THE CHARACTERIZATION AND BIODEGRADATION OF

ASPEN STERYL ESTERS AND WAXES

by

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We accept this thesis as conforming
to the required standard

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Vancouver, Canada

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ABSTRACT

Steryl esters and waxes are major contributors to pitch deposition when aspen is pulped. To overcome this problem attempts have been made to identify these compounds. The first part of this thesis focuses on the identification of these compounds using gas chromatography-mass spectrometry (GC-MS). Since the GC-MS analysis of the aspen steryl esters and waxes is a difficult task, we have only been able to identify lupeol palmitate and lupeol linoleate so far. The steryl esters and waxes fraction was hydrolyzed and the identity of the resulting sterol and fatty acid moieties established by GC-MS. β-sitosterol and β-amyrin were the two primary sterol moieties making up 34.7% and 37.5% of this fraction, respectively. Palmitic acid (C16:0) was the most abundant fatty acid making up 49.2% of the total acid material while linoleic acid (C18:2), a prominent unsaturated fatty acid in other lipid constituents, comprised 31.8% of the fatty acids in the steryl esters and waxes fraction. Standard steryl esters and waxes such as β-sitosterol palmitate and lupeol stearate were synthesized from the major hydrolyzed sterols and fatty acids identified. The aspen steryl esters and waxes fraction was spiked with the synthesized standards to confirm where these compounds eluted in the complex GC-FID chromatogram of the aspen steryl esters and waxes.

The objective of the second part of this thesis was to determine whether some wood-inhabiting fungi were capable of degrading the aspen steryl esters and waxes. Fungi that effectively degrade these compounds could possibly be used as biological treatment agents to remove steryl esters and waxes from aspen wood prior to pulping. Aspergillus luchuensis and Cunninghamella
*elegans* were the two most effective fungi in the degradation of aspen steryl esters and waxes in liquid culture, consuming 3.0 and 2.2 mg/mL in 7 days, respectively. These fungi grew by hydrolyzing the steryl esters and waxes into their sterol and fatty acid components.

*A. luchuensis* and *C. elegans* were also grown on steryl esters and waxes in the presence of glucose or triglycerides in order to understand how these organisms behaved on complex substrates such as aspen wood. As expected, both fungi consumed the glucose prior to modifying the steryl esters and waxes. The amount of steryl esters and waxes consumed by *A. luchuensis* and *C. elegans* was not affected by the addition of glucose. In the steryl esters and waxes cultures supplemented with triglycerides, *A. luchuensis* and *C. elegans* appeared to consume both carbon sources simultaneously.
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<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
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<td>degrees Celsius</td>
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<td>cm</td>
<td>centimeter</td>
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<td>CI</td>
<td>chemical ionization</td>
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<td>FID</td>
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<td>OSB</td>
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<tr>
<td>rpm</td>
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<td>SPE</td>
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<td>µg</td>
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<td>micromole</td>
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1. CHAPTER 1

Introduction

1.1 General Introduction

The emphasis of this project is on characterization and biological degradation of aspen steryl esters and waxes. This chapter begins with assessing the importance of aspen as a pulpwood species in Canada and the problems associated with aspen when it is pulped. The latter part of the introduction will address biological solutions to these problems, including the use of fungi. As little is known about the composition and quantity of specific components in aspen steryl esters and waxes, Chapter 2 will focus on their characterization. Chapter 3 will concentrate on the degradation of aspen steryl esters and waxes by filamentous fungi, and the last section of this thesis (Chapter 4) will address the implications of the work conducted in this project and future research.

1.2 The Importance of Aspen

Aspen (*Populus tremuloides* Michx.), once thought to be a pioneer weed species, is currently receiving attention by many sectors of the forest industry. With about 3 billion cubic meters of aspen present, it is the most abundant hardwood species in Canada (Beck et al., 1989). Most of the aspen is distributed in the western provinces (Figure 1.1), with Alberta having nearly 800 million cubic meters.
Figure 1.1 Distribution of aspen throughout Canada.

Note: Ontario data represent all poplar inventory including aspen (Beck et al., 1989).
As of 1987, only about 10% of the annual allowable cut of aspen has been harvested in western Canada, but this is beginning to change (Chen et al., 1995). Environmental pressures to reduce softwood harvests, especially in British Columbia, have heightened an interest in possible uses for aspen. For instance, the fibre-board industry is using aspen as a source of wood chips and fibres due to a lower demand for aspen from competing industries. Increased production of oriented-strand board (OSB), as well as an increasing use of aspen wafers to make OSB is occurring both in Alberta and in British Columbia (Beck et al., 1989). The pulp and paper industry is also using aspen as a fibre resource. Discarded and unusable wood from the lumber industry is traditionally the source of fibre for pulp mills; however, more efficient processing practices have resulted in a decrease in such waste wood. Reduction in softwood harvests and better use of fibre supplies by the lumber industry have left less material for pulping. In turn, the pulping industry is searching for alternate resources to supplement their present fibre supply. The low harvest and high growth rates of aspen make it an inexpensive fibre supply to papermakers (Chen et al., 1995). A typical paper sheet contains long fibres as re-enforcement and smaller, thin walled fibres and other additives as filler. Woods like lodgepole pine, spruce and hemlock have long fibres that provide adequate re-enforcement, while aspen has short, thin fibres that are a good source of filler. Aspen wood is bright, having a low quantity of color compounds, and therefore requires less bleaching than many softwood species that are pulped (Lehto and Oy, 1995). These qualities are attractive to the Canadian pulp and paper industry that must compete with countries having fast growing trees such as China, Brazil, Chile, and New Zealand. However, aspen tends to cause more pitch deposition problems than several softwoods when pulped.
1.3 Pitch Deposition

Pitch deposition is usually initiated by an aggregation of wood lipids and other wood extractives including phenolic and lignan material. Pulping additives such as calcium carbonate and sizing agents, as well as fibres or fragments of fibres, can also be incorporated into these deposits (Allen, 1980). Due to the hydrophobic nature of the wood lipids, they tend to clump together during pulping and form complex polymeric structures with some of the pulping additives. These aggregates stick to paper machine felts and pulp screens, and they often become incorporated into the paper product. Consequently, this causes the appearance of the paper to be compromised due to the presence of pitch, which reduces the value of the final product. To avoid such problems, pulp mills often halt production to remove the deposits before they cause product damage or paper breaks. Severe deposition often causes paper breaks on the paper machine, which results in hours of mill downtime to remove deposits and to restart production. Pitch deposition also increases the consumption of pulping and bleaching chemicals (Hillis, 1962). A typical kraft pulp mill producing one thousand tonnes of softwood pulp daily will spend approximately one million dollars annually to contend with pitch problems, while similar mills using aspen as a furnish, often spend 2 to 3 times more to control pitch deposition (Allen, 1988).
1.4 Wood Extractives

Lipids such as triglycerides, steryl esters, waxes and fatty acids are believed to be the major initiators of pitch deposition (Allen, 1980; Chen et al., 1995). Wood species having a high concentration of these lipids tend to cause significant deposition. This is often a problem in mechanical and thermomechanical pulping (TMP), since these pulping conditions do not change the wood lipids (Ekman and Holmbom, 1989). In addition, the few washing steps associated with mechanical pulping practices, increase the likelihood of wood lipids remaining in the pulp. Since there are several washing steps with chemical pulping and bleaching, pitch forming compounds are usually removed from the pulp. The alkaline conditions of chemical kraft pulping form soluble soaps of the triglycerides and fatty acids, so these compounds are easily washed out of the pulp (Dunlop-Jones et al., 1991). The steryl esters, waxes and sterols are unaffected by kraft pulping (Peng et al., In press). With kraft pulping, the total extractive amount is not as important as the nature of each component.

Table 1.1 compares the composition of lipids in three pulpwood species: lodgepole pine, black spruce, and aspen. Lodgepole pine, black spruce, and aspen have a concentration of 1 to 1.4% triglycerides in the sapwood with about 0.4 to 0.6% in the heartwood. Fatty and resin acids are high in the heartwood of both lodgepole pine and black spruce, while aspen has a low level of fatty acids throughout. It is important to note that aspen does not contain any resin acids (Chen et al., 1994b). There is also a substantial difference in the composition of steryl esters and waxes in aspen as compared to these two softwood species.
Table 1.1  Comparison of the lipid content in the sapwood (S) and the heartwood (H) of three Canadian pulpwoods.

<table>
<thead>
<tr>
<th>Wood Extractive</th>
<th>Aspen * (mg/g OD wood)</th>
<th>Black spruce * (mg/g OD wood)</th>
<th>Lodgepole pine ** (mg/g OD wood)</th>
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<tr>
<td></td>
<td>S</td>
<td>H</td>
<td>S</td>
</tr>
<tr>
<td>Total</td>
<td>30.9±1.2</td>
<td>25.4±1.3</td>
<td>24.3±0.2</td>
</tr>
<tr>
<td>TG</td>
<td>14.5±0.3</td>
<td>3.6±0.1</td>
<td>9.8±0.1</td>
</tr>
<tr>
<td>SE/WAX</td>
<td>6.2±0.1</td>
<td>5.2±0.2</td>
<td>2.2±0.1</td>
</tr>
<tr>
<td>FA/RA</td>
<td>0.3±0.1</td>
<td>8.3±0.3</td>
<td>3.7±0.2</td>
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</tbody>
</table>

Total = total acetone extractives; TG = triglycerides; SE/WAX = steryl esters and waxes; FA/RA = fatty acids and resin acids; mg/g OD wood = milligrams per gram of oven-dried wood.

* Data from Chen et al., 1995.

** Data from Gao et al., 1995.
In the sapwood, aspen has a concentration of steryl esters and waxes three times higher than that of lodgepole pine and black spruce. The steryl ester and wax content in the aspen heartwood is also higher than in the heartwoods of these softwoods, yet the difference is not as large as in the sapwoods.

The proportion of unsaponifiable to saponifiable extractives in hardwoods such as aspen is believed to be the culprit in deposition problems (Allen, 1988; Dunlop-Jones et al., 1991). Unsaponifiable extractives are compounds that do not form soluble soaps in alkaline conditions. They include steryl esters, waxes, sterols, and triterpene alcohols. Saponifiable extractives do form soluble soaps in alkali and include mono-, di-, and tri-glycerides, resin acids, and fatty acids (Sitholé et al., 1992). Both lodgepole pine and black spruce have a ratio of saponifiable to unsaponifiable extractives that is greater than 3:1. However, aspen has a ratio closer to 2:1 which makes it more susceptible to causing pitch problems (Dunlop-Jones et al., 1991).

1.5 Pitch Control

Seasoning, a common practice at many pulp mills, involves storing wood chips for 10 to 15 weeks, or logs for up to a year (Allen et al., 1991). During such storage, many microorganisms, either already present in the wood or from the mill yard, grow on the wood. Staining fungi, including several Ophiostoma spp., are primary colonizers of wood and consume some of the troublesome wood extractives, which result in a reduction in pitch deposition. However, these fungi also cause wood stain and require the use of additional bleaching chemicals to remove the
stain when the wood is pulped (Blanchette et al., 1992). Seasoning can also affect pulp yields due to colonization by decay fungi that degrade cellulose and hemicellulose.

Debarking logs is also a good way to reduce pitch since aspen bark contains four times the concentration of wood extractives as the aspen sapwood (Allen et al., 1991). However, during the winter months, aspen logs become difficult to debark.

Other more sophisticated pitch control treatments involve adding agents that solubilize or disperse the pitch-forming compounds. Talc is the most commonly used pitch control agent, it is a fine sand that combines with the wood lipids. The addition of talc to pulp disperses and incorporates the wood lipids into the paper as filler (Douek and Allen, 1991; Allen and Douek, 1993). This practice is usually ineffective against the severe pitch problems caused by the lipids from hardwoods such as aspen or birch.

1.6 Biological Pitch Control

Removing or modifying lipids biologically is emerging as a possible approach to controlling pitch. The two possible methods include the use of either microorganisms or enzymes produced by microorganisms. Enzymatic treatments during pulping involve adding lipases to the pulp prior to bleaching. The lipases catalyze the reaction of hydrolyzing triglycerides into fatty acid and glycerol moieties which are easily removed from the pulp with subsequent washing steps (Fischer and Messner, 1992). This treatment has been successful in reducing pitch deposition in a Japanese mill which is mechanically pulping red pine (Fujita et al., 1992). Of the lipase
preparations available, none are effective in hydrolyzing steryl esters and waxes; thus, this
treatment is ineffective when aspen is pulped.

The ‘accelerated seasoning’ of wood chips is the second possible biological treatment. This
technique consists of the removal of wood lipids by growing select microorganisms on logs or
wood chips (Brush et al., 1994). The organisms chosen for these treatments consist of
filamentous fungi that are able to grow in wood through ray cells and open resin canals, thereby
reducing the lipophilic wood extractives by assimilating them as carbon sources.

1.6.1 History of Controlled Wood Seasoning

Using fungi to treat wood chips has been proposed since the mid-1960’s. Nilsson and Asserson
found several fungi that could degrade wood resin including fungi such as Trichoderma
lignorum, Penicillium rubrum, and Gliocladium roseum (Breuil et al., 1998). Since then the
Clariant Corporation (Sandoz) has isolated several strains of Ophiostoma piliferum from wood
chip piles. The fungi isolated caused gray to black discoloration of the wood. The use of these
staining fungi to treat wood chips would require more bleaching chemicals to remove the
additional stain caused by them. As a result, the group at Sandoz, used classical genetic
techniques to retrieve a colorless strain of Ophiostoma piliferum. They grew and transferred a
gray strain for several generations, and eventually isolated a colorless strain. Following this
work, a European patent was obtained to use this colorless biological agent, under the trade name
Cartapip™, to reduce wood extractives from wood prior to pulping (Blanchette et al. 1991).
This non-pigmenting isolate consumed wood extractives to the same extent as the pigmenting
strains (Brush et al., 1994).
1.6.2 Efficiency of Biological Control

Since Cartapip™ is a sap-staining fungus, it only consumes simple sugars and some wood lipids and has a limited ability to degrade hemicellulose (Brush et al., 1994). Fungi easily use starch, glucose and other monosaccharides found in wood. Free fatty acids are also consumed by many fungi. Cartapip™ metabolizes more complex carbon sources like triglycerides by producing extracellular lipases that hydrolyze triglycerides into fatty acids and glycerols which the fungus easily assimilates. However, Cartapip™ and several other fungi have a limited ability to modify sterols, steryl esters, and waxes.

To achieve substantial reductions in lipolytic extractives, Cartapip™-treated wood chips must be stored from seven to twenty days (Blanchette et al., 1992). It has been reported that southern yellow pine chips treated for two weeks with Cartapip™ contained 60% less triglycerides and 35% less fatty acids than chips seasoned naturally for the same period (Brush et al., 1994). This fungus has been shown to grow through the ray cells, resin canals, and even through the pits separating parenchyma cells (Seifert, 1993). The addition of such a fungus reduces or prevents the growth of other naturally occurring fungi, and reduces the stain and decay problems associated with natural seasoning. Even though Cartapip™ was isolated from softwood chips, it grows well on several softwood and hardwood species.
Table 1.2 shows the percent reductions in total lipid extractives in wood chips of different species treated with Cartapip™ for two weeks. The decrease of the total extractives, as compared to naturally seasoned wood chips in the pine species, ranges around 20%, whereas the decrease on hardwoods, such as aspen and birch, is approximately 10%.

Table 1.2 Reduction of the total lipid extractives of wood chips of different wood species treated for two weeks with Cartapip™.

<table>
<thead>
<tr>
<th>WOOD SPECIES</th>
<th>PERCENT REDUCTION*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemlock/fir</td>
<td>6.5</td>
</tr>
<tr>
<td>Jack pine</td>
<td>22.2</td>
</tr>
<tr>
<td>Radiata pine</td>
<td>21.5</td>
</tr>
<tr>
<td>Southern yellow pine</td>
<td>18.1</td>
</tr>
<tr>
<td>Aspen</td>
<td>10.0</td>
</tr>
<tr>
<td>Birch</td>
<td>10.7</td>
</tr>
<tr>
<td>Cottonwood</td>
<td>14.4</td>
</tr>
<tr>
<td>Maple</td>
<td>22.5</td>
</tr>
</tbody>
</table>

*Reduction as compared to naturally seasoned control samples (Breuil et al. 1998).
More recently, Rocheleau et al. (1998) showed some promising results with a three week Cartapip™ trial on aspen wood chips (Table 1.3). The Cartapip™ treatment reduced the concentration of triglycerides and steryl esters and waxes by 25 to 35 % in aspen wood and kraft pulps as compared to natural seasoning over a three week period. The concentration of fatty acids was also decreased by 30% in aspen wood with the Cartapip™ treatment, however, the fatty acid composition between kraft pulps of seasoned and Cartapip™-treated wood remained unchanged. The physical properties such as tensile and tear strength, of paper from these pulps were unaffected by either the natural seasoning or by the Cartapip™ treatment (Rocheleau et al., 1998).

Mill-scale biological treatments of wood chips have also had some good results. In one mill trial, southern yellow pine chips were sprayed with Cartapip™ and stored for one week. This study showed a decrease in total extractives by 25%. In another mill trial, loblolly pine treated with Cartapip™ not only reduced extractives, but the mill also witnessed a 17% increase in production. This phenomenon was partially attributed to the removal of the extractives from the fibre surface, which increased fibre-to-fibre bonding and allowed for faster paper machine operating speeds. Furthermore, fewer pulping chemicals were required to get the desired brightness since Cartapip™ prevented blue-staining fungi from colonizing the wood chips (Breuil et al., 1998).
Table 1.3  Comparison of the lipid content of aspen wood and corresponding kraft pulps from aspen wood chips seasoned naturally and treated with Cartapip™ for three weeks.

<table>
<thead>
<tr>
<th>LIPID CLASS</th>
<th>WOOD (mg/g FD wood)*</th>
<th>PULP (mg/g FD pulp)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Seasoned</td>
<td>Cartapip™ treated</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>2.90 ± 0.40</td>
<td>2.20 ± 0.40</td>
</tr>
<tr>
<td>Steryl esters/</td>
<td>0.78 ± 0.04</td>
<td>0.58 ± 0.06</td>
</tr>
<tr>
<td>waxes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty acids</td>
<td>2.87 ± 0.01</td>
<td>1.90 ± 0.30</td>
</tr>
</tbody>
</table>

*(mg/g FD) refers to milligrams per gram of freeze-dried wood or pulp.

Data are from Rocheleau et al., 1998.
1.6.3 Limitations of Biological Control

Despite some successes, the effectiveness of biological treatment can be variable in mill and laboratory studies. One of the major causes of variability is directly related to the nature of wood. The extractive content of the sapwood and heartwood of several wood species has different compositions as shown in Table 1.1. High concentrations of resin acids and phenolics as found in the heartwood of some softwood species have been shown to inhibit fungal growth (Zheng et al., 1994). The poor fungal growth on aspen heartwood has been associated with the lack of nutrients, primarily nitrogen and simple sugars. The aspen sapwood concentration of total nitrogen is 80 μg/g of oven-dried wood which is about two times higher than in the heartwood. The accessibility of nitrogen in the heartwood could also be contributing to the poor growth (Wang et al., 1997). The concentration of monosaccharides in aspen sapwood is 125 μg/g of oven-dried wood whereas the heartwood only has a content of 6 μg/g of oven-dried wood (Wang et al., 1997).

Other limiting factors include the growth conditions used for the treatment. In mill trials the moisture content, temperature and oxygen content of the wood can vary depending on the season. This can affect the growth of the fungi since fungi have optimum ranges for each of these parameters. For example, fungi can grow at moisture levels of 20 to 90%, but grow more efficiently at intermediate concentrations as found in the sapwood of many wood species (~50 %). Dry summer conditions and high temperatures in wood chip piles can reduce wood moisture levels, thereby reducing fungal growth. Wood chip piles have been shown to reach 50°C in the center and top regions and since many wood-inhabiting fungi are mesophilic (grow
between 5 to 35°C), they would be unable to grow under these conditions. To control some of these environmental conditions, wood chips piles could be rotated more frequently or the wood chips could be stored in silos. This does require more financial investment and human resources.

1.7 Introduction to Steryl Esters and Waxes

Plant steryl esters and waxes have only recently been investigated even though there has been extensive research done on mammalian steryl esters. Figure 1.2 shows the basic structure of a steryl ester, a sterol molecule bonded to a fatty acid via an ester bond (Wojciechowski, 1991). Steryl esters found in plants consist of several sterols including sitosterol, stigmasterol, and campesterol esters (Dyas and Goad, 1993). The most common sterol in the plant kingdom is β-sitosterol which is frequently found in lower and higher plants. The fatty acids coupled with steryl esters tend to have 16 to 18 carbon skeletons and can be saturated or unsaturated (Wojciechowski, 1991), therefore most plant steryl esters have a molecular weight of between 630 and 700 daltons.

![Figure 1.2 β-sitosterol palmitate (C16:0) ester is typically found in plants.](image)

Figure 1.2 β-sitosterol palmitate (C16:0) ester is typically found in plants.
Since several sterol moieties and fatty acids are available for the formation of steryl esters, a large range of steryl esters is found in plants. The function of these molecules is unknown, yet they are thought to be a storage form of the sterol moieties, as most steryl esters are stored together in the cell cytoplasm in micelles. Sterols are often used by cells to elicit cell functions or to communicate with other cells. It has also been suggested that the steryl ester micelles can act as a transportation form for sterols intracellularly and intercellularly (Dyas and Goad, 1993). Another possible function of steryl esters may be a defensive response to biological attack as these compounds are toxic to many insects and fungi (Breskvar et al., 1995). The high concentration of steryl esters in aspen may fill the defensive role of resin acids, which aspen lacks.

Steryl esters and waxes are primarily found in the living components of a tree including the cambium, the apical meristem, and the roots (Dyas and Goad, 1993). Since the sapwood contains a higher proportion of living parenchyma cells, steryl esters and waxes are more prominent in the sapwood than in the heartwood of aspen which is illustrated in Table 1.1. However, the chemical composition of steryl esters and waxes in many trees such as aspen is unknown.
1.8 Objectives

Since aspen steryl esters and waxes have not been sufficiently characterized, the first objective of this research was to identify and quantify aspen steryl esters and waxes. They were analyzed in their intact form and as their hydrolyzed sterols and fatty acids. Information about these steryl esters and waxes could help in the development of pitch control strategies to reduce deposition on equipment or products. Using an ‘accelerated seasoning’ system, targeting the removal of aspen steryl esters and waxes from aspen wood, may be an effective method of contending with this deposition problem. Therefore, the second objective of this project was to screen for wood-inhabiting fungi that could degrade aspen steryl esters and waxes. The final objective was to monitor the growth behavior of two fungi on steryl esters and waxes in the presence of other carbon sources found in aspen. This included growth on steryl esters and waxes with glucose, and growth on steryl esters and waxes with triglycerides. The information obtained from these three objectives may determine whether biological treatment of aspen wood will be feasible.
2. CHAPTER 2
Characterization of Aspen Steryl Esters and Waxes

2.1 Introduction

Prior to characterization, steryl esters and waxes have to be extracted from the wood. The wood is ground into sawdust and extracted with solvents such as acetone or ethyl ether (Allen, 1980). This extract contains steryl esters, waxes, mono-, di-, and tri-glycerides, fatty acids, fatty alcohols, sterols, and some phenolic material (Sitholé, 1991; Chen et al., 1994a). Steryl esters and waxes can be separated from the other compounds using a solid-phase extraction procedure. In this procedure, the total extract is loaded onto an aminopropyl column (Bond Elut®), and the various lipid classes are eluted with a series of solvent mixtures (Figure 2.1). Since this column is polar, the first eluant contains non-polar compounds such as triglycerides, steryl esters, and waxes. This fraction is dried, dissolved in chloroform, and loaded onto a second aminopropyl column. The triglycerides are separated from the steryl esters and waxes by eluting with hexane (Chen et al., 1994a). Further separation of the other components in the wood extract can be achieved following the scheme outlined in Figure 2.1.

The most common method for analyzing steryl esters and waxes is to hydrolyze them into their constitutive components: fatty acids and sterols (Evershed et al., 1987). Both fatty acids and sterols are easily resolved using gas chromatography (GC), or reversed phase high performance liquid chromatography (HPLC). With GC, a non-polar fused silica capillary column such as a DB-5 (J & W Scientific, California) is often used to separate the fatty acids (Ekman and Holmbom, 1989).
Figure 2.1 Separation sequence of wood lipids using solid-phase extraction.

Columns 1, 2, and 3 = 3 mL aminopropyl Bond Elut® columns
Solvent A = Chloroform: Hexane (1:5)
Solvent B = Ether: Hexane (8:1)
Solvent C = Ether: Acetic Acid (98:2)
Solvent D = Hexane
Solvent E = Ether
Solvent F = Ether: Hexane (2:8)
Solvent G = Ether: Methanol (2:1)

*From Chen et al., 1994a.
However, the acids must be first converted to methyl esters in order to reduce their polarity and increase their volatility. Sterols can also be separated on non-polar GC columns and are often acetylated or silated in order to improve GC separation and increase volatility, yet this derivatization is not essential for their identification (Goad, 1991). To identify both sterols and fatty acids, the GC can be coupled to a mass spectrometer. Following the GC separation, the resolved fatty acid and sterol components are introduced directly into the mass spectrometer ion source where ionization takes place, for instance under electron impact (EI) or via chemical ionization (CI). The resulting ions are separated based on their mass to charge ratio. Since different compounds produce different spectral patterns, the mass spectra obtained are unique for specific compounds. Identification can be aided by use of mass spectra libraries. Even though sterols and fatty acids are readily characterized with this procedure, little information about the intact steryl esters and waxes is acquired by hydrolyzing these molecules (Evershed et al., 1987).

Intact steryl esters and waxes have been well studied in mammalian systems, but have only recently been studied in plants. Plant steryl esters contain several sterols, in contrast, mammalian steryl esters consist primarily of cholesterol. Due to the large number of sterol and fatty acid combinations in plants, identification becomes rather difficult.
Despite this the steryl esters and waxes of several plants including celery, *Arabidopsis*, and tobacco have been identified (Grunwald, 1975; Dyas *et al.*, 1991; Patterson *et al.*, 1993). Steryl esters and waxes from trees such as spruce and lodgepole pine have been identified and quantified as a group (Orsa and Holmbom, 1994) or as their hydrolyzed moieties (Saranpää and Piispanen, 1994; Gao *et al.*, 1995). Wallis and Wearne (1997) have recently attempted to characterize the intact steryl esters of *Eucaluptus globulus*.

GC analysis of steryl esters and waxes is often done on a non-polar column such as a DB-5 (Wakeham and Frew, 1982). This column provides good separation of esters having different length fatty acyl chains, whereas steryl esters having the same length fatty acyl chains with different levels of saturation are often not resolved. For instance, cholesterol coupled to stearic acid (C18), oleic acid (C18:1), and linoleic acid (C18:2) elute simultaneously on non-polar columns (Smith, 1983). The use of polar columns such as a Supelcowax column (Supelco Inc, Pennsylvania) resolves these esters (Rezanka, 1992). However, the stationary phase of polar columns may react with the analyte material, this may damage the column and result in variable retention times of specific components.

Due to their low volatility, steryl esters and waxes are difficult to inject by standard GC methods (Wakeham and Frew, 1982). To overcome this problem, steryl esters and waxes are often injected directly into the column or into a pre-column using a cooled on-column system. This ensures that the sample is concentrated onto the column prior to being volatilized, thus reducing loss of material or decomposition.
Because of the lack of information on the intact steryl esters and waxes of aspen wood and the technical difficulty in analyzing these molecules, we initially characterized the hydrolyzed steryl esters moieties. The information obtained on the sterols and fatty acids was used to synthesize standard steryl esters which facilitated the GC and GC-MS analysis of the intact aspen steryl esters and waxes. The investigation of the hydrolyzed steryl esters and waxes was done in collaboration with Dr. Jun pang Peng, while most of the steryl ester and wax standards were synthesized by Dr. Alessio Serreqi.

2.2 Materials and Methods

2.2.1 Sterol and Fatty Acid Standards

Commercially available sterols and fatty acids were used as standards for GC and GC-MS analytes, as well as for the synthesis of steryl esters. β-sitosterol, lupeol, palmitic acid, stearic acid, and linoleic acid were obtained from Sigma (Oakville, Ontario). The α-amyrin was obtained from ICN Biomedicals Inc (Costa Mesa, California). Reagents used for the synthesis of steryl esters were from Aldrich (Milwaukee, Wisconsin). Diazid® was obtained from Sigma and Silica gel 60 plates were from Whatman (Fairfield, New Jersey). All solvents used were HPLC grade and obtained from Fisher (Nepeau, Ontario).
2.2.2 Equipment

Analyses of steryl esters and waxes, hydrolyzed sterols, and hydrolyzed fatty acids were done on a Hewlett Packard HP 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and an HP 7673 automatic sampler. The data collection and processing were controlled by Hewlett Packard HP 3365 Series II software. Columns used include Durabond® (DB-1 and DB-5) fused capillary columns (J & W Scientific, California), and a Supelcowax 10 fused capillary column (15 m x 0.25 mm ID, 0.1 µm film) (Supelco Inc, Pennsylvania).

2.2.3 Preparation of Steryl Ester Standards

Steryl ester standards were prepared from free sterols and free fatty acids. In a typical reaction, β-sitosterol (5 mg, 12 µmol) was dissolved in methylene chloride (2 mL) with palmitic acid (7 mg, 27 µmol) and dicyclohexylcarbodiimide (DCC) (5 mg, 24 µmol). A few crystals of 4-dimethylaminopyridine (DMAP) were added as a catalyst. The reaction was stirred at room temperature for 18 hours, and monitored by thin-layer chromatography (TLC) (Section 2.2.4). Once the reaction reached completion, the β-sitosteryl palmitate was purified by SPE (Figure 2.1) and dried under nitrogen to yield a clear oil (7 mg, 11 µmol, 91% yield). The synthesized steryl esters include β-sitosterol, lupeol, and α-amyrin each coupled to palmitic (C16:0), stearic (C18:0), and linoleic (C18:2) acid, respectively.
2.2.4 Thin-layer Chromatography

Samples were spotted onto a silica gel 60 plate, and developed in hexane-diethyl ether-acetic acid (70:30:1). The plate was visualised by spraying with a molybdate oxidizing solution (5 g molybdate (VI) tetrahydrate in 10 mL sulphuric acid and 90 mL ethanol) and then heating the plate at 150°C (Chen et al., 1994a). Steryl esters and waxes run with a Rf of 0.9 while sterols and fatty acids have a Rf of about 0.3 and 0.5, respectively.

2.2.5 Sample Extraction and Fractionation

Frozen aspen wood chips were ground to fine sawdust with a Micro-Mill (Bel Arts, Pequannock, New Jersey, USA) using a 2 mm mesh. Ground fresh wood (20 g) was extracted with 350 mL of acetone in a Soxhlet apparatus for at least twelve hours at 8 cycles/h. The total extract was collected and evaporated under reduced pressure at 40°C, dissolved in chloroform and run through a magnesium sulfate column to remove any moisture. This mixture was then loaded onto 3 mL aminopropyl columns (Bond Elut®) and the major lipid classes were eluted by a series of solvents. The chloroform extracts are separated into six major classes: (1) triglycerides, (2) steryl esters and waxes, (3) fatty acids, (4) sterols, fatty alcohols, and diglycerides, (5) monoglycerides, and (6) others (Figure 2.1).
2.2.6 GC-FID Analysis of Steryl Esters and Waxes

Steryl ester and wax samples were dissolved in hexane (1 to 5 mg/mL) and 2 μL were analyzed by GC using a DB-5 fused silica capillary column (24 m × 0.25 mm ID, 0.25 μm film). The temperature program of the oven started at 50°C and was held at this temperature for 1 min. Then it was increased to 325°C at 15°C/min and held at 325°C for 45 min. The inlet and the FID were set to 335°C and helium was the carrier gas having a linear velocity of 29.4 cm/sec.

2.2.7 GC-MS Analysis of Steryl Esters and Waxes

GC-MS analysis of steryl esters and waxes was done using a Varian Saturn 2000 gas chromatograph coupled to a Varian Saturn 2000 Ion Trap mass spectrometer. The steryl esters were separated on a DB-5 high temperature column (30 m × 0.25 mm ID, 0.1 μm film). The sample was injected at an injector temperature of 60°C, it was held for 0.1 min and then increased to 300°C at 200°C/min. The initial oven temperature was set at 230°C, increased to 380°C at a rate of 6°C/min, where it was held for 11.5 min. The mass spectrometer was set in the electron impact mode (70 eV) and the mass range scanned was m/z 45-650.

2.2.8 Identifying Standard Steryl Esters in the Aspen Sample

The aspen steryl esters and waxes fraction was spiked with 200 μg/mL of each synthesized steryl ester. These spiked samples were analyzed by GC-FID (Section 2.2.6), while the synthesized standards were analyzed by GC-FID and GC-MS (Section 2.2.7).
2.2.9 Hydrolysis of Steryl Esters and Waxes

The steryl esters and waxes fraction from the wood was saponified using an ethanolic solution of potassium hydroxide. Aspen steryl esters and waxes (10 mg) were refluxed in 0.5 M potassium hydroxide in 90% ethanol (5 mL) until the reaction was completed (5 to 6 h), as verified by TLC (Section 2.2.4). The solutions were then acidified to pH 2.0 with 1 M HCl and extracted with chloroform. The extracts were run through a magnesium sulphate column and dried under a stream of nitrogen with mild heating (Gao et al., 1995). The components were separated through fractionation by SPE (Figure 2.1) to yield a mixture of 4 and 6 mg of free fatty acids and free sterols, respectively.

2.2.10 Methylation of Free Fatty Acids

Fatty acids were methylated by reaction with diazomethane in ether. The diazomethane solution was generated by reacting N-methyl-N-nitroso-p-toluene sulfamide (Diazid®) (2-4 g in 4 mL of ethyl ether) with 11 M potassium hydroxide (4 mL) in ethanol (2 mL). A stream of nitrogen was used to deliver the diazomethane to an ice cooled ether solution (100 mL) until a yellow colour persisted. In a typical reaction, fatty acids (1 to 5 mg) were dissolved in ethyl ether/methanol (3:1, 1 mL) and the diazomethane solution was added until the yellow colour persisted. Then the solvent was gently removed under nitrogen and the methylated fatty acids were redissolved in ethyl ether to a concentration of 1 mg/mL.
2.2.11 GC-FID Analysis of Free Sterols and Fatty Acids

The free sterols and fatty acids were dissolved in hexane (1 to 5 mg/mL), and 2 µL of these solutions were resolved on a DB-5 capillary column (24 m × 0.25 mm ID, 0.25 µm film). The temperature program started at 140°C and increased at 5°C/min to 180°C, then at 10°C/min to 300°C, where it was held for 40 min. Helium was used as the carrier gas at linear velocity of 32.7 cm/sec. The inlet temperature was set at 325°C and the FID was at 360°C.

2.2.12 GC-MS Analysis of Free Sterols and Fatty Acids

The hydrolyzed sterols were analyzed by comparing their electron impact mass spectra with those from the literature, and from the Chemical Abstracts Service (CAS) database which contains mass spectra data of many compounds. The GC-MS used was a Hewlett Packard HP 5890 Series II gas chromatograph equipped with a DB-5 fused silica capillary column (15 m × 0.25 mm ID, 0.25 µm film) with a VG-TR mass selective detector. The ion source was set at 70 eV and the mass range scanned was from m/z 50-450.

The fatty acids were analyzed as their methyl esters by GC-MS, using the above DB-5 column and conditions, and were identified by comparing their mass spectra with those in the MS database (CAS).
2.3 Results and Discussion

2.3.1 GC-FID Analysis of Aspen Steryl Esters and Waxes

Initially, we tried to separate the steryl esters and waxes using a 30 m DB-1 and a 24 m DB-5 column. The retention times of the steryl esters and waxes on these columns were between 60 to 90 min with acceptable resolution. Shorter DB-1 columns (5 and 15 m) were also examined, however, the resolution was poor. A more polar column, 15 m Supelcowax 10, was tested as well. The retention times on the polar column were up to two hours and the resulting peaks were broad. These results suggested that the esters were interacting too strongly with the column’s stationary phase. In fact, remnants of steryl esters came off the column in subsequent runs.

Further method development with the DB-5, 24 meter column produced a better separation of this intact fraction. Injection of the sample posed some problems. First, 2 µL of 10 mg/mL of aspen steryl esters and waxes had to be injected to get an acceptable FID response. Under standard splitless injection conditions a significant portion of the steryl esters and waxes decomposed. This is usually corrected by doing an on-column injection of the sample, however, our GC was not designed to perform this procedure.
Therefore, a cool splitless injection was used to introduce the sample to the column. By lowering the column temperature from 140°C to 50°C during injection, we were able to reduce the sample concentrations to 2 mg/mL (Wakeham and Frew, 1982). The oven temperature program started at 50°C and increased at a rate of 15°C/min up to 325°C. The peak separations were not affected by the rapid increase in temperature as compared to slower rate increases, therefore we were able to shorten our runs to 65 min in contrast to 90 min used in the original methodology. The best aspen steryl esters and waxes separation is presented in Figure 2.2. The major peak (peak 1) at 35.5 min and the doublet (peak 2 and 3) at 37.7 and 38.0 min each accounted for approximately 30% of the total area of aspen steryl esters and waxes as determined by GC. This chromatogram also contained several small overlapping peaks between 40 and 55 min.
Figure 2.2 Aspen steryl esters and waxes separated by GC on a DB-5, 24 meter column.
2.3.2 Hydrolyzed Sterol Analysis

Since intact aspen steryl esters and waxes were difficulty to identify by GC-MS, an alternative approach was used. The compounds were hydrolyzed and the released sterols and fatty acids were analyzed. GC-FID analysis of aspen hydrolyzed sterols revealed 5 major peaks (Figure 2.3). Identification of these peaks by GC-MS indicated three triterpene alcohols and two sterols. The structure and quantities of these compounds are presented in Table 2.1. The three triterpene alcohols, α-amyrin, lupeol, and β-amyrin, have a molecular weight (MW) of 426 daltons; and the two sterols identified, β-sitosterol and α-sitosterol, have a MW of 414 and 426 daltons, respectively. Common ions in the electron impact mass spectra of sterols and triterpenes include the molecular ion $M^+$ and the $[M-H_2O]^+$ (Goad, 1991).

β-sitosterol was the prominent sterol constituting about 35% of the hydrolyzed aspen steryl esters and waxes. β-sitosterol is found in many plants, therefore it was not surprising to find it in high concentrations in the steryl ester fraction of aspen. Analysis of steryl esters in celery shoots and roots has also shown that 25 to 45% of the total steryl ester fraction contained β-sitosterol (Dyas et al., 1991).

Of the three triterpene alcohols, β-amyrin was the most abundant, comprising 37.5% of the hydrolyzed steryl esters and waxes. Lupeol and α-amyrin were poorly separated; lupeol (peak 3) was identified on the shoulder of the large α-amyrin peak (peak 2). Dailey et al. (1997) observed a similar separation with lupeol and α-amyrin peaks from the silated hydrolyzed sterols of *Amaranthus palmeri* steryl esters.
Figure 2.3  GC chromatogram of hydrolyzed sterols and triterpene alcohols from aspen separated on a DB-5, 24 meter column.

Peaks: 1. β-sitosterol, 2. α-amyrin, 3. lupeol, 4. β-amyrin, 5. α-sitosterol.
Table 2.1 Properties of the hydrolyzed aspen sterols and triterpene alcohols.

<table>
<thead>
<tr>
<th>Sterol or Triterpene alcohol</th>
<th>Structure</th>
<th>Molecular Weight</th>
<th>GC Retention Time (min)</th>
<th>Amount mg/kg OD wood*</th>
<th>Percent of the total hydrolyzed sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-sitosterol</td>
<td><img src="image" alt="β-sitosterol Structure" /></td>
<td>414</td>
<td>30.70</td>
<td>596.4</td>
<td>34.7</td>
</tr>
<tr>
<td>α-amyrin</td>
<td><img src="image" alt="α-amyrin Structure" /></td>
<td>426</td>
<td>31.56</td>
<td>251.3</td>
<td>14.6</td>
</tr>
<tr>
<td>Lupeol</td>
<td><img src="image" alt="Lupeol Structure" /></td>
<td>426</td>
<td>31.85</td>
<td>83.1</td>
<td>4.8</td>
</tr>
<tr>
<td>β-amyрин</td>
<td><img src="image" alt="β-amyrin Structure" /></td>
<td>426</td>
<td>32.98</td>
<td>643.3</td>
<td>37.5</td>
</tr>
<tr>
<td>α-sitosterol</td>
<td><img src="image" alt="α-sitosterol Structure" /></td>
<td>426</td>
<td>34.10</td>
<td>142.7</td>
<td>8.3</td>
</tr>
</tbody>
</table>

* mg/kg OD wood = milligrams per kilogram of oven-dried wood (determined by GC chromatogram area).
2.3.3 Hydrolyzed Free Fatty Acid Analysis

The methyl esters of the hydrolyzed fatty acids were also analyzed by GC-MS (Figure 2.4). Most of the fatty acids present in the steryl esters and waxes contained between 16 to 22 carbons and over 65% of them were saturated (Table 2.2). Palmitic acid (C16:0) made up nearly 50% of the hydrolyzed acid fraction in aspen, whereas linoleic acid (C18:2) was the most abundant unsaturated acid and contributed to 31.8% of the acid material. The hydrolyzed fatty acid fraction also contained trace amounts of phenolic compounds. Most of the fatty acid moieties in plant steryl esters and waxes consist of 12 to 22 carbons (Dyas and Goad, 1993), and this was also the case with aspen wood. The fatty acid moieties in lodgepole pine steryl esters and waxes contain between 16 to 20 carbons as well (Gao et al., 1995).

The acyltransferase enzyme, is responsible for the esterification of sterols and fatty acids in plants, and is specific to which acids it esterifies (Dyas and Goad, 1993). Fatty acids with a 16 carbon backbone are more likely to be esterified than those with an 18 carbon backbone while unsaturated acids have a higher rate of esterfication than saturated acids of the same length. This might influence the production of aspen steryl esters and waxes since palmitic acid (C16:0) is the most prominent acid group, and linoleic acid (C18:2) is more abundant than stearic acid (C18:0). This phenomenon also appeared with celery steryl esters and waxes which have a high proportion of palmitic and linoleic acids (Dyas et al., 1991).
Figure 2.4 GC-MS analysis of hydrolyzed fatty acids of aspen steryl esters and waxes.

Table 2.2 Fatty acids from hydrolyzed aspen steryl esters and waxes.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>GC Retention Time (min)</th>
<th>Concentration mg/g OD wood*</th>
<th>Percent of the total hydrolyzed fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-Methyl-tetradecanoic acid (15:0)</td>
<td>5.65</td>
<td>21.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>7.22</td>
<td>705.0</td>
<td>49.2</td>
</tr>
<tr>
<td>Margaric acid (17:0)</td>
<td>8.88</td>
<td>19.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Linoleic acid (18:2)</td>
<td>10.05</td>
<td>455.8</td>
<td>31.8</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>10.72</td>
<td>108.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Eicosanoic acid (20:0)</td>
<td>13.47</td>
<td>63.7</td>
<td>4.4</td>
</tr>
<tr>
<td>2,4 Bis(1-methylethyl)-phenol</td>
<td>14.12</td>
<td>6.4</td>
<td>0.4</td>
</tr>
<tr>
<td>2,2'-Methylenebis 6-1,1-dimethyl ethyl)-phenol</td>
<td>14.30</td>
<td>12.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Docosanoic acid (22:0)</td>
<td>15.47</td>
<td>29.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Bis(2-ethylhexyl) ester of 1,2-benzene-di-carboxylic acid</td>
<td>15.55</td>
<td>10.7</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*mg/g OD wood = milligrams per gram of oven-dried wood (determined by GC chromatogram area)
2.3.4 Mass Spectra Analysis of Synthesized Steryl Esters and Waxes

The information acquired from the hydrolyzed aspen steryl esters and waxes was used to synthesize standards of some of the major intact steryl esters and waxes found in aspen. Under electron impact mass spectra (EIMS) conditions, six of seven synthesized steryl ester standards did not produce a molecular ion (M⁺) (Table 2.3). Although molecular ions were not detected, the fragmentation pattern of steryl esters having the same sterol backbone had similar spectra since the esters fragmented into their sterol moieties and fatty acyl components.

The β-sitosterol esters contained abundant m/z 255 and 396 ions. The m/z 396 is β-sitosterol [RCOH-H₂O]⁺ and the m/z 255 is representative of the sterol moiety without a side-chain (Evershed et al., 1989). EIMS of steryl esters results in a loss of the fatty acyl chain (Evershed et al., 1989); however, with β-sitosterol stearate and lupeol stearate an m/z 283 ion was observed which is likely due to the stearic acid ion [R'CO₂]⁺. As expected, β-sitosterol linoleate had a slightly different fragmentation pattern than the other β-sitosterol esters due to the two double bonds in the fatty acid moiety.

All the lupeol samples contained prominent m/z 189 and 393 fragments. Both lupeol palmitate and stearate had similar fragmentation with the m/z 189 being the base peak whereas lupeol linoleate had the m/z 409 ion as its base peak. The m/z 409 fragment is due to lupeol that has lost water [RCOH-H₂O]⁺. The slightly different fragmentation with lupeol linoleate is also likely due to its unsaturated fatty acid group.
Table 2.3 Electron Impact (70 eV) mass spectra profiles of synthesized steryl esters.

<table>
<thead>
<tr>
<th>Steryl ester</th>
<th>Molecular Weight</th>
<th>m/z data of ions observed (% relative intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-sitosteryl palmitate</td>
<td>652</td>
<td>91(50), 105(55), 145(100), 160(49), 213(38), 255(62), 381(90), 396(55)</td>
</tr>
<tr>
<td>β-sitosteryl stearate</td>
<td>680</td>
<td>91(30), 105(42), 147(90), 160(38), 213(38), 255(66), 275(30), 283(15), 381(100), 396(90)</td>
</tr>
<tr>
<td>β-sitosteryl linoleate</td>
<td>676</td>
<td>91(92), 147(100), 160(68), 213(35), 255(48), 381(90), 396(90), 414(1)</td>
</tr>
<tr>
<td>lupeol palmitate</td>
<td>664</td>
<td>95(52), 121(48), 147(40), 189(100), 203(48), 257(12), 297(20), 365(20), 393(20), 409(15)</td>
</tr>
<tr>
<td>lupeol stearate</td>
<td>692</td>
<td>95(52), 121(50), 147(40), 189(100), 203(48), 282(10), 297(25), 365(30), 393(20), 409(18)</td>
</tr>
<tr>
<td>lupeol linoleate</td>
<td>688</td>
<td>95(90), 121(62), 147(50), 189(71), 203(52), 259(42), 365(28), 393(18), 409(100)</td>
</tr>
<tr>
<td>α-amyrin palmitate*</td>
<td>664</td>
<td>135(10), 189(13), 203(12), 218(100), 409(15), 441(16), 468(73), 530(55), 647(33), 662(30)</td>
</tr>
</tbody>
</table>

*Mass spectrum obtained via probe sample.
Of all the steryl esters synthesized, only α-amyrin palmitate produced a molecular ion (m/z 664). As expected this molecule also fragmented into its sterol moiety (m/z 409) while the m/z 218 ion represented the base peak. Lower electron voltages (20 eV) may be more effective in retrieving a molecular ion of each of the other esters examined, however, analysis of other plant steryl esters at such low voltages produced only trace quantities of the molecular ion (Dyas et al., 1991). Other techniques such as chemical ionization mass spectrometry (CIMS) have been successful employed to obtain molecular ions of steryl esters as well as fragmented ions of the sterol and fatty acid moieties (Evershed et al., 1989; Rezanka, 1992).

2.3.5 GC-FID Experiment of Spiked Aspen Steryl Ester and Wax Fraction

By spiking the aspen steryl ester and wax fraction with the standard steryl esters (β-sitosterol, lupeol, and α-amyrin esters), several constituents in the fraction could be identified (Figure 2.5). Peak 1 at 35.5 min contained β-sitosterol palmitate ester. Lupeol palmitate was present in the left shoulder of the large doublet peak at 37.7 min (peak 2) while α-amyrin palmitate was found in the right shoulder of the doublet peak at 38.0 min (peak 3). Since lupeol only consists of approximately 5% of the sterols in aspen steryl esters and waxes, this doublet probably contains several other esters to compensate for the inflated amount of material in this peak.
Figure 2.5 GC-FID chromatograms of aspen steryl esters and waxes (SE/W) spiked with 0.2 mg of lupeol palmitate and amyrin palmitate.

Peaks: 2. lupeol palmitate, and 3. α-amyrin palmitate. Similar spiking experiments revealed other standards in the following peaks: 1. β-sitosterol palmitate, 4. β-sitosterol stearate/β-sitosterol linoleate, and 5. lupeol stearate/lupeol linoleate.
β-sitosterol linoleate and stearate both co-eluted on a right shoulder of the triplet peaks at 42.4 min (peak 4), and lupeol stearate and lupeol linoleic ester eluted at 45.5 min (peak 5). Steryl esters from eucalyptus wood, having the same sterol coupled to fatty acyl groups that differed only by the degree of saturation, have also been shown to elute simultaneously with the use of DB-5, non-polar GC columns (Wallis and Wearne, 1997).

All three palmitate esters elute before corresponding esters with longer chain fatty acids. For example, β-sitosterol palmitate elutes before both β-sitosterol stearate and β-sitosterol linoleate. Dyas et al. (1993) also found that β-amyrin palmitate eluted before β-amyrin linoleate under similar conditions. Therefore, palmitate esters of α-sitosterol and β-amyrin should likely elute before stearic and linoleic esters of these compounds.

The three β-sitosterol esters eluted faster than the three corresponding lupeol esters while α-amyrin palmitate and stearate ran slower than the corresponding esters of lupeol. This is probably a trend with steroyl esters having these sterols combined with other fatty acids.
2.3.6 GC-MS Analysis of Aspen Steryl Esters and Waxes

Even though the GC separation of the aspen steryl esters and waxes was acceptable, the mass spectra of this sample gave little or no usable data. With GC-MS, it was difficult to resolve peaks using similar conditions as with the GC system. This could be due to the vacuum that is present at the interface between the GC and the MS.

Nevertheless, analyses using a Varian Saturn GC-MS equipped with a high temperature DB-5, 30 meter column gave the best results. Figure 2.6 shows that the total ion chromatogram (TIC) was similar to that on the Hewlett Packard GC shown in Figure 2.2. Lupeol palmitate (peak 3) and lupeol linoleate (peak 5) which had retention times of 20.37 min and 21.73 min respectively, were identified in this analysis. Although molecular ions were not observed in the mass spectra, a comparison to the mass spectra of synthesized standards of lupeol palmitate and linoleic confirmed the identity of these peaks (Table 2.3).

The mass spectra data of some of the resolved aspen steryl esters and waxes are shown in Table 2.4. Even though some of the resolved compounds resembled our synthesized standards or steryl esters and waxes reported in the literature, many of the compounds were not identified. Characteristic ion fragments of two or more cited steryl esters were detected in the mass spectra of many of the separated steryl esters and waxes. This suggests that either the aspen steryl esters and waxes were not sufficiently resolved or that some of the steryl esters and waxes decomposed on the GC column.
Figure 2.6 GC profile of aspen steryl esters from a Varian Saturn GC-MS with a 30 meter high temperature DB-5 column.

Peaks: 1. and 2. unknown, 3. lupeol palmitate, 4. unknown, 5. lupeol linoleic, 6. unknown.
Table 2.4 Electron Impact (70 eV) mass spectra of selected aspen steryl esters and waxes.

<table>
<thead>
<tr>
<th>Steryl ester Peak</th>
<th>GC Retention Time</th>
<th>m/z data of ions observed (% relative intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19.86</td>
<td>69(10), 109(15), 159 (10), 187 (10), 241(15), 296(5), 393(100), 409(1)</td>
</tr>
<tr>
<td>2</td>
<td>20.06</td>
<td>81(15), 95(22), 147(18), 175(18), 189(50), 203(100), 218(28), 393(30)</td>
</tr>
<tr>
<td>3 (lupeol palmitate)</td>
<td>20.37</td>
<td>95(47), 121(40), 147(48), 189(100), 203(68), 257(15), 297(18), 365(10), 393(28), 409(20)</td>
</tr>
<tr>
<td>4</td>
<td>21.20</td>
<td>95(32), 109(30), 147(25), 187(20), 217(18), 298(15), 394(100), 409(18)</td>
</tr>
<tr>
<td>5 (lupeol linoleate)</td>
<td>21.77</td>
<td>95(58), 119(55), 147(55), 189(100), 203(75), 297(10), 393(40), 409(50)</td>
</tr>
<tr>
<td>6</td>
<td>21.97</td>
<td>95(48), 135(50), 147(42), 175(38), 232(18), 273(15), 379(15), 408(52)</td>
</tr>
</tbody>
</table>
2.3.7 Summary

In this investigation, attempts were made to characterize some of the major compounds in the steryl ester and wax fraction of an aspen extract. However, GC separation coupled to FID and MS identification of the intact steryl esters and waxes was difficult. Upon hydrolysis of these compounds, it was found that aspen steryl esters consisted of two sterols and three triterpene alcohols that were coupled to saturated fatty acids (C16 to C18). Steryl ester standards synthesized during this study were used to identify several esters in the aspen steryl ester and wax fraction.
3. CHAPTER 3
Degradation of Aspen Steryl Esters and Waxes by Filamentous Fungi

3.1 Introduction

Growth and development of filamentous fungi in wood are influenced by the composition and the structure of wood, and by environmental factors. Fungi normally colonize wood at moisture contents of 40 to 80\%, which are common (Eaton and Hale, 1993). In addition, fungi grow into the wood ultrastructure more efficiently than other non-filamentous microorganisms including bacteria. Consequently, fungi are the predominant microorganisms invading wood, and therefore are better candidates than bacteria for the ‘accelerated seasoning’ of wood chips (Zabel and Morrell, 1992). The ideal fungus for the biological treatment of aspen wood chips should remove triglycerides, steryl esters, waxes, and fatty acids, as these compounds are the major contributors to aspen pitch problems. There are also other variables to consider in selecting the appropriate organism. Since wood is a heterogeneous substratum with different concentrations of extractives, nitrogen, and water, an ideal fungus should be able to grow or survive under those conditions. Changes in temperature from season to season as well as high temperatures in wood chip piles require a fungus that can withstand substantial changes in its environment. An effective organism should not degrade the wood fibre as the treated wood chips will be used as a furnish to produce pulp products. Finally, the chosen fungus should be non-pathogenic and should not produce toxic metabolites (Chen et al., 1994b). Fungi that meet the above criteria would have the potential to be used industrially as biotreatment agents on aspen wood chips.
Many fungi consume triglycerides and fatty acids readily (Brush et al., 1994), however, steryl esters and waxes are complex carbon sources, and are therefore not degraded by most microorganisms (Chen et al., 1994b). Steryl esters are storage forms of sterols, which are often toxic to fungi (Udea et al., 1990). This toxicity is mainly due to the accumulation of sterols in fungal cytoplasmic membranes, which affects the cell membrane composition and inhibits fungal growth (Breskvar et al., 1995). A few bacterial strains belonging to the genus Arthrobacter, Bacillus, Mycobacterium, and Streptomyces degrade sterols to carbon dioxide and water (Marsheck et al., 1972). While no fungi have been found to degrade sterols or steryl esters and waxes, a considerable amount of information on the modification of sterols by fungi has been reported.

The most common steroid transformation observed is the hydroxylation of the sterol backbone (Mahato and Majumdar, 1993). For example, the filamentous fungus, Rhizopus nigricans, hydroxylates non-fungal sterols at the 11 position on the sterol backbone (Figure 3.1). This can be considered a defense mechanism against the toxicity of these molecules.

Figure 3.1 Hydroxylation of β-sitosterol by R. nitricans at the 11 position of the steroid molecule to detoxify the sterol backbone.
When the hydroxylation reaction is inhibited, the fungus is unable to grow even in the presence of glucose (Breskvar et al., 1995). Some fungi like *R. nigricans* hydroxylate only the 11 position in the steroid molecule whereas other fungi like some *Penicillium* species can hydroxylate several positions on the sterol backbone. With the addition of hydroxyl groups to the sterol backbone, the sterols become more hydrophilic and are therefore unable to interfere with the fungal membranes. These changes enable the microorganisms to grow in the presence of sterols without utilizing them as a carbon source (Seidel and Horhold, 1992).

Another sterol modification used by fungi (*Fusarium solani*) is the degradation of the sterol side-chain. In fact, the pharmaceutical industry often uses organisms that cleave the side-chain to produce steroid precursors for the synthesis of commercially available steroids (Seidel and Horhold, 1992; Mahato and Majumdar, 1993).

As no wood-inhabiting fungi have previously been found to grow on aspen steryl esters and waxes, we screened 25 wood-inhabiting fungi on liquid media containing these compounds. Two fungi (*Aspergillus luchuensis* and *Cunninghamella elegans*) that effectively grew on the steryl esters and waxes, were grown on triglycerides and glucose, two other non-structural carbon sources available in aspen. In addition, these fungi were grown on steryl esters and waxes in the presence of either triglycerides or glucose. Analysis of growth on these mixed substrates will determine whether these fungi used one carbon source preferentially, or whether both carbon sources were used simultaneously or sequentially.
3.2 Materials and Methods

3.2.1 Steryl Esters and Waxes Isolation from Wood

Frozen aspen wood chips were ground with a Micro-Mill (Bel Arts, Pequannock, New Jersey, USA) using a 2 mm mesh. The freshly ground wood (200 g) was extracted with 1.5 L of acetone for 24 h in a 4 L Erlenmeyer flask. This mixture was filtered and the extract was collected and evaporated under reduced pressure at 40°C. Due to the large amounts of steryl esters and waxes required, total extracts (1 ~ 2 g) were dissolved in chloroform and loaded onto a large silica column (5 cm diameter x 15 cm height). The lipids were eluted with hexane and ethyl ether (50:1) into 75 mL fractions. Fractions showing steryl esters and waxes, via thin-layer chromatography (Section 2.2.4), were collected and evaporated. This procedure yielded 1.5 mg of steryl esters and waxes per gram of fresh wood (300 mg).

3.2.2 Steryl Ester and Wax Emulsion

Purified aspen steryl esters and waxes (300 mg) were dissolved in 100 mL of acetone and 20 mL of deionized water was added dropwise with mixing. More acetone was added to the solution if it became opaque during the addition of the water. The acetone was then removed with a rotary evaporator at 30°C. The result was a stable 2% emulsion of steryl esters and waxes in water.
3.2.3 Fungal Cultures and Fungal Growth

The fungi used were obtained from the American Type Culture Collection (ATCC), the Forintek Canada Corporation, and the University of Alberta Microfungus Collection and Herbarium.

All fungi were pre-grown in 500 mL Erlenmeyer flasks containing 80 mL of liquid minimal medium (B-medium) with micronutrient, vitamins, and 2% soluble starch (Abraham et al., 1993). The minimal medium contained, per liter: 0.4 g CaCl$_2$.H$_2$O, 1.0 g KH$_2$PO$_4$, 0.8 g anhydrous Na$_2$HPO$_4$, 0.5 g MgSO$_4$.7H$_2$O, 1.6 g NH$_4$NO$_3$, 3.0 g potassium hydrogen phthalate, and 1 mL of 1000-times concentrated micronutrient solution (Vogel, 1956). The pH of the medium was adjusted to 6.1 and autoclaved. One mL of 1000-times concentrated, filter-sterilized vitamin stock was then added (Montenecourt and Eveleigh, 1977). After four days of growth at 25°C, the cultures were harvested, homogenized and centrifuged for 10 min at 8,000 rpm. Each pellet was washed with deionized water, recentrifuged, and resuspended in deionized water. Fungal biomass was determined by filtering 1 mL of homogenized culture through a dry, pre-weighed glass microfiber filter (Whatman). The filter was then dried in a microwave (High setting for 10 min), allowed to cool in a dessicator, and weighed.

3.2.4 Screening for Fungi on Aspen Steryl Esters and Waxes

The total volume of a culture was 2 mL consisting of the following: 1 mL of B-medium, 0.8 mL of 2% steryl esters and waxes emulsion, and 0.2 mL of 1 mg/mL of fungal mycelium which was determined by the mycelium dry weight of the pre-culture (Section 3.2.3). The cultures were
grown for seven days at 25°C on a rotary shaker at 250 rpm. All the fungi were grown in triplicate.

3.2.5 Analyses of Steryl Esters and Waxes after Fungal Inoculation or Growth

The fungal cultures were extracted with hexane (3 x 3 mL). The extracts were combined, passed through 3 mL magnesium sulfate columns, and then dried under nitrogen with mild heating. The samples were weighed to determine the amount of steryl esters and waxes remaining. The extracts were dissolved in hexane (1 mL), and 2 µL of the sample was analyzed using a HP 5980 gas chromatograph containing a DB-5 capillary column (24 m x 0.20 mm ID, 0.25 µm film). The programmed temperature started at 250°C, then increased at 10°C/min to 350°C, where it was held for 20 min. The injector temperature was 320°C while the detector (FID) was set at 360°C. The biomass was determined by filtering the extracted cultures through pre-tared glass microfibre filters, which were then dried and weighed (Section 3.2.3).

3.2.6 Growth of Fungi on Mixed Substrates

In these experiments, we used the same medium as above (Section 3.2.4) with the addition of another carbon source. In the glucose and steryl esters and waxes experiment, 1% glucose was added to the B-medium (Section 3.2.3). In the triglycerides and steryl esters and waxes experiment, both the triglycerides and the steryl esters and waxes were emulsified in separate round bottom flasks using the procedure explained in Section 3.2.2. The triglycerides mixed cultures consisted of 1 mL of B-medium, 0.8 mL of 2% steryl esters and waxes, 0.4 mL of 2%
triglycerides, and 0.2 mg of inoculum. The fungi were grown for 14 days, in triplicate, at 25°C and 250 rpm in a rotary shaker.

3.2.7 Analysis of the Substrate in Mixed Cultures

Following fungal growth, 0.2 mL of a cholesterol solution (2.5 mg/mL) in acetone was added as an internal standard. The cultures containing triglycerides and steryl esters and waxes were extracted with chloroform:hexane (1:5) while cultures with glucose and steryl esters and waxes were extracted with hexane (Section 3.2.5). The sterols, fatty acids, and steryl esters and waxes were all separated using SPE (Section 2.1). The steryl esters and waxes were weighed while the sterols and fatty acids were quantified by GC with comparison to the internal standard. The extracted cultures were filtered through pre-tared glass microfibre filters and the mycelia were dried and weighed (Section 3.2.3).

The supernatent was analyzed for glucose by anion exchange high performance liquid chromatography (HPLC). A Dionex DX-500 HPLC system (Dionex, Sunnyvale, CA, USA) having a CarboPac PA-1 column controlled by Peaknet 4.3 software was used. A Spectra System AS3500 autoinjector (Spectra Physics, Fremont, CA, USA), injected 20 μl of sample that were eluted with deionized water at a flow of 1 mL/min. A Dionex ED40 electrochemical detector was used to detect glucose which has a retention time of 25 min under these conditions.
3.2.8 Growth of Fungi on Aspen Sterols with or without Glucose

In the first experimental set, hydrolyzed sterols from aspen steryl esters and waxes (Section 2.2.4) were used as the sole carbon source. The cultures consisted of 1 mL B-media, 0.8 mL of 1.2% sterol emulsion, and 0.2 mL of 1 mg/mL of inoculum (A. luchuensis). Each of these cultures were grown in triplicate at 25°C and 250 rpm for 14 days.

In the second experiment set, A. luchuensis was grown on a mixture of glucose and sterols. The cultures consisted of 1 mL B-medium with 1% glucose, 0.8 mL of hydrolyzed sterols (0.05 to 0.4%) and 0.2 mg of inoculum (A. luchuensis). Each culture was grown in triplicate for 3 days with the growth conditions explained above.

Following growth, all the cultures were extracted with chloroform:hexane (1:5) having cholesterol as an internal standard. The amount of sterol remaining was measured by comparison to cholesterol (Section 3.3.7), and the biomass was determined as explained in Section 3.2.3.

3.2.9 Growth of Fungi on Fatty Acids

The cultures contained 1 mL of B-medium, 0.8 mL of stearic (C18:0) or linoleic (C18:2) acid at a concentration between 0.2 to 0.8% (w/v), and were inoculated with 0.2 mg of A. luchuensis. Each culture was grown in triplicate for four days at 25°C and 250 rpm. Following growth, 0.1 mL of pentanoic acid (5 mg/mL) dissolved in ethyl acetate was added and used as the internal standard. The cultures were extracted with ethyl acetate (3 x 3 mL). The extract was dried, weighed, and analyzed by GC using the conditions described in Section 2.2.11.
3.3 Results and Discussion

3.3.1 Extracting and Preparing the Substrate

Screening fungi for their ability to degrade aspen steryl esters and waxes could be done either on liquid media or on wood. Since the chemical composition of wood is variable, having different concentrations of lipids, extractives, and nitrogen transversely and longitudinally, it would be difficult to assess the effectiveness of the fungi that degrade steryl esters and waxes in wood. Fungal growth in wood (2 to 3 weeks) is slower than in liquid media (1 week); in addition, fungal growth on wood is measured indirectly by ergosterol, a membrane sterol produced during fungal growth (Gao et al., 1993). Ergosterol is extracted from the wood and quantified using HPLC. Because ergosterol levels in the fungal membranes do not increase proportionally during different fungal growth stages, this measurement is only an index used to estimate fungal growth (Gao et al., 1993). Screening fungi on liquid media was more efficient than on wood, as substantial fungal growth in liquid media could be achieved in 4 to 7 days. The biomass was measured directly by weighing the dry mycelium, which was easier and more accurate than quantifying the ergosterol.

The first difficulty encountered in screening fungi in liquid media was to prepare a stable steryl esters and waxes emulsion. Hydrophobic substrates are often emulsified or solubilized into aqueous media with the addition of surfactants (Tiehm, 1994). For example, the addition of surfactants has been shown to increase the degradation of polycyclic aromatic hydrocarbons by fungi, since the substrate was made more easily accessible to the microorganisms (Volkering et al., 1995).
We used three common surfactants, Triton X 100, Tween 80, and Tween 20, to disperse the insoluble steryl esters and waxes in water. Stable emulsions of steryl esters and waxes were obtained at surfactant concentrations of 10% and higher. At these concentrations the growth of most fungi was affected. Even when added at lower concentrations (1%), surfactants have been known to affect microbial growth by disrupting the fungal membranes (Aronstein, 1991). Therefore, we did not use surfactants to emulsify the aspen steryl esters and waxes.

Stable emulsions without the use of surfactants were made by dissolving the steryl esters and waxes in acetone, and adding 30% more water than was required for a 2% emulsion. The acetone and the excess of water were removed with a rotary evaporator. To ensure sterile conditions, the resulting 2% emulsion of steryl esters and waxes was removed from the rotary evaporator with the air intake being covered with a cotton plug soaked in ethanol. The homogeneity of this emulsion was assessed by taking three separate 1 mL aliquots, extracting and quantifying the steryl esters and waxes. The concentration of the recovered steryl esters and waxes was within 5% error with all three samples.
3.3.2 Screening Fungi able to Degrade Aspen Steryl Esters and Waxes

Among the 25 wood-inhabiting fungi selected on aspen steryl esters and waxes, at least 2 fungi from each of the following four fungal divisions were examined: ascomycetes, basidiomycetes, zygomycetes, and deutereomycetes (imperfecti). Fungi that commonly colonize aspen (*Peniophora polygonia*) or that are capable of modifying sterols and steryl esters and waxes (*Aspergillus sp.*) were screened. Other fungi tested included fungi that consume wood resin (*Ophiostoma sp.*) and fungi known to degrade other complex hydrocarbons such as polycyclic aromatic hydrocarbons (PAH).

Table 3.1 shows the consumption of steryl esters and waxes and the production of biomass for each fungus that was examined. None of the ascomycetes including *Cephaloascus albidus* and *Ophiostoma piliferum* (Cartapip™) could effectively grow on the steryl esters and waxes. Two basidiomycetes, *Bjerkandera adusta* and *Phanerochaete chrysosporium*, grew well on this substrate. These two fungi have also been shown to grow effectively on other complex carbon sources like polycyclic aromatic compounds (Bezalel *et al.*, 1996). *Cunninghamella elegans*, a zygomycete, consumed 2.2 mg/mL of the steryl esters and waxes in 7 days. Many of the imperfecti fungi screened, *Ceriporopsis subvermispora*, *Cladosporium resinae*, *Peniophora polygonia*, *Penicillium thomii*, and *Trichoderma viride*, did not grow on the steryl esters and waxes. All the *Aspergillus* species tested grew on the steryl esters and waxes. Among these *Aspergillus luchuensis* degraded more steryl esters and waxes (3 mg/mL) than any of the other fungi tested.
Table 3.1 Fungal degradation of 0.8% aspen steryl esters and waxes (SE/W) after 7 days of growth.

<table>
<thead>
<tr>
<th>MICROORGANISM</th>
<th>SE/W CONSUMPTION (mg/mL)*</th>
<th>BIOMASS (mg/mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ASCOMYCETES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ophiostoma piliferum (Cartapip™)</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Ophiostoma crassivaginatum (Alberta)</td>
<td>0.1 ± 0.2</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Cephaloascus albidus (ATCC)</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Xylobolus frustulatus (ATCC)</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td><strong>BASIDIOMYCETES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bjerkandera adusta (Forintek)</td>
<td>1.8 ± 0.1</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Phanerochaete chrysosporium (Forintek)</td>
<td>2.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Trametes versicolor (ATCC)</td>
<td>0.8 ± 0.4</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>Peniophora polygonia (Forintek)</td>
<td>0.6 ± 0.2</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Ceriporiopsis subvermispora (Madison)**</td>
<td>0.3 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Sistotrema brinkmannii (Forintek)</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td><strong>ZYGOMYCETES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cunninghamella elegans (ATCC)</td>
<td>2.2 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Mortierella isabellina (ATCC)</td>
<td>0.3 ± 0.2</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td><strong>DEUTEREOMYCETES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus luchuensis (ATCC)</td>
<td>3.0 ± 0.5</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>Aspergillus awamori (ATCC)</td>
<td>1.5 ± 0.4</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Aspergillus flavus (ATCC)</td>
<td>0.7 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Aureobasidium pullulans (Forintek)</td>
<td>2.0 ± 0.3</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>Gliocladium roseum (Forintek)</td>
<td>1.8 ± 0.3</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Cladosporium resinae (ATCC)</td>
<td>0.4 ± 0.3</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Lecythophora hoffmannii (Alberta)</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Penicillium thomii (Alberta)</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Phialophora alba (ATCC)</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Phialophora melinii (ATCC)</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Scytalidium circinatum (ATCC)</td>
<td>0.4 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Stachybotrys cylindrospora (ATCC)</td>
<td>0.1 ± 0.1</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Trichoderma viride (Forintek)</td>
<td>0.1 ± 0.2</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

* (mg/mL) refers to milligrams per mL of culture.

** This culture was obtained from the USDA Forest Products Laboratory, Madison, Wisconsin.

Uncertainties are standard deviations on three repeats.
After fungal growth, the remaining steryl esters and waxes were extracted and analyzed by GC-FID to determine whether changes were occurring in the structure of these compounds (Figure 3.2: line A, B, C, and D). Fungi that did not grow on the steryl esters and waxes were unable to modify the substrate, therefore the GC profile of these compounds was the same as the untreated steryl esters and waxes (line A). The relative concentration of steryl esters and waxes decreased with the more efficient fungi such as Cunninghamella elegans and Gliocladium roseum; however, the steryl esters and waxes composition did not change as observed by GC-FID. Fungi that grew on this substrate released sterols that accumulated in the medium. These sterols were observed by GC-FID with a retention time between 3 and 4 min. The alkaline hydrolyzed sterols (line D) showed four primary sterols in aspen steryl esters and waxes. Most fungi like C. elegans (line C) hydrolyzed the steryl esters and waxes into all four sterol moieties while G. roseum (line B) only accumulated three of the major sterols. The first peak of the sterol group which corresponds to β-sitosterol (Section 2.3.2) was missing when G. roseum grew on this substrate. This fungus either did not hydrolyze esters containing β-sitosterol or the β-sitosterol was taken up by the fungus following hydrolysis of β-sitosterol esters. It is likely that C. elegans and G. roseum have different mechanisms for degrading aspen steryl esters and waxes.
Figure 3.2 GC-FID analysis of aspen steryl esters and waxes on a DB-5, 24 meter column following 7 days of growth with two effective fungi.

A = untreated aspen steryl esters and waxes; B = steryl esters treated with Gliocladium roseum; C = steryl esters treated with Cunninghamella elegans; and D = sterols from hydrolyzed aspen steryl esters and waxes.
3.3.3 Monitoring the Growth of \textit{A. luchuensis} and \textit{C. elegans} on Steryl Esters and Waxes

The fungi were grown on steryl esters and waxes for 14 days. The fungal biomass and the amount of steryl esters and waxes remaining in the media were monitored throughout the growth in order to understand how these organisms degrade these compounds. Both fungi consumed 2.7 mg/mL of steryl esters and waxes over the 14 day incubation (Figure 3.3 A and B). In the first two days, \textit{A. luchuensis} and \textit{C. elegans} did not grow; therefore, there was no increase in biomass and no change in the concentration of the substrate. The organisms grew most actively between day 2 to 7; however, the growth patterns were slightly different.

From day 2 to 7, \textit{A. luchuensis} grew gradually with an increase of 1.7 mg/mL in biomass, while during the second week, there was an increase in biomass of only 0.8 mg/mL. The concentration of steryl esters and waxes in the medium had decreased by 1.7 mg/mL at day 7, and by another 0.9 mg/mL between day 7 to 14.

\textit{C. elegans} accumulated 2.4 mg/mL of biomass and degraded 2.7 mg/mL of steryl esters and waxes in the first 7 days of growth. Following day 7, \textit{C. elegans} did not grow or change the composition of the steryl esters and waxes.
Figure 3.3 Consumption of steryl esters and waxes and biomass produced by *Aspergillus luchuensis* (A) and *Cunninghamella elegans* (B) when grown on 0.8% aspen steryl esters and waxes for 14 days.

Error bars are the standard deviation of three repeats.
The accumulation of toxic metabolites could be responsible for the reduction in growth of \textit{A. luchuensis} and the cessation in growth of \textit{C. elegans} after 7 days. The hydrolysis of 2.5 mg/mL of steryl esters and waxes will liberate 1.5 mg/mL of sterols and 1.0 mg/mL of fatty acids. Since fatty acids are easily assimilated by fungi at low concentrations (Brush \textit{et al.}, 1994), the released acids are probably not the cause of the cessation of growth on the steryl esters and waxes. Although low, the concentration of sterols liberated could be toxic to these fungi. Therefore, the profile of sterols remaining in the media, following the growth of \textit{A. luchuensis} and \textit{C. elegans} on steryl esters and waxes, were analyzed by GC-FID (Figure 3.4). \textit{A. luchuensis} produced several sterol metabolites, whereas \textit{C. elegans} does not appear to modify the released sterols. \textit{A. luchuensis} was likely transforming the sterols in order to overcome their toxicity, while \textit{C. elegans} may have lacked the enzymes responsible for modifying these compounds. This could also explain why \textit{A. luchuensis} was still growing after 7 days, whereas \textit{C. elegans} did not grow any further.

In the following part of this project, we selected the most promising fungus (\textit{A. luchuensis}), and we attempted to explain the growth pattern on steryl esters and waxes by growing this fungus on the different constituents of these compounds.
Figure 3.4 GC-FID analysis of hydrolyzed sterols on a DB-5, 24 meter column following growth of *Aspergillus luchuensis* (A) and *Cunninghamella elegans* (B) on 0.8% steryl esters and waxes.
3.3.4 Growing *Aspergillus luchuensis* on Hydrolyzed Sterols with and without Glucose

In order to understand why all the steryl esters and waxes were not degraded by the fungi, we determined whether *A. luchuensis* was able to use aspen sterols as a sole carbon source. The complete hydrolysis of 0.8% steryl esters and waxes used in the above growth experiments (Section 3.3.3) gives 0.5% sterols and 0.3% fatty acids. Thus, the minimal B-medium was supplemented with 0.5% aspen sterols. When incubated in this medium for 14 days, *A. luchuensis* did not grow or modify the sterols. This result suggested that the sterols were either toxic to the fungus, or were not a carbon source for the fungus. To resolve these hypotheses, *A. luchuensis* was grown for three days on a medium containing different concentrations of sterols (0.05% to 0.4%) supplemented with an easily metabolizable carbon source (0.5% glucose).

When *A. luchuensis* grew on this medium, it produced the same biomass (1.6 mg/mL) at all the sterol concentrations (0.05% to 0.4%). The biomass was equivalent to the biomass obtained on 0.5% glucose alone (Figure 3.5). This suggested that the increasing level of sterols provided did not inhibit fungal growth and that *A. luchuensis* could not use the sterols as a sole carbon source as there was no increase in fungal biomass. Surprisingly, the concentration of sterols remaining in the medium following fungal growth was lower than the original concentration present in the medium. It was hypothesized that the fungus could have accumulated the sterols in their hyphae or into cellular structures. Another possibility was that the fungus was modifying the sterols in such a way that they could not be extracted with hexane.
Figure 3.5 *Aspergillus luchuensis* grown for three days on 0.5% glucose with varying concentrations of hydrolyzed aspen sterols.

Error bars are the standard deviation of three repeats.
3.3.5 Growing *Aspergillus luchuensis* on Saturated and Unsaturated Fatty Acids

*A. luchuensis* was also grown for three days in varying concentrations of saturated (stearic acid) or unsaturated (linoleic acid) fatty acids to investigate whether these acids could be used as a carbon source or if they affected fungal growth (Figure 3.6). The fungus appeared to grow on both stearic (C18:0) and linoleic (C18:2) acid.

*A. luchuensis* produced 0.47 mg/mL and 0.67 mg/mL of biomass on media containing 0.2% and 0.4% linoleic acid, respectively. Even though the concentration of linoleic acid was doubled from 0.2% to 0.4%, the biomass achieved was not doubled. A similar result was observed between the 0.4% and the 0.8% cultures. *A. luchuensis* was not inhibited by linoleic acid concentrations up to 0.8%; however, growth was affected with increasing linoleic acid concentrations. Following the three days of growth, all the cultures contained only trace amounts of linoleic acid which was measured by GC.

With stearic acid, *A. luchuensis* accumulated only 0.28 mg/mL of biomass with the 0.2% culture. There was an increase in biomass to 0.42 mg/mL with 0.4% stearic acid, but no further increase in growth above this concentration. Concentrations of 0.6 to 1 mg/mL of stearic acid remained in the media with cultures having 0.4% and 0.8% of this acid.
Figure 3.6 Biomass produced by *Aspergillus luchuensis* when grown for three days in varying concentrations of linoleic and stearic acid.

Error bars are the standard deviation of three repeats.
*A. luchuensis* grew better on linoleic acid than on stearic acid under each fatty acid concentration examined, thus linoleic acid was a better carbon source for this organism than stearic acid. Saturated fatty acids are a difficult carbon source for fungi to metabolize, and they have been shown to inhibit fungal growth (Garg and Muller, 1993). Therefore, the slow consumption of steryl esters and waxes by fungi could be partially attributed to the high proportion of saturated acids in the steryl esters and waxes fraction of aspen (Section 2.3.3).

### 3.3.6 Growing *A. luchuensis* and *C. elegans* on Mixed Substrates

Aspen wood contains more than a single carbon source including simple sugars (glucose) and lipids (fatty acids, triglycerides, steryl esters and waxes). Therefore, it was important to assess the behavior of the effective fungi, *A. luchuensis* and *C. elegans*, on steryl esters and waxes when another carbon source was present in the culture medium.

### 3.3.6.1 Growing *A. luchuensis* and *C. elegans* on Glucose and Steryl Esters and Waxes

Glucose, a simple sugar, is found in aspen wood at a concentration of 0.01% oven-dried (OD) wood, while steryl esters and waxes reach concentrations as high as 0.8% OD wood (Wang et al., 1997). In this experiment, a steryl esters and waxes to glucose ratio of 2:1 was used to evaluate the growth of *A. luchuensis* and *C. elegans* on steryl esters and waxes when simple carbon sources were also available.
Figure 3.7 shows *Aspergillus luchuensis* growth on steryl esters and waxes with glucose. When *A. luchuensis* grew on glucose alone, an exponential growth phase was observed between 16 to 48 h, with an increase in biomass to 1.9 mg/mL. This phase was followed by a stationary growth phase. A similar growth pattern was observed when the fungus was grown on glucose and steryl esters and waxes. This suggested that the fungus consumed the glucose first. The disappearance of glucose was verified by HPLC (Figure 3.8). During the first 48 h, there was no change in the concentration of the steryl esters and waxes confirming that the fungus assimilated the glucose before it hydrolyzed this more complex fraction. Between day 2 and 7, the concentration of steryl esters and waxes decreased by 1.8 mg/mL while the biomass reached a level of 2.8 mg/mL. *A. luchuensis* did not grow or degrade any more of the substrate after day 7. During the first 7 days of growth, the decrease in steryl esters and waxes in the presence of glucose (1.8 mg/mL) was similar to the decrease of the steryl esters and waxes when they were the sole carbon source (1.7 mg/mL). However, under the mixed substrate conditions, *A. luchuensis* accumulated 1.1 mg/mL more biomass by day 7 than when grown on the steryl esters and waxes alone for 7 days. The limiting factor for the growth of this fungus on the steryl esters and waxes could be due to lack of essential nutrients in the medium. The increased biomass in the mixed substrate experiment may have completely consumed or limited the accessibility of the remaining nutrient resources (nitrogen or the essential vitamins and minerals) in the medium. Another possibility is that the large amount of biomass generated in these small 2 mL cultures could be affecting the aeration of the cultures, thereby affecting fungal growth.
Figure 3.7 Steryl esters and waxes remaining and biomass accumulation following growth of Aspergillus luchuensis on 0.5% glucose and 0.8% steryl esters and waxes for 10 days.

Errors are the standard deviation of three repeats.
Figure 3.8 HPLC analysis of the glucose remaining following growth of *Aspergillus luchuensis* in 0.8% steryl esters and waxes with 0.5% glucose.
On glucose and steryl esters and waxes, *C. elegans* growth was similar to that of *A. luchuensis* (Figure 3.9). *C. elegans* consumed 2.8 mg/mL of steryl esters and waxes from day 2 to 7 with an increase in biomass to a level of 2.8 mg/mL. This fungus did not consume any more steryl esters and waxes after day 7. *C. elegans* reduced the proportion of steryl esters and waxes in the presence of glucose (2.8 mg/mL) to the same extent as when these lipids were the sole carbon source (2.7 mg/mL). The biomass produced by this fungus under both growth conditions was greater than 2.4 mg/mL; therefore, the cessation of growth by *C. elegans* after day 7 could be a result of the high levels of biomass in these cultures. Other factors could also be contributing to a reduction in growth.

The accumulation of byproducts in the medium following 7 days of incubation could be the culprit for reduced growth on the glucose mixed substrate by *A. luchuensis* and *C. elegans*. Therefore, we determined the amounts of sterols and fatty acids present in the medium throughout the incubation period. With the 2 mg/mL of steryl esters and waxes that were hydrolyzed by *A. luchuensis* by day 7, there should be 1.2 mg/mL of sterols and 0.8 mg/mL of fatty acids released. The major hydrolyzed fatty acids of aspen steryl esters and waxes were not detected which suggests that either the organism consumed them quickly or that our analysis was not sensitive enough to detect small quantities of these acids. Sterols accumulated throughout the incubation, with only 0.33 mg/mL of sterols in the medium following 7 days of growth (Table 3.2). As mentioned earlier, these compounds could also have been modified by the fungus, and therefore be more difficult to extract from the aqueous media. It is also possible that the sterols accumulate in the fungi, thereby affecting fungal growth.
Figure 3.9  Steryl esters and waxes remaining and biomass accumulation after growth of Cunninghamella elegans on 0.5% glucose alone and on 0.5% glucose with 0.8% steryl esters and waxes for 14 days.

Errors are the standard deviation of three repeats.
Table 3.2 Concentration of sterols liberated by *Aspergillus luchuensis* when grown on 0.8 % steryl esters and waxes in the presence of 0.5 % glucose.

<table>
<thead>
<tr>
<th>Days of Incubation</th>
<th>Hydrolyzed Sterols (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>7</td>
<td>0.33 ± 0.06</td>
</tr>
<tr>
<td>10</td>
<td>0.54 ± 0.09</td>
</tr>
</tbody>
</table>

3.3.6.2 Growing *A. luchuensis* and *C. elegans* on Triglycerides

Triglycerides are also a common class of lipids found in aspen wood. Thus measuring the growth of *A. luchuensis* and *C. elegans* on triglycerides was essential to assess the potential of these organisms as biotreatment agents on aspen wood chips. As expected, both fungi were able to metabolize the triglycerides (Figure 3.10).

*A. luchuensis* consumed 2.0 mg/mL of the 4.0 mg/mL of triglycerides gradually over 14 days with a final biomass of 1.4 mg/mL. Whereas *C. elegans* consumed only 1.3 mg/mL of triglycerides with an increase in biomass of 0.6 mg/mL in the first week. After day 7, *C. elegans* did not grow or consume any more triglycerides. However, it is important to note that the results with *C. elegans* could be somewhat misleading as only 2 mg/mL rather than 4 mg/mL of triglycerides were introduced into the starting cultures.
Figure 3.10 Concentration of triglycerides remaining and accumulation of biomass when *A. luchuensis* (A) was grown on 0.4% triglycerides, and when *C. elegans* (B) was grown on 0.2% triglycerides for 14 days.

Error bars are the standard deviation of three repeats.
Both fungi consumed triglycerides and produced biomass throughout the first 7 days of incubation. When steryl esters and waxes were the only carbon source, the fungi did not grow or modify the substrate during the first two days of incubation. This suggests that *A. luchuensis* and *C. elegans* can metabolize triglycerides more easily than steryl esters and waxes.

### 3.3.6.3 Growing *A. luchuensis* and *C. elegans* on Steryl esters and Waxes with Triglycerides

*Aspergillus luchuensis* and *Cunninghamamella elegans* were also grown on mixed medium containing steryl esters and waxes with triglycerides. A concentration of 0.8% steryl esters and waxes to 0.4% triglycerides was used in the medium. This proportion is similar to that in aspen wood. The growth pattern on this mixture of substrates was different from that observed on steryl esters and waxes with glucose.

*A. luchuensis* active growth up to 2 days was followed by a stationary phase for 2 days, and then by a gradual increase in the biomass up to day 14 (Figure 3.11). The biomass increased to 1.7 mg/mL by day 7, and by another 1.3 mg/mL between day 7 to 14. However, the fungus hydrolyzed only 1 mg/mL of the triglycerides in the culture with the mixed substrate, in contrast to 2 mg/mL in the culture in which they were the only carbon source. It is possible that some of the triglycerides were bound to the steryl esters and waxes, thus limiting the triglycerides availability to the fungus. The steryl esters and waxes concentration was reduced by 1.9 mg/mL in the first 7 days, and by another 0.8 mg/mL in the next 7 days. The decrease in steryl esters and waxes in this experiment (1.9 mg/mL) by day 7, was similar to the decrease (1.8 mg/mL) attained when glucose was provided as the alternate carbon source.
Figure 3.11 Concentration of triglycerides and steryl esters and waxes remaining and the accumulation of biomass when *Aspergillus luchuensis* was grown on 0.8% aspen steryl esters and waxes and 0.4% triglycerides.

Errors are the standard deviation of three repeats.
Following day 7, *A. luchuensis* continued to degrade the substrates in the triglyceride mixed cultures, while the fungus did not consume any steryl esters and waxes, in the steryl ester and wax medium supplemented with glucose. The higher concentration of biomass accumulated by day 7 in the glucose mixed cultures (2.7 mg/mL) as compared to 1.7 mg/mL in the triglyceride mixed cultures, could be limiting further growth of this fungus. The higher level of biomass could affect accessibility of essential nutrients to the fungus and aeration of these cultures.

The concentrations of the fatty acids and sterols remaining in the medium following growth with *A. luchuensis* were also measured. As in the mixed glucose experiment, fatty acids were not detected and sterols increased gradually (Table 3.3).

**Table 3.3 Concentration of sterols released into the medium following growth of Aspergillus luchuensis on 0.8% steryl esters and waxes in the presence of 0.4% triglycerides.**

<table>
<thead>
<tr>
<th>Days of Incubation</th>
<th>Hydrolyzed Sterols (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>7</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>14</td>
<td>0.50 ± 0.05</td>
</tr>
</tbody>
</table>
*C. elegans* showed a different growth pattern on the steryl esters and waxes with triglycerides than *A. luchuensis* (Figure 3.12). This fungus grew gradually up to day 4 with a slight increase in biomass and a decline in both triglycerides and steryl esters and waxes. *C. elegans* grew substantially from day 4 to day 7, consuming 6 mg/mL of steryl esters and waxes and trace amounts of triglycerides. In the second week of incubation, this fungus did not grow or consume any more lipids. *C. elegans* degraded twice as much steryl esters and waxes with triglycerides present than with steryl esters and waxes with added glucose or with steryl esters and waxes alone. These results are difficult to explain and further experiments are ongoing to attempt to resolve this problem.

### 3.3.7 Summary

Of the twenty-five fungi screened on aspen steryl esters and waxes, 7 fungi could grow effectively on this medium. These fungi tended to hydrolyze the aspen steryl esters and waxes into sterol and fatty acid moieties. *A. luchuensis* and *C. elegans* were the best performers on this substrate consuming between 2 to 3 mg/mL of steryl esters and waxes in 7 days. These two fungi also degraded triglycerides which are commonly found in aspen and are associated with pitch deposition. When grown on steryl esters and waxes supplemented with glucose, *A. luchuensis* and *C. elegans* consumed the glucose first; however, they did not appear to degrade either triglycerides or steryl esters and waxes preferentially.
Figure 3.12 Concentration of triglycerides and steryl esters and waxes remaining and the accumulation of biomass when *Cunninghamella elegans* was grown on 0.8% aspen steryl esters and waxes and 0.4% triglycerides.

Errors are the standard deviation of three repeats.
4. CHAPTER 4
Concluding Remarks and Future Work

The increasing value of Canadian softwoods, due to strict harvesting measures, has resulted in increased use of hardwoods, such as aspen and birch. When pulped, aspen and birch cause severe pitch deposits, which are mainly attributed to the high concentration of unsaponifiable extractives like steryl esters and waxes. Since the composition of aspen steryl esters and waxes is unknown, the first part of this thesis attempted to identify the major components in this fraction.

Identification of the intact steryl esters and waxes by GC-MS was difficult, as mass spectra of many of the separated esters and waxes contained fragment ions of two or more steryl esters and waxes. Hydrolyzing aspen steryl esters and waxes into their sterol and fatty acid constituents revealed that \( \beta \)-sitosterol and \( \beta \)-amyrin were the two principal sterol moieties; and palmitic acid (C16:0), stearic acid (C18:0), and linoleic acid (C18:2) were the major fatty acids.

Future work on the characterization of the intact aspen steryl esters and waxes requires further fractionation of these compounds. This could be achieved with liquid chromatography or thin-layer chromatography techniques. For example, silver ion (argentation) chromatography, which consists of impregnating silica gel with silver ions, separates compounds based on their degree of saturation (Evershed et al., 1987). Therefore, steryl esters and waxes having saturated and
unsaturated fatty acids could possibly be resolved. The analysis of the steryl esters and waxes should be easier with fewer compounds present in each fraction. Argentation chromatography has been successfully employed to identify the steryl esters and waxes of plants like celery and Arabidopsis (Dyas et al., 1991).

Further development of the GC techniques may also facilitate analysis of aspen steryl esters and waxes. Using shorter GC columns, 10-15 m rather than 24-30 m, and a lower GC oven temperature program may be required to prevent decomposition of steryl esters and waxes prior to mass detection.

Characterization and quantification of the major aspen steryl esters and waxes would have simplified the second major component of this project which involved the fungal treatment of aspen steryl esters and waxes.

Seven of the 25 wood-inhabiting fungi tested were able to hydrolyze the aspen steryl esters and waxes into sterol and fatty acid moieties. Aspergillus luchuensis and Cunninghamella elegans, were investigated for their ability to degrade aspen steryl esters and waxes in mixed substrate conditions, as found in aspen wood. Both fungi preferentially consumed glucose before steryl esters and waxes. However, when triglycerides were provided together with the steryl esters and waxes, these fungi were able to consume both carbon sources simultaneously. Even though these organisms appear to grow on steryl esters, waxes and triglycerides, the amount of lipids consumed by the fungi was low, and may not be sufficient to treat aspen wood chips.
Further work should involve the growth of *A. luchuensis* and *C. elegans* on aspen wood in the laboratory and examining the changes in the wood lipids. These fungi will likely consume simple carbon sources like glucose and fatty acids preferentially. However, the ultrastructure of wood may make these easier carbon resources inaccessible, allowing the fungi to metabolize more complex molecules including steryl esters, waxes, and triglycerides. Paper (handsheets) should be produced from the fungal-treated wood, and tested for strength and quality, to ensure that these fungi do not affect the wood fibers.

Other experiments using biological treatment of steryl esters could focus on the mechanisms by which the esters are degraded. For this, a model compound such as β-sitosterol palmitate could be used as a substrate for the fungi (*A. luchuensis* and *C. elegans*). The metabolites produced and released into the medium and accumulating in the mycelium could be extracted and analyzed. However, analysis of metabolites in the mycelium may be difficult, but they could possibly be monitored by growing the fungi on a radiolabelled or a fluorescence labelled steryl ester.

The research conducted in this project has provided some information about the nature of aspen steryl esters and waxes and the degradation of these compounds by some wood-inhabiting fungi. The steryl esters and waxes fraction was more complex than we expected, and therefore should be further identified before continuing with the biodegradation studies of these compounds.
REFERENCES


