. The combinations of the parameters used in the experiment were as follows:

- Pulpzyme 1% (Enzyme_A) and Pulpzyme 2% (Enzyme_B) (ows);
- No surfactant (A) and 0.1% (v/v) surfactant (B);

- No EDTA (A) and 0.5 mmol EDTA (B);
- No vanillin (A) and 0.2% (v/v) vanillin (B)

For the control sample the SCF's were treated in buffer solution only without adding enzyme, chelating agent, surfactant or vanillin.

The experiment was conducted at 60 °C and pH8 (optimum temperature and pH for Pulpzyme HC enzyme) for 90 min in a shaking water bath. After treatment, the SCF's were washed, dried and conditioned for 24 hours before the weight loss was measured. Three replications were averaged. The separated supernatant solution was saved for analyzing the carbohydrates present in the solution using GC-MS. Table 6 gives the weight loss recorded for the SCF's using different combinations of the parameters.

Table 6: Average weight loss obtained for SCF's treated using Pulpzyme HC enzyme.

Treatment	Surfactant (%)	EDTA (mmol)	Vanillin (%)	Wt loss (%)
Control	No	No	No	5.80
1% Pulpzyme	No	No	No	5.73
1% Pulpzyme	0.1	No	No	6.40
1% Pulpzyme	0.1	0.5	No	5.73
1% Pulpzyme	0.1	0.5	0.2	9.13
1% Pulpzyme	No	0.5	0.2	5.80
1% Pulpzyme	No	No	0.2	6.47
1% Pulpzyme	No	0.5	No	7.60
1% Pulpzyme	0.1	No	0.2	6.20
2% Pulpzyme	No	No	No	5.67

2% Pulpzyme	0.1	No	No	6.40
2% Pulpzyme	0.1	0.5	No	6.93
2% Pulpzyme	0.1	0.5	0.2	5.13
2% Pulpzyme	No	0.5	0.2	7.47
2% Pulpzyme	No	No	0.2	6.67
2% Pulpzyme	No	0.5	No	7.60
2% Pulpzyme	0.1	No	0.2	5.27

From Table 6, of all the combinations used on SCF's only the combination of enzyme (1%), in presence of surfactant, EDTA and vanillin substrate gave a significant weight loss compared to the control sample. But even this weight loss was much less than the weight loss obtained for conventionally treated (sodium hydroxide treated) SCF's. Therefore, though studies conducted by other researchers found that the addition of vanillin and chelating agents increased the enzyme action, this was not the case in our samples.

The recalcitrant nature of the SCF's could be due to the presence of lignin, a complex phenylpropane structure that is difficult to degrade. Therefore, for an effective enzymatic treatment, a pre-treatment that attacks the lignin and opens up the structure to enhance the accessibility of the SCF structure to enzymes was required.

The supernatant solutions that were separated from the treated SCF's were processed to prepare the samples to be passed through GC-MS. Towards the end of the preparation cycle I realized that I had used a lesser quantity of water to wash the Dowex column than the required volume. Due to this, I had collected incomplete sugar samples for GC-MS analysis with parts of the sugar sample left on the column. I had to discard the results that I had obtained for these samples from GC-MS.

2. Laccase Enzyme

In the initial part of the experiment SCF's were treated with laccase enzyme in presence of mediators (ABTS, guaiacol) at different concentrations of 0.5%, 2.5% and 5% on weight of SCF's, using different enzyme concentrations (2U/g, 20 U/g and 40 U/g) and treatment times of 1 hr, 4 hr, and 6 hr. The experiment treatments were stopped after studying for the low and high enzyme concentrations as the weight loss obtained for the samples were low. For guaiacol only, the high parameter combination was tried and, since this resulted in low weight loss, no further treatments were done using this mediator. The experimental combinations studied are given in Table 7:

Table 7: Experiments done using the enzyme mediator combinations on SCF's

No.	Laccase (U/g)	Mediator (w/v)	Time (hr)	Surfactant
1	-	-	1,4,6	Yes
2	2	0.5% ABTS	1,4,6	Yes
3	2	2.5% ABTS	1,4,6	Yes
4	2	5% ABTS	1,4,6	Yes
5	2	0.5% ABTS	1,4,6	No
6	2	2.5% ABTS	1,4,6	No
7	40	5% ABTS	1,4,6	Yes
8	40	5% Guaiacol	1,4,6	Yes

Table 8 gives the average weight loss recorded for the SCF's treated with laccase enzyme and mediators.

Table 8: Weight loss (%) obtained for SCF's treated with laccase mediator system combinations

No.	Treatment Time		
	1 hr	4 hr	6 hr
1	4.03	4.35	4.42
2	5.17	5.03	5.56
3	4.5	4.57	4.58
4	3.27	3.21	3.03
5	3.19	2.71	2.97
6	2.74	3.17	3.35
7	4.96	5.96	4.22
8	3.0	4.27	4.04

One reason for a low weight loss could be that the treatment was not performed in a pressurized reactor under oxygen pressure, as was done in some previous research (Garcia, Camarero et al. 2003; Fillat and Roncero 2009; Valls, Vidal et al. 2010). Also, time could be a factor affecting the results obtained as previous work by others had continued the experiment for 12 hours, or left the samples sitting in the liquor bath for 24 hours. The type of mediator used could also be a factor as most of the effective experiments that reported high level of delignification were done using a laccase-mediator called 1-hydroxybenzotriazole (HBT). The drawback for this mediator is that HBT, a member of the N-OH compounds, and their reaction products, are suspected to have toxicity (Camarero, Ibarra et al. 2007; Gutierrez, Rencoret et al. 2007).

C. Microwave (MW) Treatment only

Temperature profiles for the MW were prepared with respect to substrate (water, NaOH), power (10%, 30%, 50%) and treatment time (5 min, 7 min). The data obtained for these experiments are given in Figure 34 and Figure 35. From Figure 34, it is clear that 10% power did not raise the temperature significantly. While the 50% power provides a higher heating rate, this setting caused the water to evaporate, leading to reduction in volume of the sample at the end of the treatment. No significant difference ($p=0.7135$) was found between the 5 min and 7 min temperature profiles. At 30% power, the ideal temperature was reached with limited evaporation occurring. Therefore, 30% power was chosen for the experiment. In Figure 35, the temperature profiles for 0.5% NaOH and 4% NaOH solutions at 30% power are shown to be similar, the difference being statistically non-significant. The profile for pure water under similar conditions was slightly lower but followed the same trend. In order to evaluate the effect of MW pre-treatment on SCF's, 30% power was selected along with the two different treatment times (2.5 min and 5 min) and four different NaOH concentrations (v/v) (0.5%, 1%, 2% and 4%). The results obtained in the experiments were compared to conventional treatments in terms of weight loss, reduction of sugar yield and chemical modifications. These results are given under next section D. Combined Microwave and Enzymatic Treatments.

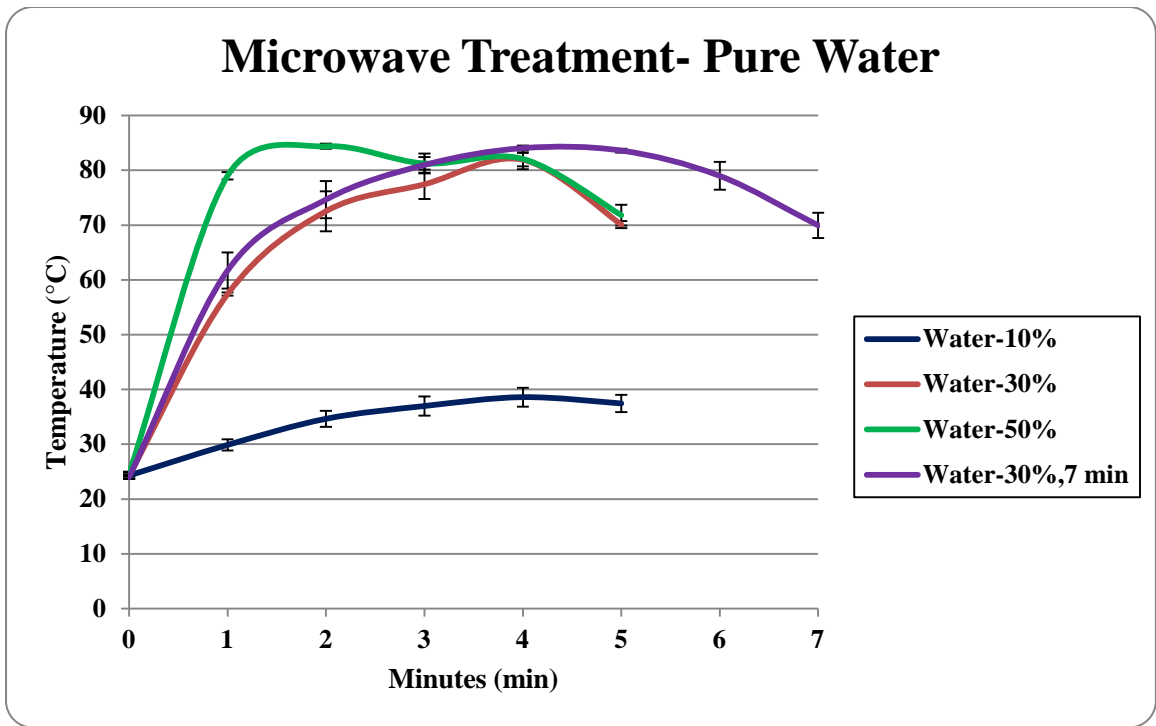


Figure 34: Temperature profiles obtained for pure water at 10, 30 and 50 percent power.

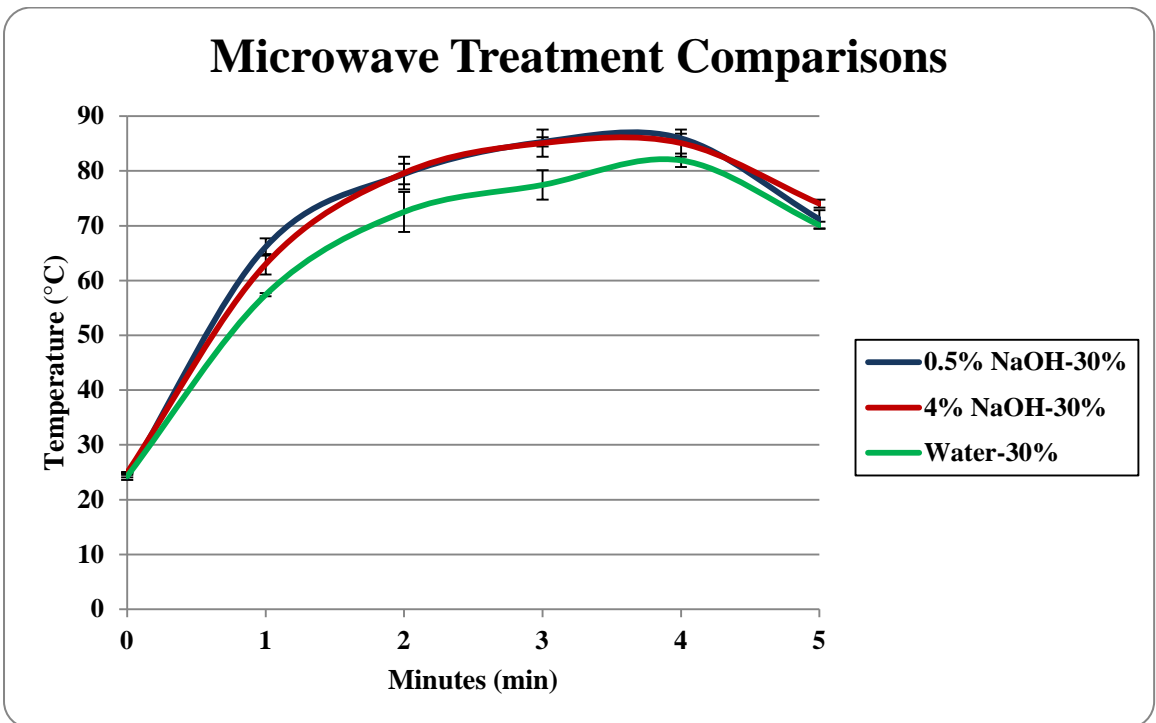


Figure 35: Temperature profiles for pure water versus NaOH solutions at 30 percent power.

D. Combined Microwave (MW) and Enzyme Treatments

1. Xylanase Enzyme

a. Weight Loss

From the initial evaluation of the different treatments to be used on SCF's, the combination of 1% (ows) Pulpzyme, 0.5 mmol EDTA, 0.1% (v/v) surfactant and 0.2% (v/v) vanillin produced significant weight loss and this treatment combination was used. This set of experiments consisted of the following:

- I.
 - a. Treatment of seed coat fragments with NaOH solutions at 0.0 (pure water), 0.5, 1.0, 2.0, and 4.0 percent NaOH for 2.5 min with microwave (2.5_MW) irradiation.
 - b. For each of the conditions in (Ia), the supernatant solution was saved, seed coat fragments were washed, dried and conditioned, and weight losses calculated.
 - c. The same procedure in (Ia) and (Ib) was followed on seed coat fragments, but with 5.0 min of microwave (5.0_MW) irradiation.
- II.
 - a. Next, the procedure in (Ia) was duplicated. The 2.5_MW irradiated seed coat fragments in different NaOH concentration solutions were washed and further subjected to enzymatic treatment consisting of 1% Pulpzyme, 0.5 mmol EDTA, 0.1% nonionic surfactant (Triton X100) and 0.2% vanillin for 90 min.
 - b. For each of the conditions in (IIa), the supernatant solution was saved, the seed coat fragments were washed, dried and conditioned, and weight losses calculated.
 - c. The same procedure in (IIa) and (IIb) was followed on seed coat fragments, but with 5.0 min of microwave (5.0_MW) irradiation.

Figure 36 and Figure 37 give the weight loss results for the treatments that used 2.5_MW and 5.0_MW treatments, respectively.

For the 2.5_MW treatments (Figure 36), an increase in weight loss was observed with an increase in NaOH concentration (2.5_MW-NaOH). MW-NaOH combined with enzyme treatment (MW-NaOH-Enz), created more weight loss than MW-NaOH treatment alone. When pure water was used as the medium in the microwave an average weight loss of 5.4% (MW-Water) was obtained. This weight loss increased to 10.13% when the MW-Water treated SCF's were further treated with Pulpzyme HC enzyme (MW-Water-Enz). For MW-NaOH treatments, the weight loss obtained for the SCF's increased from 14.6% to 43.4% with an increase in the NaOH concentration from 0.5% to 4%. When the MW- NaOH treated SCF's were further subjected to an enzyme treatment (MW-NaOH-Enz), the total weight loss increased from 24.4% (0.5% NaOH) to 50.44% (4% NaOH). Therefore, by combining the enzyme treatment with MW-NaOH an additional increase in weight loss of between 7.02% and 17.4% was observed. The maximum differential weight loss for MW-NaOH-Enz was obtained for 1% NaOH treated SCF's (17.4%) while the differential weight loss obtained for 4% NaOH treatment (7.02%) was comparatively less. One of the reasons for this decrease in differential weight loss at higher NaOH concentration could be that at higher concentrations of NaOH there was a corresponding increase in dissolution of non-degraded polysaccharides, peeling-off reactions, alkaline hydrolysis and decomposition of polysaccharides (Berggren 2003). Therefore, the starting substrate present for the enzymatic treatment was reduced, which in turn resulted in fewer changes occurring after enzymatic treatment.

For the 5 min treatment (Figure 37), results similar to the 2.5 min treatment were observed. For a pure water medium (MW-Water), the weight loss increased when the SCF's were treated enzymatically after 5 minutes of microwave. For microwave treatment using a NaOH medium and no enzyme treatment (MW-NaOH), the weight loss for the SCF's increased from 18.69%

(0.5% NaOH) to 51.10% (4% NaOH). For microwave treatment followed by an enzymatic treatment (MW-NaOH-Enz), the weight loss increased from 31.47% to 54.93% with respect to NaOH concentration. For the combined treatments the weight loss difference obtained between microwave treatment and enzyme treatment followed the same trend as the 2.5 min treatment. A maximum difference of 13.97% was obtained for 1% NaOH treated samples while a low difference of 3.83% was obtained for 4% NaOH treated SCF's. Compared to SCF's treated for 2.5 min the SCF's treated for 5 min exhibited higher weight losses.

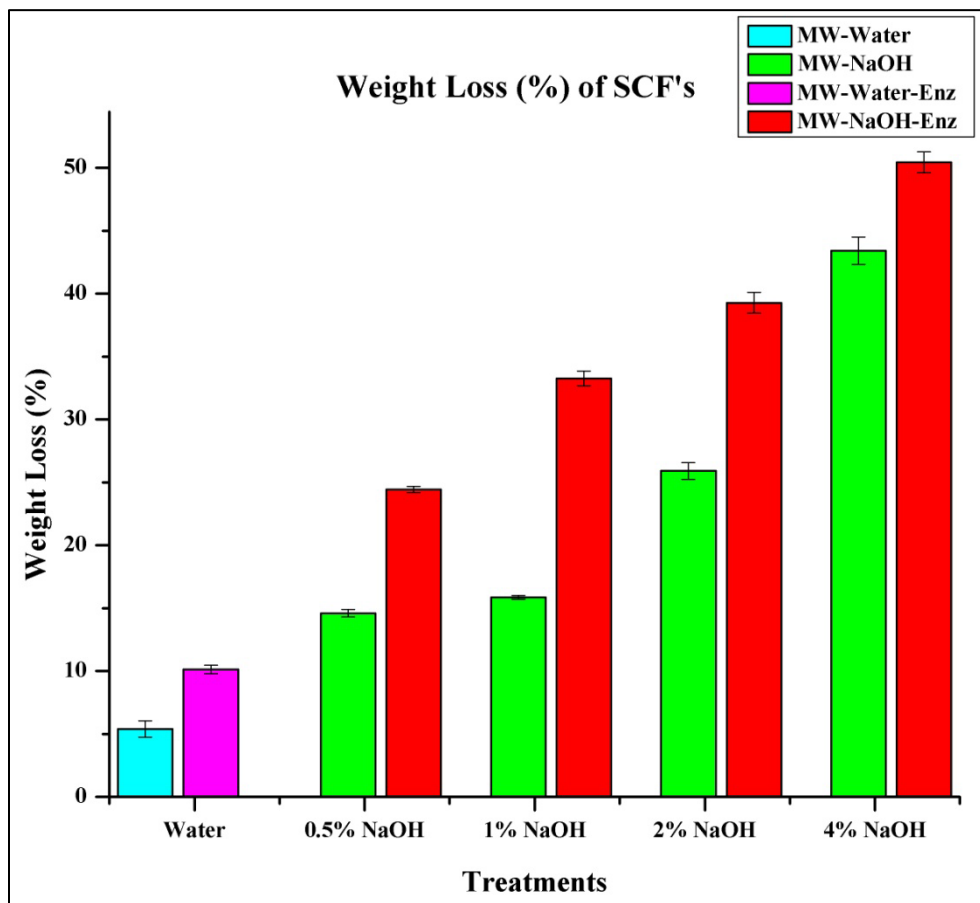


Figure 36: Weight loss obtained from two sets of treatments on SCF's. (i) 2.5 min microwave treatment with NaOH and water (ii) 2.5 microwave treatment followed by an enzymatic treatment for 90 min.

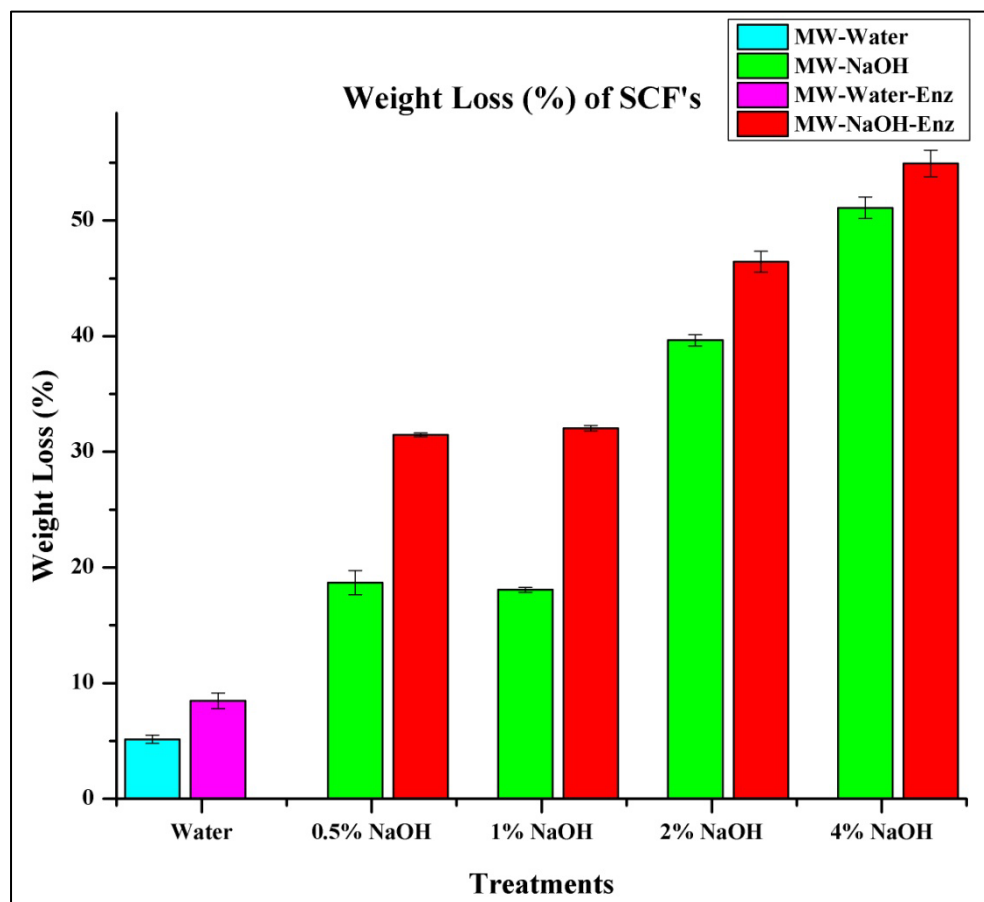


Figure 37: Weight loss obtained from two sets of treatments on SCF's (i) 5 min microwave treatment with NaOH and water (ii) 5 min microwave treatment followed by an enzymatic treatment for 90 min.

b. Analysis of sugars removed: gas chromatography and mass spectroscopy (GC-MS)

The monosaccharides present in the supernatant liquid from treatments were derivatized by silylation. The active hydrogens in -OH, -COOH, -NH, -NH₂, and -SH groups were replaced with trimethyl-silyl groups (Figure 38) to mask the polar groups and thus increase their volatility. Derivatization was required to convert non-volatile carbohydrates into volatile compounds that

were more thermally stable and easily detected by the gas chromatography (Campbell, Goheen et al. 2011).

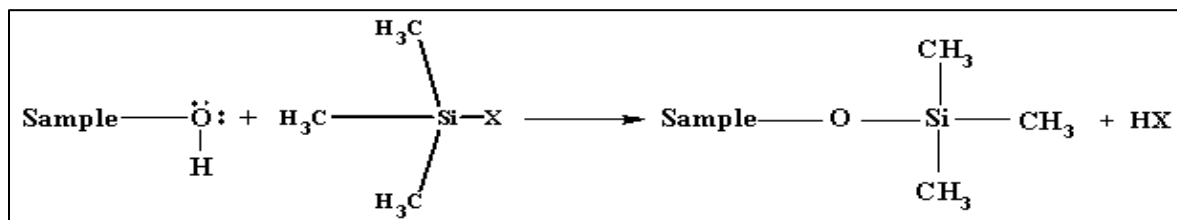


Figure 38: General reaction mechanism for the formation of trialkyl-silyl derivatives (Orata 2012)

Tri-Sil HTP Reagent composed of hexamethyldisilzane (HMDS), trimethylchlorosilane (TMCS) and highly pure pyridine was used to produce TMS derivatives of the monosaccharides. The potential peaks produced on the chromatogram by the derivatives formed using these reagents were easy to record. From the results obtained the peak areas for individual sugar residues present in the supernatant liquid at 2.5 min and 5 min microwave treated samples were calculated. Of the residues, only arabinose, xylose, glucose and galacturonic acid were of significant yield, while the other sugar residues were of negligible amount. Therefore, only these four sugar residues were considered in the analysis section. Xylose and arabinose are five carbon monosaccharides present as components of hemicelluloses. Glucose is a six carbon monosaccharide obtained from breakdown of cellulose, while galacturonic acid is a six carbon monosaccharide obtained from pectin.

Figure 39 represents the chromatogram obtained for sugar standards. These included arabinose (Ara), xylose (Xyl), glucose (Glu), galactose (Gal), galacturonic acid (Gal A), glucuronic acid (Glu A) and myo-inisitol, an internal standard (ISTD). This internal standard was used to determine the retention time for each monosaccharide. Figure 40 represents the relative intensities obtained from SCF's that were conventionally treated with 4% NaOH. The peak areas

for monosaccharides were obtained from the chromatogram by calculating the area of the peaks based on their retention time and were separately graphed to compare the amount of monosaccharides released by each treatment. The greater peak area of monosaccharides correlates to more action on the substrate by the treatment.

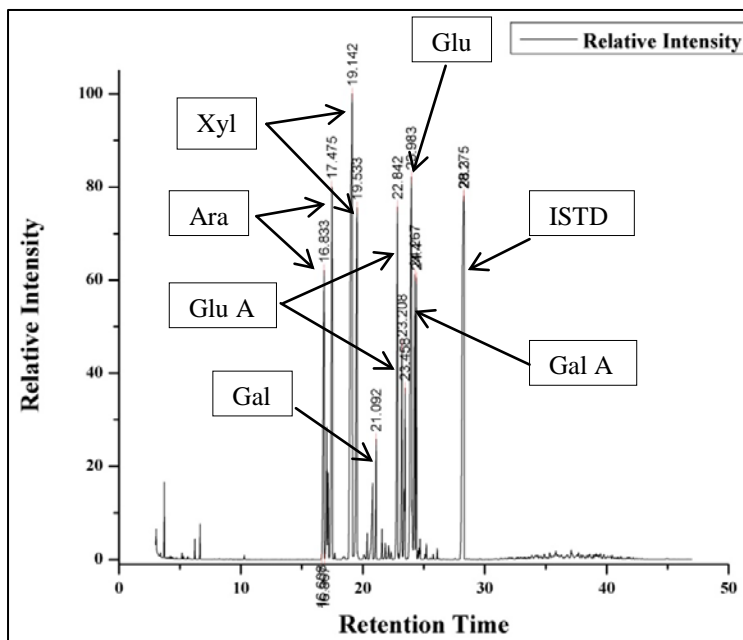


Figure 39: Gas chromatogram for sugar standards. Standards included arabinose, xylose, glucose, galacturonic acid, glucuronic acid, and internal standard.

The peak area of sugar residues obtained from the 2.5 min microwave treatment with NaOH (2.5_MW-NaOH), the enzymatic treatment following microwave and conventional treatments (0.5% NaOH and 4% NaOH) are given in Figure 41 to Figure 44. The amount of arabinose residue produced by combining the MW-NaOH and enzyme treatments (MW-NaOH-Enz) equals to the sum of residues obtained for the MW-NaOH treatment and enzyme treatment following microwave. From Figure 43, an increasing trend is observed for the amount of

arabinose released in the supernatant liquid with respect to NaOH concentration used in the microwave treatment. For pure water, a very low level of arabinose residue was obtained. Both at 0.5% and 4% (v/v) NaOH concentrations, microwave combined enzyme treatments (MW-NaOH-Enz) produced more arabinose residue when compared to conventional treatments at 0.5% and 4% NaOH concentration respectively.

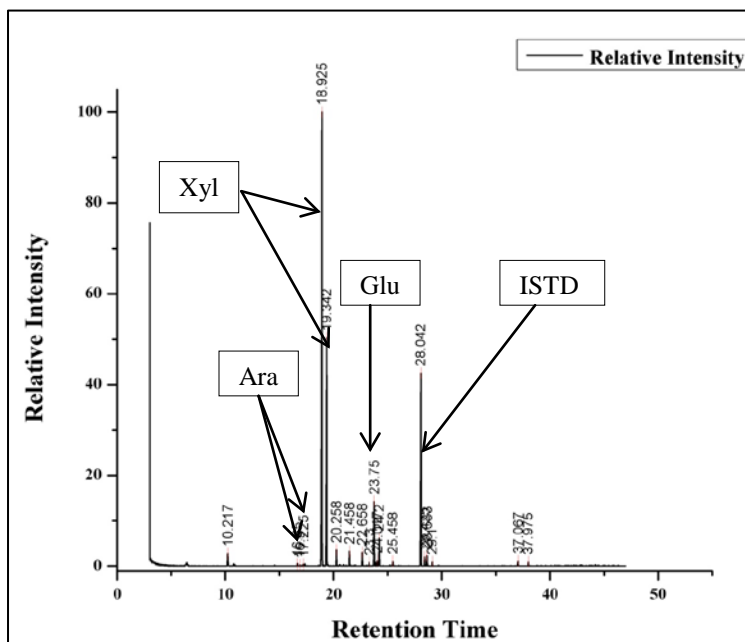


Figure 40: Gas chromatogram obtained for a 4% NaOH conventionally treated SCF's.

Figure 42 shows the xylose residue produced by various treatments. When water alone was used as the medium a very low xylose residue was released. The amount of xylose produced increased with an increase in NaOH concentration used in the microwave treatment. The microwave combined enzyme treatment (MW-NaOH-Enz) produced less xylose compared to corresponding conventional treatments.

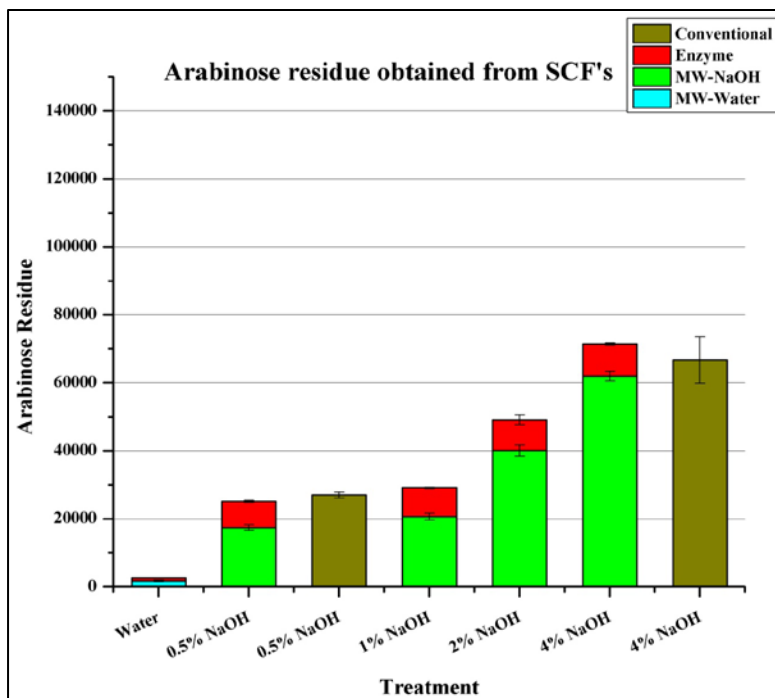


Figure 41: Arabinose residue obtained from SCF's after different treatments at 2.5 microwave treatment time.

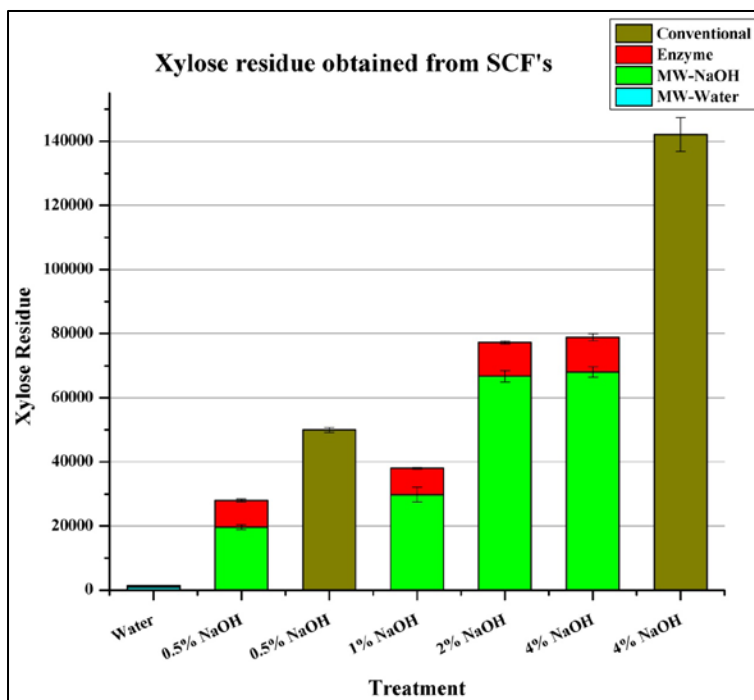


Figure 42: Xylose residue obtained from SCF's after different treatments at 2.5 microwave treatment time.

Figure 43 gives the amount of glucose residue produced by the various treatments applied to SCF's. Microwave treatment (MW-NaOH) and microwave combined enzyme treatment (MW-NaOH-Enz) generated a very low amount of glucose residue compared to conventional treatment. This indicates that the effect of the combined treatment on cellulose is minimal compared to conventional treatment.

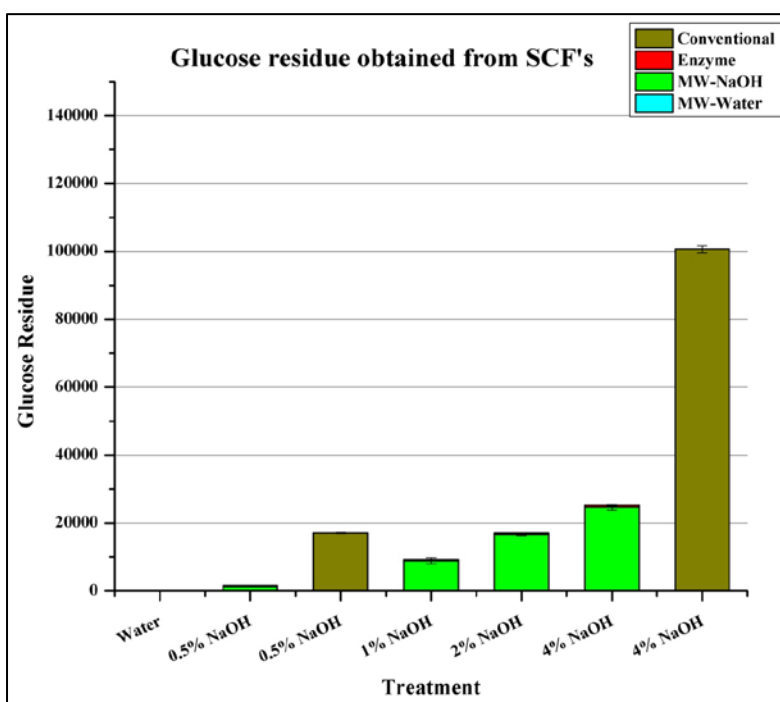


Figure 43: Glucose residue obtained from SCF's after different treatments at 2.5 microwave treatment time.

Figure 44 shows the amount of galacturonic acid released after various treatments applied to SCF's. The amount of residue generated is significantly less for all the treatments, with no residue produced for the microwave combined enzyme treatment using NaOH (MW-NaOH-Enz) and water (MW-Water-Enz).

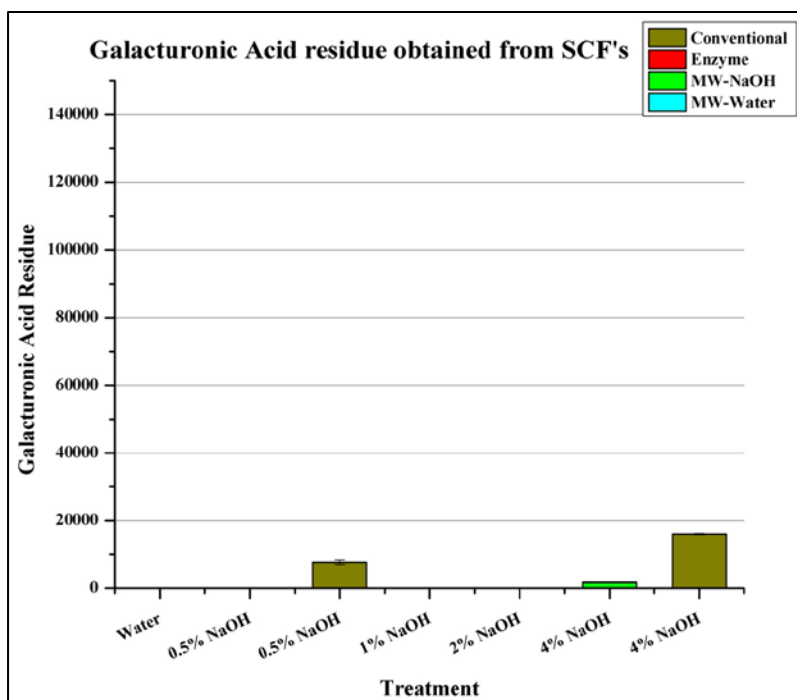


Figure 44: Galacturonic acid residue obtained from SCF's after different treatments at 2.5 microwave treatment time.

From the residues obtained after 2.5 min microwave treatment time, we can conclude that the arabinose and xylose are the sugars that are mainly removed from SCF's. In the case of conventional treatment, glucose is also removed. Since Pulpzyme is a xylanase enzyme the treatment helps to extract only hemicellulose components. This is an important factor to consider as conventional treatment affects all the components present in a substrate resulting in loss of strength as seen in case of excess removal of glucose as a result of hydrolysis of cellulose.

Figure 45 represents arabinose residue obtained by treatment of the supernatant liquid using 5 min microwave treatment. Similar to 2.5 min microwave treatment, an increasing trend in arabinose residue was observed with respect to NaOH concentration. Also the amount of sugar residue obtained for the microwave combined enzyme treatment was comparatively more than

that obtained for conventional treatment. The amount of residue removed for 5 min microwave treatment was comparatively more than that for the 2.5 min treatment time.

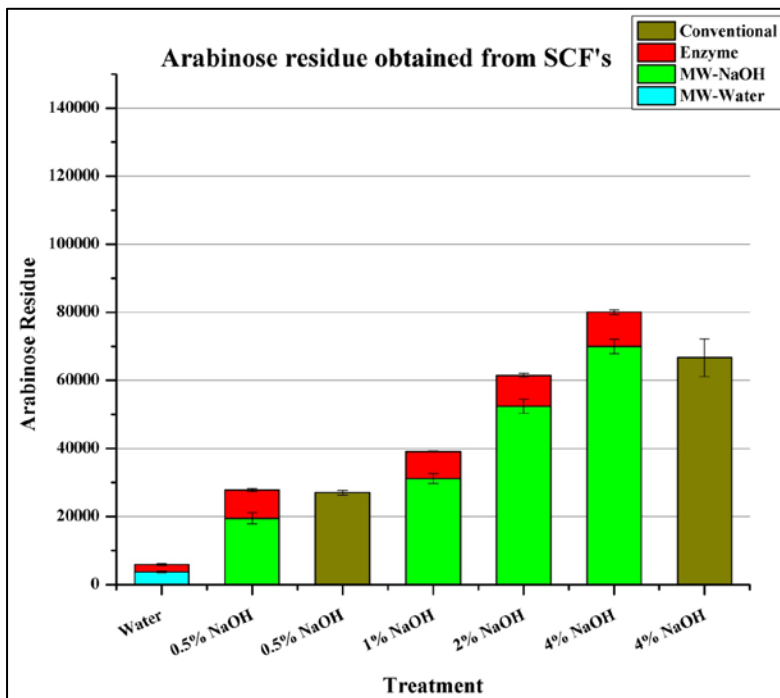


Figure 45: Arabinose residue obtained from SCF's after different treatments at 5 microwave treatment time.

Figure 46 represents the xylose residue obtained from SCF's treated with 5 min microwave treatment. Again an increasing trend in the amount of residue produced with NaOH concentration was observed in this case. Similar to the 2.5 min treatment, the microwave combined enzyme treatment produced less residue when compared to the conventional treatment. At the same concentrations, the residue produced at 5 min microwave treatment time was more than that produced at 2.5 min.

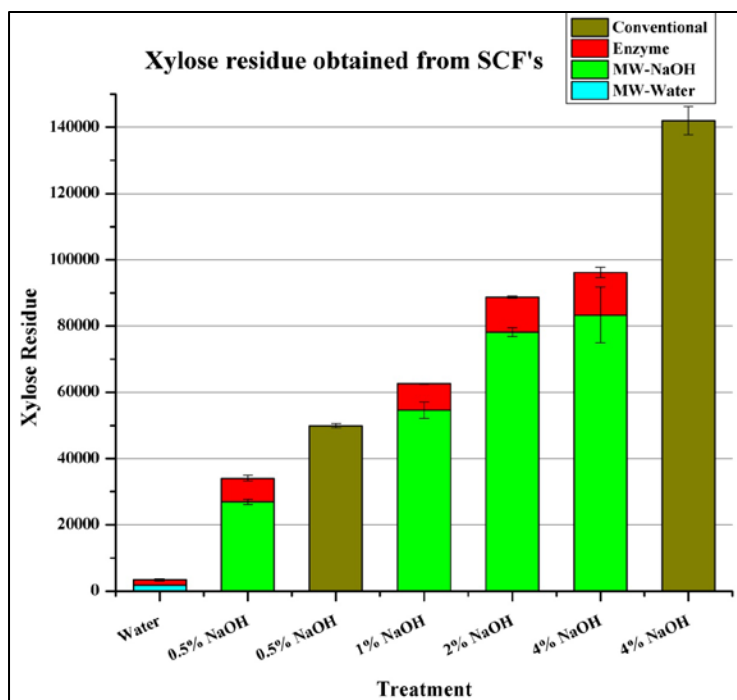


Figure 46: Xylose residues from SCF's at 5 minutes microwave treatment

Figure 47 represents the amount of glucose produced when the SCF's were treated at 5 minutes microwave treatment time. The amount of glucose generated increased with an increase in NaOH concentration. The amount of glucose generated by conventional treatment was still higher than that produced by the microwave and microwave combined enzyme treatment. When compared to 2.5 min microwave treatment, the 5 min microwave treatment generated more glucose. Figure 48 represents the amount of galacturonic acid produced by various treatments at 5 min microwave treatment. Compared to other sugars produced under similar conditions, a low amount of galacturonic acid residue was generated.

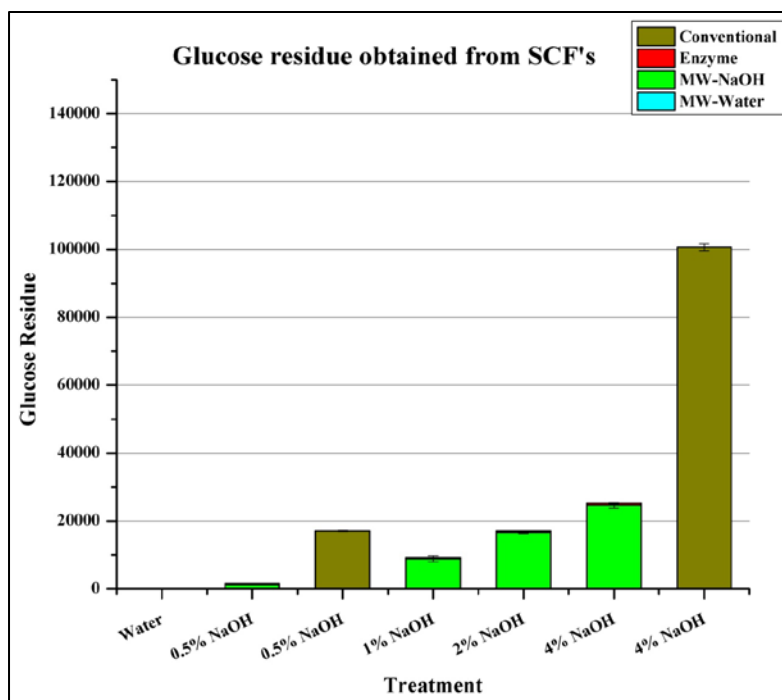


Figure 47: Glucose residue from SCF's after treatments at 5 minutes with microwave.

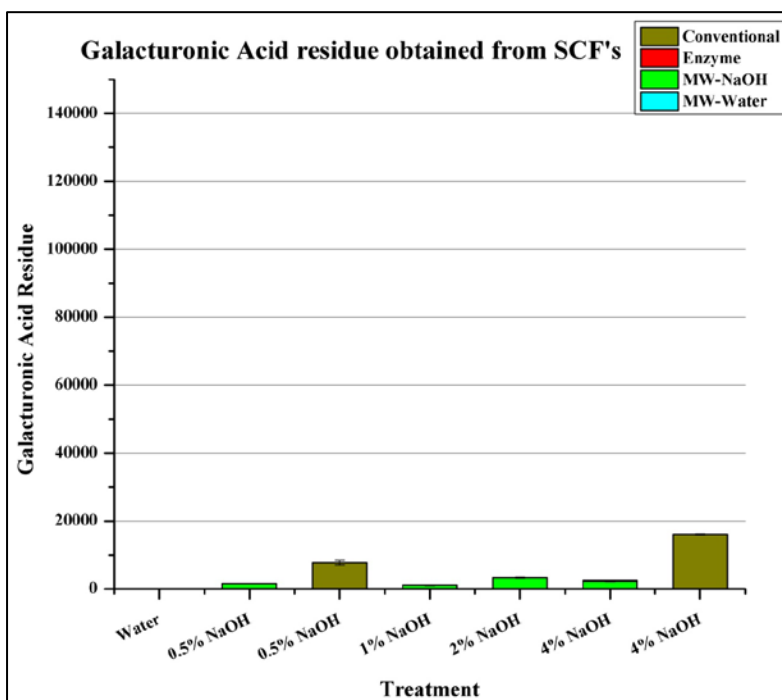


Figure 48: Galacturonic acid residue from SCF's after 5 minutes microwave treatment.

Arabinose and xylose were the main sugars produced when SCF's were treated for 5 minutes. Greater amounts of sugars were generated at 5 minutes treatment compared to 2.5 minutes treatment. Microwave treatment also resulted in greater amounts of glucose with higher NaOH concentrations. *Therefore a higher concentration of NaOH at 5 min treatment time is redundant as it only results in more hydrolysis of cellulose.*

c. FTIR Spectroscopy

Fourier transform infrared spectroscopy (FTIR) was used to investigate chemical changes in the seed coat fragments after microwave treatment and combined microwave-enzyme treatment. All major bands were selected as seen in Figure 49 and the components to which these peaks were tentatively assigned are as given in Table 9.

Table 9: FTIR band wavelength assignments to known components in SCF's (Faix 1991; Fengel and Wegener 2003; Pandey and Pitman 2003; Muller 2008; Rana 2008; Pucetaite 2012)

Wave No. (cm ⁻¹)	Assignment
1740, 1732	C=O stretch in xylans (hemicelluloses)
1649, 1643	Absorbed O-H (water) and conjugated C-O
1596, 1505	C=C stretch of aromatic ring in lignin
1465, 1460	C-H deformation in lignin and carbohydrates
1424	C-H deformation in lignin and carbohydrates
1372	C-H deformation in cellulose and hemicelluloses
1328	S ring plus G ring condensed
1235, 1230	Syringyl ring and C= stretch in lignin and xylans
1167, 1157	C-O-C vibration in cellulose and hemicelluloses
1122	Aromatic skeletal vibration and C-O stretch

1053, 1032	C-O vibration in cellulose and hemicelluloses
897	C-H deformation in cellulose

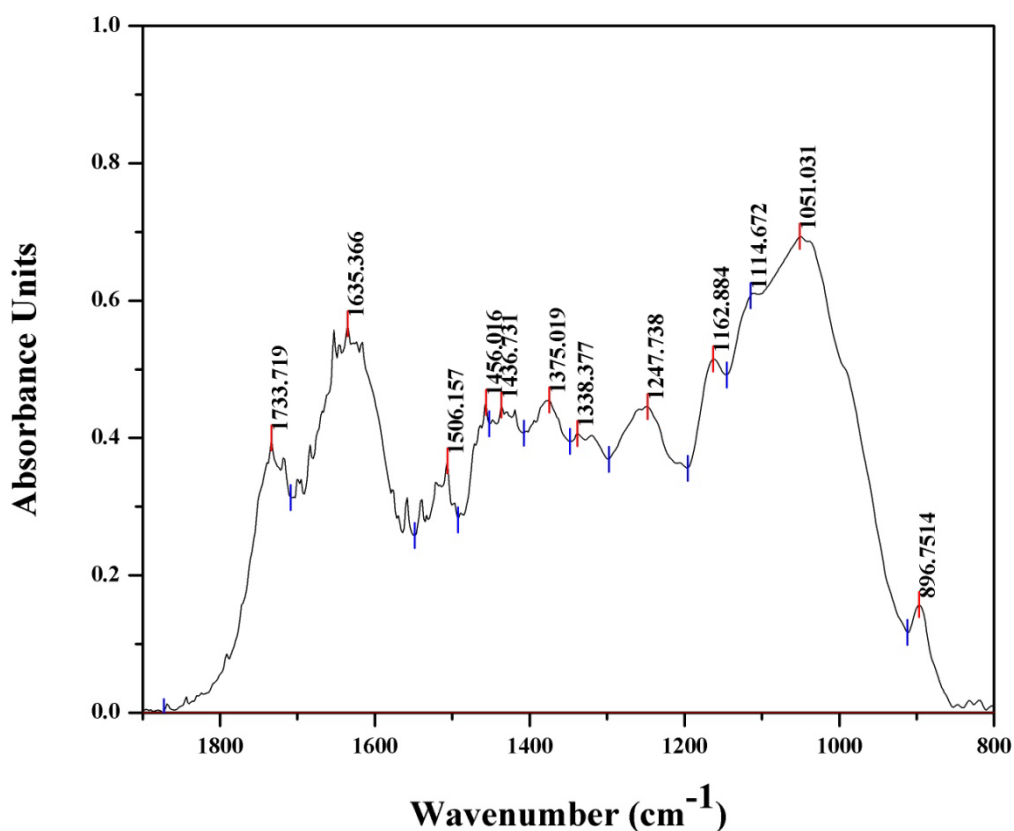


Figure 49: Mean normalized FTIR spectrum of untreated seed coat fragments in the fingerprint region (1900-800 cm^{-1}).

The FTIR data collected was initially preprocessed by averaging and normalizing the spectra to compensate for any variations that might occur due to small differences in sample size used in collecting the spectra. First and second derivatives of the each spectrum were obtained using Origin software. Figure 50 shows first and second derivatives obtained for untreated SCF's using Savitzky-Golay Smoothing of 2nd order polynomial of 15 and 20 points respectively.

To investigate the FTIR spectra's in more detail principal component analysis was performed using second order derivatives of vector normalized spectra and the factor loadings were calculated using Origin software. Figure 50 shows the PCA constructed for the average spectral data obtained for the SCF's. Figure 51 gives the factorial loading obtained for PC2 component of the 2nd derivative data set.

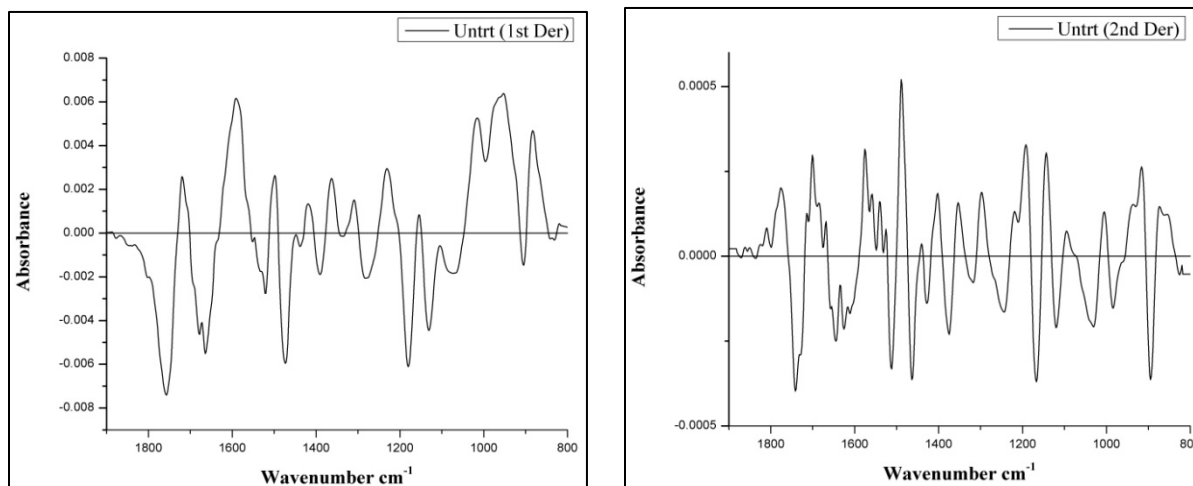


Figure 50 : First and second derivatives obtained for untreated seed coat fragments using FTIR

The PCs formed clearly separates the spectral data into 2 groups along PC2 in the above figure. To investigate the source of variation in this analysis, factor loadings were also analyzed. PC1 component explained 80.23% of the variation while 2nd and 3rd factors explained 11.19% and 5.53% of the total variation. As seen in Figure 52, all the samples were positive along the first factor loading (PC1) while PC2 was able to group them into 2 groups of positive and negative factor loadings along PC2. The band assignments of the factor loadings are given in Table 9 and indicate presence of important compounds as listed in the table. PC4 when considered with PC2 also helps in segregating the data into groups as seen in Figure 53.

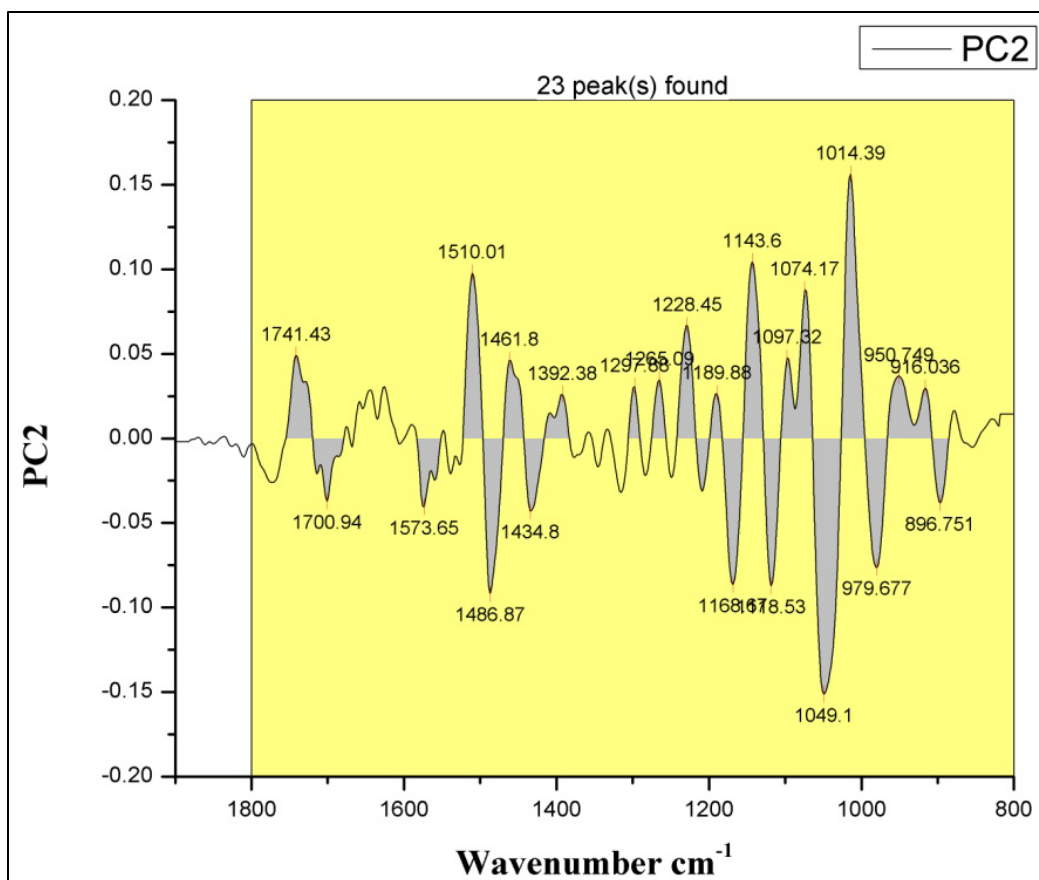


Figure 51: Factor loadings of the second factor obtained by PCA. The ten most pronounced valleys and hills representing the ten main peaks are given in Table 10 below.

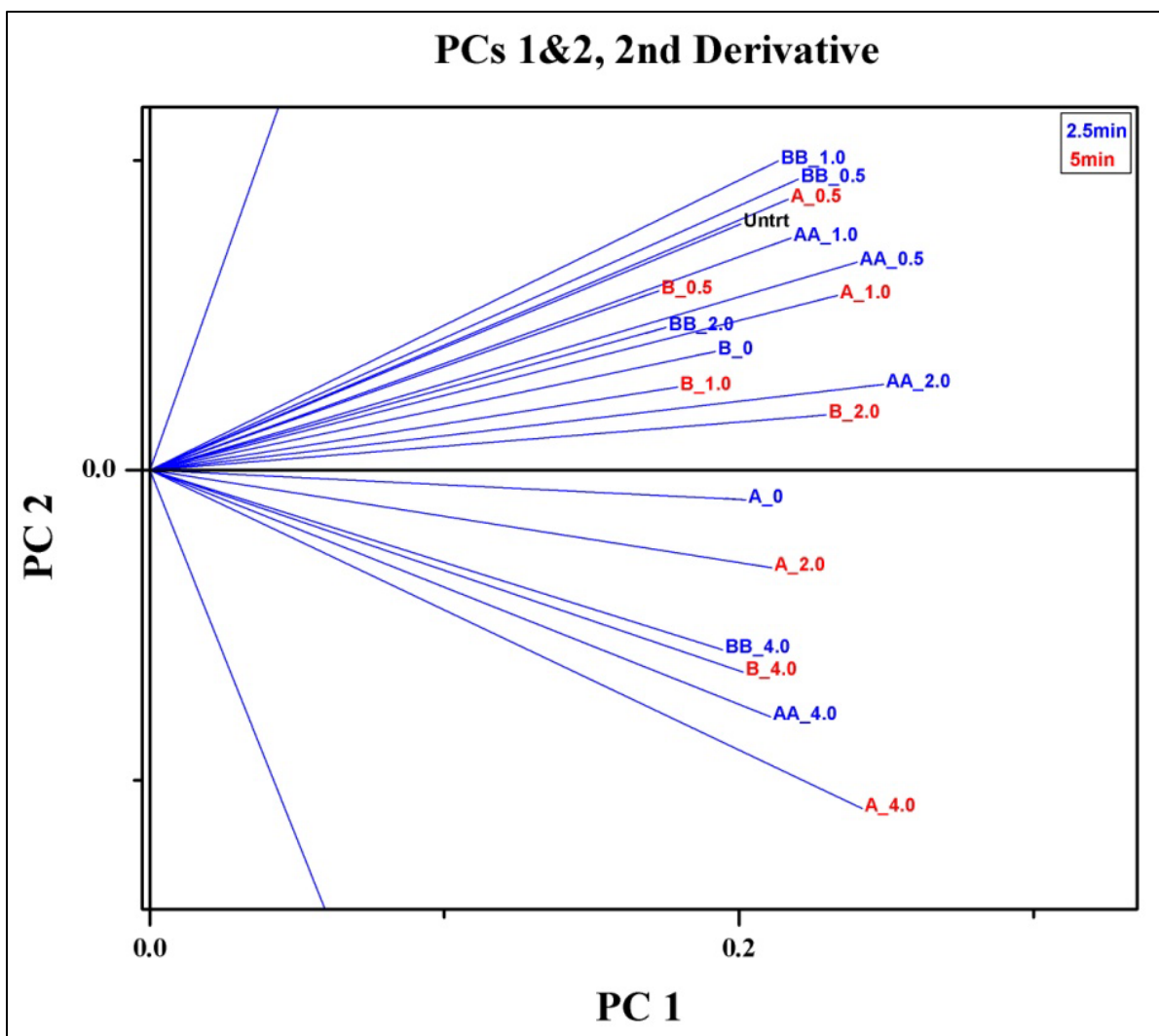


Figure 52: Principal components for 2nd order derivatives of spectral data. Blue data points indicate the 2.5 min microwave treatment time while the red indicates 5 min microwave treatment time. 'A' series represent microwave combined with enzymatic treatments while 'B' represents microwave treatment alone.

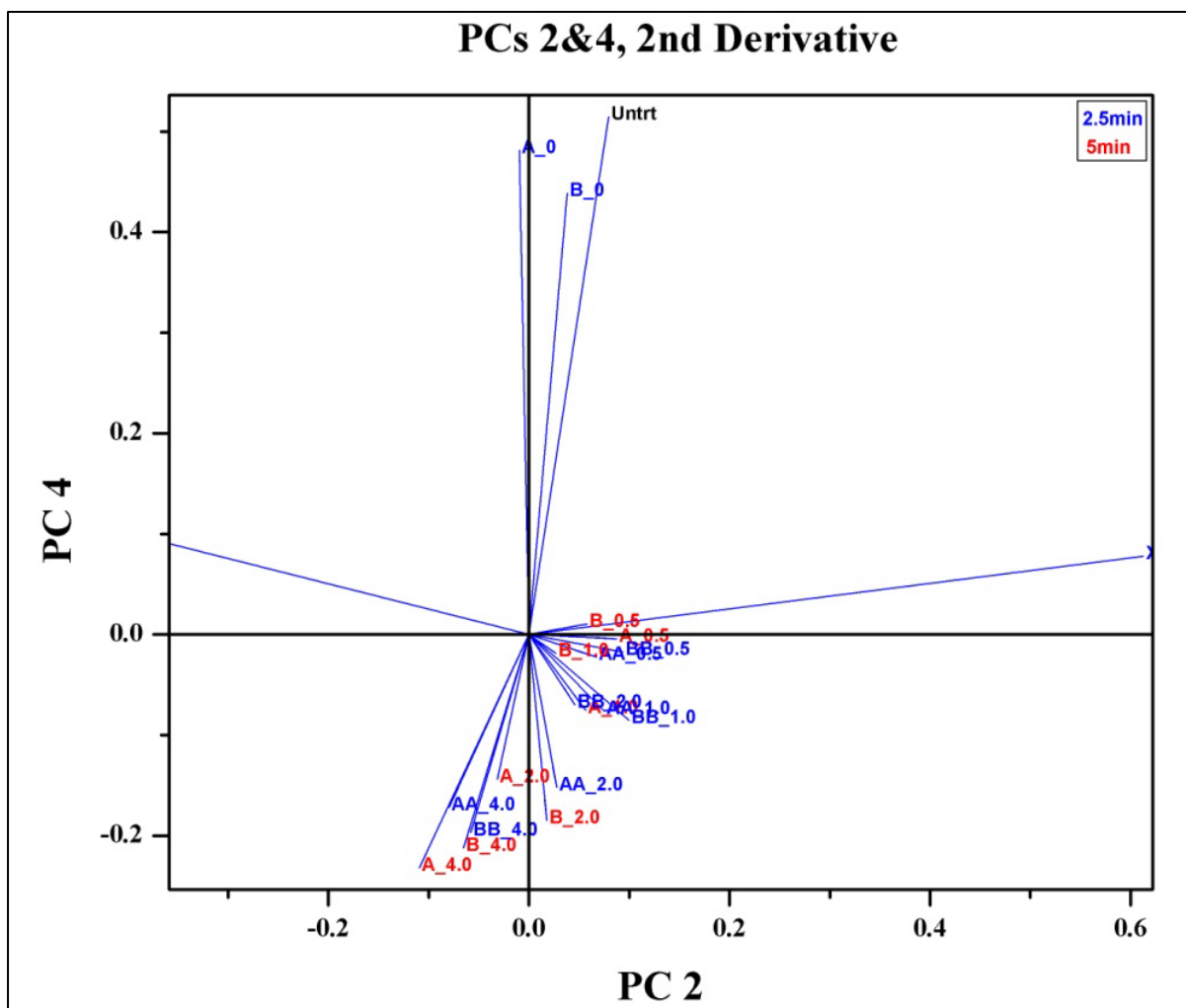


Figure 53: Principal components for 4th order derivatives of spectral data. Blue data points indicate the 2.5 min microwave treatment time while the red indicates 5 min microwave treatment time. 'A' series represent microwave combined with enzymatic treatments while 'B' represents microwave treatment alone.

From the PC graphs we can clearly demarcate the spectral data obtained from higher NaOH concentration as they are located together in the same quadrant away from untreated sample. The only consideration is the 2% NaOH microwave treated combined with enzyme treatment that is present in the 4% NaOH samples quadrant. This indicates that the 2% NaOH treatment produced results comparable to the higher concentrations when microwave treatment was combined with the enzymatic treatment. Table 10 gives the list of the main spectral peaks

obtained from the factor loadings that were predominantly responsible for separation of the groups as seen in the PC plot in the previous figures. Most of the band assignment for the PC's for the spectra's were in the hemicellulose and cellulose components.

Table 10: Band assignments of significant PC2 and PC4 components of the second order derivative samples that help in segregation of the samples into groups.

Wave Number		Band Assignment
PC2_2 nd Der	PC4_2 nd Der	
1014		C-O stretch in cellulose and hemicellulose
1049		C-O vibrations in cellulose and hemicellulose
1144		Cellulose C-O-C
1510		C=C stretch of aromatic ring in lignin
1074		C-O stretch in cellulose
1487		C-C stretch, CH and OH wag in cellulose and hemicellulose, CH deformation in lignin
1119		Aromatic skeletal vibrations and C-O stretch
1169		C-O-C vibrations in cellulose
1229		Syringyl ring and C= stretch in hemicellulose
978		C-O stretch in a ring in cellulose and hemicellulose
	1512	C=C stretch of aromatic ring in lignin
	1489	C=C stretch of aromatic ring in lignin
	1572	Aromatic skeletal vibrations and C-O stretch
	1163	C-O-C vibration in cellulose and hemicellulose
	1462	C-H deformation in lignin and carbohydrates
	1597	C=C stretch of aromatic ring in lignin
	1188	C-O-C vibration in cellulose and hemicellulose
	1043	C-O stretch in cellulose and hemicellulose
	1144	Cellulose C-O-C
	1124	C-H deformation in lignin

Hierarchical Cluster Analysis (HCA) was used in assessing the similarities or dissimilarities between samples based on the intrinsic interrelationship between structure and property of samples. HCA is useful in organizing the observed data into groups by placing

similar subjects in the same group thus maximizing similarities within a cluster. This in turn maximized the dissimilarities between groups and the subjects in different groups are less compatible with each other. A hierarchical tree diagram called dendrogram was constructed with the clusters linked at increasing levels of dissimilarity. Therefore, at the beginning, the samples present in a cluster had similar characteristic features but as the clusters amalgamated larger clusters of increasing dissimilar compounds were formed. The spectral data of all samples were subjected to cluster analysis where different clustering methods such as nearest neighbor, furthest neighbor, centroid, median and Ward's were tested on the data. The best result was obtained using Ward's method for calculating the distance of the matrix based on Euclidean distance.

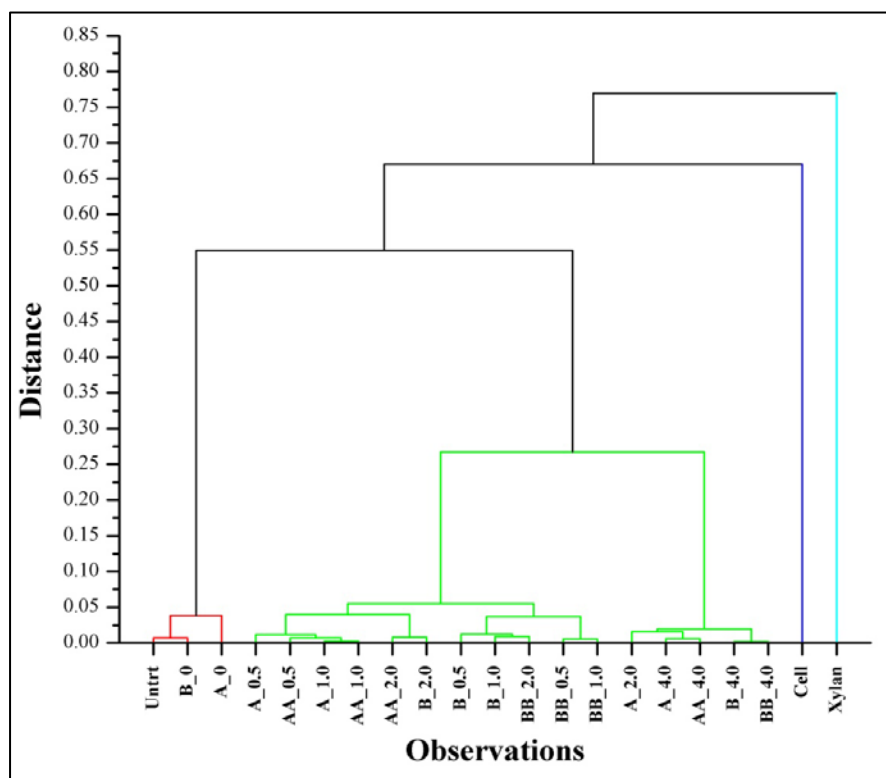


Figure 54: Cluster analysis by Ward's of the FTIR spectra of seed coat fragments. FTIR spectra used in this analysis were pre-processed with normalization and second order derivation.

From the dendrogram in Figure 54, untreated SCF's were present in a cluster away from treated sample clusters thus indicating that treatment introduced changes in SCF's. The second cluster next to the untreated SCF's cluster contained the SCF's processed at low NaOH concentrations followed by enzymatic treatment. The next set of clusters contained SCF's processed using only microwave at low NaOH concentration. The 2 % NaOH microwave combined with the enzyme treated samples seemed to exhibit characteristic features similar to 4% NaOH microwave treated and microwave combined with enzyme treated samples at 2.5 min and 5 min.

To further analyze the changes occurring due to the treatments done on SCF's in the components (hemicellulose, cellulose and lignin) of the samples their ratios were constructed for each treatment. From Table 10 on band wavelength assignment, 1732 cm^{-1} peak corresponds to hemicellulose, 1505 cm^{-1} peak corresponds to lignin and peak at 897 cm^{-1} corresponds to cellulose. Therefore, ratios of peaks heights at 1732 cm^{-1} to 897 cm^{-1} (hemicellulose/cellulose), 1505 cm^{-1} to 897 cm^{-1} (lignin/cellulose) and 1732 cm^{-1} to 1505 cm^{-1} (hemicellulose/lignin) were constructed. Figure 55, represents the ratio obtained for hemicellulose to cellulose contents in the SCF's. The untreated SCF, pure water medium microwave sample (B_0) and pure water microwave combined with enzyme sample (A_0) represented having high levels of hemicellulose while the remaining treated samples both microwave only (B's) and microwave combined with enzyme (A's) exhibited reduction in hemicellulose content due to degradation. Two percent NaOH microwave treatment combined with enzyme treatment (A_2.0) actually created lesser amount of hemicellulose after being subjected to the treatment. Pure cellulose (Cell) and xylan (Xylan) used as reference samples were not comparable with the SCF samples as observed in the PCA analysis in the earlier section.

The hemicellulose to lignin ratio obtained by dividing the peak heights at 1732 cm^{-1} to 1505 cm^{-1} is given in Figure 56. Trends similar to the hemicellulose to cellulose in previous section are continued in this ratio. Pure water medium samples exhibited high levels of hemicellulose in them (A_0, B_0) whereas treated samples had a reduced level of hemicellulose to lignin ratio.

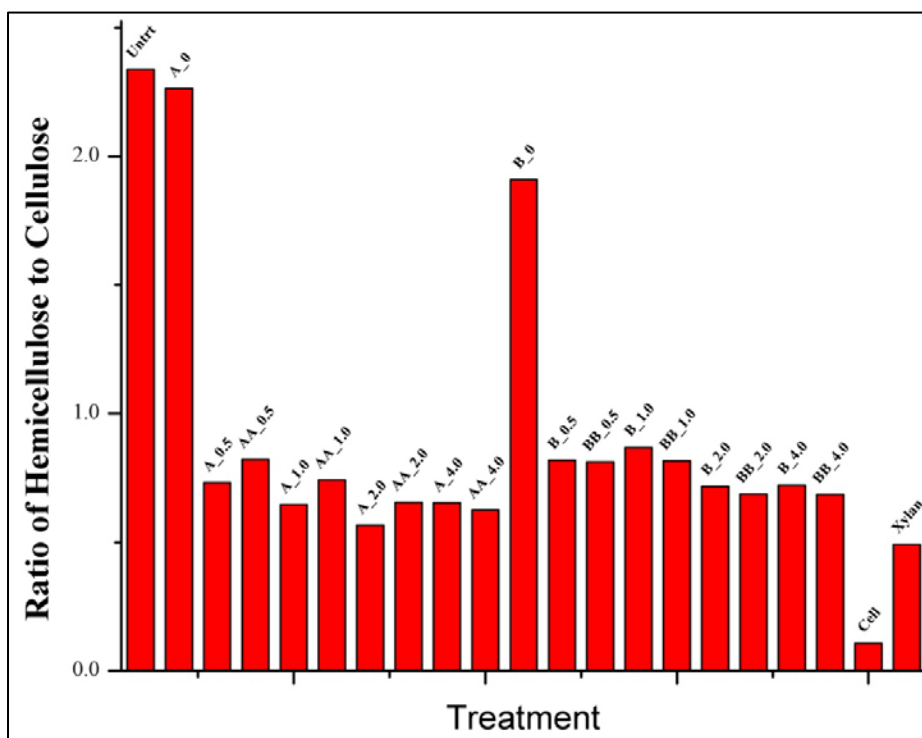


Figure 55: Ratio of peak heights at 1732 cm^{-1} to 897 cm^{-1} representing the hemicellulose to cellulose ratio in the SCF's

In Figure 57 lignin to cellulose ratio for the SCF's is displayed. Lignin was less susceptible to degradation from the treatments as less amount of lignin was affected by the treatments done on SCF's. At higher NaOH concentrations, the increase in peak height ratio for the lignin to cellulose ratio could be because the treatment removed more of cellulose from the

samples as observed from the GC-MS results obtained for glucose residue at 4% NaOH treatment.

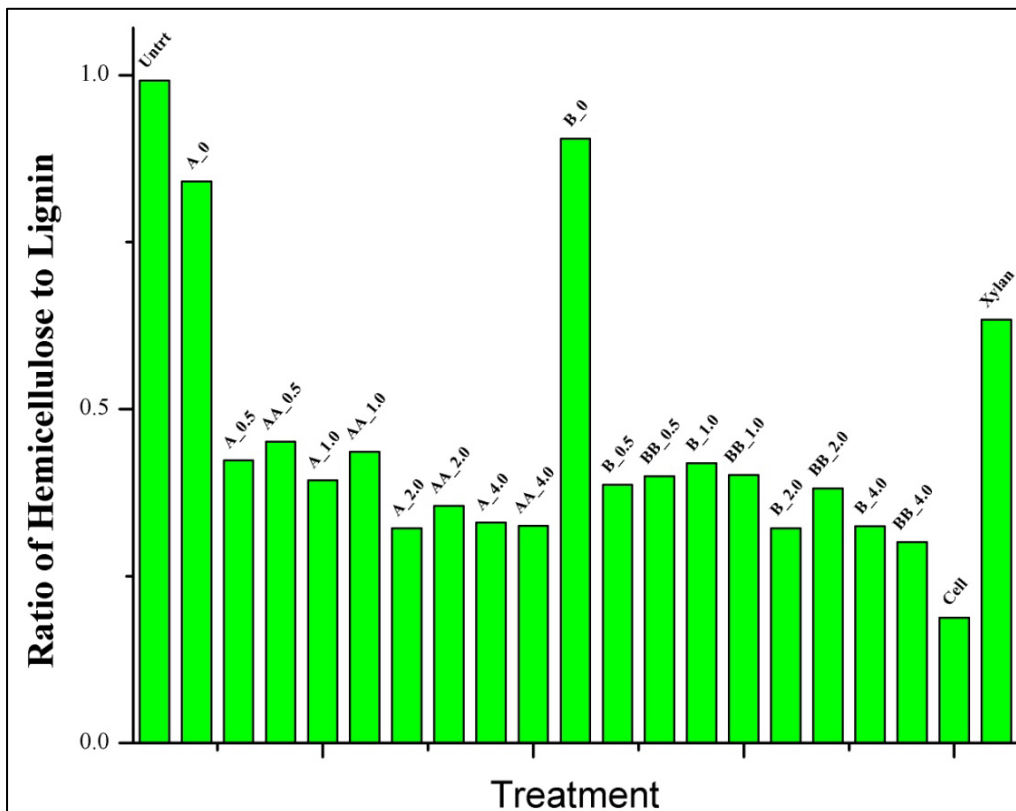


Figure 56: Ratio of peak heights at 1732 cm^{-1} to 1505 cm^{-1} representing the hemicellulose to lignin ratio in the SCF's

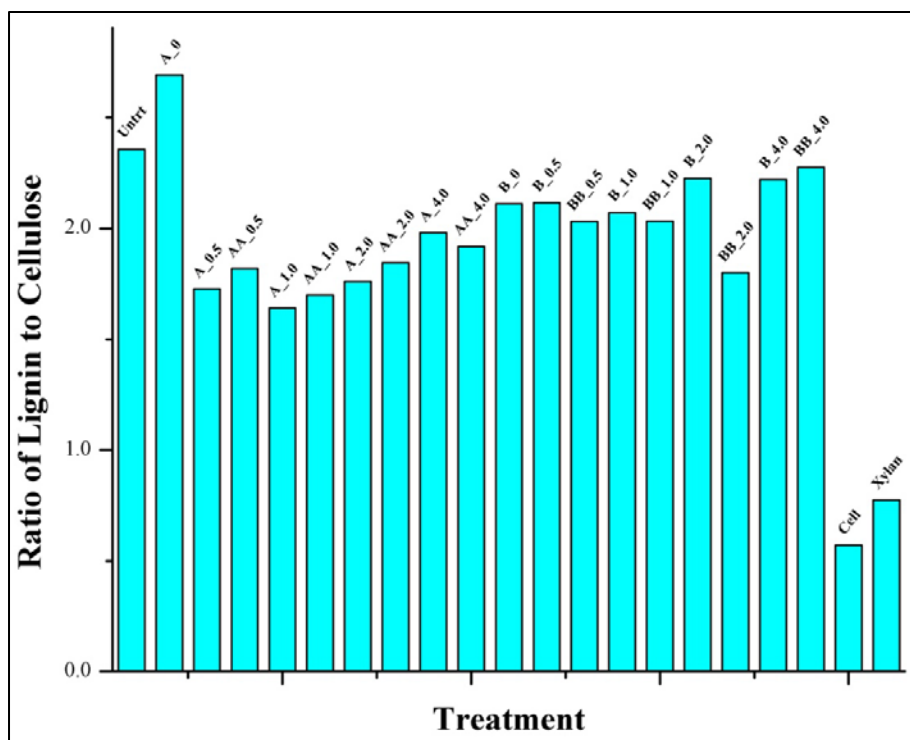


Figure 57: Ratio of peak heights at 1505 cm^{-1} to 897 cm^{-1} representing the lignin to cellulose in the SCF's

Based on the results obtained from weight loss, GC-MS and FTIR for the SCF's we can draw the following conclusions.

- SCF's that were processed using a pure water medium in the microwave (MW-Water) and microwave combined with xylanase enzyme (MW-Water-Enz) were not effective. Their results were close to that of untreated SCFs. There were low weight losses and very low amount of sugar residues. In FTIR, these two treatments (A_0 and B_0) again exhibited properties similar to untreated SCF's and therefore were linked to the untreated sample in the HCA dendrogram. When the changes in the composition of samples were determined by calculating the ratios of hemicellulose, cellulose and lignin, these two samples again displayed ratios similar to untreated sample. Therefore, we can conclude that pure that water as a medium for microwave is not effective.

- Microwave pretreatment before enzymatic treatment had a positive impact on the enzyme action on SCF's. Higher weight losses for SCF's occurred for those microwave treated by NaOH compared to SCF's microwave-treated in pure water. NaOH concentration also had a major impact. A increase in weight loss was observed with an increase in NaOH concentration. Combining NaOH and microwave treatment contributed in considerable increase in the weight loss obtained for the SCF's. Increase in microwave treatment time from 2.5 min to 5 min also had a positive impact on weight loss as it resulted in an increase in weight loss obtained for the SCF's. When compared to 2% NaOH conventional treatment of SCF's the microwave combined with enzyme treatment produced higher weight losses for 2% NaOH at 2.5 min and 5 min treatment times. In GC-MS analysis of the sugar residues released by the treatments the microwave combined with enzyme treatment (MW-NaOH-Enz) released greater amounts of arabinose and xylose similar to the amount released by the NaOH conventional treatments and lower amounts of glucose than the conventional treatment. Similar to weight loss, the increase in treatment time from 2.5 min to 5 min increased the sugar yield in the supernatant solutions. In PCA results of the FTIR data, the spectra were divided into two groups with untrt, A_0.5, AA_0.5, AA_1.0, etc low NaOH concentration treatments having positive scores whereas the remaining treatments such as A_0, A_2.0, A_4.0, AA_4.0, B_4.0, BB_4.0 having negative scores for PC2. In Figure 55, PC2 again resulted in similar separation of the spectral data while PC4 separated the data between untreated and treated samples. Using the loading factors the individual peaks causing this variation were found and is as given in Table_. From the factorial loadings it is evident that compounds representing carbohydrates and lignin played a

major role in PC2 and PC4 separation of the high concentration treatment group from the remaining treatments. Further, when ratios were compared the reduction in hemicellulose was found to be more prominent than those for lignin and cellulose. The cluster analysis separated the treatments into different clusters where A_2.0 (2% NaOH microwave combined with enzyme treatment) was linked to the higher concentration group away from untreated SCF.

- Cellulose (cell) and hemicellulose (xylan) samples used as references were completely different and had no similarities with the SCF samples as seen from the PCA and HCA results.

2. *Laccase Enzyme*

a. **Weight Loss**

The initial treatments of SCF's using a laccase-mediator system did not provide effective results in terms of weight loss. Therefore, the laccase enzyme treatment was combined with NaOH microwave treatment to enhance the degradation of lignin. Since laccase enzyme attacks lignin it was initially used for treating SCF's and was followed up with enzymatic treatment to facilitate breakdown of lignin. The laccase mediator treatment including treating SCF's with 40U/g of laccase enzyme in presence of 5% ABTS mediator, 0.05% surfactant, for 0.5 hr, 1 hr, and 2 hr in a buffer set at a pH of 6.5. At the end of the treatment the SCF's were separated from the supernatant solution, washed and transferred to 0.5% and 1% NaOH solution to be irradiated using microwave for 5 min. Also a cocktail of mediators was used to enhance the laccase enzyme action in lignin biodegradation and study the impact of the mediator on enzyme action. A 1:1 molar ratio of ABTS and vanillin were combined to be used as a mediator in the reaction. Table 11 gives the list of treatments initially done to seed coat fragments using laccase mediator

system. Table 12 gives the average weight loss recorded for the SCF's treated with laccase mediator system following the experiment listed in the previous table. The weight loss obtained for the SCF's increased considerably after combining the enzymatic treatment with microwave treatment.

Table 11: Experiments involving laccase mediator system used on SCF's

No.	Laccase (U/g)	Mediator	Time (hr)	Surfactant	NaOH (5 min)
1.	40	5% ABTS	0.5, 1, 2	Yes	0.5%
2.	40	5% ABTS	0.5, 1, 2	Yes	1%
3.	40	5% ABTS (0.97mM), 1.5% vanillin (0.97 mM)	0.5, 1, 2	Yes	1%
4.	40	5.14% ABTS (1mM), 1.72% vanillin (1mM)	0.5, 1, 2	Yes	1%

Table 12: Average weight loss obtained for SCF's treated with LMS.

No.	Treatment Time		
	0.5 hr	1 hr	2 hr
1	20.12	20.30	20.30
2	24.61	25.26	24.40
3	22.66	23.11	23.06
4	21.96	23.61	24.04

b. Analysis of Lignin Removed

i. Klason Treatment

The National Renewable Energy Laboratory (NREL) procedure was followed to determine the lignin content in SCF samples. Seventy two percent concentrated sulfuric acid (H_2SO_4) was used to degrade the samples and the percentages of acid insoluble lignin (AiSL) and acid soluble lignin (ASL) were determined. An important factor that was all the steps involved in the experiment had to be done within the time limit. If any part of the experiment extended beyond the time limit then the final results obtained would be completely different. This was exemplified by problems with the vacuum suction to be used on the samples. After autoclave at 121 °C for one hour the samples had to be cooled and filtered to separate sample from the acid solution. This process had to be completed within 20 min. There were problems with the vacuum suction and some of the samples took over an hour and a half to complete the separation and washing of the samples (Table 13). In these cases the results were clearly wrong for the acid insoluble lignin values (the highlighted sections in Table 13). In the table, the highlighted samples have a higher amount of lignin content than the starting material (untreated SCF's). These data had to be rejected. The vacuum suction was not working correctly for this experiment.

Table 13: Results obtained for Klason lignin analysis. A0- no enzyme, A1- 2 U/g of laccase, B0- no mediator, B1- 0.5%, B2- 2.5 %, B3- 5% ABTS, C1- 1hr, C2- 4hr, C3- 6 hr.

Treatment	AiSL	ASL	Total
Untreated SCF's rep1	24.72	3.25	27.97
Untreated SCF's rep2	25.7	1.83	27.7
Untreated SCF's rep3	23.8	2.82	26.62

Untreated SCF's rep4	31.73	2.20	33.93
Untreated SCF's rep5	40.77	2.58	42.95
Untreated SCF's rep6	33.08	2.17	36.13
NaOH conventional 0.5%	16.94	2.27	19.21
NaOH conventional 0.5%	17.36	2.23	19.59
NaOH conventional 2%	14.32	2.22	16.54
NaOH conventional 2%	14.35	2.23	16.58
NaOH conventional 4%	14.35	2.96	17.31
NaOH conventional 4%	14.15	3.06	17.21
A0B0C1 with surfactant	29.97	2.3	32.27
A0B0C2 with surfactant	30.48	2.12	32.6
A1B1C1 without surfactant	22.43	2.68	25.11
A1B1C2 without surfactant	22.57	2.44	25.01
A1B3C1 with surfactant	26.44	4.19	30.63
A1B3C2 with surfactant	22.85	2.2	25.05
A1B3C3 with surfactant	23.43	2.36	25.79

ii. Pyrolysis Molecular Beam Mass Spectrometry (py-MBMS)

The Klason method was a time consuming process that gave values that were inconsistent with expected values. Therefore, py-MBMS was used to determine the lignin composition in the seed coat fragments. This process was a quicker and a more consistent method of analysis for lignin. The SCF's were pyrolyzed at very high temperatures and the residues of pyrolysis were analyzed using a mass spectrometer. Total lignin content and S/G ratio were calculated using PCA (Principal Component Analysis).

The problem faced with this method was that the instrument did not give consistent results on the same sample that were run on two different days. Therefore, the results obtained on day one could not be compared with the results obtained on day two, as they were not consistent. In addition, the results obtained from conventional NaOH treated samples showed a higher percentage of lignin content than the untreated SCF's, a result that could not be correct. The percentages of lignin determined by the py-MBMS method are given in the Table 14. The lignin percentage obtained using py-MBMS were not compatible with the results obtained with Klason lignin analysis (Table 13).

Table 14: Percentage of lignin results obtained using py-MBMS on SCF's tested on different days

Day	Treatment	Lignin (%)	Avg. Lignin (%)
1	Untreated	24.7, 23.6, 24.9, 24.5	24.4
2	Untreated	19.2, 19.5, 18.7, 18.1, 19.2, 18.5	18.87
3	Untreated	16.7, 16.5, 17.5, 16.7, 16.3, 16.9	16.77
4	Untreated	16.8, 17.2	17.0
4	0.5% NaOH, Conventional	18.2, 18.5	18.35
4	2% NaOH, Conventional	17.0, 17.5	17.25
4	4% NaOH, Conventional	15.9, 15.7	15.8
5	Untreated	17.5, 17.7, 17.1	17.43
5	0.5% NaOH, Conventional	17.8, 18.0, 17.7	17.83
5	2% NaOH, Conventional	16.3, 16.5, 17.2	16.67
5	4% NaOH, Conventional	14.9, 15.6, 15.8	15.43
6	Untreated	17.6, 18.4, 18.2	18.07
6	0.5% NaOH, Conventional	17.3, 17.5, 18.2	17.67
6	2% NaOH, Conventional	18.3, 17.6, 18.6	18.17
6	4% NaOH, Conventional	16.7, 17.4, 17.1	17.07

iii. Pyrolysis Gas Chromatography Mass Spectrometry (Py/GC-MS)

Pyrolysis molecular beam mass spectrometry (py-MBMS) resulted in non-consistent results. Since using py-MBMS to identify the lignin composition was clearly problematic, pyrolysis gas chromatography mass spectrometry (py/GC-MS) was utilized to determine the lignin composition in SCF's. In this method the samples were pyrolyzed and passed through a GC-MS instrument to separate volatile compounds and determine the lignin content. The total run time for each sample was 53 min. The important peaks that were identified for py/GC-MS are as given in Table 15.

Table 15: Coding given for the main peaks considered for calculating the lignin in the SCF's.

Peak	Name
G1	Guaiacol
G2	methylguaiacol
G2_1	ethylguaiacol
G3	vinylguaiacol
G4	allylguaiacol
G5	vanillin
G6	4-propenylguaiacol
G7	coniferyl alcohol
S1	Syringol
S2	methylsyringol
S2_1	ethylguaiacol
S3	vinylsyringol
S4	allylsyringol
S5	Syringaldehyde
S6	4-propenylsyringol

The peak areas for the lignin related peaks were determined based on their retention times in gas chromatography.

Table 16: Base area for lignin peaks in SCF's obtained using py/GC-MS

Untreated				0.5% NaOH Conventional Treatment		
No.	Ret. Time	Peak	Area	Ret. Time	Peak	Area
1	13.202	G1	177253	13.207	G1	593704
2	16.185	G2	138509	19.549	G3	706947
3	19.543	G3	766344	20.563	S1	1521792
4	20.547	S1	1353170	22.969	S2	155886
5	22.959	S2	216594	23.039	G6	447217
6	23.028	G6	459008	24.888	S2_1	148796
7	24.881	S2_1	505168	25.827	S3	918492
8	25.823	S3	853786	26.669	S4	77827
9	26.66	S4	62667	28.021	S5	65839
10	28.865	S6	420333	28.873	S6	575936
11				34.82	S7	35440
Average			4952832	Average		5247876
2% NaOH Conventional Treatment				4% NaOH Conventional Treatment		
No.	Ret. Time	Peak	Area	Ret. Time	Peak	Area
1	13.205	G1	464290	13.213	G1	330778
2	16.201	G2	163429	16.217	G2	130162
3	19.549	G3	623436	19.56	G3	639201

4	20.557	S1	1211541	20.572	S1	1104738
5	22.964	S2	180801	22.981	S2	122041
6	23.03	G6	345355	23.038	G6	346144
7	24.888	S2_1	139628	24.901	S2_1	148620
8	25.825	S3	734992	25.844	S3	881411
9	26.67	S4	60473	26.682	S4	45559
10	28.021	S5	35409	28.028	S5	51049
11	28.869	S6	443978	28.885	S6	544838
Average			4403332	Average		4344541

From the averages obtained for the py/GC-MS peaks in Table 16 0.5% NaOH treated sample seemed to have more lignin than untreated sample, which was unlikely to be true. Due to this unresolved consistency this method was not pursued further.

iv. Surface Enhanced Raman Spectroscopy

In the initial trials, different standards for the SCF's were tested. Syringyl (S) and guaiacyl (G) lignins in pure forms were obtained from complex carbohydrate research center (CCRC), pure cellulose and xylan samples were purchased from Sigma Aldrich. Untreated, and 0.5%, 2%, 4% NaOH-treated SCF's of 1 mg/ml were suspended in ethanol. The required amount of samples was transferred to the wells in the array plate and left to air dry. Collected data was processing using R-statistics). Principal component of analysis (PCA) was conducted for the data set. Also, further dilutions of ethanol background, and untreated SCF's were done to analyze any difference existing with respect to concentration. The results obtained from the analysis of the components using PCA are as seen in Figure 58.

Where

▼ represents the treated samples

* represents the ethanol samples,

■ represents G-Lignin

+ represents the S-Lignin

Figure 58 gives the PLS-DA (partial least square discriminant analysis) for lignin standards containing S- and G- lignin. In Figure 58a, treated samples were clearly separated from standard samples, but overlapped with a few ethanol samples. In Figure 58b similarly, ethanol samples overlapped with few of the treated samples. In Figure 58c, G-lignin was easily separated from the rest of the samples while in Figure 58d, S-lignin was easily separated from the remaining samples in the group. The sensitivity (true positive samples) and specificity (true negative samples) for the data is given in Table 17. Specificity measures the proportion of negatives that are correctly identified. Values closer to 1.000 are more preferred for this method.

Table 17: Sensitivity and specificity values for each sub-group in the main group of treatment, ethanol, G-lignin and S-lignin.

	TREAT	ETHANOL	G-LIG	S-LIG
Sensitivity (CV)	1.000	1.000	1.000	1.000
Specificity (CV)	0.936	0.952	1.000	1.000
Class. Err (CV)	0.0318471	0.0239362	0	0
RMSECV	0.199264	0.204214	0.120680	0.482578

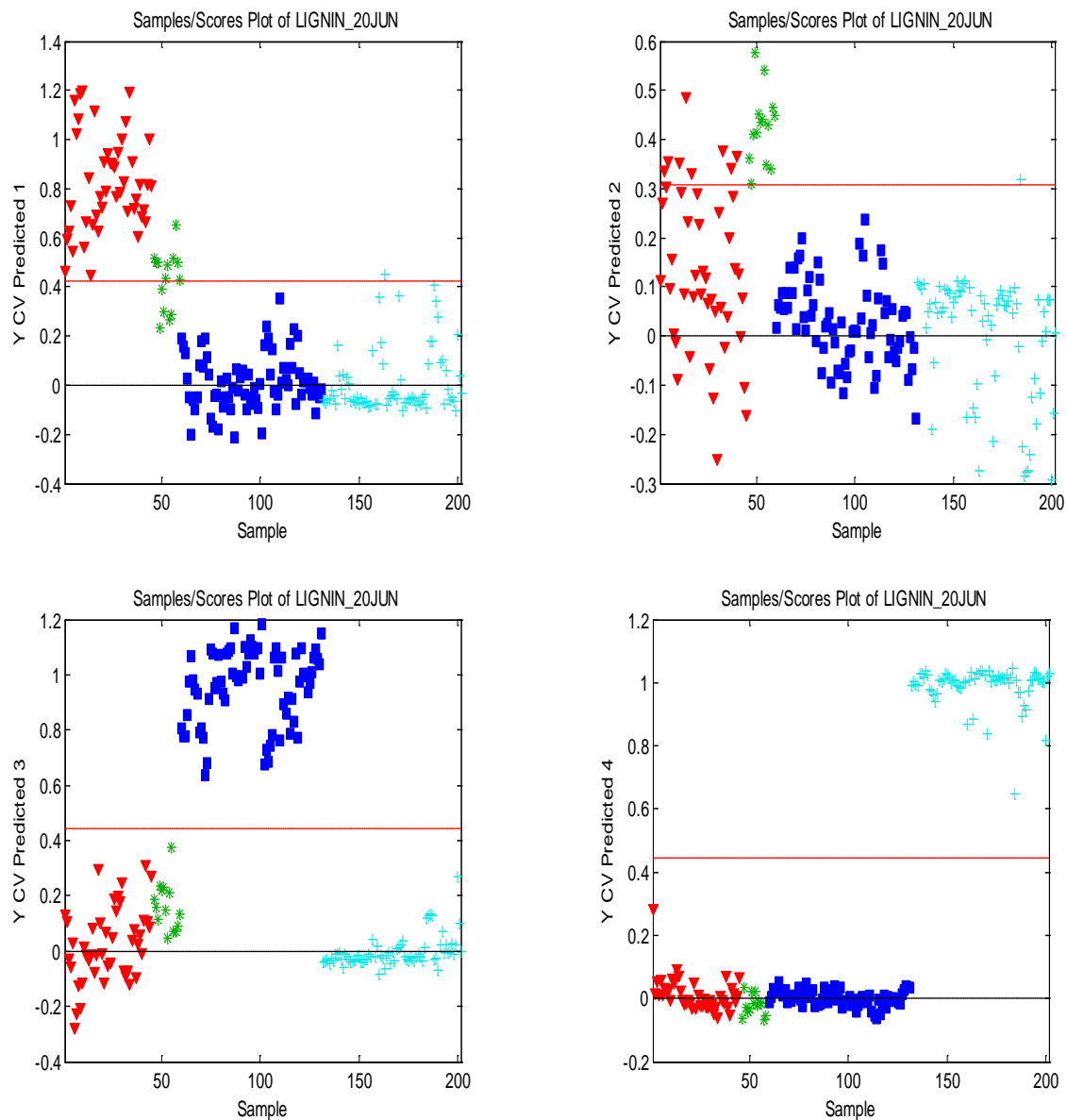


Figure 58: PLS-DA obtained by doing principal component analysis on SCF samples, and lignin standards containing S- and G- lignin (output obtained using Matlab)

The main problem in using S- and G-lignins as standards was that when PLS was used to build a regression model between concentrations and SERS signal intensities a linear correlation was not obtained between them (Figure 59). A correlation coefficient R^2 of only 0.570 was

obtained for this model. Therefore, this combination cannot be used to predict the concentration of the lignin based on Raman intensities.

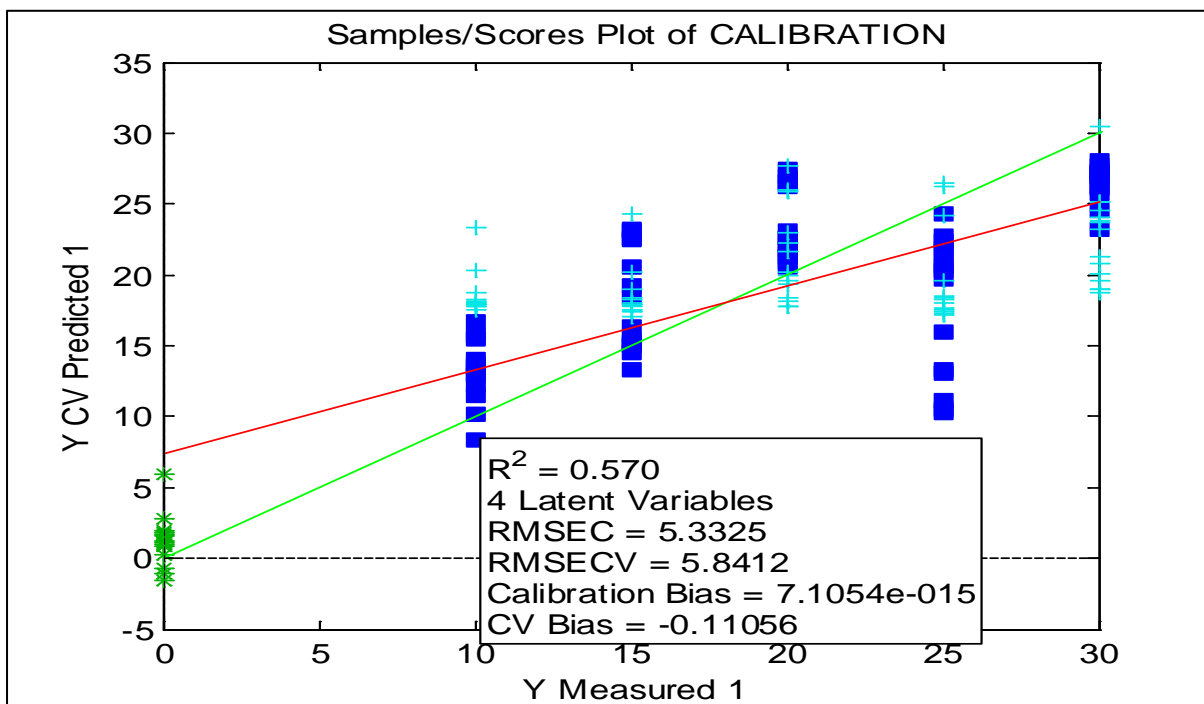


Figure 59: PLS model for S-and G- lignins was constructed for known lignin concentrations vs predicting the concentrations.

In a second trial, mixtures of pure lignin and cellulose were used as standards to represent the minimum and maximum amounts of lignin present in SCFs (lignin amounts determined using Klason analysis). Lignin and cellulose were mixed in the combination of 15% lignin-85% cellulose and 27% lignin-73% cellulose and the effect of using ethanol to make this mixture was also studied. Chemometrics was used to convert the data by preprocessing data (Figure 60) and constructing PCA and HCA for the samples.

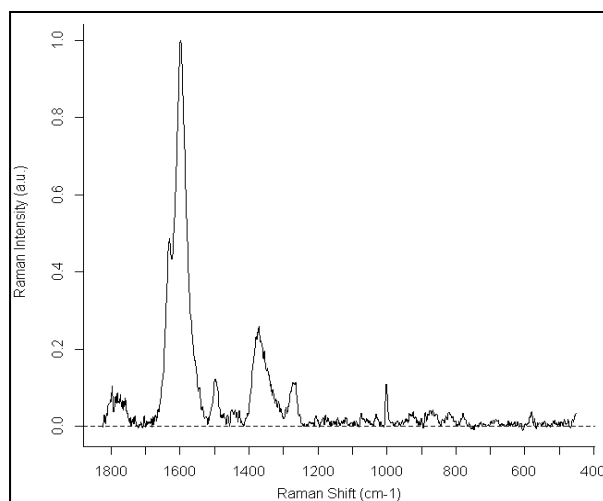


Figure 60: Spectral peak obtained after normalizing and smoothing the curve using R-Statistics.

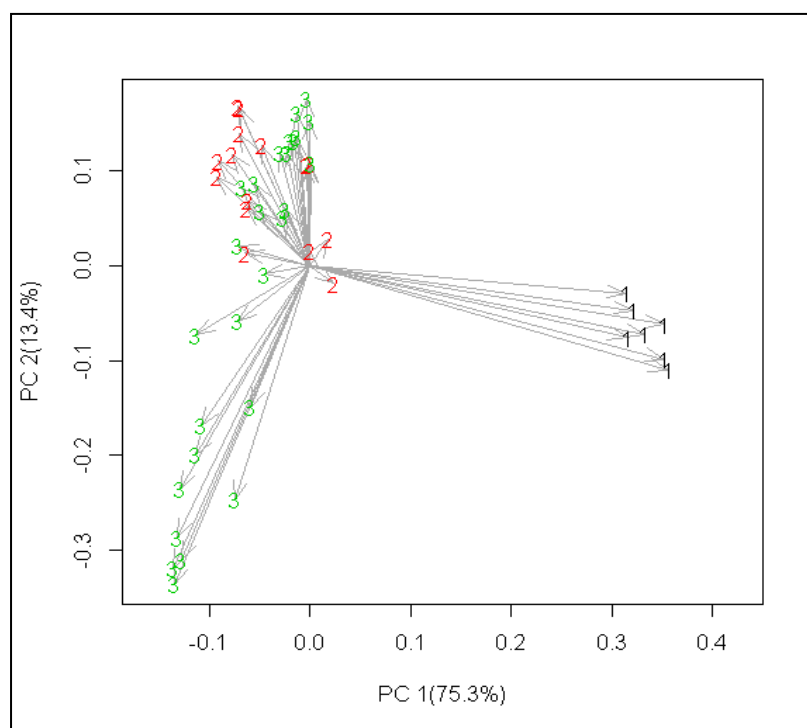


Figure 61: Principal component analysis performed on data obtained from Raman spectroscopy.

PCA analysis was done on the results obtained from Raman spectroscopy to determine if there was any major difference in samples containing different concentrations of lignin, and if

ethanol had any impact on the results obtained from the reference material. In Figure 61, (1) represents ethanol, (2) represents both 15% lignin and 27% lignin samples while (3) represents both 15% lignin and 27% lignin in ethanol.

From Figure 61, ethanol was found to have less impact on the results obtained for the lignin samples. Though some of the lignin and lignin-ethanol samples overlapped on the PC2 axis, there was a clear separation between lignin samples and lignin-ethanol samples. From Figure 62 cluster analysis of the data also clearly separated the data set into clusters of ethanol, lignin-ethanol and lignin samples. The conclusion was that these sets of data give separate but related results.

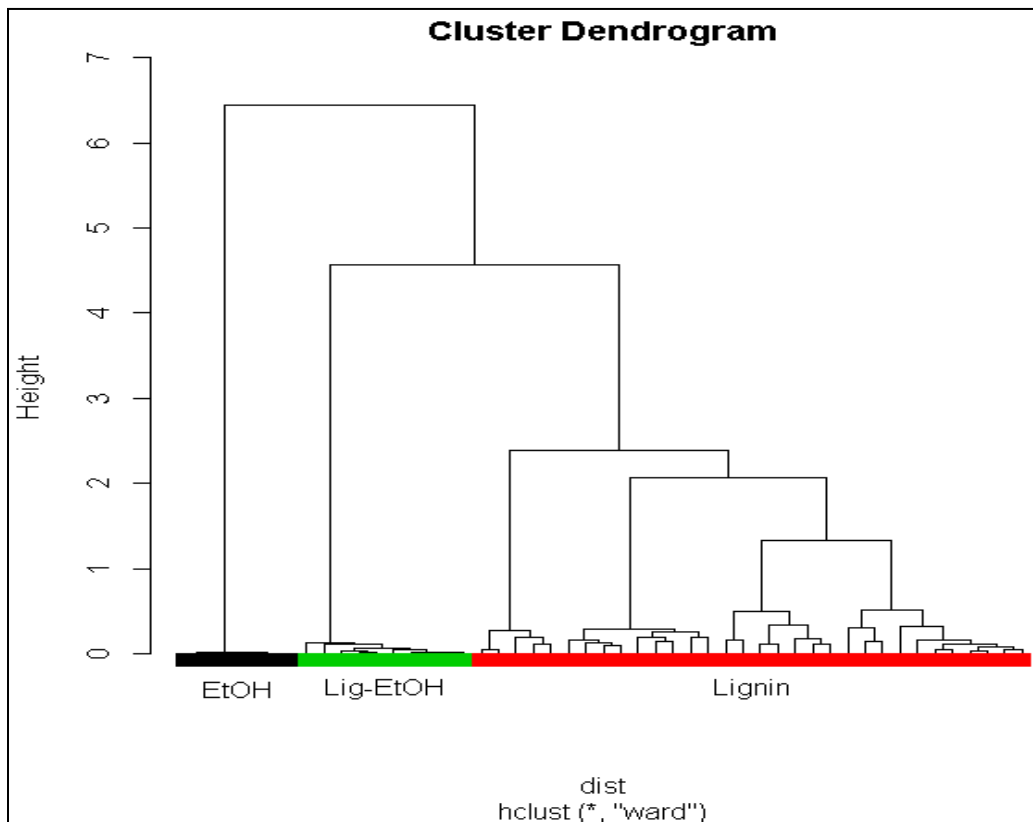


Figure 62: HCA of the lignin standards clustered in three groups of ethanol, lignin-ethanol and lignin respectively.

PLS model was used to build the regression model between lignin concentrations using in the study and SERS signals obtained from using Raman spectroscopy. A linear correlation was obtained between the lignin concentrations with a correlation coefficient of $R^2 = 0.870$, as seen in Figure 63.

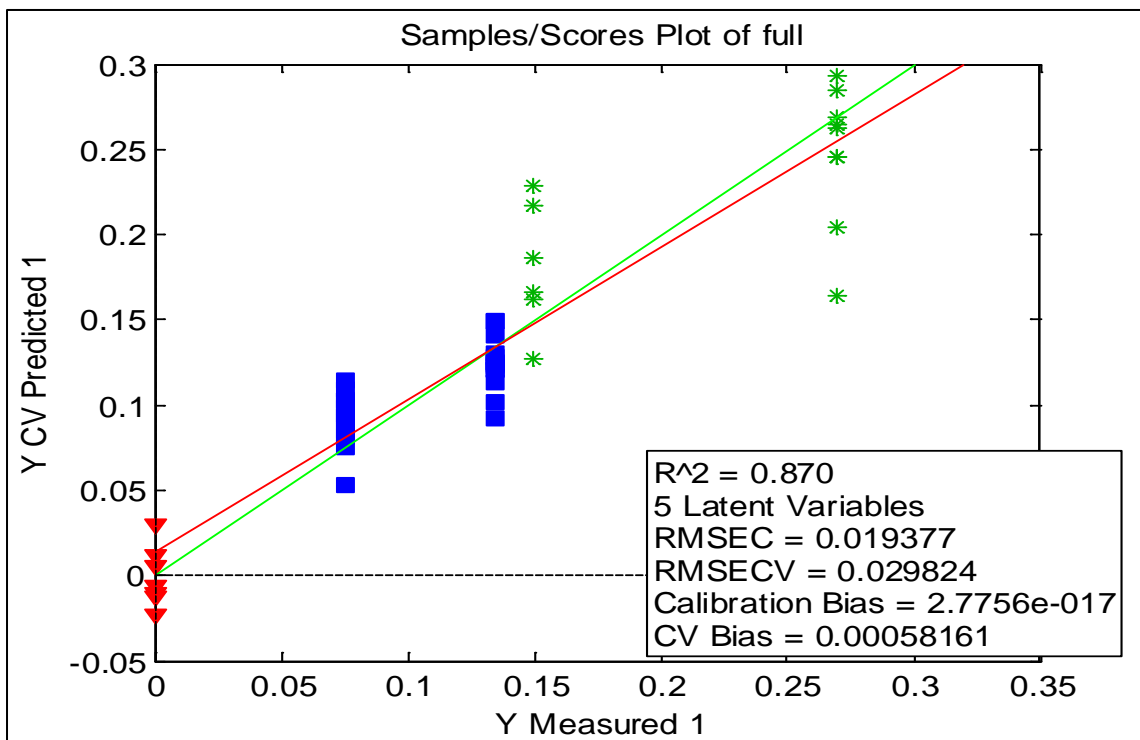


Figure 63: PLS model for lignin was constructed for known lignin concentrations vs predicting the concentrations.

Since the initial study using pure lignin and cellulose as standards gave positive results the experiment was expanded to include lignin concentrations between 15% and 27% (minimum and maximum amount of lignin present in seed coat fragment). At this point, when the experiment was repeated using SERS to collect spectral data for different concentrations of

lignin standards, the data obtained (not included) was varying and there was no correlation between concentrations of lignin measured.

CHAPTER 5

CONCLUSIONS

The overall objective of this research was to investigate the feasibility of designing an effective treatment incorporating enzymes to aid the complete breakdown of seed coat fragments which are present as impurities in cotton greige fabric. The specific objectives were to

- Analyze the effect of conventional sodium hydroxide treatment on cotton seed coat fragments.
- Screen enzymes that were effective in hydrolyzing the hemicellulose and lignin, two of the major components found in seed coat fragments.
- Determine if the components used in the enzymatic treatment boosted the enzyme action on seed coat fragments.
- Investigate pre-treatments that increased the effectiveness of enzymes and reduced the concentration of sodium hydroxide required to breakdown the seed coat fragment structure.

Seed coat fragments were used as the substrate in this research. They typically require a high NaOH concentration and a long treatment time to be effectively detached from the fabrics. Xylanase (Pulpzyme[®] HC) and laccase enzymes were selected for this research to specifically attack hemicellulose (pentosan) and lignin, the two of the major components present in the SCF's, the other being cellulose (48 percent). Cellulase enzyme was an option that was not considered in this research because cellulase enzymes degrade the cellulose in the cotton fibers, thus reducing the tensile strength of a fabric.

In her previous work, Csiszar (Csiszar, Losoncz et al. 2006) used xylanase enzyme as a pre-treatment to alkaline treatment. She used spinning blowroom waste as a model, which contains a mixture of stalk, leaves and seed coat fragments as her starting substrate.

From the extensive literature review done some conclusions drawn were:

- Enzymes play a significant role in the removal of hydrophobic material from the surface of cotton in fabric.
- Various enzymes such as amylases, pectinases, cellulases and catalase enzymes have been in use in textile wet processing, creating less impact on the environment compared to conventional treatments.
- The chemical composition of seed coat fragments significantly differs from that of other impurities present in cotton fabric.
- The presence of lignin in seed coat fragments makes the seed coat fragment recalcitrant in nature and highly difficult to eliminate from the fabric.
- Conventional scouring using sodium hydroxide produces good results in breaking down of seed coat fragments, but due to the negative impact on the environment a substitute treatment that is more eco-friendly is needed.
- Enzymes may be an effective alternative to conventional methods for treatment of seed coat fragments provided that the lignin in the chemical structure can be altered to increase enzyme access to the hydrolyzable inner core of seed coat fragments.

The key findings from the research work are summarized as follows:

1. Conventional Treatment

- a. A 60 minute sodium hydroxide treatment was done on seed coat fragments using 0.5 percent, 2 percent and 4 percent sodium hydroxide solutions at a treatment

temperature of about 100 °C. Weight losses of 24.4 percent, 37.1 percent and 59.6 percent were obtained, respectively, for the NaOH treated seed coat fragments.

2. Xylanase Enzyme

- a. Xylanase enzyme treatment was done on seed coat fragments at pH 8 at 60 °C for 90 minutes using Pulpzyme HC enzyme. This treatment was not very effective as a very low weight loss of 5.7 percent resulted. Use of additives such as ethylenediamine-tetra-acetic acid (EDTA) and vanillin resulted in only a slight increase (6.0 percent and 9.2 percent) in weight loss. I did not find the use of additive compounds to effectively boost the enzyme action on seed coat fragments as was reported by other researchers. By adding EDTA to the enzyme solution, Csiszar obtained a 4 percent increase in weight loss for seed coat fragments and by adding vanillin, Kaya obtained a 15 percent increase in xylan hydrolysis for oat spelt xylan substrate (Kaya, Heitmann et al. 2000; Csiszar, Losonczi et al. 2001).
- b. When microwave-water was used on seed coat fragments the result obtained was insubstantial. Low weight losses of 5.4 percent and 5.1 percent were obtained for 2.5 minutes and 5.0 minutes microwave treatment. These values obtained were not of statistical significance. When microwave-water treated seed coat fragments were further subjected to enzymatic treatment, the results were a slight increase in weight loss (2.5 minutes: 10.1 percent and 5.0 minutes: 8.5 percent).
- c. Use of microwave-NaOH as a pre-treatment on SCF's before enzymatic treatment gave promising results with increased weight losses. For a 2.5 minutes treatment, weight losses of 14.6 percent and 43.4 percent were obtained for NaOH concentrations of 0.5 percent and 4 percent, respectively. For a 5.0 minutes treatment,

- weight losses of 18.7 percent and 51.1 percent were obtained for 0.5 percent NaOH and 4 percent NaOH concentrations, respectively. When sodium hydroxide solution was used in microwave it considerably increased the microwave effect due to its high dipole moment (6.89D) which was absent in the case of pure water (2.12D).
- d. For enzymatic treatment following microwave-NaOH treatment, higher weight losses occurred. At 2.5 minutes MW-NaOH-Enz treatment the weight losses were 24.4 percent and 50.4 percent for 0.5 percent and 4 percent NaOH, respectively. At 5.0 minutes MW-NaOH-Enz treatment for 0.5 percent and 4 percent NaOH the weight losses were 31.5 percent and 55.0 percent, respectively.
 - e. When compared to the weight loss (37.1 percent) obtained for conventional 2 percent NaOH treatment, the weight loss obtained for MW-NaOH-Enz treatment at 2.5 minutes (39.2 percent) and 5.0 minutes (46.4 percent).
 - f. In principal component analysis of FTIR data, the 5.0 minutes microwave-2% NaOH-Enz sample is present in the region of concentrated microwave-4% NaOH-Enz sample. Therefore, by combining microwave with enzyme treatment a lower concentration of sodium hydroxide produced results similar to that from higher concentrations.
 - g. Gas chromatography mass spectroscopy results showed that the microwave-2% NaOH-Enz sample resulted in the removal of hemicellulose exemplified by xylose and arabinose sugar residues in a range close to that of the microwave-4% NaOH-Enz sample. For microwave-2% NaOH-Enz treatment, the relative peak area for xylose was 88,767 while for arabinose it was 61,500. For microwave-4% NaOH-Enz

treatment, the relative peak areas obtained for xylose and arabinose were 91,831 and 80,033, respectively.

3. Laccase Enzyme

- a. The initial study using a laccase-mediator system on seed coat fragments was not found to be effective in creating substantial weight loss. The treatments resulted in weight losses between 2.7 percent – 6.0 percent.
- b. Reasons for this low weight loss could be that the treatment was not performed in a pressurized reactor under oxygen pressure, and HBT mediator was not used. As reported in some of the papers, a 600kPa pressurized reactor with 2.7% odp of HBT resulted in nearly 50% delignification. It was found that HBT mediator is potentially toxic to be used in treatments.
- c. When a microwave treatment was combined with laccase treatment it resulted in increasing the weight loss obtained for the seed coat fragments. A laccase-mediator system (40U/g laccase enzyme, 5 percent ABTS mediator) treatment applied for one hour in bubbling oxygen at room temperature, when combined with 5.0 minute microwave-NaOH treatment at 1% NaOH concentration, increased the weight loss obtained for the seed coat fragments to 25.3 percent.

4. Problems faced in analysis of lignin

- a. A standard Klason procedure by National Renewable Energy Laboratory (NREL) was used to determine the percent lignin present in SCF's after treatment. This method presented several problems involving the functioning of the vacuum pump, and hence was discontinued.

- b. Pyrolysis molecular beam mass spectroscopy (Py-MBMS) was used to attempt to determine the amount of lignin remaining in samples after each treatment. This method gave inconsistent results which varied widely for the same sample tested on different days.
- c. Pyrolysis gas chromatography and mass spectroscopy (Py/GC-MS) was also applied to seed coat fragments. This gave inconsistent results, with treated seed coat fragments giving higher lignin content than untreated seed coat fragment (for 0.5% NaOH conventional treated SCF. The lignin content present in the SCF had a relative peak area of 5,247,876 units whereas untreated SCF had a relative peak area of 4,952,832 units). This method was not pursued further.
- d. For surface enhanced Raman spectroscopy (SERS), the difficulty was in finding the right type of reference material required to construct the standards for lignin. Though the initial study gave positive results on expanding the study the results obtained were inconsistent to draw any statistical conclusions.

Overall, the conclusion to be drawn from this research is that enzymes alone cannot be used to effectively breakdown seed coat fragments due to their complex structure. Additional treatments are required to boost the enzyme action. In this research, when microwave treatment was combined with xylanase enzyme treatment, the results were comparable to conventional treatment. The sodium hydroxide concentration and treatment temperature required to produce the results were reduced from that usually required in conventional treatments.

For laccase enzyme, though the **concept** was ideal for seed coat fragments, based on presence of lignin in their composition, analytical methods that can give reliable quantitative values for lignin are needed.

Future Work

Future research should:

- Optimize the xylanase enzyme treatment parameters.
- Sodium hydroxide is not a preferred chemical to be used due to its negative environmental impact. Therefore new polar compounds with a high dipole moment that have less impact on the environment have to be found to replace sodium hydroxide solution in the microwave treatment.
- Use microwave to carry out enzyme treatments directly in the microwave thus reducing the treatment time required to carry out the process.
- Optimize the treatment conditions for laccase-mediator systems to be applied to seed coat fragments.

Identify an easy and applicable quantitative method to analyze lignin content present in seed coat fragments and draw conclusions based on the quantities present in the sample.

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APPENDIX

R-Statistics

R is an open source programming and software environment used for statistical computing and graphics. It is a pre-compiled binary version software that is freely available under the GNU General Public License and is provided for various operating systems. It is a popular programming language that offers a lot of statistical and graphical functions to plot high quality graphs, perform linear and nonlinear modeling, conduct statistical tests, etc. R mainly uses a command line interface but also provides several graphical user interfaces. It is mostly used by statisticians for statistical computation with performance benchmarks compared to MATLAB. User created packages which allow additional options such as use of specialized statistical techniques, interactive graphical presentations, different import/export options, etc extends the capabilities of R. Besides the core packages that get installed with R, an addition of 5300 packages are available at the Comprehensive R Archive Network (CRAN) for installation based on the users requirements (Wikipedia 2012).

Chemometrics

Chemometrics is described as a highly interfacial discipline for extracting information from chemical systems by data-driven means. It is widely used to solve problems in core data-analytic disciplines such as chemistry, biochemistry, medicine, biology and chemical engineering fields. Highly multivariate data resulting from instruments such as IR, UV/Vis spectroscopy, MS, NMR, atomic emission/absorption, etc are analyzed by chemometrics. Techniques such as principal components analysis (PCA), hierarchical cluster analysis (HCA)

and partial least-square (PLS) can be applied to the multivariate data sets to reveal hidden patterns in the complex data by reducing the information to a more comprehensible form (Wikipedia 2012).

Principal Component Analysis (PCA)

PCA is a statistical analysis tool used to reduce the dimensionality of high-dimensional space of multivariate data. The main idea is that most of the high dimensional data has superfluous variables that are highly correlated containing similar information. Therefore, using PCA will decompose the original data into a subspace of reduced dimension containing as much information as possible. In this process, redundancy and noise variables are highly decreased and new variables that consist of linear combinations of the original data are defined with the first variable aligned in the direction containing most variation. Every subsequent variable is orthogonal to the previous variable in the direction containing the remaining variation. New variables are termed as principal components (PCs) or latent variables (LVs), and the coordinates of the samples in the new space are referred to as scores while the new dimensions are called as loadings. After finding the latent variables, all the samples in the initial PCs can be plotted while ignoring the higher order PCs as only a few PCs capture most of the variation in the data set (Wehrens 2011).

Hierarchical Cluster Analysis (HCA)

HCA is a clustering algorithm that constructs a hierarchy or tree like structure by joining together similar observations in the data set. Hierarchical cluster can either be agglomerative or divisive. In agglomerate cluster, individual clusters will be merged into larger clusters based on the Euclidian distance between the clusters. Data that are close together in a two dimensional space will be clustered together to form clusters and are considered to be equally belonging to

the cluster. Different methods such as single linkage, complete linkage, Ward's method, etc are available for clustering (Dorrien 2006).

The different packages used in this analysis include: hyperSpec, chemometrics, chemometrics with R, pls, ggplot2, plotrix, MASS, rrcov, etc.

Data code ((Wehrens 2011) written in R is as follows:

```
# hyperSpec for Lignin samples

# To read baseline corrected data file in .txt form and check the class of each column
file = read.table ("C:\\Documents and Settings\\drenuka\\My Documents\\Lignin_data.txt",
header = TRUE, dec = ".", sep = "\\t")
sapply(file, class)

# convert data into hyperSpec Object and validate it
lignin = new ("hyperSpec", wavelength =file[,1], spc =t(file [,-1]),
data = data.frame(sample = colnames (file [,-1])),
labels = list (.wavelength = "Raman Shift (cm-1)", spc = "Raman Intensity (a.u.)")) # convert
data into hyperSpec Object and validate it

# Check the validity of the new object formed
chk.hy (lignin)
validObject (lignin)
colnames (lignin)
lignin$sample

# Pre-process of baseline corrected data set:

plot (lignin[10], wl.reverse = TRUE)
lignin = spc.loess (lignin, seq(452, 1824, 0.5))      # Spectra smoothing
lignin
plot (lignin[10], wl.reverse = TRUE)

factor<- apply(lignin,1,max)                          # Normalization of data
lignin = sweep (lignin,1,factor, "/")

lignin.centered <-sweep (lignin, 2, mean, "-")        #Mean centering of data
plot (lignin.centered[10], wl.reverse = TRUE)

# To plot average of replications:

plotspc (lignin [8:13], func=mean, label.spc, wl.reverse= TRUE, col = "red")      # A_Lig
plot (lignin [14:25], func = mean,wl.reverse= TRUE, add = TRUE, col = "black")    #B_Lig
```

```

plot (lignin [23:34], func=mean, wl.reverse= TRUE, add = TRUE, col = "purple")
#A_Lig_EtOH
plot (lignin [35:43], func=mean, wl.reverse= TRUE, add = TRUE, col = "green") #B_Lig_EtOH
plot (lignin [1:7], func=mean, wl.reverse= TRUE, add = TRUE, col = "red") # EtOH

```

PCA analysis on normalized and mean centered data set using SVD

```

datac <- as.matrix (lignin.centered[1:49,,452~1824])
str(datac)
dim(datac)
row.names(datac)
data.svd <- svd(datac)
data.scores<- data.svd$u%*% diag(data.svd$d)
data.loadings <- data.svd$v

```

```

data.vars <-data.svd$d^2 / (nrow(datac)-1)
data.totalvar <- sum(data.vars)
data.relvars <- data.vars / data.totalvar
variances <- 100*round(data.relvars, digits =3)
variances[1:5]

```

```

extra<- c(rep(1,7), rep(2,15), rep(3,27)) # Add classes to the data set
datac.classes<- extra

```

PCA- Scores plot

```

plot(data.scores[,1:2], type = "n",
xlab = paste("PC 1(", variances[1], "%)", sep = ""),
ylab = paste ("PC 2(", variances[2], "%)", sep = ""))
abline (h=0, v=0, col = "gray")
points (data.scores[,1:2], col = datac.classes,
pch = datac.classes)

```

Transpose data set

```

datatt <- t(datac)
str(datatt)
colnames(datatt)
datat.svd <- svd(datatt)
datat.scores<- datat.svd$u%*% diag(datat.svd$d)
datat.loadings <- datat.svd$v

```

```

datat.vars <-datat.svd$d^2 / (nrow(datatt)-1)
datat.totalvar <- sum(datat.vars)
datat.relvars <- datat.vars / datat.totalvar

```



```
variances <- 100*round(datat.relvars, digits =3)
variances[1:5]
```

PCA- Loadings plot

```
plot (datat.loadings[,1]* 1.2, datat.loadings[,2], type = "n",
      xlab = paste ("PC 1(", variances[1], "%)", sep = ""),
      ylab = paste ("PC 2(", variances[2], "%)", sep = ""))
arrows(0,0, datat.loadings[,1], datat.loadings[,2],
       col = "darkgray", length = .15, angle = 20)
text(datat.loadings[,1:2], col = classes, labels = classes)
```

To determine the number of PCs to select

```
par(mfrow = c(2,2))
barplot(data.vars[1:10], main = "variances",
        names.arg = paste ("PC", 1:10))
barplot (log(data.vars[1:10]), main = "log(variances)",
        names.arg = paste("PC", 1:10))
barplot(data.relvars[1:10], main = "Relative variances",
        names.arg = paste ("PC", 1:10))
barplot (cumsum(100 * data.relvars[1:10]),
        main = "Cumulative variances(%)",
        names.arg = paste ("PC", 1:10), ylim = c(0, 100))
```

HCA clustering

```
dataM <- as.matrix (lignin.centered[1:49,,452~1824])
str(dataM)
dist <- pearson.dist (dataM)
dend <- hclust(dist, method = "ward")
par (xpd = TRUE, mar =c (5.1, 4,4,3))
plot (dend, hang =-1, labels =FALSE)
dataM$clusters <- as.factor (cutree (dend, k =3))
cols <- c ("darkblue", "orange", "#C02020")
levels (dataM$clusters) <- c("EtOH", "Lignin", "Lig-EtOH")
mark.dendrogram (dend, dataM$clusters)
dataM$clusters
```

```
datac.dist <- dist(datac)
datac.hcward <- hclust(datac.dist, method = "ward")
plot (datac.hcward, labels= classes)
table(classes, datac.hcward$cluster)
```

To draw scores comparing different classes

```
plot(data.scores[,1:2], type = "n",
```

```
xlab = paste("PC 1(", variances[1], "%)", sep = ""),
ylab = paste ("PC 2(", variances[2], "%)", sep = "")
abline (h=0, v=0, col = "gray")
points (data.scores[,1:2], col = datac.diff,
pch = datac.diff) # (to print in colored symbols) either this
```

```
diff<- c(rep(1,7), rep(2,15), rep(3,12), rep(4,6), rep (2,6))
datac.diff<-diff
points (data.scores[,1:2], pch = datac.diff)
plot (seq_along (diff), diff, pch = as.numeric (diff))
```