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Date April 15 1982
ABSTRACT

Growing economic and environmental concerns have resulted in a move to reduce fresh water utilization at mechanical pulp and paper mills. This involves retaining and reusing process waters, which consequently provides benefits such as lower effluent treatment costs, energy savings, and improved environmental performance. However, mills experience major problems when attempting water system closure, such as the accumulation of dissolved and colloidal substances (DCS) within the water system. The buildup of DCS can lower paper quality, increase rates of corrosion, and reduce paper machine runnability. Consequently, efficient and cost effective treatment strategies are required for the removal of these contaminants in order for mills to achieve effective water system closure. Unfortunately, existing treatment technologies are far from ideal, and as a result, fresh water usage at mills remains high.

Our group has previously demonstrated the potential of a fungal and enzyme treatment strategy for the removal of DCS from mill process waters. To further the development of this treatment strategy, the work conducted in this thesis initially focused on fungal growth and enzyme production, followed by an evaluation of enzyme thermostability. Fungal growth was then carried out in a larger scale (18 L) bioreactor to identify potential changes to extracellular enzyme production as a result of scale-up. The enzymes produced in both cases were used to treat fresh white water and pulp from a mechanical pulp and paper mill, and changes to white water, fiber, and paper properties were determined.

The white-rot fungus, Trametes versicolor, utilized in this treatment strategy did not grow well at 45 or 60°C, and the production of cellulolytic, hemicellulolytic, lipolytic, and oxidative enzymes were significantly reduced when compared to the values detected after growth at 30°C. However, these same enzymes were found to maintain substantial percentages of their original activity when incubated at 65°C for extended periods of time. The scale-up of fungal growth in a bioreactor produced very comparable enzyme activities to those determined previously in shake flask cultures.

Enzymatic treatment of fresh white water and pulp resulted in changes to both white water and fiber properties. The average colloidal particle size within the treated
white water was reduced when compared to untreated white water, while the average molecular weight of the phenolic compounds present in the white water increased. Additionally, the average zeta potential of the colloidal particles was decreased in the treated water, indicating reduced colloidal particle stability. The changes made to the white water contaminants as a result of the enzymatic treatments significantly enhanced DCS removal by precipitation when an alum post treatment step was employed. Mechanical pulp added to the enzyme treated white water showed increased surface charge when compared to pulp blended with the corresponding control water.

Handsheets were prepared from enzyme treated or control pulps and were formed in enzyme treated or control white waters, with or without alum post treatment. Paper consolidation and dry strengths were unaffected by any of the treatments, as was illustrated by the very similar densities, scattering coefficients, and tensile, tear, burst, and zero-span indices measured. Enzyme treatment of white water and pulp, followed by alum post treatment significantly enhanced paper surface properties by lowering handsheet roughness and porosity. However, enzyme addition hindered paper optical properties, resulting in lower brightness values. The loss in pulp brightness was largely overcome after a two-stage hydrogen peroxide brightening sequence was applied. Brightening resulted in the enzyme treated pulp reaching a comparable final brightness to that of bleached control pulp.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$e_{420}$</td>
<td>molecular extinction coefficient at 420 nanometers</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>°C/min</td>
<td>degrees Celsius per minute</td>
</tr>
<tr>
<td>µeq/g</td>
<td>microequivalents per gram</td>
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<tr>
<td>µL</td>
<td>microliter</td>
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<tr>
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<td>micrometer</td>
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<td>$A_{404}$</td>
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<tr>
<td>ABTS</td>
<td>2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>Alum</td>
<td>aluminum sulphate</td>
</tr>
<tr>
<td>AOX</td>
<td>absorbable organic halogens</td>
</tr>
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<td>Ave</td>
<td>average</td>
</tr>
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<td>BOD</td>
<td>biological oxygen demand</td>
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<tr>
<td>C9</td>
<td>nine carbon unit</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>CMC</td>
<td>carboxymethyl cellulose</td>
</tr>
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<td>COD</td>
<td>chemical oxygen demand</td>
</tr>
<tr>
<td>DADMAC</td>
<td>diallyl-dimethyl-ammonium-chloride</td>
</tr>
<tr>
<td>DAF</td>
<td>dissolved air flotation</td>
</tr>
<tr>
<td>DCS</td>
<td>dissolved and colloidal substances</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminepenta-acetic acid</td>
</tr>
<tr>
<td>$E_1$</td>
<td>first alkaline extraction stage</td>
</tr>
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<td>energy-dispersive X-ray analysis</td>
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<td>ethylenediaminetetra-acetic acid</td>
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<td>electron spectroscopy for chemical analysis</td>
</tr>
<tr>
<td>g</td>
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</tr>
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<td>grams per mole</td>
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<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
</tr>
<tr>
<td>H₂O₂</td>
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</tr>
<tr>
<td>H₂SO₄</td>
<td>sulphuric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>ISO</td>
<td>international standards organization</td>
</tr>
<tr>
<td>kPa·m²/g</td>
<td>kilopascal square meters per gram</td>
</tr>
<tr>
<td>km</td>
<td>kilometer</td>
</tr>
<tr>
<td>L</td>
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</tr>
<tr>
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<td>liters per minute</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
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m
square meters per kilogram
m²/kg
m³
cubic meter
mg/L
milligrams per liter
MgSO₄
magnesium sulphate
min
minute
mL
milliliter
mL/min
milliliters per minute
mM
millimolar
mN·m²/g
millinewton square meters per gram
MTBE
methyl tert butyl ether
mV
millivolt
MVR
mechanical vapour recompression
N
normal
N₂
nitrogen gas
NaOH
sodium hydroxide
Na₂S₂O₃
sodium thiosulphate
N·m/g
Newton meters per gram
nm
nanometer
P₂O₅
phosphorus pentoxide
PCD
particle charge detector
PES-Na
polyethene sodium sulphonate
PNPL
p-nitrophenol laurate
PNPP
p-nitrophenol palmitate
QP
chelation-peroxide
QPP
chelation-peroxide-peroxide
rpm
revolutions per minute
s/100 mL
seconds per 100 milliliters
SD
standard deviation
SDS
sodium dodecyl sulphate
SCCM
standard cubic centimeters per minute
TDCS
total dissolved and colloidal substances
TE
total extractives
THF
tetrahydrofuran
TMP
thermomechanical pulping
TNE
total non-extractables
TOC
total organic carbon
U/L
units per liter
UV
ultraviolet
v/v
volume per volume
wt/v
weight per volume
ACKNOWLEDGMENTS

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CHAPTER I. Introduction and Research Objectives

1.1 Background

Mechanical pulp and paper mills require large volumes of fresh water for their operation, as well as adequate receiving waters for effluent discharge. Effluents are usually released from the mill without significant water recovery following treatment to reduce components that impact negatively on the environment. Until recently, water conservation measures had not been implemented at many mills. However, there is a growing trend in the industry to conserve water through recycling. Economic and environmental concerns have initiated these changes, with total containment of process waters on-site being the ultimate goal. Stringent discharge limits, lower chemical costs, reduced fiber loss, and energy savings have all contributed to the move towards closed water systems (Cronin, 1996; Dexter, 1996; Farlow, 1996).

At mechanical pulp and paper mills fresh water is no longer free, as it has been in the past. Collection and delivery of water to a mill has costs, as does treatment and release of the process waters and, as a result, water has changed from a disposable commodity to a valuable resource (Dexter, 1996). Accordingly, improvements in equipment and processes have reduced the amounts of water required for operation, which overall have proven to be economically beneficial. At some paper mills, increased closure of paper machine white water systems has been implemented. This has not only reduced effluent treatment costs, but has also benefited the mills by reducing fiber, chemical additive, and energy losses (Springer et al., 1985). In order to achieve substantially higher savings, mills will need to adopt closed water systems that produce no, or very limited effluent discharge, and consequently require minimal intake of fresh
water. For this to occur, both white water and effluent must be recycled back into the process water at points where fresh water is still being consumed.

Additionally, regulations governing the quality of water discharged from pulp and paper mills have become more stringent. Failure to meet these regulations is undesirable and can result in large fines or even termination of operation. As a result, mills spend considerable sums of money operating effluent treatment systems. Not only would a closed water system greatly reduce these ever increasing operational costs, it would also lead to savings in many other areas. However, accomplishing this goal has proven quite difficult for the majority of existing mills as current technologies for cleaning process waters are expensive and/or inefficient.

Even with economic and environmental drivers leading pulp and paper mills towards closed water systems, the many anticipated and unanticipated problems associated with reducing fresh water use have limited implementation. As less fresh water enters a mechanical pulp and paper mill and a greater amount of water is recycled, an unwanted accumulation of contaminants within the water system occurs (Zhang et al., 1999). This buildup of contaminants, including compounds such as dissolved and colloidal substances (DCS), can create numerous problems within mill processes and with product quality. It has been suggested that the buildup of DCS is the major factor limiting reduced fresh water use, as these substances can cause problems such as increased deposits, corrosion, microbial growth, reduced drainage rates, and unwanted odour generation (Cronin, 1996; Farlow, 1996). At integrated pulp and paper mills, the carryover of DCS to the papermachine is also a serious concern, as DCS accumulation can interfere with paper making chemicals and reduce paper quality (Lindström et al.,
In order for mills to close their water systems, an efficient and economic process that reduces contaminant levels while accommodating the various processes and furnishes is required.

One emerging treatment strategy that directly employs fungi and their extracellular enzymes to remove contaminants from mechanical pulp and paper mill process water has shown good potential. Recently, the efficacy of this strategy to remove DCS from white water has been demonstrated (Zhang et al., 1998; Zhang et al., 2000). However, specific questions concerning the potential impacts to white water, fiber, and paper properties resulting from the enzymatic treatments need to be addressed before this strategy can become a commercial reality. Therefore, the primary focus of this thesis was to increase the knowledge base pertaining to the production and thermostability of the extracellular enzymes liberated during fungal growth on white water, and their subsequent effect on specific white water, pulp and paper properties.

1.2 Thermomechanical Pulping and Papermaking

Mechanical pulps are produced using mechanical refining energy to separate wood chips into individual fibers, which in turn can be used to make paper or other end products. The most common type of mechanical pulping is thermomechanical pulping (TMP), which adds heat in the form of steam to help liberate the fibers and improve pulp characteristics (Smook, 1992). The TMP process typically employs two refining stages; the first is carried out at elevated temperature and pressure to promote fiber separation, while the second was traditionally done at ambient temperature to enhance fiber properties (Figure 1-1). However, most modern TMP systems now utilize pressurized secondary refining in order to maximize energy recovery. The high temperatures and
Figure 1-1 Representative layout of a TMP newsprint mill. Black arrows indicate the flow of fiber throughout pulping and papermaking; grey arrows indicate the flow of process waters throughout the integrated mill.
pressures used help limit fiber cutting, which results in papers with improved strength properties (Smook, 1992). After refining, the pulp is diluted with hot water for latency removal before screening and thickening. The pulp can then be brightened, and finally sent to the paper mill for papermaking.

At the paper mill, the incoming pulp is initially diluted with fresh water and mixed with various chemical additives to enhance papermachine runnability and alter specific paper properties (Figure 1-1). Paper is then produced using one of the many different papermachine designs available. A common Fourdrinier-type papermachine utilizes a pressurized head box, which discharges a uniform spray of highly diluted fibers onto a constantly moving forming fabric (Biermann, 1996). The water is quickly removed by suction along the Fourdrinier table, resulting in sheet formation. The paper is next fed through a press section and a dryer section to further consolidate the sheet and increase interfiber bonding. Finally, the paper is sent through a calender stack to reduce thickness and increase surface smoothness, before being wound onto a reel for sale.

Mechanical pulp and paper mills predominantly utilize light coloured softwoods, such as various species of spruce, pine, fir, hemlock, and balsam as fiber sources. Softwoods are composed of cellulose (40-45%), hemicelluloses such as galactoglucomannan (5-8%), (galacto)glucomannan (10-15%), and arabinoglucuronoxylan (7-10%), as well as lignin (26-32%), extractives (1-5%), and trace levels of inorganic materials (Sjöström, 1993). Unlike chemical pulps, lignin is retained in mechanical pulps in order to maximize yield, but consequently produce papers with lower strength properties due to the presence of fewer cellulose fibers per gram of paper, and a corresponding reduction in interfiber bonded area (Biermann, 1996). To compensate, the longer length fibers of
softwoods are generally favoured over the shorter ones typically found in hardwoods. Furthermore, oxidation of the lignin results in yellowing of the pulp and paper with exposure to air and light. These drawbacks have largely restricted mechanical pulps to the production of non-permanent papers such as newsprint. However, the increasing competition for fiber resources, combined with relatively low production costs have led to large increases in world production over the past 20 years.

The flow of water throughout an integrated pulp and paper mill can vary greatly from one mill to the next. Generally, the largest inputs of fresh water into a mill are used for pulp dilution prior to papermaking, and for shower waters during papermaking (Figure 1-1). This water is recycled to varying extents around the paper mill, while some of the low-fiber effluent may be transferred to the pulp mill for pulp dilution after refining. During mechanical pulp processing, 93-97% of the starting wood material is recovered in the final product (Sjöström, 1993). The remaining 3-7% of the original wood is released into the water primarily during pulping, which translates to a loss of 30-70 kg/ton of pulp produced. As a result, the process waters collected in the pulp mill after pulp dilution, screening, cleaning, and thickening tend to be the most heavily contaminated. Furthermore, increased temperature and processing time promotes greater dispersion of wood-derived components into the water. Pulp brightening also releases additional material into the process water and can alter the physio-chemical nature of the contaminants already present (Thornton, 1994).

White water is a term used to describe most of the process waters at mechanical pulp and paper mills. The name arises from the white, cloudy appearance resulting from the release and dispersion of DCS from the wood, as well as from fibers and fines
remaining within the water after pulp separation has occurred. Depending on the exact flow of water throughout a particular mill, white water may be a combination of papermachine water, water from pulp screening, cleaning, and thickening, chip washer water, and bleach plant filtrates.

1.3 Problems Associated with Closure of Water Systems at Mechanical Pulp and Paper Mills

A wide range of DCS can be found in the white waters of integrated newsprint mills. The components dissolved and dispersed during mechanical pulping are comprised of carbohydrates, lignin, extractives, and inorganics (Figure 1-2) (Wearing et al., 1985a; Zhang et al. 1999). Papermachine white water may contain additional substances including lignin derivatives, starches, sizing agents, defoamers, dyes, surfactants, slimicides, and synthetic polymers (Vendries and Pfromm, 1988). At mills with open water systems, the concentration of these contaminants is kept low due to the dilution effect of incoming fresh water. However, system closure at mills leads to increased contaminant levels. A closed white water system can contain more than 3% total solids, of which more than 70% are in either dissolved or colloidal forms (Cronin, 1996). Suspended solids, such as fibers and fines, makeup the remaining 30%. The net effect of process water closure is increased DCS and ionic concentration.

The individual contaminants have different effects on paper strength and optical properties (Wearing et al., 1985a). Lignin is believed to bind to fibers rendering the surfaces hydrophobic. This inhibits interfiber hydrogen bonding and therefore reduces sheet strength. As well, lignin and lignin-like substances can lower paper brightness by redeposition onto the pulp (Pranovich et al., 1995). This is due to the chemistry of lignin,
Figure 1-2. Typical compounds dissolved and dispersed from wood into process waters at mechanical pulp and paper mills. (Sjöström, 1993)
which is comprised of phenylpropane building blocks containing quinone structures that inherently give rise to colour. On the other hand, most carbohydrates within the white water are hydrophilic, but are also capable of strong sorption onto pulp, thereby improving strength properties by acting as bridging agents to aid bonding between fibers (Laffend and Swenson, 1968). In contrast, dissolved inorganics can have variable effects on paper strength. For example, anions appear to have little influence on paper properties, but can increase demand for chemical additives, whereas cations decrease fiber swelling, and alter retention, flocculation, and sheet formation. Additionally, the buildup of dissolved inorganics can lead to serious corrosion problems in the mill (Springer et al., 1986).

The accumulation of extractives in process waters is and has been one of the biggest concerns for pulp and paper mills. Lipophilic extractives, commonly referred to as pitch or resin, are mainly composed of fatty acids, resin acids, steryl esters, and triglycerides (Örså and Holmbom, 1994). These compounds tend to be hydrophobic in nature and form associations with each other when released to the process waters. Lipophilic extractives cause papermachine operators significant problems, as these materials lower papermachine runnability and product quality (Lindström et al., 1977; Wearing et al., 1985a). The buildup of these compounds can cause pitch deposition on the papermachine, specks in the paper, decreased wet-web and dry strengths, interference with cationic process chemicals, and impaired sheet brightness. Besides lipophilic extractives, wood also contains hydrophilic extractives such as lignans, sterols, and other phenolic compounds that are more or less soluble in the process waters. These compounds also accumulate with water system closure and can lower paper strength.
properties (Zhang et al., 1999). Unfortunately, removal of these dissolved substances from white water is both difficult and costly.

The buildup of resin and fatty acids in process waters may cause the most detrimental problems for mills. Water surface tension is largely controlled by the presence of materials having both hydrophobic and hydrophilic functionalities. In white water, these acids are ionized resulting in compounds that contain both chemical groups. This hinders sheet formation by interfering with the forces that normally pull fibers together, thus reducing bond strength and bonded area (Springer et al., 1986). Consequently, wet-web strength properties of the形成 paper are significantly reduced, resulting in increased sheet breaks.

The accumulation of DCS in process waters can lead to other problems for mechanical pulp and paper mills, and ultimately hinder high-speed papermaking processes. In order to maintain production on modern high speed papermachines, variables such as pH, temperature, and white water composition need to be tightly controlled (Garver et al., 1997). Increased DCS in the white water can cause runnability problems including deposition on the press role, felt plugging, and reduced drainage rates. The buildup of DCS can also reduce the efficiency of retention aids. All of these reduce paper machine efficiency, lower paper quality, and initiate felt picking.

Multiple research projects have investigated the effects of DCS on paper properties (Brandal and Lindheim, 1966; Lindström et al., 1977; Wearing et al., 1985a; Zhang et al., 1999; Bessonoff et al., 2000). One study investigated handsheet strength properties of two typical newsprint furnishes when formed in water contaminated with dissolved and colloidal substances (Wearing et al., 1985a). It was shown that wet-web
tensile strength was impaired as a result of lower water surface tension and reduced fiber-fiber interactions. Surface tension was found to decrease as a function of total DCS concentration. The dry strength properties were also affected, which was attributed to both decreased bonded area and decreased bond strength. Handsheet brightness was also lowered. More recently, it has been shown that the accumulation of DCS during white water recycling impaired both the physical and optical properties of the resulting paper (Zhang et al., 1999; Zhang et al., 2000). Specifically, the presence of dissolved resin and fatty acids lowered wet-web strength properties, and hindered sheet consolidation, as was reflected by reduced sheet density, internal bonding, and tensile strength. It was also shown that the accumulation of colloidal particles significantly lowered paper porosity, water absorption, and brightness.

The effect that process water contaminants have on paper produced from secondary fiber has also been examined (Springer et al., 1985; Springer et al., 1986). Different organic and inorganic model compounds were added during handsheet production to investigate their effects on sheet tensile index. The addition of organic contaminants including kraft lignin, water soluble wood extractives, and defoamer all resulted in lower sheet strength. With the exception of defoamer, the effects of the organic contaminants reached maximums at low addition levels (<4 ppm). Kraft lignin and defoamer addition lowered sheet strength properties by reducing the interfiber bond strength per unit area. It was apparent that lignin and defoamer sorption onto the fiber surfaces interfered with bonding. As for the various inorganic contaminants added, only iron was found to significantly lower sheet strength. Sodium and calcium did not, while alum had only a minimal adverse effect on tensile index. The addition of iron was found
to disrupt sheet formation by enhancing fiber flocculation, which resulted in nonuniform areas of high and low fiber content. As well, the addition of iron significantly decreased fiber swelling, thereby reducing the interfiber bonded area. These studies further illustrate the need for contaminant removal at mills attempting to close their water systems.

The accumulation of dissolved inorganics in a white water system was simulated to investigate possible effects on recycled linerboard (Vendries and Pfromm, 1998). With increased water recycling, a corresponding increase in the concentrations of dissolved inorganic and organic materials was observed. The fibers were found to have a maximum adsorption capacity for aluminum, sulfur, sodium, chloride, and calcium that was low enough to allow a steady buildup to occur. Increases in colloidal substances, fibers, and fines were also noted. Since it is difficult to control many of the inorganics present in a water system, the high ionic strengths attained with further recycling have the potential to cause sheet properties to deteriorate to unacceptable levels, as well as cause corrosion problems within the mill.

Along with the direct concerns over DCS accumulation in white water and their affects on pulp and paper properties, it has also been recognized that mills will need to place a greater emphasis on limiting microbiological growth within water systems. The buildup of organic materials in white water serves to increase biological activity by providing greater nutrient sources for bacteria (Gudlauski, 1996). Microbial growth can lead to severe slime deposition and corrosion, the formation of volatile gases, and to the production of organic acids that affect fiber bonding. Paper quality can be reduced by the introduction of defects from microbial deposits, and sheet strength can be affected as a
result of fiber spoilage. Papermachine runnability problems such as sheet breakage and screen plugging may also increase with higher levels of growth. As mills move towards closure, changes in process water contaminants and decreased levels of dissolved oxygen will shift microbial populations to include a higher percentage of anaerobes. These bacteria can create new problems for mills by producing odourous gases and greater amounts of acid in comparison to aerobes. Moreover, the exact problems created by a changing bacterial population within a water system are very difficult to predict.

1.4 The Nature of Colloidal Particles in Pulp and Paper Mill Process Waters

The nature of the colloidal particles in the process waters at mechanical pulp and paper mills is complex. Unfortunately, a clear understanding of colloidal particles is difficult to ascertain due to the number of components present in white water, and to the different physio-chemical mechanisms that may occur during dispersion of those components from the pulp that ultimately lead to particle formation. Colloidal particles are primarily composed of lipophilic extractives, lignin, and inorganic materials, with rapid dispersion of extractives and lignin occurring mainly during the first refining stage (Back and Allen, 2000). Due to the fact that these components are more or less hydrophobic, colloidal particles are formed upon release into water. Initially, the different compounds may be separate from each other, however, the various processes at a mill cause relatively homogeneous particles to form quickly.

Colloidal particles tend to be in a dispersed state, with particle stability controlled by both electrostatic and steric stabilization mechanisms (Sundberg et al., 1994a; Sundberg et al., 1996). Natural surfactants, such as ionizable resin and fatty acids located on the particle surfaces help to partially stabilize the more hydrophobic fats and sterols
located inside the particles. The resulting carboxylate groups present on the surface gives rise to particles with negative surface charges, which in turn results in particles having zeta potentials. As the colloidal particles move throughout a mill, they can either be deposited on equipment surfaces or on pulp fibers. Ideally, mills want to minimize deposition on equipment, and improve the amount of particles carried through with the final product or to the effluent. However, the chance for colloidal particles to flocculate and deposit on equipment increases when the concentration of free particles is high. It has been shown that simple electrolytes such as CaCl₂, as well as hemicelluloses, especially glucomannans, and polygalacturonic acids can increase particle stability and at the same time induce higher rates of aggregation (Sundberg et al., 1994a; Sundberg et al., 1994b; Holmbom et al., 1995; Sundberg et al., 1996). These components may help bridge colloidal particles to the negatively charged fiber surfaces, and therefore reduce deposition on machinery.

The concentration of colloidal particles present in white water is dependant on the location within the mill (Wearing et al., 1985b). Concentrations tend to be highest after initial refining and are controlled by factors such as temperature, pH, and for highly recycled white water, the concentration of particles already present within the water (Örså et al., 1995). It has been suggested that the dissolution of organic material from wood can decrease as levels within the water increase after many recirculations (Jahren et al., 1999). At one integrated TMP mill, researchers investigated DCS variation in papermachine white water and found that the concentration of colloidal particles was highest at the head box, and decreased through the flat box to the fourth press (Garver et al., 1997). This suggests that as web consistency increases during paper formation, more
of the colloidal particles become entrapped in the sheet. However, with increased closure and consequently increased concentrations of particles, levels will likely rise beyond the removal capacity of the sheet.

1.5 Mechanical Treatments for Contaminant Removal from Pulp and Paper Mill Process Waters

At present, mechanical vapour recompression (MVR) is the only established process to reduce effluents to very low levels at mechanical pulp mills (Stevenson, 1992; Gerbasi et al., 1993). This strategy involves the collection of multiple condensate streams from evaporators, followed by biological treatment and filtration before the water is returned to the process (Stevenson, 1990). In this process, the contaminants are concentrated and sent to the furnace before the remaining solids are collected, cooled, and finally land filled. Unfortunately, this process has major drawbacks, particularly its capital-intensive nature, and therefore is unlikely to be installed in existing mills (Wiseman et al., 1996).

In paper mills, disc filters are often used to recover fiber from paper machine waters, thereby partially cleaning the water prior to reuse (Cronin, 1996). Disc filters have the advantage of not being affected by slight changes in volume or concentration of DCS within the white water. Additional filtration, such as an Algas filter, following the disk filter can further improve water recycling processes (Webb, 1997). Although a clarification system can also be used to remove some of the suspended solids in the white water, such systems only remove larger particles, and DCS continue to buildup with reduced fresh water use. These types of mechanical treatment alone are sufficient for the production of brown paper grades, which are not as sensitive to problems such as colour
or surface properties. However, the production of high-grade newsprint and other quality printing papers requires extensive cleaning of the ensuing process waters.

Along with filtration and retention, the removal of contaminants from papermachine white waters also occurs by flocculation and deposition (Garver et al., 1997). During sheet formation, a substantial decrease in the concentration of anionic species and UV-absorbing components occurs, while some extractives, clay, and inorganic salts show only small changes. As a result, these compounds can quickly accumulate to unsuitably high levels.

Another technology that is in limited use at some mechanical pulp and paper mills is membrane filtration (Webb, 1997). Membranes are filters that have very small pore sizes and can therefore remove greater amounts of contaminants (Cronin, 1996). Reverse osmosis, nanofiltration, ultrafiltration, and microfiltration are the most popular membrane technologies currently used. Membranes function by concentrating solids into smaller volumes of water before treatment and disposal. However, this process is not ideal because contaminants are simply concentrated rather than degraded. Furthermore, installation is expensive and membrane plugging is very common.

Other physical/chemical treatment strategies for contaminant removal from mechanical pulp and paper mill process waters have recently been evaluated. Dissolved-air flotation (DAF) technology has shown the capacity to remove suspended solids and colloidal particles from mill process waters (Anon., 1986; Krofta et al., 1987; Thurley et al., 1996). This strategy dissolves air into the water using high pressure, which results in solids rising to the water surface with the generated bubbles, where they are subsequently skimmed off. Dissolved-air flotation has been successfully applied in deinking mills,
however, it is much less effective at removing contaminants from pulp mill white waters, especially dissolved substances (Richardson et al., 1996). As a result of this and the high associated energy costs, this technology is not widely used.

The use of organically tailored heulandite minerals was recently examined for the removal of DCS from recycled white water (Bouffard and Duff, 1999). Heulandite aggregates treated with hexadecyltrimethylammonium cations efficiently removed resin and fatty acids, as well as other low-molecular weight organic compounds. However, most higher-molecular weight colloidal particles are not affectively removed. This is likely due to the large size and hydrophobic-hydrophilic nature of these particles. This drawback, as well as the large doses of tailored heulandite required, limits the implementation of this technology.

Oxidation treatments using reactants such as hydrogen peroxide and ozone have been shown to remove both organic and inorganic contaminants from mechanical pulp mill sewer waters and effluent (Amoth, 1992; Roy-Arcand et al., 1995; Roy-Arcand et al., 1996). Ozone treatments in particular are able to greatly reduce the DCS levels and colour of mill effluents. However, the high cost of ozone generation currently makes this technology uneconomical for most mills.

1.6 The Potential of Biological Treatment to Remove Contaminants from Pulp and Paper Mill Process Waters

An emerging alternative to physical or chemical treatment is the use of microorganisms to degrade DCS within pulp and paper mill process waters. Various biological treatments are currently being assessed and show great potential. The use of fungi, particularly white-rot fungi, and fungal enzymes to reduce colour, toxicity, BOD
and COD in kraft pulp mill effluents is well established (Archibald et al., 1990; Galeno and Agosin, 1990; Feijoo et al., 1995). For example, spent liquor from the first alkaline extraction stage \((E_1)\) following chlorination contains polymeric, chlorinated, and oxidized lignin derivatives primarily responsible for effluent colour (Sundman and Kirk, 1981). Studies have shown that the white-rot fungus *Phanerochaete chryosporium* is capable of removing 60-80\% of this colour, while reducing BOD and COD by 40\% and 60\%, respectively (Sundman and Kirk, 1981; Eaton et al., 1982). This observed decolourization results from a combination of chromophore destruction and decomposition of the polymers to low-molecular weight, colourless, soluble/volatile products. Fungal treatments of \(E_1\) effluent after chlorination have also been shown to reduce the chlorine content per \(C_9\) lignin unit by 30-45\%. It is believed that methylation and oxido-reduction reactions are involved in dechlorination, with benzyl alcohol and veratrole being the main products generated. Other work has identified the white-rot fungus *Trametes versicolor* as a potent decolourizing agent (Pallerla and Chambers, 1995). Treatment of bleach plant effluents with encapsulated fungi resulted in a 76-83\% removal of colour, and a 43-59\% reduction in AOX.

More specifically, the use of enzymes to treat kraft mill effluents has been examined. The direct application of oxidative enzymes to mill effluents, as opposed to treatment with the intact fungi that produced the enzymes, has been shown to improve treatment efficiency (Davis and Burns, 1990; Ferrer et al., 1991; Davis and Burns, 1992; Al-Kassim et al., 1994a). Lignin and horseradish peroxidases, and laccases have received the most attention, with the decreases in effluent colour, BOD, COD, and chlorine content observed during fungal treatments attributed to the activities of these
types of enzymes. As these oxidative enzymes are relatively non-specific in terms of substrates, they have the benefit of being applicable for the treatment of a large variety of aromatic contaminants. Lignin peroxidases, horseradish peroxidases, and laccases have all been shown to catalyze the polymerization of aromatic compounds in kraft mill effluents, resulting in significantly reduced effluent colour (Davis and Burns, 1990; Ferrer et al., 1991; Davis and Burns, 1992). Both free and immobilized enzymes were able to oxidize various phenolic compounds to aryloxy radicals, which then polymerized into larger structures. It has been shown that the resulting products have low solubility and are readily precipitated from solution, thus demonstrating the potential for concurrent removal of aromatic pollutants and colour from process waters (de Jong et al., 1992; Nicell et al., 1992; Al-Kassim et al., 1994b; Nicell et al., 1995).

In contrast to the extensive amount of work that has been carried out on the fungal and enzymatic treatment of kraft mill effluents, little has been reported on the biological treatment of mechanical pulp and paper mill process waters. Some of the few studies that have been carried out include the use of activated sludge systems to treat white water at a TMP mill and a corrugating medium mill (Eder et al., 1992). This strategy allowed for good reductions in both BOD and COD. Another system that is currently being assessed involves the biological treatment of in-line waters to remove process water organics. In this case an anaerobic bed reactor that was inserted into a closed water circuit at a paper mill was shown to decrease the levels of DCS present in the white water (Pichon et al., 1996). A recycled fiber board mill has also successfully installed a combined anaerobic and aerobic treatment system to remove organic and inorganic impurities from its highly closed process water system (Habetsm et al., 1997). Biofilm processes have recently
gained wide interest as a method of treating pulp and paper mill process waters due to their potential for high loading (Ødegaard et al., 1994; Brock-Due et al., 1994). For example, an aerobic moving-bed process is currently being used in the full-scale treatment of effluent from an integrated newsprint mill (Brock-Due et al., 1997). Unfortunately, all of these treatment strategies are expensive, slow, and consume energy as large water volumes must first be cooled to temperatures around 35°C to allow for sufficient microorganism growth.

The use of bacteria for TMP white water treatment has also been evaluated (Jahren et al., 1999). One attractive option is the use of thermophilic bacteria that grow optimally at temperatures of 55°C or higher. This alleviates the need to cool process waters, which would enable recycling of the treated water without additional energy requirements. In laboratory experiments, anaerobic and aerobic bacterial treatments were integrated into a model closed water system. The anaerobic reactor removed most of the organic material released, including carbohydrates, lignin-like material, and 84% of the soluble COD. The aerobic reactor was less effective at removing carbohydrates and soluble COD from the original process water. However, steady increases in total soluble COD were noted after multiple recirculations when either anaerobic or aerobic treatment was incorporated into the water system. This was presumably due to the buildup of more recalcitrant compounds.

Mechanisms for contaminant removal from process waters have been proposed for aerobic bacterial treatment. Levels of extractives such as resin and fatty acids can be effectively reduced by adsorption onto biomass, and degraded by bio-oxidation and air oxidation (Liu et al., 1996). These acids are believed to quickly bind to the sludge in the
bioreactor and are then gradually broken down, primarily through bio-oxidation. Due to these oxidation steps, aerobic treatment is capable of removing resin and fatty acids much more efficiently than anaerobic treatment. However, for other contaminants the opposite may be true. This suggests that a combined anaerobic/aerobic treatment system might provide the best compromise.

Some enzyme systems are also effective at reducing contaminant levels in white water at mechanical pulp and paper mills. Triglycerides present in colloidal particles have been identified as one of the most troublesome components that cause pitch problems. Lipase enzymes are most active at oil-water interfaces of emulsified substrates, and therefore are effective against colloidal particles in process waters (Macrae and Hammond, 1985). The action of this class of enzyme is thought to destabilize colloidal particles through the destruction of triglycerides and other ester bonded extractives, and therefore limit deposition on equipment surfaces (Irie et al., 1990). In newsprint mill trials, the addition of a mix of three enzymes, resinase A from Aspergillus, lipase AYL from Candida ruosa, and lipase OF from Candida cylindracea, to the pulp greatly reduced paper machine cleaning frequency from 2.5 to 0.21 times per day (Fujita et al., 1992). Holes and spots on the paper caused by pitch were also reduced by over 65%. The degree of triglyceride hydrolysis was determined to be about 75%.

Pectinases also have the potential to decrease DCS levels in process waters at mechanical pulp and paper mills (Thornton, 1994). It has been shown that as much as 50% of the anionic polyelectrolytes present in peroxide-bleached mechanical pulp suspensions are polygalacturonic acids, which can bind to both colloidal particles and fiber. Enzymatic treatments of pulp or filtrate using commercial pectinases have been
found to degrade polygalacturonic acids to galacturonic acids, thereby greatly reducing their ability to form complexes with cationic paper making additives. This strategy, as well as the use of other enzyme systems, shows good potential for further development.

1.7 Combined Fungal and Enzyme Treatment for Contaminant Removal From Pulp and Paper Mill Process Waters

Most biological treatment technologies attempting to remove DCS from process waters have not yet proven to be efficient and/or cost effective for mechanical pulp and paper mills. A newly emerging strategy to circumvent the DCS problem(s) is the employment of fungi and fungal enzymes to degrade the compounds accumulating during water system closure. Recently, it has been demonstrated that fungi and fungal enzymes are capable of degrading or altering many of the detrimental DCS present in white waters into less harmful products and biomass (Zhang et al., 1998; Zhang et al., 2000). In short, this strategy utilizes enzymes produced during fungal growth on small streams of white water, and subsequently releases the enzymes into the main process water stream where most of the treatment occurs. The main advantage of this technology is that only a small stream of process water requires cooling to allow for adequate fungal growth. As the fungus is grown directly on white water without added nutrients or pH adjustment, the resulting enzymes produced are well suited for the degradation of the DCS present in the process waters. Furthermore, the extracellular enzymes produced are able to catalyze reactions with the DCS much faster when compared to the fungi. The enzymes are also much more robust than the fungi and demonstrate some ability to remain active at the elevated temperatures typically found in mills water systems. Thus, this strategy appears
very promising as a means of alleviating some of the problems associated with water system closure.

The white-rot fungus *Trametes versicolor* was identified as having potential for fungal and enzyme treatment of mechanical pulp and paper mill process water based on its demonstrated ability to degrade the contaminants within white water, including extractives (Cai *et al*., 1998). A comprehensive screen of 21 white- and brown-rot fungi revealed that growth of the white-rot fungi *Bjerkandera* sp., *Dichomitus squalens*, *Phanerochaete chrysosporium*, and *Trametes versicolor* was not inhibited by the contaminants within white water. Further investigation of these four fungi demonstrated their capacity for efficient growth directly on white water. Specifically, the growth of *Trametes versicolor* resulted in the greatest removals of both TOC and total extractives from the white water and was therefore chosen for further use in the development of this treatment strategy.

In subsequent work we have shown that *Trametes versicolor* produces a wide range of extracellular enzymes when grown on mechanical pulp mill white waters (Zhang *et al*., 1998; Zhang *et al*., 2002). After analysis, the enzymes were categorized into general groups: oxidative enzymes primarily containing laccases, and hydrolytic enzymes that included lipases and cellulases/hemicellulases. It is believed that the combination of these different enzymes, especially the laccases and lipases, enables the fungus to efficiently degrade or alter many of the DCS present. As a result, it has since been recognized that a better understanding of the many factors controlling extracellular enzyme production during fungal growth on white water is fundamental to the further development of this treatment strategy.
The production of laccases by white-rot fungi grown on various substrates has been well documented (Eriksson et al., 1990; Szklarz et al., 1989; Bourbonnais et al., 1995; Manzanares et al., 1995). Laccases are glycosylated polyphenol oxidases that catalyze the reduction of one dioxygen molecule to two molecules of water, while simultaneously oxidizing local aromatic substrates (Thurston, 1994). *Trametes versicolor* grown on unsupplemented mechanical pulp mill white waters has been shown to produce considerable laccase activities (Zhang et al., 2002). One group investigating the regulation of laccase expression in *T. versicolor* determined copper and nitrogen levels to be factors that control laccase gene transcription (Collins and Dobson, 1997). As the concentration of copper or nitrogen in the fungal cultures increased, a concurrent increase in laccase gene transcription occurred. The addition of various compounds such as 1-hydroxybenzotriazole and 2,5-xylidine also increased laccase activity. Similarly, *Trametes villosa* has been reported to have genes encoding multiple laccase isozymes that are differentially regulated, with some being constitutively expressed and others being inducible (Yaver et al., 1996). Other research has focused on identifying inducers of laccase synthesis by different white-rot fungi. One group found that syringaldazine addition to *Coriolus hirsutus* grown under submerged cultivation conditions improved laccase yield by a factor of ten (Koroljova-Skorobogat'ko et al., 1998). Improved laccase induction in *Ceriporiopsis subvermispora* and *Cyathus stercoreus* has also been demonstrated with the addition of various low-molecular weight aromatic compounds (Sethuraman et al., 1998). Of the many compounds tested, 3,4-dimethoxycinnamic acid triggered the greatest increases in laccase production by factors of 2.54 and 2.90 above the controls, respectively. These results suggest that the small phenolic compounds, such
as lignans and sterols, present within mill process waters are effective at promoting laccase production.

Extracellular lipase production has been extensively studied for many different fungi, and depending on species, can be induced by various carbon or nitrogen sources, especially oils (Pal et al., 1978; Jacobsen et al., 1989; Papaparaskevas et al., 1992; Salleh et al., 1993; Hädrich-Meyer and Berger, 1994). However, unlike laccases the many factors controlling lipase production in white-rot fungi are as of yet not well classified. Lipases are glycerol ester hydrolases that hydrolyze the ester bonds between glycerol and the acyl moieties (Vorderwülbecke et al., 1992). Due to the hydrophobic nature of triglycerides and other ester bonded substances, lipases are most active at oil-water interfaces (Macrae and Hammond, 1985). In fact, lipase activity against emulsified substrates is considerably higher when compared with activity towards dissolved esters (Verger, 1984). In recent work, *Trametes versicolor* grown on mechanical pulp and paper mill process water was observed to control lipase activity in response to the availability of specific substrates (Zhang et al., 2002). When grown on white water lacking colloidal particles, and therefore lacking nearly all ester bonded extractives, *T. versicolor* produced very low levels of lipase in comparison to when grown on white water containing colloidal particles. Thus, the presence of ester bonded extractives appears to be an important stimulus for the production of lipase.

The production and regulation of cellulolytic enzymes has been studied for many types of fungi. The O-glycosyl hydrolases are a widespread group that hydrolyze the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety (Eriksson et al., 1990). Generally, white-rot fungi are thought
to produce these enzymes constitutively, as they use these enzymes for primary metabolism. Mechanical pulp and paper mill process waters contain considerable amounts of dissolved carbohydrates and when *Trametes versicolor* was grown on white water, both cellulolytic and hemicellulolytic enzymes were readily produced (Zhang et al., 2002). The combination of these enzymes was able to remove nearly all of the carbohydrates present, initially utilizing glucose and mannose, and later galactose.

When the culture filtrates produced by *Trametes versicolor* during growth on white water were used to treat fresh white water, a significant decrease in total DCS was observed. This decrease was primarily attributed to the removal of extractives. Assessing the extractives specifically, the lignans, sterols, steryl esters, and triglycerides were all reduced by over 90%, while 20-50% of the resin and fatty acids were removed. Laccase enzymes are thought to be responsible for catalyzing the removal of lignans and sterols. These low molecular weight phenolic compounds were polymerized into high molecular weight lignin-like material. The ability of laccases to catalyze fatty acid degradation has also been recently demonstrated, and this may have contributed to the decreased fatty acid content within the white water (Zhang et al., 2002b). Lipase activity is thought to have decreased the level of ester bonded extractives, including triglycerides and steryl esters. High cellulolytic and hemicellulolytic activities were also detected, which were likely responsible for hydrolyzing cellulose and hemicellulose moieties within the white water into monomeric sugars. These promising results illustrate the potential of this strategy as a means of alleviating the problems associated with water system closure at mechanical pulp and paper mills.
1.8 Enzymatic Fiber Modification of Mechanical Pulp

The proposed fungal and enzyme treatment strategy will release enzymes directly into the main water systems at mechanical pulp and paper mills. This process should not only facilitate DCS removal from white water, but may also allow for enzymatic fiber modification to alter specific paper properties. Over the last two decades there has been a substantial amount of research conducted attempting to apply different enzymes to modify mechanical pulps in order to enhance product quality and reduce refining energy demands. Previous research has demonstrated that the application of different enzymes directly to mechanical pulp is capable of altering a variety of pulp and paper properties (Kirk and Jeffries, 1996; Bajpai, 1999; Wong and Mansfield, 1999). For example, the actions of cellulolytic and laccase enzymes on fibers have been investigated and show good potential for increased use. Considering these same types of enzymes have been identified in the culture filtrates produced by T. versicolor grown on white water, it is possible that fungal and enzyme treatment will generate additional benefits for mills implementing this technology.

Specifically, the ability of cellulolytic enzymes to alter specific mechanical pulp properties has been explored. Cellulase and xylanase treatments have aimed at enhancing fiber bonding, as well as lowering refining energy consumption. The goal herein is to increase fiber fibrillation with the addition of enzymes and thereby improve interfiber bonding and sheet strength. However, it appears that different enzyme preparations have quite diverse effects on mechanical fibers. For example, some cellulolytic enzymes have been shown to increase fibrillation, while others appear to remove fibrils and smooth the fiber surfaces (Jeffries and Lins, 1990; Jackson et al., 1993). It is possible that
defibrillation is responsible for the observed increased freeness of secondary fiber, resulting in higher drainage rates without significant reductions to strength properties (Bhardwaj et al., 1995; Stork et al., 1995). This improvement in pulp dewatering has allowed for increased papermachine speeds. It is currently unknown how the cellulolytic enzymes produced by *T. versicolor* actually modify mechanical fibers, but further research may reveal their potential for expanded use at mechanical pulp mills.

The potential for laccase treatments of mechanical pulps has also been recently investigated. The application of laccase to deconstructed wood chips reduced the refining energy required to achieve a given freeness and at the same time increased tensile and tear strengths of the ensuing paper (Mansfield, 2002). It was suggested that the improved paper strength properties resulted from some form of lignin activation, which has also been shown to increase auto adhesion between fibers during the production of medium density fiberboard (Felby et al., 1997). Laccase catalyzed modifications to lignin on fiber surfaces also have the potential to improve the bleachability of different mechanical pulps. Research utilizing Douglas-fir derived pulp showed that laccase treatments increased the bleached brightness, while significantly reducing hydrogen peroxide consumption (Chandra et al., 1998). These results indicate that fiber modification with laccase is capable of enhancing paper strength and optical properties, which warrants additional research in the future.

### 1.9 Research Objectives

The mechanical pulp and paper industry has and continues to face economic and environmental pressures to reduce fresh water usage by improving processes and recycling process waters. However, it is apparent that current technologies to remove
contaminants from process waters are inadequate and/or too expensive to implement. As a result, water system closure at nearly all existing mills has yet to be realized. Our group has recently demonstrated that a combined fungal and enzyme treatment strategy has the potential to meet the requirements of mills and allow for increased system closure. The primary objective of this thesis is to further develop this treatment strategy by providing a better understanding of the fungal enzymes released into the process waters, and their effects on white water, fiber, and paper properties.

This thesis will address: 1) Extracellular enzyme production by *Trametes versicolor* grown on white water at elevated temperatures. 2) The thermostabilities of the extracellular enzymes produced. 3) An evaluation of scaled-up fungal growth in a bioreactor. 4) Physio-chemical modifications to colloidal particles and dissolved substances within the white water resulting from enzyme addition. 5) Enzymatic changes to white water and fiber properties. 6) The influence of enzymatic treatment on paper properties. The results presented in the following chapters of this thesis attempt to answer these unknowns, and allow for further evaluation of this treatment strategy.
CHAPTER II. Materials and Methods

2.1 Mill White Water and Pulp Samples

Howe Sound Pulp and Paper Ltd. provided the white water and pulp samples used for this research. The white water was collected from the cloudy white water chest of the disk thickener, while the pulp was collected from the main TMP line after the latency chest. The chip supply used at that time was a mixture of interior spruce/pine/fir and coastal hemlock/balsam from British Columbia. The mill consumes on average 43 m$^3$ of fresh water per tonne of newsprint produced, which translates into 25800 m$^3$ of fresh water per day.

2.2 Fungal Growth on White Water and Enzyme Production

2.2.1 Small Scale Fungal Growth in Flasks

*Trametes versicolor* was grown at 30°C as a fungal mat on 500 mL of malt extract broth (Difco) in a 2 L Erlenmeyer flask without shaking for 7 days. The mat was then separated from the broth, thoroughly washed with sterile distilled water, and then homogenized with an additional 50 mL of water in a Waring blender for 20 seconds. The homogenate (4 mL) was used to inoculate 125 mL of sterile white water in 500 mL Erlenmeyer flasks, which were then incubated at 30°C, 45°C, or 60°C with shaking at 150 rpm. After 2, 3, 5, 7, and 9 days of growth, the fungal mycelium was removed by filtration through a fine nylon mesh, and the culture filtrate containing extracellular enzymes was collected. A minimum of 5 flasks were combined for each collection day and assayed immediately to determine enzyme activities, and/or used to treat fresh white water.
2.2.2 Large Scale Fungal Growth in a Bioreactor

As before, *Trametes versicolor* was grown at 30°C as a fungal mat on malt extract broth (Difco) without shaking for 7 days. After the growth period, the mats were separated from the broth, thoroughly washed with sterile distilled water, homogenized with additional water in a Waring blender for 20 seconds, and finally combined in a sterile glass bottle for a total volume of approximately 600 mL. The homogenate was used to inoculate 16 L of sterile white water in an 18 L capacity LH Fermentation 2000 Series bioreactor (Emeryville, CA, USA) (Figure 2-1). The reactor was maintained at 30°C, and continually stirred with the mixer set at 600 rpm. Air was fed into the headspace above the water surface at 5 L/min, which resulted in dissolved oxygen levels ranging between 30-40%. After 1 and 2 days of growth, 50 mL culture filtrate samples were collected, and assayed to determine enzyme activities. After the 3rd day, the bioreactor was emptied, and the culture filtrate was collected after fungal mycelium removal by filtration through a fine nylon mesh. This filtrate was used immediately to treat fresh white water, as well as assayed to determine enzyme activities.

2.3 Determination of Enzyme Activities Produced by *Trametes versicolor*

2.3.1 Laccase Assay

Laccase activity was determined spectrophotometrically by the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Wolfenden and Willson, 1982). The assay reaction mixture contained 0.1 mL of the fungal culture filtrate added to 0.5 mM ABTS (Sigma) in 0.1 M sodium acetate buffer (pH 4.8) for a total volume of 3 mL. The reaction was monitored as the increase in $A_{420}$ ($\varepsilon_{420} = 3.6 \times 10^{4}$ M$^{-1}$ cm$^{-1}$) over 10 minutes at 50°C. At least 4 replicates were done for each measurement. Laccase activity
Figure 2-1. 18 L capacity LH Fermentation 2000 Series bioreactor used for large scale growth of *Trametes versicolor*.
was then expressed in units per L of fungal culture filtrate, where 1 unit equals 1 μmol of ABTS oxidized per minute.

2.3.2 Lipase Assay

Lipase activity was measured spectrophotometrically by monitoring the quantity of p-nitrophenol liberated from either p-nitrophenol palmitate (PNPP) (Sigma) or p-nitrophenol laurate (PNPL) (Sigma) (Winkler and Stuckmann, 1979). The reaction mixture included 0.1 mL of 15 mM PNPP or PNPL dissolved in 2-propanol added dropwise to 1.5 mL of 0.1 M sodium acetate buffer (pH 5.5) containing 0.4% (v/v) Triton X-100 (Bio-Rad Laboratories) and 0.2% (w/v) gum arabic (Sigma). After heating the substrate emulsion to 50°C for 15 minutes, 0.4 mL of the fungal culture filtrate was added to obtain a total volume of 2 mL. The reaction was monitored as an increase in A₄₀₄ over 30 minutes, with a minimum of 5 replicates done for both PNPP and PNPL. A p-nitrophenol standard curve was used to convert absorption into concentration. The two substrates yielded very similar results, and therefore the lipase activities presented are averages of the values obtained for PNPP and PNPL. One unit of lipase activity corresponds to 1 μmol of p-nitrophenol liberated from PNPP or PNPL in one minute, per L of fungal culture filtrate.

2.3.3 Cellulase and Hemicellulase Assays

Cellulolytic and hemicellulolytic enzyme activities were determined by the generation of reducing sugars from different substrates (Miller, 1959). These substrates included 1% carboxymethylcellulose (CMC) (Sigma) to measure endoglucanase activity, 0.5% konjac glucomannan (Megazyme) to measure glucomannanase activity, 0.5% locust
bean gum galactomannan (Sigma) to assay galactomannanase activity, and 0.5% xylan from birchwood (Sigma) to determine xylanase activity. The reaction mixtures contained 0.5 mL of fungal culture filtrate added to 0.5 mL of substrate in 0.05 M sodium citrate buffer (pH 4.8). The reaction mixtures were incubated for 30 minutes at 50°C, at which time 3 mL of dinitrosalicylic acid (DNS) solution was added, mixed, boiled for 5 minutes, and diluted with 20 mL of distilled water prior to determination of absorbance at 540 nm. For all enzymes assessed, at least 4 replicates were done, and controls that lacked enzyme or substrate were run concurrently. Glucose, mannose, and xylose standard curves were used to convert absorbance into concentration of sugars released from CMC, gluco and galactomannans, and xylan, respectively. Endoglucanase, gluco and galactomannanase, and xylanase activities were then expressed as units per L of fungal culture filtrate, where 1 unit equals 1 μmol of glucose, mannose, or xylose released per minute.

2.3.4 Proteinase Assay

Proteinase activity was determined using Protazyme AK tablets (azarine-crosslinked casein) (Megazyme). Protazyme AK tablets were dissolved in 1 mL of 0.1 M sodium phosphate buffer (pH 7.0) containing 1% (w/v) SDS (Sigma), and allowed to equilibrate for 5 min at 40°C. Exactly 1 mL of fungal culture filtrate was added to each dissolved Protazyme AK tablet and allowed to incubate for 10 min at 40°C with continuous agitation. The reaction was terminated by the addition of 10 mL 2% (w/v) trisodium phosphate (pH 12.3) with vigorous stirring on a vortex. The reaction tubes were then allowed to stand at room temperature for 2 minutes prior to filtration through Whatman No. 1 filters. The absorbance of the filtrates was measured at 590 nm against a
substrate blank. Proteinase activity is expressed in protease units per L of culture filtrate, where one protease unit is defined as the amount of enzyme that produces the equivalent of 1 µmol of tyrosine per minute from a soluble casein at pH 7.0 and 40°C. The conversion formula used was: Protease (U/L) = 42.0 × A_{590} + 2.9, which was derived from the fungal serin proteinase K.

2.4 Determination of Changes to Enzyme Activities at 65°C

The heat tolerances of laccase, lipase and cellulolytic/hemicellulolytic enzymes at 65°C were determined from culture filtrates produced after 3 or 9 days of fungal growth at 30°C. For both, 500 mL of the culture filtrates were heated to 65°C in 1 L Erlenmeyer flasks with mixing (150 rpm) and immediately assayed as described in the previous sections to determine initial enzyme activities. Over a 10 hour period, the culture filtrates were maintained at the same conditions (65°C) and assayed to identify changes to the various activities. The data is reported as percentage decreases of initial enzyme activities. This evaluation was done in duplicate.

2.5 Enzymatic Treatments of White Water and Pulp

2.5.1 Small Scale Enzyme Treatments of White Water and Pulp

Enzymatic treatments of fresh white water were carried out at 65°C for either 1 or 2 hours using the culture filtrate produced after 3 days of fungal growth at 30°C. Initially, 1.3 L of white water was heated to temperature (65°C) in a 4 L Erlenmeyer flask, at which time 0.65 L of preheated fungal culture filtrate was added (2:1 ratio of white water to culture filtrate), and maintained at 150 rpm mixing. After 1 or 2 hours, the 2 L total volume was divided equally into four 1 L Erlenmeyer flasks, and mechanical
pulp was then added to each to attain 1% consistency. These four flasks were mixed (150 rpm) at 65°C for an additional 1, 2, 4, or 6 hours, after which the pulp was separated from the white water by filtration through a 300-mesh nylon filter. The resulting white water and pulp samples were used for all subsequent experimentation. For all cases, control flasks containing white water without added enzyme were run in parallel. The entire procedure was carried out in duplicate.

2.5.2 Large Scale Enzyme Treatments of White Water and Pulp

Large scale enzymatic treatments of fresh white water were carried out for 2 hours at 65°C using the culture filtrate produced by Trametes versicolor grown in a bioreactor. Initially, 6.5 L of white water and 3.5 L of culture filtrate were heated to temperature, and then combined in a 20 L bucket. Duplicate buckets were prepared in order to produce 20 L total volume of treated white water. The buckets were mixed at 150 rpm for 2 hours, and then mechanical pulp was added to each to attain 1% consistency. The buckets were mixed (150 rpm) at 65°C for an additional 2 hours, after which time the pulp was separated from the white water by filtration through a 300-mesh nylon filter. Finally, the water from the two buckets was combined and used subsequently for papermaking. Control buckets containing white water and pulp without added culture filtrate were also concurrently prepared.

2.6 Alum Post Treatment of Enzyme Treated White Water

Large scale white water treatment was carried out a second time as outlined above, except for the addition of an alum (aluminum sulphate) post treatment step following enzyme treatment. In this case, the collected white water (20 L) after pulp
removal was again heated to 65°C, and mixed with a Caframo Type RZR 50 stirrer (Wiarton, Ont., Canada). Alum was added (400 mg/L), and after 5 seconds of stirring at high speed, the stirring rate was gradually reduced to zero over the next 15 seconds. Any precipitate that formed was allowed to settle overnight, and the remaining water was decanted off and used for papermaking. A control bucket containing white water without added enzyme was also prepared.

2.7 Determination of Physical Properties of White Water and Pulp

2.7.1 Particle Size and Zeta Potential Analysis

Particle size and zeta potential distributions of enzyme treated and control white waters were measured using a Mastersizer 2000 Particle Size Analyzer and a Zetasizer 2000 Zeta Potential Analyzer (Malvern Instruments Ltd., Malvern, Worcs., England), respectively, following manufacture’s instructions. Prior to measurement, the water samples were centrifuged at 500 × g for 30 min and decanted to remove any suspended solids. The particle size distributions were plotted against both percentage volume and number over the measurement range, while the zeta potential analysis presented is the average of the distributions measured. For each sample, particle size and zeta potential evaluations were measured in triplicate.

2.7.2 Molecular Weight Distribution of Phenolic Compounds

Gel Permeation Chromatography (GPC) was used to determine the molecular weight distribution of phenolic components present in both control and treated white water samples. GPC analyses were carried out on a Waters 625 liquid chromatography system (Millipore Corp., Milford, Mass., USA), using a series of four TSK-GEL columns
(G1000 HXL, G3000 HXL, G4000 HXL and G6000 HXL, Varian, Sunnyvale, CA, USA) with molecular weight cut-offs of $1 \times 10^3$, $6 \times 10^4$, $4 \times 10^5$, and $4 \times 10^7$, respectively. Briefly, 20 mL samples of white water were first freeze dried, and then acetylated by stirring with 1 mL pyridine and 1 mL acetic anhydride overnight. The samples were then filtered and evaporated in the presence of excess ethanol. The samples were dried with $\text{P}_2\text{O}_5$ in a desiccator, and then were redissolved in 5 mL of tetrahydrofuran (THF), and finally injected (20 μL) into the liquid chromatography system. Tetrahydrofuran was used as the eluant, which was set at a flow rate of 1 mL/min, and detected using a Waters 486 UV detector at 280 nm. Calibration of molecular weight was made with polystyrene standards (Toyo Soda Manufacturing Co., Ltd., Tokyo, Japan) under the same conditions.

2.7.3 Colloidal Particle and Fiber Charge Determination

Particle and fiber charge were measured using a Müber Particle Charge Detector, PCD 03 (Müber Analytic Inc., Marietta, GA, USA), connected to a Müber PCD-Titrator. The titrations were conducted following the method of Wågberg et al. (1989), and the Müber PCD 03 instruction manual (1998). For each of the white water samples, 5.0 g was weighed, and then 20.0 g of either 0.001 N Polybrene (1,5-dimethyl-1,5-diazaundecamethylene-poly-methobromide) (Sigma), or 0.001 N poly-DADMAC (polydiallyl-dimethyl-ammonium-chloride) (Müber) were added. Polybrene adsorption was used to calculate total charge, while poly-DADMAC adsorption was used to calculate surface charge. To measure fiber charge, 100 mg (based on oven-dry weight) was added to the corresponding 5 g white water sample. The water samples, with or without pulp, were stirred with polyelectrolyte for 30 min, and then centrifuged at $500 \times g$ for 30 min.
The liquid was decanted off, and then titrated with 0.001 N PES-Na (polyethene sodium sulphonate) (Mütek). The reported fiber charges were calculated as the difference between the white water samples with and without pulp. For each sample, the measurements were done in duplicate.

2.8 Chemical Characterization of White Water Samples

2.8.1 Total Dissolved and Collodial Substances (TDCS)

The TDCS in the different white waters was determined gravimetrically. For each, 20 mL samples were oven dried at 105°C to a constant weight. Four replicates were done for each of the white waters.

2.8.2 Total Extractives (TE) and Total Non-Extractables (TNE)

The TE and TNE in the different white waters were determined gravimetrically. For each sample, 20 mL was extracted three times with 25 mL MTBE. The solvent phase containing extractives was collected and evaporated using mild heat and N₂. The aqueous phase and the evaporated solvent phase were then oven dried at 105°C to a constant weight. Three replicates were done for each of the white waters.

2.8.3 Carbohydrates

The carbohydrates present in the different white waters were quantified using a Dionex DX-500 HPLC system (Dionex, Sunnyvale, CA, USA) controlled by Peaknet 5.10 software. Initially, 40 mL white water samples were extracted three times with 50 mL MTBE, with the aqueous phases then freeze dried for 3-4 days. After drying, samples were transferred to 125 mL Erlenmeyer flasks and 30 mL of 4% H₂SO₄ was added. The samples were then autoclaved at 121°C for 1 hour, cooled, and filtered before
analysis by HPLC. Anion-exchange chromatography of monosaccharides was carried out using a Carbopac PA-1 column, which was equilibrated with 10 mM NaOH, and regenerated after each sample with 250 mM NaOH. A Dionex AS3500 auto injector was used to inject 10 µL of sample, and the sugars were eluted using Nanopure water at a flow rate of 1 mL/min. The monosaccharides were monitored using a Dionex ED40 electrochemical detector (gold electrode), with parameters set for pulsed amperometric detection of sugars, as recommended by the manufacturer. Post column addition of 0.2 M NaOH to the flow stream before the detector was done at a rate of 0.5 mL/min. Arabinose, galactose, glucose, xylose, and mannose standard curves were used to convert electrical signal into concentration of each sugar. The total carbohydrate reported for each sample was calculated as the sum of the monosaccharides present.

2.8.4 Lignin

The lignin content of the different white waters was determined as acid-insoluble lignin (TAPPI Test Method T 222). For each sample, 40 mL of white water was extracted three times with 50 mL MTBE and then freeze dried for 3-4 days. The dried samples were transferred to 125 mL Erlenmeyer flasks and autoclaved at 121°C for 1 hour with 30 mL of 4% H₂SO₄. The samples were then filtered through medium porosity (10-15 µm pore size) filtering crucibles, and washed with 150 mL distilled water. The crucibles containing lignin were dried at 105°C to constant weight to determine the amount of lignin present. Four replicates were done for each of the white waters.

2.8.5 Extractives

The extractives present in the different white waters were analyzed following a
slightly modified method of Örså and Holmbom (1994). Initially, 4 mL water samples were extracted three times with 5 mL MTBE. The solvent phases containing extractives were transferred to 8 mL screw capped vials and were evaporated using mild heat and N2, and then further dried in a vacuum desiccator. Next, the samples were silylated by adding 50 μL trimethylchlorosilane (Fluka) and 100 μL bis-(trimethylsilyl)-trifluoroacetamide (Fluka) under a N2 environment. The vials were sealed and heated to 60°C for 30 min, after which time 200 μL of toluene was added. The samples were then transferred to GC vials for analysis using a Hewlett Packard 5890 series-2 GC equipped with a Hewlett Packard 7673 injector. The GC column used was a DB-1 narrow bore capillary column (0.25 mm × 5 m, J&W Science) coated with polymethyl siloxane (0.25 μm film thickness). Helium was used as the carrier gas (20 mL/min), and the injection temperature was 300°C. The total running time for each sample was 24.5 minutes. Initially, the temperature was 100°C, and then raised after 1.5 minutes to 115°C at a rate of 30°C/min, held for 1 minute, then raised again to 300°C at a rate of 10°C/min. The final temperature was maintained for 3 minutes. The detector (FID) temperature was maintained at 320°C for the entire run. Abietic acid (Sigma), linoleic acid (Sigma), betulin (Aldrich), cholesteryl linoleate (Sigma), and 1,2-dipalmitoyl-3-oleoyl-rac-glycerol (Sigma) were used as internal standards, representative of the resin acids, fatty acids, lignans and sterols, steryl esters, and triglycerides, respectively. The retention times used to determine resin and fatty acid content ranged from approximately 2 to 7 minutes, lignan and sterols from 7 to 9 minutes, steryl esters from 10 to 17 minutes, and triglycerides from 17.5 to 21 minutes.
2.8.6 Ash Content

The ash contents of the different white waters were determined gravimetrically. For each water, four replicates of 25 mL were first oven dried at 105°C in crucibles, and then placed into a muffle furnace and maintained at 525°C until the samples had been completely combusted, as indicated by the absence of black particles (~ 6 hours). The crucibles were allowed to cool in a desiccator before weighing.

2.9 Handsheet Preparation

Handsheets were prepared according to standard TAPPI Test Method T 205 sp-95, with slight modification. Both enzyme treated pulp and untreated control pulp were used to make handsheets. Sheets were formed in the 4 different white waters, those being enzyme treated white water, enzyme treated white water with added alum, control white water, and control white water with added alum. Initially, the moisture contents of the two pulps were determined, and suitable amounts of each were weighed to allow for the formation of 15 handsheets (60 g/m² oven dry weight) in the 4 white waters, as well as for freeness determination. The enzyme treated pulp was disintegrated in either the enzyme treated white water, or the enzyme treated white water with added alum. The control pulp was disintegrated in either the control white water, or the control white water with added alum. After disintegration, the stocks were diluted with corresponding white water, and then equally divided for sheetmaking and freeness measurement. Freeness was determined according to the TAPPI Test Method T 227 om-94. Prior to sheetmaking, the handsheet machine was thoroughly cleaned and rinsed with white water, and then filled with approximately 15 L of the appropriate white water, which was
recirculated throughout the sheetmaking process. The handsheets were prepared in one pass, with the initial 5 sheets discarded to ensure uniform fines content.

2.10 Handsheet Properties

Handsheets physical properties were tested according to standard TAPPI Test Method T 220 sp-96. The non destructive measurements of handsheet grammage (TAPPI T 410 om-98), thickness (TAPPI T 411 om-97), light-scattering and absorption coefficients (TAPPI T 425 om-96), smoothness (TAPPI T 538 om-96), brightness (TAPPI T 452 om-98), and porosity (TAPPI T 460 om-96) were examined first. The sheets were then cut according to standard procedure, and destructively tested for tensile strength (TAPPI T 494 om-96), bursting strength (TAPPI T 403 om-97), tearing resistance (TAPPI T 414 om-98), and zero-span breaking strength (TAPPI T 231 cm-96).

2.11 Pulp Brightening

Enzyme treated and control pulps were brightened with hydrogen peroxide using a QP (chelation-peroxide) or QPP (chelation-peroxide-peroxide) sequence. Twelve grams (dry weight) of both pulps were weighed, brought to 5% consistency using distilled water, and then chelated with 0.2% EDTA (Rose Scientific) at 45°C for 1 hour. The chelations were performed in sealed plastic bags that allowed the pulps to be thoroughly mixed by hand. After 1 hour, the pulps were washed twice with 120 mL distilled water through a Buchner funnel with Whatman #1 filter paper. The pulps were next divided into three equal amounts of 4 g dry weight prior to brightening. Brightening was carried out in plastic bags at 80°C and 10% consistency for 2 hours using 0.2% DTPA (Sigma), 0.5% MgSO₄, 1.5% NaOH, and 3% H₂O₂ (Fisher). Initially, the DTPA
and MgSO₄ were combined, dissolved in distilled water, and then mixed with the pulps. The NaOH was also dissolved in distilled water, and then combined with H₂O₂ prior to addition to the pulps. The pulps were frequently mixed by hand during the 2 hours, and then washed twice with 40 mL distilled water through a Buchner funnel with Whatman #1 filter paper. For the pulps brightened using a QPP sequence, the brightening stage was repeated twice prior to air-drying. The exact H₂O₂ concentration and H₂O₂ consumption during brightening were determined by titration with Na₂S₂O₃. Pad brightness was measured according to standard TAPPI Test Method T 452 om-98.
CHAPTER III. Enzyme Production by *Trametes versicolor* Grown on White Water and Changes to Enzyme Activities as a Result of Elevated Temperature

3.1 Introduction

Combined fungal and enzyme treatment for contaminant removal from mechanical pulp and paper mill process waters employs both fungi and the enzymes produced as a result of fungal growth. As mentioned earlier, the proposed strategy will divert a relatively small stream of white water to allow for extracellular enzyme production with these enzymes then released into the main water system where a majority of the treatment will occur. Although all of the same components found in wood are present in pulp and paper mill process waters, fungal growth and enzyme production is considerably different when *Trametes versicolor* is grown on these liquid substrates. In order to increase the knowledge required to move this treatment technology towards commercial reality, enzyme production by *T. versicolor* grown on white water was further investigated. Cellulase, hemicellulase, lipase, and laccase activities were monitored when fungi were grown at 30°C, 45°C, and 60°C over a nine day period to determine possible impacts of increased temperature. Additionally, the culture filtrates were heated and maintained at 65°C to determine the thermostabilities of these same enzymes. Finally, *T. versicolor* was grown on white water in a bioreactor to investigate enzyme production at a significantly larger scale.

3.2 Enzyme Production by *Trametes versicolor* Grown on White Water at 30°C, 45°C, and 60°C

Although *Trametes versicolor* is capable of removing significant amounts of DCS from white water, a combined treatment strategy is believed necessary due to the long
retention times required by the fungi in comparison to the extracellular enzymes, and to the limited temperature range needed to allow for adequate fungal growth. It is thought that the elevated temperatures typically found in mill water systems would be detrimental to enzyme production, and therefore the water diverted for fungal growth will likely require some cooling. In order to investigate the effect that temperature has on enzyme production, *T. versicolor* was grown on white water at 30°C, 45°C, and 60°C.

When grown at 30°C over a nine day period, *T. versicolor* appeared to grow well on unsupplemented white water. Within three days, the culture filtrates had become much more transparent when compared to the cloudy state of the original white water. The activities of the cellulolytic and hemicellulolytic enzymes assayed, including endoglucanase, glucomannanase, galactomannanase, and xylanase all steadily increased during the first seven days of growth, however, very little continued production was observed between day seven and nine (Figure 3-1). Lipase activity showed a steady increase over the entire nine day period, whereas laccase activity reached a maximum at day three, and then decreased significantly.

When *T. versicolor* was grown on white water at 45°C or 60°C, the fungi did not appear to grow well, and the culture filtrates remained cloudy after nine days at both temperatures. At 45°C, endoglucanase, glucomannanase, galactomannanase, and xylanase activities were slightly lower than corresponding activities produced up to day three at 30°C. However, these activities did not further increase as they did at the lower temperature, but instead remained fairly constant over the nine day growth period (Figure 3-2). Lipase activity produced at 45°C was low and did not increase beyond the levels produced at the lower temperature. Laccase activity was not detected at 45°C. At 60°C,
Figure 3-1. Extracellular enzyme activities detected in *Trametes versicolor* culture filtrates after growth on white water at 30°C.
Figure 3-2. Extracellular enzyme activities detected in *Trametes versicolor* culture filtrates after growth on white water at 45°C.
endoglucanase, glucomannanase, galactomannanase, and xylanase activities were again slightly lower than corresponding activities produced at 45°C up to day three, but then these activities slowly declined until termination at day nine (Figure 3-3). At the highest growth temperature, neither lipase nor laccase activities were detected.

After examining fungal growth and enzyme production at the different growth temperatures, it was apparent that *T. versicolor* does not perform well on white water at elevated temperatures. It is clear that the higher temperatures were detrimental to extracellular enzyme production, especially laccase, which was not detectable after growth at 45°C. In general, mechanical pulp mill white waters tend to range between 50 and 60°C in temperature for most processes. Considering laccase and lipase activities are required for the removal of many of the different contaminants, it is apparent that water diverted for fungal growth will require cooling. A temperature that permits maximum enzyme production, especially laccase and lipase, is critical to the overall efficiency of this treatment strategy.

At 30°C, *T. versicolor* grew well on white water and produced the highest enzyme activities. Furthermore, most of the enzymes produced appeared to be very stable within the culture filtrates and maintained their activities at this temperature. It was clear that the hydrolytic enzyme activities assayed, including endoglucanase, glucomannanase, galactomannanase, and lipase did not show any decreases over the nine day growth period, while the xylanase activity showed only a minor decrease from day seven to nine. The laccase activity did decrease significantly after reaching a maximum at day three. However, it is probable that this resulted from a combination of decreased laccase production by *T. versicolor* due to the absence of small phenolic compounds in the white
Figure 3-3. Extracellular enzyme activities detected in *Trametes versicolor* culture filtrates after growth on white water at 60°C.
water after three days of growth, and to the inherently less stable nature of these oxidative enzymes. It is therefore likely that a temperature of or close to 30°C will provide the optimal growth environment for this technology should it be incorporated into a mill. As a result, all subsequent fungal growth on white water was carried out at 30°C.

### 3.3 Changes to Enzyme Activities When Heated and Maintained at 65°C

Another factor critical to fungal and enzyme treatment of mechanical pulp and paper mill process waters is the ability of the liberated enzymes to remain active and degrade DCS when released into the main water streams. In order for this strategy to be economically feasible for mills, only small amounts of white water will be cooled to produce extracellular enzymes, thereby minimizing additional energy requirements for reheating process waters. Typically, water temperatures range between 50 and 60°C. However, temperatures can reach 65°C or higher for some processes at different mills. To allow for efficient removal of DCS from mill water systems, the enzymes must be stable to some extent at these elevated temperatures. To investigate changes in enzyme activities as a result of temperature, culture filtrates produced by *T. versicolor* after three or nine days of growth on white water at 30°C were heated and maintained at 65°C.

An examination of the cellulolytic and hemicellulolytic enzyme activities revealed that exposure to 65°C resulted in a quick initial decrease, followed by a much slower decline in activity. Endoglucanase activity in both the three and nine day culture filtrates lost greater than 50% of the original activity in one hour (Figure 3-4). Likewise, glucomannanase activity in the three day culture filtrate was decreased by slightly over 50% in one hour. However, the activity present in the nine day filtrate appeared to decrease more rapidly with less than 10% of the original activity remaining after one hour.
Figure 3-4. The effect of incubation at 65°C on the loss of endoglucanase activity present within culture filtrates produced by *Trametes versicolor* after growth on white water for 3 or 9 days.
Figure 3-5. The effect of incubation at 65°C on the loss of glucomannanase activity present within culture filtrates produced by *Trametes versicolor* after growth on white water for 3 or 9 days.
Similarly, changes to galactomannanase and xylanase activities also followed this trend. The three day culture filtrate lost approximately 40% of its galactomannanase activity and 32% of its xylanase activity within one hour, whereas the nine day culture filtrate had its corresponding activities decrease by 86% and 65% over the same time, respectively (Figure 3-6, Figure 3-7). For all four of these activities, the three day culture filtrate retained a greater percentage of its original activity when compared to the nine day culture filtrate. The three day culture filtrate retained approximately 22% of original endoglucanase and glucomannanase activities, over 40% of galactomannanase activity, and 45% of xylanase activity after ten hours at 65°C. The nine day culture filtrate retained approximately 8%, 2%, 7% and 17% of its respective original activities after ten hours.

The change to lipase activity when culture filtrate was heated and maintained at 65°C demonstrated a similar profile to those observed for the cellulolytic and hemicellulolytic activities. In this case, only the lipase activity present within the nine day culture filtrate was monitored due to the very low levels found in the three day filtrate. Again, exposure to 65°C resulted in a fast initial decrease, followed by a much more gradual loss of activity (Figure 3-8). After approximately 1.5 hours, lipase activity was decreased by 50%, and after ten hours roughly 28% of the original activity remained.

The change to laccase activity as a result of heating to 65°C was somewhat different to that observed for either the cellulolytic/hemicellulolytic or lipase enzymes. For both the three and nine day culture filtrates, the initial decrease in activity was more gradual in comparison to the other enzyme activities monitored. Laccase activity in the three and nine day culture filtrates decreased by 50% after approximately three hours, and
Figure 3-6. The effect of incubation at 65°C on the loss of galactomannanase activity present within culture filtrates produced by *Trametes versicolor* after growth on white water for 3 or 9 days.
Figure 3-7. The effect of incubation at 65°C on the loss of xylanase activity present within culture filtrates produced by *Trametes versicolor* after growth on white water for 3 or 9 days.
Figure 3-8. The effect of incubation at 65°C on the loss of lipase activity present within culture filtrate produced by *Trametes versicolor* after growth on white water for 9 days.
Figure 3-9. The effect of incubation at 65°C on the loss of laccase activity present within culture filtrates produced by *Trametes versicolor* after growth on white water for 3 or 9 days.
steadily decreased over the next seven hours (Figure 3-9). In contrast to the hydrolytic enzymes, the nine day culture filtrate retained a greater percentage of its original activity than did the three day filtrate. After ten hours at 65°C, slightly less than 30% of the original activity remained in the nine day culture filtrate, whereas only 5% remained in the three day filtrate.

The ability of enzymes to remain active at elevated temperatures is influenced by many factors. It is well known that the loss of activity at higher temperature is due to protein denaturation and the consequent loss of native conformation (Lehninger et al., 1993). Exposure to heat can disrupt the hydrogen bonds, and hydrophobic, ionic, and van der Waals interactions that maintain proper secondary and tertiary structure critical to enzyme function. Some enzymes have a much higher tolerance to heat than others, which is due in part to their intrinsic nature and/or interactions with external species. For example, the primary amino acid sequence can confer increased thermostability by promoting tighter packing and greater numbers of disulphide bonds. The carbohydrate content can play a role, as shown by the loss of thermostability of cellulolytic enzymes as a result of partial deglycosylation (Hayashida and Yoshioka, 1980). Furthermore, improved enzyme stability can result from interactions with various ions or compounds present, including substrates, as well as from interactions with other proteins, and self-aggregation (Maheshwari et al., 2000).

Like many different organisms, fungi are capable of, and regularly produce multiple isoforms of extracellular enzymes, each of which may be optimally suited to an array of different conditions. Thermophilic fungi have been shown to produce multiple forms of cellulolytic, lipolytic, and oxidative enzymes that have unique properties.
McHale and Coughlan, 1981; Huge-Jensen et al., 1987; Coughlan and McHale, 1988; Berka et al., 1997; Schülein, 1997; Prabhu and Maheshwari, 1999). The observed changes in activities at 65°C suggest the possibility that *Trametes versicolor* also produces multiple isoforms of the enzymes investigated. Both cellulolytic and lipase activities decreased initially upon exposure to 65°C, but then maintained a constant amount of activity for an extended period of time. One explanation may be that some of the isoforms present were not thermostable, and therefore quickly lost activity, while others present were stable and remained active at this elevated temperature. Laccase activity decreased in a more continual manner. However, the changes observed within the first five hours exceeded the changes over the last five hours, again suggesting the presence of some isoforms with higher thermostability than others.

The activities present within three and nine day culture filtrates were monitored at 65°C to investigate the thermostability of the enzymes liberated during different stages of fungal growth. The three day culture filtrate was chosen because it showed maximum laccase activity, while the nine day filtrate was chosen for its high levels of cellulolytic and lipase enzymes. It is interesting to note that a greater percentage of original laccase activity was lost from the three day culture filtrate as opposed to the nine day filtrate, whereas the nine day culture filtrate lost greater percentages of cellulolytic activities when compared to the three day filtrate. For both the cellulolytic and laccase enzymes, the higher the original activity, the greater the overall percentage loss. This further suggested that a majority of these enzymes produced above certain base levels were not overly stable at higher temperature.
From these results, it is clear that the extracellular enzymes produced by *Trametes versicolor* grown on white water can retain significant levels of activity when incubated at 65°C. Cellulolytic, hemicellulolytic, lipase, and laccase enzymes all retained some activity after extended periods of time at this elevated temperature. The temperature of 65°C was chosen in order to simulate the more extreme conditions potentially found at mills, as process waters are normally somewhat cooler. It is therefore likely that these enzymes will remain active for significant periods of time when released into the water systems at mills, especially if initially released into areas that are more moderate in temperature. In order to further investigate these effects, all subsequent enzymatic treatments of fresh white water were carried out at 65°C.

3.4 Proteinase Activities Produced by *Trametes versicolor* Grown on White Water at 30°C

The levels of proteinase activity produced by *Trametes versicolor* grown on white water over nine days at 30°C were also monitored to further investigate enzyme stability. Proteinases are degradative enzymes that are capable of cleaving proteins into small peptides and amino acids, and are classified to a number of different groups. For example, they have been classified on the basis of catalytic function, pH optimum, site of cleavage, or requirement of a free thiol group (North, 1982). The production of high levels of extracellular proteinase could potentially result in the unwanted breakdown of the various enzymes needed for DCS removal from white water. To determine possible levels of enzyme cleavage, azurine-crosslinked casein was used as a substrate to monitor proteinase activity.
During the nine day growth period, *Trametes versicolor* produced limited levels of extracellular proteinase. From day two to nine, proteinase activity marginally increased. However, levels remained very low over the entire growth period (Table 3-1). In fact, the change in activity from the three day culture filtrate to the nine day filtrate was only 0.3 U/L. These low levels suggest that proteinase is not a major enzyme required by *T. versicolor* for growth on white water. It is therefore likely that proteinase plays a very minor role, if any, in decreasing the activities of the other extracellular enzymes liberated into the white water during growth.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Growth Time (days)</th>
<th>Proteinase Activity (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungal culture filtrate</td>
<td>2</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>4.1 ± 0.2</td>
</tr>
</tbody>
</table>

Table 3-1. Proteinase activities detected in the culture filtrates produced by *Trametes versicolor* after growth on white water at 30°C. (± SD)

3.5 **Scaled-up Enzyme Production by *Trametes versicolor* Grown on White Water in a Bioreactor**

Mechanical pulp and paper mills use large volumes of water that can be contaminated with high concentrations of DCS, especially as mills move towards system closure. In order to effectively remove these materials, a sufficient amount of culture filtrate produced by *Trametes versicolor* will be required for release into the water.
systems at mills. For this to occur, *T. versicolor* will need to be grown in a large scale bioreactor incorporated into the water system of the mill. However, fungal growth and enzyme production observed on small volumes of white water in the lab may be quite different to growth and enzyme production achieved at an industrial scale. To investigate scale-up, fungi were grown on white water in an 18 L bioreactor at 30°C to determine possible impacts to extracellular enzyme production.

It was apparent that the fungal growth and enzyme production profiles obtained in the 18 L bioreactor were very similar to those previously obtained in the smaller scale shake flask studies. *Trametes versicolor* was grown on white water for three days and, as observed before, the culture filtrate became noticeably clearer and less opaque after this growth period. By day three, the measured endoglucanase, glucomannanase, and lipase activities were very similar to those produced on the small scale, while galactomannanase activity was nearly 8 U/L higher, and xylanase activity approximately 19 U/L lower when produced on a larger scale (Figure 3-10). The laccase activity produced in the bioreactor was over 20 U/L higher after three days, potentially due to the constant aeration provided during growth in the bioreactor. The extracellular proteinase activity present in the bioreactor culture filtrate was very similar to that found previously (Table 3-2).

The results obtained from the scaled-up growth of *T. versicolor* in a bioreactor were very promising as the fungi grew well and produced extracellular enzymes in a similar manner to that obtained after growth in individual flasks. A three day period was chosen based on previous small scale growth in order to maximize laccase activity while minimizing overall growth time. In fact, laccase activity was found to be significantly higher after three days of growth, which should potentially aid in furthering the efficiency
Figure 3-10. Extracellular enzyme activities detected in *Trametes versicolor* culture filtrate after growth on white water in an 18 L bioreactor at 30°C.
of this fungal and enzyme treatment strategy. In the future, larger bioreactor scale-ups will be required before implementation at a mill occurs. However, these initial results indicate that *T. versicolor* is capable of efficient growth and enzyme production on a larger scale.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Growth Time (days)</th>
<th>Proteinase Activity (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungal culture filtrate</td>
<td>1</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.9 ± 0.2</td>
</tr>
</tbody>
</table>

Table 3-2. Proteinase activities detected in the culture filtrate produced by *Trametes versicolor* after growth on white water in an 18 L bioreactor at 30°C. (± SD)

3.6 Conclusions

The white-rot fungus *Trametes versicolor* was chosen for fungal and enzyme treatment of mechanical pulp and paper mill process waters because of its ability to grow on white water without added nutrients or pH adjustment. During growth, the fungi produced a range of enzymes that degraded or polymerized many of the contaminants present within the white water. However, it was clear that *T. versicolor* could not grow effectively at elevated temperatures, thus confirming the need to cool the process waters before using it as the growth substrate for enzyme production. However, the extracellular enzymes produced by the fungi were able to retain significant levels of activity after prolonged (10 hours) incubation at 65°C. It is likely that the lower temperatures of around 50 to 60°C expected in most mill white water systems will result in even greater
enzyme thermostability. Lastly, the observed growth and extracellular enzyme production by *T. versicolor* on a larger scale was very encouraging. The ability of this fungus to grow well in larger bioreactors is essential to this treatment strategy, and these initial results indicate that *T. versicolor* is capable of doing so.
CHAPTER IV. Fungal Enzyme Treatments of White Water and Pulp – Impact on White Water and Fiber Properties

4.1 Introduction

It is well known that the accumulation of dissolved and colloidal substances in process waters at mechanical pulp and paper mills has serious implications. Our previous research has demonstrated the ability of fungal and enzyme treatment to remove many of these contaminants from white water, potentially allowing for greater reuse and recycle of the water (Zhang et al., 1998; Zhang et al., 2000). However, before this treatment technology can be considered for implementation at a mill, the impact on both white water and fiber properties as a result of enzyme addition must be elucidated. To identify such changes, enzymes produced by T. versicolor grown on white water for three days at 30°C were collected and used to treat fresh white water and mechanical pulp at 65°C. The white water and fiber were then separated and the specific properties of each were examined. Subsequently, scaled-up enzyme treatment of white water and pulp was carried out at 65°C using the bioreactor produced culture filtrate in order to investigate larger scale treatment.

4.2 Changes to Colloidal Particles as a Result of Enzymatic Treatments of White Water

The buildup of colloidal particles in mechanical pulp and paper mill process waters can cause a multitude of problems, including increased pitch deposits and reduced paper quality, specifically hindering surface and optical properties. Our previous research has demonstrated the ability of fungal enzymes to reduce total extractive levels, especially those present within colloidal particles (Zhang et al., 2000). In order to gain a
better understanding of how enzyme treatments affect the nature and stability of colloidal particles in white water, the size distributions and zeta potentials of the particles were measured. Initially, the culture filtrates were used to treat fresh white water for either one or two hours at 65°C, after which mechanical pulp was added and mixed for an additional one, two, four, or six hours at the same temperature. The white water and pulp were then separated in order to roughly simulate the treatment events that may occur at a mill.

When fresh white water was treated for one hour at 65°C with the culture filtrate, a reduction in the average colloidal particle size was observed (Figure 4-1). In the control white waters nearly all of the colloidal particles present ranged between 0.09 and 0.6 μm, while enzymatic treatment clearly reduced this size. The colloidal particles ranged from 0.09 to slightly over 0.25 μm in the treated waters. The white water treated for two hours at 65°C revealed distributions that were very similar to those produced after one hour treatment, indicating most of the reactions occurred within the first hour of interaction (Figure 4-2). It was apparent that the length of time that the supplemented mechanical pulp was mixed with treated water had no impact on particle size distribution. For both the one and two hour treated white waters, the addition of pulp for one, two, four, or even six hours resulted in distributions that were nearly identical to each other. As a result, only the distributions produced after one or six hours of pulp mixing are shown for simplicity (Figure 4-1, Figure 4-2).

Particle size distribution was also plotted against the actual volume that the colloidal particles occupied. Relatively speaking, there are very few larger particles present, but as the radius of a spherical object has a cubic relationship with its volume, larger colloidal particles occupy a much greater volume than do smaller ones.
Figure 4-1. Colloidal particle size distributions (by % number) in white water treated for 1 hour at 65°C with enzymes produced by *Trametes versicolor* with subsequent pulp addition for 1 or 6 additional hours.
Figure 4-2. Colloidal particle size distributions (by % number) in white water treated for 2 hours at 65°C with enzymes produced by *Trametes versicolor* with subsequent pulp addition for 1 or 6 additional hours.
Comparing the treated and control white waters after one hour at 65°C, it was clear that enzyme addition catalyzed the removal of particles between 0.2 and 0.5 μm in size (Figure 4-3). This resulted in the generation of a bimodal population of colloidal particles; one having an average size of approximately 0.16 μm, and the other approximately 0.56 μm. It was again apparent that the length of time that pulp was mixed with white water did not significantly alter particle size distribution. The white water treated for two hours at 65°C produced very similar particle size distributions to those produced after one hour of treatment (Figure 4-4).

The zeta potentials of the colloidal particles present within treated and control white waters were measured to investigate changes to particle stability. It was determined that enzyme addition to white water acts to decrease the average zeta potential of the particles present. After the one hour treatment and subsequent pulp additions at 65°C, the average zeta potential measured was -15.7 mV, while the corresponding control waters had an average potential of -20.5 mV (Table 4-1). The white water treated for two hours with subsequent pulp additions resulted in an average zeta potential of -15.8 mV, while the two hour controls averaged -20.1 mV. Both the one and two hour enzyme treatments resulted in similar decreases of approximately 4.5 mV, further suggesting that a majority of the reactions occurred within the first hour.

Changes to colloidal particle size and zeta potentials have potential implications for mechanical pulp and paper mills. These results clearly indicate that fungal enzyme treatment of white water alters both the nature and stability of the particles present. Reduced colloidal particle size alone has the potential to improve paper surface and printing properties, as smaller particles can potentially fill the spaces and voids between
Figure 4-3. Colloidal particle size distributions (by % volume) in white water treated for 1 hour at 65°C with enzymes produced by *Trametes versicolor* with subsequent pulp addition for 1 or 6 additional hours.
Figure 4-4. Colloidal particle size distributions (by % volume) in white water treated for 2 hours at 65°C with enzymes produced by *Trametes versicolor* with subsequent pulp addition for 1 or 6 additional hours.
Table 4-1. Average zeta potentials determined for enzyme treated and control white waters. (± SD)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pulp Addition</th>
<th>Zeta Potential (mV)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated (1 hr)</td>
<td>1 hr</td>
<td>-15.8</td>
<td>4.69</td>
</tr>
<tr>
<td></td>
<td>2 hrs</td>
<td>-16.1</td>
<td>4.74</td>
</tr>
<tr>
<td></td>
<td>4 hrs</td>
<td>-15.7</td>
<td>4.77</td>
</tr>
<tr>
<td></td>
<td>6 hrs</td>
<td>-15.2</td>
<td>4.72</td>
</tr>
<tr>
<td></td>
<td>Ave.</td>
<td>-15.7 ± 1.0</td>
<td>4.73</td>
</tr>
<tr>
<td>Control (1 hr)</td>
<td>1 hr</td>
<td>-21.1</td>
<td>4.79</td>
</tr>
<tr>
<td></td>
<td>2 hrs</td>
<td>-20.6</td>
<td>4.75</td>
</tr>
<tr>
<td></td>
<td>4 hrs</td>
<td>-20.2</td>
<td>4.74</td>
</tr>
<tr>
<td></td>
<td>6 hrs</td>
<td>-19.9</td>
<td>4.80</td>
</tr>
<tr>
<td></td>
<td>Ave.</td>
<td>-20.5 ± 1.1</td>
<td>4.77</td>
</tr>
<tr>
<td>Treated (2 hrs)</td>
<td>1 hr</td>
<td>-15.9</td>
<td>4.72</td>
</tr>
<tr>
<td></td>
<td>2 hrs</td>
<td>-15.7</td>
<td>4.68</td>
</tr>
<tr>
<td></td>
<td>4 hrs</td>
<td>-16.0</td>
<td>4.69</td>
</tr>
<tr>
<td></td>
<td>6 hrs</td>
<td>-15.5</td>
<td>4.73</td>
</tr>
<tr>
<td></td>
<td>Ave.</td>
<td>-15.8 ± 0.5</td>
<td>4.71</td>
</tr>
<tr>
<td>Control (2 hrs)</td>
<td>1 hr</td>
<td>-20.7</td>
<td>4.81</td>
</tr>
<tr>
<td></td>
<td>2 hrs</td>
<td>-20.1</td>
<td>4.78</td>
</tr>
<tr>
<td></td>
<td>4 hrs</td>
<td>-19.8</td>
<td>4.73</td>
</tr>
<tr>
<td></td>
<td>6 hrs</td>
<td>-19.6</td>
<td>4.83</td>
</tr>
<tr>
<td></td>
<td>Ave.</td>
<td>-20.1 ± 1.1</td>
<td>4.79</td>
</tr>
</tbody>
</table>
fibers more completely, thereby improving paper uniformity. Reduced colloidal particle stability also has the potential to help alleviate pitch problems at mills, as demonstrated by previous research (Irie et al., 1990; Fujita et al., 1992). In newsprint mill trials, decreased particle stability resulting from enzyme addition significantly reduced pitch deposition on equipment surfaces. It is likely that our fungal and enzyme treatment strategy will have a similar effect.

4.3 The Effect of Enzymatic Treatment on Phenolic Compounds

Phenolic compounds are found as both dissolved and dispersed molecules in process waters at mechanical pulp and paper mills. Our group has previously shown that over 70% of the lignin-like material, and greater than 25% of the steryl esters were contained within the colloidal particles (Zhang et al., 2000). In contrast, nearly all of the lignan and sterols, the more hydrophilic extractives, were dissolved in the white water. The accumulation of these and other dissolved substances can result in significant problems for mills, including reduced paper strength properties. Unfortunately, removal of dissolved substances from large volumes of white water is both difficult and costly.

In an attempt to ascertain the effect of enzyme addition on the phenolics present in white water, the molecular weight distributions of these compounds were measured. As before, fresh white water was treated with culture filtrate for one or two hours at 65°C, and then mixed with pulp for an additional one, two, four, or six hours. The predominant molecular weight populations present in the untreated control waters ranged from approximately 500-1000 g/mol (Figure 4-5, Figure 4-6). This range is indicative of lignan monomer and dimer type compounds. In contrast, it was apparent that the enzyme addition catalyzed an increase in molecular weight, generating a main peak above 2500
Figure 4-5. Molecular weight distributions of phenolic compounds present in white water treated for 1 hour at 65°C with enzymes produced by *Trametes versicolor* with subsequent pulp addition for 1 or 6 additional hours.
Figure 4-6. Molecular weight distributions of phenolic compounds present in white water treated for 2 hours at 65°C with enzymes produced by *Trametes versicolor* with subsequent pulp addition for 1 or 6 additional hours.
g/mol. Again, there was very little difference between the molecular weight distributions of white water treated for one or two hours, and only distributions produced after one or six hours of pulp mixing are shown, as this did not impact molecular weight significantly (Figure 4-5, Figure 4-6).

It is apparent that enzyme treatment of white water increased the molecular weight distribution of the phenolic compounds present. The capacity to do so has potential implications for mechanical pulp and paper mills, as it has been shown that enzyme polymerized aromatic compounds have reduced solubility and are readily precipitated from process waters (Nicell et al., 1992; Al-Kassim et al., 1994b; Nicell et al., 1995). For mills that wish to remove these materials, the task becomes significantly easier as solubility decreases. Alternatively, for mills that are less concerned with this issue, such as mills that produce lower brightness papers, the polymerized phenolics can be removed by entrapment during paper formation, and concurrently contribute to sheet basis weight.

4.4 Changes to White Water and Pulp Charge as a Result of Enzyme Treatments

Both white water and pulp charge are issues of concern at mechanical pulp and paper mills. Depending on the desired characteristics of the end product, mills add a variety of different chemical additives to tailor specific paper properties. These chemicals, including fillers and retention aids, come at a cost, and therefore mills want to minimize their loss during processing. To do this, the various chemicals need to be retained on the fibers and forming network. Since mechanical pulp fibers carry negative charges, many mills utilize positively charged chemicals to maximize retention, and
therefore minimize loss to the effluent. However, the anionic nature of the white water is such that additives are lost due to binding with particles instead of fiber.

To investigate changes to white water and fiber charge as a result of enzyme treatment, polyelectrolyte titrations were used to determine both total and surface charge of the control and enzyme treated waters and fibers. Again, fresh white water was treated for either one or two hours at 65°C, and subsequently mixed with mechanical pulp for an additional one, two, four, or six hours. It was apparent that cationic demand increased when the white water was treated with enzymes. Both of the polyelectrolytes employed showed increases of approximately 0.22 μeq/g for the one and two hour treatments (Table 4-2). The addition of pulp for different lengths of time did not have a significant impact on cationic demand, and therefore the values obtained were averaged. Enzymatic treatment of white water resulted in an average total charge of 2.38 μeq/g, while the corresponding controls had an average charge of 2.16 μeq/g. Normally, increased cationic demand is unwanted at pulp and paper mills for the reasons mentioned above. However, concurrent increases to pulp fiber charge overshadow this change in water charge.

In contrast to cationic demand, enzyme treatment resulted in a very favourable increase in pulp fiber charge. Mechanical pulp was found to have an average total charge of 39.98 μeq/g, and an average surface charge of 21.90 μeq/g after mixing with white water treated with culture filtrate for one or two hours (Table 4-3). Mechanical pulp that was mixed with corresponding control white waters had an average total charge of 35.29 μeq/g, and an average surface charge of 17.22 μeq/g. Furthermore, the length of time that the pulp fiber was mixed with white water did not significantly affect the measured
<table>
<thead>
<tr>
<th>Sample</th>
<th>Pulp Addition</th>
<th>Total Charge (μeq/g white water)</th>
<th>Surface Charge</th>
</tr>
</thead>
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<td></td>
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<tr>
<td></td>
<td>6 hrs</td>
<td>2.36</td>
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</tr>
<tr>
<td>Ave.</td>
<td>2.37 ± 0.04</td>
<td>2.07 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Control (1 hr)</td>
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<td>1.88</td>
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<tr>
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<td>2 hrs</td>
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<td>2.39 ± 0.03</td>
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<td></td>
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<tr>
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<td>6 hrs</td>
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<td>2.17 ± 0.03</td>
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Table 4-2. Cationic demands determined for enzyme treated and control white waters. (± SD)
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<th>Sample</th>
<th>Pulp Addition</th>
<th>Total Charge (μeq/g pulp*)</th>
<th>Surface Charge</th>
</tr>
</thead>
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<td><strong>21.94 ± 0.32</strong></td>
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<td>17.89</td>
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<td>Treated (2 hrs)</td>
<td>1 hr</td>
<td>39.94</td>
<td>21.47</td>
</tr>
<tr>
<td></td>
<td>2 hrs</td>
<td>40.24</td>
<td>21.35</td>
</tr>
<tr>
<td></td>
<td>4 hrs</td>
<td>39.55</td>
<td>22.39</td>
</tr>
<tr>
<td></td>
<td>6 hrs</td>
<td>39.58</td>
<td>22.20</td>
</tr>
<tr>
<td>Ave.</td>
<td></td>
<td><strong>39.83 ± 0.53</strong></td>
<td><strong>21.85 ± 0.52</strong></td>
</tr>
<tr>
<td>Control (2 hrs)</td>
<td>1 hr</td>
<td>34.90</td>
<td>16.02</td>
</tr>
<tr>
<td></td>
<td>2 hrs</td>
<td>34.80</td>
<td>16.98</td>
</tr>
<tr>
<td></td>
<td>4 hrs</td>
<td>34.51</td>
<td>17.60</td>
</tr>
<tr>
<td></td>
<td>6 hrs</td>
<td>36.25</td>
<td>17.90</td>
</tr>
<tr>
<td>Ave.</td>
<td></td>
<td><strong>35.12 ± 0.77</strong></td>
<td><strong>17.13 ± 0.83</strong></td>
</tr>
</tbody>
</table>

* Based on oven dry weight

Table 4-3. Total and surface charges for mechanical pulp mixed with enzyme treated and control white waters. (± SD)
fiber charges. Both surface and total charge of the pulp were increased by enzyme addition on average 4.68 and 4.70 μeq/g, respectively. The increased surface charge was very similar to the increased total charge, suggesting modification that occurred primarily on the fiber surfaces.

Increases to fiber surface charge likely occurred for a number of reasons. It is well known that mechanical pulp fibers carry negative charges that arise primarily from the ionization of carboxylic acid groups present in both cellulose and hemicellulose (Sjöström, 1989). It is probable that the cellulolytic and hemicellulolytic enzymes present cleaved some of the carbohydrate polymers on the fiber surfaces exposing a greater number of carboxylic groups. It is also probable that enhanced deposition of hemicellulose moieties and colloidal particles onto fiber surfaces as a result of enzymatic treatment may have occurred. The fact that the fiber charge was increased to a much greater extent than was the cationic demand of white water raises interesting implications. Higher surface charge has the potential to reduce the amount of additives required by promoting stronger binding and retention of chemical to the fiber. Additionally, this may also improve paper strength properties by promoting stronger chemical crosslinking of fiber.

4.5 Scaled-up Enzymatic Treatment of White Water and Pulp

The ability to treat the large volumes of process water utilized by mechanical pulp and paper mills is critical to this fungal and enzyme treatment strategy. Our initial work demonstrated the efficiency of extracellular enzymes produced by *Trametes versicolor* to catalyze the removal of many of the DCS present within white water. In order to investigate treatment on a larger scale, the culture filtrate produced after three days of
fungal growth on white water in a bioreactor at 30°C was used to treat fresh white water at 65°C, and then subsequently mixed with mechanical pulp. Due to the observation that the treatment times used previously did not result in significantly different white water or fiber properties, scaled-up treatments of white water were carried out for two hours, after which mechanical pulp was added and mixed for an additional two hours. An alum post treatment step was also incorporated after enzymatic treatment to exploit any physiochemical changes made to the colloidal particles and phenolic compounds, thereby potentially increasing removal of these contaminants.

When used to treat fresh white water, the bioreactor produced culture filtrate demonstrated the ability to alter the chemical nature of the white water. It was apparent that the original and control white waters were quite similar in levels of non-extractables, while a decrease in total extractives primarily accounted for a 60 mg/L reduction in total dissolved and colloidal substances (TDCS) (Table 4-4). A slight reduction in carbohydrate content, specifically galactose and mannose, as well as a small increase in ash content was noted in the control white water, likely due to deposition and release of these materials from the pulp. The reduction in total extractives in the control water was probably due to a combination of redeposition onto the added pulp and loss of some of the more volatile compounds with exposure to heat. However, when comparing control to treated white water, it was obvious that enzyme addition had a much greater impact on water composition than did pulp addition or elevated temperature alone. The lignin content in the treated water was determined to be more than double that of the control water, and a concurrent reduction of greater than half the total extractives was observed. It is quite likely that some of the extractives were removed during fungal growth, as
### Table 4-4. The chemical compositions of original white water, control and enzyme treated white waters, as well as treated and control white waters with added alum. (± SD)

<table>
<thead>
<tr>
<th>Component</th>
<th>White water</th>
<th>Control</th>
<th>Treated</th>
<th>Treated + Alum</th>
<th>Control + Alum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TDCS</td>
<td>1410 ± 7</td>
<td>1350 ± 8</td>
<td>1327 ± 13</td>
<td>1190 ± 8</td>
<td>1480 ± 12</td>
</tr>
<tr>
<td>Non Extractables</td>
<td>1063 ± 20</td>
<td>1055 ± 6</td>
<td>1193 ± 20</td>
<td>1057 ± 15</td>
<td>1192 ± 15</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>771 ± 19</td>
<td>753 ± 23</td>
<td>716 ± 22</td>
<td>706 ± 21</td>
<td>754 ± 20</td>
</tr>
<tr>
<td>Arabinose</td>
<td>82 ± 3</td>
<td>92 ± 4</td>
<td>85 ± 3</td>
<td>84 ± 3</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>Galactose</td>
<td>228 ± 5</td>
<td>208 ± 7</td>
<td>207 ± 5</td>
<td>210 ± 6</td>
<td>206 ± 6</td>
</tr>
<tr>
<td>Glucose</td>
<td>151 ± 5</td>
<td>182 ± 5</td>
<td>158 ± 5</td>
<td>152 ± 4</td>
<td>179 ± 6</td>
</tr>
<tr>
<td>Xylose</td>
<td>27 ± 2</td>
<td>36 ± 2</td>
<td>34 ± 3</td>
<td>33 ± 3</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Mannose</td>
<td>283 ± 4</td>
<td>235 ± 5</td>
<td>232 ± 6</td>
<td>227 ± 5</td>
<td>242 ± 4</td>
</tr>
<tr>
<td>Lignin</td>
<td>101 ± 8</td>
<td>109 ± 9</td>
<td>233 ± 5</td>
<td>15 ± 5</td>
<td>77 ± 7</td>
</tr>
<tr>
<td>Ash</td>
<td>160 ± 6</td>
<td>180 ± 9</td>
<td>181 ± 7</td>
<td>283 ± 13</td>
<td>304 ± 12</td>
</tr>
<tr>
<td>Total Extractives</td>
<td>343 ± 7</td>
<td>285 ± 10</td>
<td>130 ± 5</td>
<td>110 ± 7</td>
<td>275 ± 9</td>
</tr>
<tr>
<td>Resin/Fatty acids</td>
<td>94.3 ± 2.2</td>
<td>84.3 ± 3.0</td>
<td>67.5 ± 4.7</td>
<td>57.2 ± 5.0</td>
<td>86.5 ± 2.3</td>
</tr>
<tr>
<td>Lignans/Sterols</td>
<td>104.2 ± 2.1</td>
<td>108.0 ± 3.2</td>
<td>0</td>
<td>0</td>
<td>103.8 ± 1.5</td>
</tr>
<tr>
<td>Steryl esters</td>
<td>32.0 ± 2.9</td>
<td>32.3 ± 1.5</td>
<td>0</td>
<td>0</td>
<td>31.1 ± 1.6</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>10.1 ± 0.6</td>
<td>9.7 ± 1.2</td>
<td>0</td>
<td>0</td>
<td>10.2 ± 0.6</td>
</tr>
</tbody>
</table>

Table 4-4. The chemical compositions of original white water, control and enzyme treated white waters, as well as treated and control white waters with added alum. (± SD)
observed previously (Zhang et al., 2002). However, the extensive decrease observed in the treated water indicates more than a simple dilution effect. It was also noted that the carbohydrate content of the enzyme treated water was lower than the control, which in this case likely resulted from the removal of glucose and other sugars within the culture filtrate during fungal growth.

The addition of an alum post treatment step after enzyme treatment further altered the white water composition. The alum treatment rapidly resulted in the formation of a large number of flocs within the enzyme treated white water. The flocs then quickly settled out. The resulting water was visibly much more transparent than either the white water treated with enzymes alone, or the control water (Figure 4-7). Upon addition of alum to the control white water, flocs did not form and the water remained cloudy in nature, even when twice the amount of alum was added. Comparing the enzyme treated water to the enzyme treated water with alum post treatment, a significant decrease in TDCS from 1327 to 1190 mg/L was noted (Table 4-4). This decrease was primarily due to the removal of lignin, although slight reductions in carbohydrate and total extractive contents were also observed. There was a large increase in the ash content of the enzyme treated water with subsequent post treatment, which was due to residual alum remaining in the water after precipitate removal. As suggested by visual assessment, it was apparent that alum post treatment of the control white water had a much lower impact than it did on the enzyme treated water. Compared to control white water alone, the control white water with added alum had very similar carbohydrate and total extractive contents, while the ash content was considerably higher. Alum addition did lower the lignin content in
Figure 4-7. Pictures of the white waters used for papermaking in Erlenmeyer flasks (A), and in 20 mL test tubes (B). Waters: 1-original white water, 2-control white water, 3-control white water with added alum (400 mg/L), 4-control white water with added alum (800 mg/L), 5-fungal enzyme treated white water, and 6-fungal enzyme treated white water with added alum (400 mg/L).
the control water. However, the levels did not approach those remaining in the enzyme treated water after post treatment.

To further examine extractive removal from the white water as a result of enzyme addition, the extractives present within the different waters were analyzed by gas chromatography. It was apparent from the GC profiles that the extractives in the original, control, and alum post treated control white waters were very similar, which was confirmed when the concentrations of these compounds were determined (Figure 4-8, Table 4-4). Alternatively, the enzyme treated white water and enzyme treated water with alum post treatment had markedly different extractive profiles. These two waters showed a significant absence of lignans and sterols (7 to 9 min), steryl esters (10 to 17 min), and triglycerides (17.5 to 21 min), while the resin and fatty acid content (2 to 7 min) was decreased by 20%. It is unclear if this drop in resin and fatty acid content was due to enzymatic degradation or rather their removal from the culture filtrate during fungal growth. However, it is clear that the other extractives present in the fresh white water were largely removed by the enzyme treatments. As well, alum post treatment of enzyme treated white water removed an additional 15% of the remaining resin and fatty acids.

In order to determine the impact that the scaled-up enzyme treatment and alum post treatment might have on the phenolic compounds present in white water, the molecular weight distributions were again measured. Original, control, and alum post treated control white waters were found to have similar distributions that corresponded well with the distributions obtained previously from the untreated waters (Figure 4-9). Again, the main population of phenolics present ranged from approximately 500 g/mol to slightly over 1000 g/mol in molecular weight. In the treated water, enzyme addition
Figure 4-8. GC profiles of the extractives present in original white water, control white water, control water with added alum, enzyme treated white water, and enzyme treated water with added alum. Inset: Magnified view of GC profiles from 10-22 min.
Figure 4-9. Molecular weight distributions of phenolic compounds present in original white water, control white water, control water with added alum, enzyme treated white water, and enzyme treated water with added alum.
caused the polymerization of many of these small phenolics, which produced a peak above 2500 g/mol, as observed previously. This change in molecular weight distribution was further illustrated by the near complete removal of the small phenolics, lignans and sterols, and the concurrent increase of lignin-like substances within the enzyme treated white water (Table 4-4). It was clear that alum addition to the enzyme treated water facilitated the removal of most of the polymerized phenolics, leaving only a minor peak at approximately 500 g/mol (Figure 4-9). The molecular weight distribution measured for the enzyme treated water with alum post treatment was very positive as it demonstrated the ability to remove many of these contaminants from the white water.

Alum was chosen for post treatment after enzyme treatment because of its common use in the pulp and paper industry, as well as its known ability to coagulate contaminants within process waters (Bennett et al., 1988; Stephenson and Duff, 1996; Omoike and vanLoon, 1999). Generally, alum is used to treat both incoming fresh water and effluent, and may be added during papermaking for internal sizing. When dissolved in water, alum forms a number of positively charged species that tend to complex with the negatively charged materials present. As a result of this charge neutralization, the organic contaminants are destabilized in the water, are precipitated, and can then become linked together to form larger flocs. It is very likely that these same phenomena occurred during alum addition to the enzyme treated white water. The observation that alum addition to control water did not induce floc formation indicates enzyme treatment greatly enhances post treatment.

Scaled-up enzymatic treatment of white water using the culture filtrate produced by Trametes versicolor grown in a bioreactor appears promising. The demonstrated
ability of a larger scale treatment to remove the majority of the lipophilic extractives and polymerize nearly all of the dissolved phenolics present in white water is an important step in the development of this treatment strategy. It was apparent that the changes to white water properties as a result of scaled-up treatment were very comparable to those resulting from treatment on a smaller scale. It was also apparent that the enzyme addition altered the physio-chemical nature of the contaminants present, as shown by enhanced removal with alum post treatment. The stabilities of the remaining extractives and phenolic compounds within the water were decreased by enzyme treatment, and this facilitated their removal through precipitation. The resulting water had greatly reduced extractive and lignin contents, which should in itself allow for greater water reuse and recycle in a mill.

4.6 Conclusions

The research outlined in this chapter demonstrated the ability of fungal enzyme treatment of mechanical pulp and paper mill white water to alter both white water and fiber properties. White water and pulp treatments on a small scale resulted in decreased average colloidal particle size, increased particle zeta potential, increased molecular weight of the phenolic compounds and increased fiber surface charge. It was clear that the majority of these reactions occurred within the first hour, as indicated by the very similar changes made after one or two hours of treatment. Decreased colloidal particle size has the potential to improve paper surface properties, while concurrent increased fiber charge will potentially lower additive requirements by improving chemical-fiber bonding. It is well known that increased particle zeta potential and molecular weight of phenolics reduces the stability of these compounds within the white water, which in turn
facilitates their easier removal. This became evident after scaled-up enzyme treatment and alum post treatment, which resulted in the precipitation of the majority of these contaminants. In fact, the scaled-up enzyme treatment using the bioreactor produced culture filtrate was very comparable to the previous results obtained from the small scale white water treatments. The larger scale treatment resulted in the removal of most of the lipophilic extractives, as well as the polymerization of small phenolics into larger lignin-like structures. It was obvious that the reactions that occurred had altered the physiochemical nature of the contaminants present, and after alum post treatment, produced white water with significantly lower levels of TDCS. It seems likely that the demonstrated ability to remove DCS from white water can be achieved at a much larger scale and ultimately implemented at a mill. This work indicates that combined fungal and enzyme treatment should not only provide an effective means for mills to further move towards water system closure, but may also serve to enhance certain fiber/paper properties.
CHAPTER V. Large Scale Fungal Enzyme Treatment of White Water and Pulp – Impact on Paper Properties

5.1 Introduction

The demonstrated ability to remove DCS from mechanical pulp and paper mill white water using the extracellular enzymes produced by Trametes versicolor appears very promising. The research outlined in the previous chapters of this thesis revealed that enzymatic treatment of white water and mechanical pulp altered specific properties of each in a positive fashion. Nearly all of the wood extractives present within the white water were impacted by enzyme addition, either being degraded or polymerized, while the fiber surface charge was increased. However, the changes made to both the white water and pulp properties will, to some extent, undoubtedly affect paper properties. It was apparent that the enzymatic treatment altered the physio-chemical nature of the colloidal particles and phenolic substances within the white water. It is possible that the redeposition of these materials onto the fiber may have an impact on paper quality. As well, changes made to the fibers themselves as a result of enzyme addition may also have an effect on the paper.

In order to investigate possible impacts on paper properties as a result of enzymatic treatment of white water and pulp, handsheets were prepared and tested. Initially, the culture filtrate collected after three days of fungal growth in a bioreactor was used to treat fresh white water for two hours at 65°C, after which time mechanical pulp was then added and mixed at the same temperature for two additional hours. Subsequently, the white water and pulp were separated and then used for papermaking. Concurrently, white water and pulp treatment were mirrored, with the inclusion of alum.
post treatment of the white water. In the latter case, after the water and pulp had been separated, the white water was reheated to 65°C before alum addition occurred. Two controls lacking fungal enzymes were also prepared, again one with alum post treatment and one without. The four resulting white waters and pulps were then used to produce handsheets, which in turn were tested for specific dry strength, surface, and optical properties.

5.2 Impact on Paper Dry Strength Properties as a Result of Enzyme Treatments

Fiber strength, fiber length, and interfiber bonding largely control handsheet dry strength properties. Obviously, makers of newsprint want to maximize these properties in order to limit potential runnability problems of modern high-speed printing presses, and reduce the overall basis weight of their final product. The densities and dry strength properties determined for the four types of handsheets produced were in fact very similar, indicating that both enzyme treatment of white water and pulp, and alum post treatment of white water did not significantly impact paper strength (Table 5-1). The measured densities of the handsheets produced with either control or enzyme treated pulp were not significantly different, suggesting that sheet consolidation was not affected by the different treatments. Furthermore, interfiber bonding was not significantly altered in any of the handsheets, which is reflected in the very similar tensile, tear, and burst indices. Finally, the average strength of the individual fibers, as measured by zero-span, was again not significantly changed in any of the four cases.

It is clear from these results that enzyme treatment of white water and pulp did not alter handsheet strength properties. It was evident from the work outlined in the previous chapter that the addition of fungal enzymes altered the physio-chemical nature
Table 5-1.  Dry strength properties of handsheets formed in control white water, control water with added alum, treated white water, and treated water with added alum. (± SD)

<table>
<thead>
<tr>
<th>Property</th>
<th>Control</th>
<th>Control + Alum</th>
<th>Treated</th>
<th>Treated + Alum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness (μm)</td>
<td>182.5 ± 1.0</td>
<td>190.8 ± 1.9</td>
<td>178.2 ± 1.9</td>
<td>179.6 ± 2.5</td>
</tr>
<tr>
<td>Density (g/cm³)</td>
<td>0.326 ± 0.002</td>
<td>0.326 ± 0.003</td>
<td>0.321 ± 0.003</td>
<td>0.331 ± 0.005</td>
</tr>
<tr>
<td>Tensile index (N·m/g)</td>
<td>32.6 ± 0.8</td>
<td>29.6 ± 1.5</td>
<td>31.3 ± 1.8</td>
<td>31.9 ± 0.4</td>
</tr>
<tr>
<td>Tear index (mN·m²/g)</td>
<td>7.2 ± 0.3</td>
<td>7.2 ± 0.3</td>
<td>7.0 ± 0.4</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>Burst index (kPa·m²/g)</td>
<td>2.1 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Zero-span (km)</td>
<td>8.7 ± 0.5</td>
<td>8.4 ± 0.6</td>
<td>8.4 ± 0.3</td>
<td>8.4 ± 0.4</td>
</tr>
</tbody>
</table>

of most of the extractives and increased the fiber surface charge. This increased fiber charge was likely due to the combination of changes made to the fibers themselves and to the redeposition of extractives. However, these changes did not affect the ability of the fibers to consolidate and form interfiber hydrogen bonds during sheet formation. This was clearly demonstrated by the very similar tensile, tear, and burst strengths measured for the four paper types (Table 5-1). Further, the fiber strength was not affected by the addition of enzymes. Based on the previous literature concerning the beneficial effects of enzymatic fiber modification, it was somewhat unexpected that more of a positive effect was not seen. However, it was of greater importance that fiber strength and interfiber bonding were not altered to the point of negatively impacting paper dry strength properties.

5.3 Impact on Paper Surface Properties as a Result of Enzyme Treatments

Air resistance and surface roughness were also determined to assess the surface properties of the various handsheets. It was apparent that the air resistance values of
handsheets produced using enzyme treated pulp and white water with alum post treatment were significantly higher than the other paper types (Table 5-2). The other three paper types were found to have similar air resistances. Considering that the densities of the four paper types were all very similar, it is probable that this increased air resistance was a result of the remaining colloidal particles and lignin-like substances filling the interfiber spaces in a more uniform manner, as opposed to increased sheet consolidation. All of the other handsheets produced were formed in the presence of contaminants of greater size, those being the larger colloidal particles within the control white waters or the polymerized phenolic compounds within the enzyme treated white water, which appeared unable to fill the interfiber spaces as effectively. Improvements to handsheet roughness also followed this same trend, with enzyme addition and alum post treatment increasing sheet smoothness (Table 5-2).

<table>
<thead>
<tr>
<th>Property</th>
<th>Control</th>
<th>Control + Alum</th>
<th>Treated</th>
<th>Treated + Alum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air resistance (s/100 mL)</td>
<td>42 ± 3</td>
<td>48 ± 3</td>
<td>45 ± 5</td>
<td>65 ± 2</td>
</tr>
<tr>
<td>Roughness (SCCM*)</td>
<td>1739 ± 100</td>
<td>1738 ± 98</td>
<td>1725 ± 120</td>
<td>1604 ± 86</td>
</tr>
</tbody>
</table>

* Standard cubic centimeters per minute

Table 5-2. Surface properties of handsheets formed in control white water, control water with added alum, treated white water, and treated water with added alum. (± SD)

It was apparent that a combination of enzyme addition and alum post treatment of white water were required for enhancement of air resistance and roughness values. The addition of alum to the control water did not alter deposition or sheet formation to the extent that it significantly impacted either of these paper properties. Enzyme treatments
of white water were shown to decrease the average colloidal particle size, while increasing the molecular weight of the phenolic compounds present (Figures 4-1 to 4-6). However, this alone also did not change the air resistance or roughness. Only after the application of both enzymes and alum were these paper properties significantly modified. It is likely that the combined effects of alum induced precipitation for the removal of large polymerized phenolic compounds and alum crosslinking of smaller colloidal particles into the pores and interfiber spaces during paper formation acted to improve air resistance and roughness values.

The demonstrated ability to increase air resistance and sheet smoothness has the potential to improve printing properties. Increased air resistance indicates decreased sheet porosity, which helps prevent excessive ink penetration and resulting show-through (Wilson, 1998). Our previous work using untreated white water and pulp for papermaking also found that colloidal particle deposition decreased porosity, however, a large increase in sheet roughness was noted (Zhang et al., 1999). In this case, decreased surface roughness was likely a result of reduced colloidal particle size in the enzyme treated white water. Paper with improved smoothness and surface uniformity is desirable in that it can help produce quality image reproductions when printed on. Printing on rough paper can introduce microspots of reflected white light within the image, causing lower apparent ink density and print contrast (Wilson, 1998). Thus, it appears that enzymatic treatment of white water and pulp followed by alum post treatment has the potential to improve the printability of the resulting paper.
5.4 Impact on Paper Optical Properties as a Result of Enzyme Treatments

Optical properties of the different handsheets were also measured to identify other potential impacts as a result of the enzyme treatments. When the enzyme treated and control pulps were examined after separation from the white water, it was apparent that enzyme addition resulted in darker pulps when compared to the controls. This was verified when handsheet brightness was measured (Table 5-3). Handsheets produced from control treatments had an average brightness of 50.8 % ISO, while sheets produced using control pulps with alum post treatment had a brightness of 47.5 % ISO. In contrast, a sharp decrease in brightness was noted for handsheets made using enzyme treated pulp, demonstrating a brightness of 40.1 % ISO. There was nearly a four point gain in brightness to 43.9 % ISO when the enzyme treated pulp and alum post treated white water were used to produce handsheets. Unfortunately, the brightness was still significantly lower than was obtained with the controls.

<table>
<thead>
<tr>
<th>Property</th>
<th>Control</th>
<th>Control + Alum</th>
<th>Treated</th>
<th>Treated + Alum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brightness (% ISO)</td>
<td>50.8 ± 0.2</td>
<td>47.5 ± 0.2</td>
<td>40.1 ± 0.5</td>
<td>43.9 ± 0.2</td>
</tr>
<tr>
<td>Scattering coefficient</td>
<td>67.9</td>
<td>66.8</td>
<td>69.3</td>
<td>66.6</td>
</tr>
<tr>
<td>(620 nm, m²/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absorption coefficient</td>
<td>5.5</td>
<td>6.8</td>
<td>12.4</td>
<td>9.1</td>
</tr>
<tr>
<td>(457 nm, m²/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5-3. Optical properties of handsheets formed in control white water, control water with added alum, treated white water, and treated water with added alum. (± SD)
Scattering and adsorption coefficients were also determined for the four paper types (Table 5-3). The scattering coefficients of the handsheets made from either enzyme treated or control pulps were all quite similar, further indicating enzyme addition did not significantly alter the fiber bonding or sheet density. In contrast, the calculated adsorption coefficients followed the same trend as handsheet brightness, with values for the sheets made from control pulps significantly lower than those for the enzyme treated pulps. This confirmed that lower handsheet brightness resulted from increased light adsorption.

Brightness is a very important concern at pulp and paper mills and, as a result, mills spend considerable amounts of money on bleaching and chemical additives to produce white paper products. The observed decreases in paper brightness are of course unwanted and were likely caused by redeposition of lignin-like material and colloidal particles onto the fibers and forming paper. Additionally, some of the lignin within the pulp may have been oxidized during the enzyme treatments, further decreasing brightness. Comparing only the handsheets produced from control pulp and control white water with or without alum post treatment, it was apparent that the addition of alum resulted in decreased paper brightness. This suggested that the alum helped bridge some of the colour producing contaminants in the white water onto the fiber during paper formation. As for the enzyme treated pulps, it was clear from the brightness values obtained that enzyme addition resulted in even greater deposition of these coloured materials onto the fibers. In the enzyme treated white waters, nearly all of the phenolic compounds were polymerized into higher molecular weight structures. During papermaking, it was evident that some of these larger molecules were trapped with the
forming sheet, resulting in handsheets with the lowest average brightness. Alum post treatment of enzyme treated white water removed a majority of these larger lignin-like structures, and when used for papermaking it produced sheets with improved brightness. However, removal of phenolics from the white water did not overcome the brightness decreases caused by redeposition during the enzyme treatments. For this reason, it would likely be beneficial to add an alum treatment step before the mechanical pulp is mixed with the enzyme treated white water.

5.5 Lignin Content and Pulp Brightening with Hydrogen Peroxide

To further investigate the redeposition of colour producing contaminants onto the fiber, which resulted in decreased pulp brightness, the lignin content and bleachability of the enzyme treated and control pulps were determined. The pulps were brightened with hydrogen peroxide using either a single or two-stage sequence. Brightness pads were then produced and any potential increases to pulp brightness were measured.

From the initial visual inspection of the enzyme treated and control pulps, the decreased brightness of the enzyme treated pulp suggested an increased lignin content. It was thought that changes made to the white water contaminants and fiber surfaces as a result of enzyme addition caused increased deposition of lignin-like materials onto the pulp. However, when the lignin contents were determined, the values obtained for the enzyme treated and control pulps were not significantly different (Table 5-4). It is possible that the deposition of a very small amount of lignin-like material was responsible for the brightness decrease observed, and the procedure for lignin content determination simply lacked the sensitivity required (Table 5-5). Furthermore, exposure to strong acid and high temperature may have disrupted some of the redeposited material.
to the point of being undetectable. Alternatively, enzyme catalyzed oxidation of the lignin present on the fiber surfaces may have been largely responsible for the decreased pulp brightness. In the future it may be useful to use a method such as electron spectroscopy for chemical analysis (ESCA) that allows for very accurate determinations of lignin and extractive coverages of mechanical fiber surfaces (Bendzala et al., 1995; Koljonen et al., 1997).

<table>
<thead>
<tr>
<th>Pulp Sample</th>
<th>Lignin Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.00 ± 0.13</td>
</tr>
<tr>
<td>Enzyme Treated</td>
<td>29.17 ± 0.06</td>
</tr>
</tbody>
</table>

Table 5-4. Lignin content of control and enzyme treated pulps. (± SD)

Hydrogen peroxide was next applied to the enzyme treated and control pulps to identify potential changes to bleachability resulting from enzyme addition. When the control pulp was brightened, the first stage increased brightness by 14.4 points above unbrightened pads for an average of 64.3 % ISO, while the second brightening stage further increased brightness to 69.1 % ISO (Table 5-5). The pads produced from enzyme treated pulp had an initial brightness of 41.4 % ISO, but after a single brightening stage averaged 61.9 % ISO, an improvement of 20.5 points. The second brightening stage further increased pad brightness to 67.8 % ISO, which is close to the average value obtained for the control pulp. These results indicate that a two stage peroxide brightening sequence may restore the loss in pulp brightness due to enzymatic treatment of white
water and pulp. Under identical conditions, both brightening stages increased brightness of the enzyme treated pulp to greater extents when compared to brightness increases for the control pulp. This was likely due to higher concentrations of lignin-like materials on the enzyme treated fiber surfaces that were more accessible to the peroxide.

<table>
<thead>
<tr>
<th>Pulp Sample</th>
<th>Brightening Sequence</th>
<th>Brightness (%ISO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Unbrightened</td>
<td>49.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>QP</td>
<td>64.3 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>QPP</td>
<td>69.1 ± 1.3</td>
</tr>
<tr>
<td>Enzyme Treated</td>
<td>Unbrightened</td>
<td>41.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>QP</td>
<td>61.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>QPP</td>
<td>67.8 ± 0.6</td>
</tr>
</tbody>
</table>

Table 5-5. Brightness of pads produced from control or enzyme treated pulps and brightened with hydrogen peroxide. (± SD)

5.6 Conclusions

Large scale enzyme treatment of white water and pulp did impact paper properties to some extent, as did alum post treatment of white water. It was clear that the addition of enzymes and/or alum did not significantly alter sheet consolidation, interfiber bonding, or individual fiber strength. This was an important observation in that any reductions to paper strength properties would be unacceptable to the mill operator. When the paper surface properties were measured, it was noted that both enzyme treatment and alum post treatment were required to appreciably change air resistance and roughness of the paper. It is likely that the removal of the enzyme polymerized phenolics and redeposition of
smaller colloidal particles resulting from alum addition combined to decrease paper porosity and roughness. These changes are quite positive in that they have the potential to improve printing properties. Finally, paper optical properties were negatively impacted by the enzyme treatments. It is likely that the decreased paper brightness resulted from a combination of lignin oxidation and increased deposition of colour producing contaminants onto the fiber during the enzyme treatments. However, the application of a two-stage hydrogen peroxide brightening sequence resulted in enzyme treated pulp with comparable final brightness to brightened control pulp. These results, combined with the previous results demonstrating the ability of enzymes to remove DCS from white water, continue to suggest the potential of this fungal and enzyme treatment strategy for contaminant removal from mechanical pulp and paper mill process waters.
CHAPTER VI. Concluding Remarks

6.1 Conclusions

The primary objective of the work conducted in this thesis was to gain a more complete understanding of fungal and enzyme treatment for mechanical pulp and paper mill process water remediation. Fungal growth and extracellular enzyme production were extensively investigated, with concurrent examination of the thermostabilities of the liberated enzymes. Scale-up was attempted in a bioreactor in order to compare larger scale growth to that observed in shake flasks. The enzymes produced in both cases were then used to treat fresh white water, and mechanical pulp and the resulting changes to specific white water, fiber, and paper properties were determined. The goal was to help move this treatment strategy towards becoming a commercial reality by elucidating specific aspects critical to its future implementation.

It was clear from the initial growth experiments that the white-rot fungus utilized in this treatment strategy, *Trametes versicolor*, did not produce sufficient extracellular enzymes when grown at elevated temperatures. At 45 or 60°C, enzyme production was significantly inhibited in comparison to the levels obtained at 30°C. When this treatment technology is incorporated into a water system typically operating between 50 and 60°C it is apparent, from these results, that cooling of the white water diverted for fungal growth will be required. However, it was also evident that the enzymes collected were to varying extents thermostable at 65°C, confirming the ability of these enzymes to remain active at the high temperatures found in the water systems at mills. Cellulase/hemicellulase, lipase, and laccase enzymes quickly lost some activity upon exposure to 65°C, but a significant percentage of the enzymes were able to maintain...
activity for extended periods of time. This suggested that a proportion of the enzymes produced were more tolerant to high temperature than others, which may allow for the buildup of appreciably high levels when released into a recirculating water system. Furthermore, the more moderate temperatures normally present at mills, as well as the presence of many different substances within the water system, may help to further stabilize the enzymes and improve water treatment.

The observed enzyme production by *T. versicolor* grown in a larger scale bioreactor was very similar to that determined previously for smaller scale growth. The levels of cellulolytic/hemicellulolytic and lipase enzymes in both cases were quite comparable, while laccase activity present in the bioreactor produced culture filtrate was significantly higher than that measured in the shake flasks. This was very promising in that future implementation of this treatment strategy is in part dependent on the ability to produce sufficient amounts of enzymes to treat the large volumes of white water at mills. These initial results demonstrate this potential and suggest that substantial enzyme production in larger bioreactors is possible.

When the extracellular enzymes were used to treat fresh white water and pulp on a small scale, specific changes to both white water and pulp properties were noted. The addition of enzymes resulted in a reduction in the average colloidal particle size, decreased particle zeta potential, increased molecular weight of phenolic compounds, and increased water and fiber charge. The treatments were conducted at 65°C, and it was apparent that most of the changes made to water and fiber properties occurred very rapidly. The ability to quickly catalyze reactions at high temperature is desirable, and further demonstrates the potential for efficient white water treatment. Fast reaction rates
were also evident when white water and pulp treatments were scaled-up using the bioreactor produced culture filtrate. Larger scale treatment resulted in the removal of most lipophilic extractives, including the near total removal of ester bonded extractives and, as before, increased the molecular weight of the phenolic compounds present.

Overall, the changes made to the white water and pulp properties as a result of enzymatic treatment were positive. Enzyme addition altered the physio-chemical nature of the contaminants present and this facilitated easier removal when alum post treatment was applied. Phenolics with increased molecular weight resulted in compounds with decreased stability within the water, which enabled nearly complete removal of these compounds using alum induced precipitation. As well, reduced colloidal particle stability, as indicated by increased zeta potential, also enhanced removal of some of the remaining lipophilic extractives. The slight increase in white water cationic demand after the enzyme treatments was unwanted. However, the concurrent increase in fiber surface charge far overshadowed the higher water charge. Enzymatic changes made to the fibers, and enhanced redeposition of altered contaminants were likely responsible for this increased charge, which has the potential to reduce chemical costs by improving chemical-fiber bonding.

Changes to specific paper properties as a result of enzyme treatment of white water and pulp were also investigated. It was evident that neither enzyme addition nor alum post treatment significantly altered handsheet dry strength properties. The treatments did not change sheet consolidation, as revealed by similar handsheet densities and scattering coefficients for each of the paper types. Further, interfiber bonding was unaffected, as is reflected in the similar tensile, tear, and burst indices. On the other
hand, paper surface properties were significantly affected by the combination of enzyme addition and alum post treatment. The ensuing handsheets were found to have smoother surfaces and lower porosity. It is likely that the removal of polymerized phenolics and enhanced deposition of smaller colloidal particles during papermaking as a result of alum addition combined to improve these handsheet properties. The production of smoother, more uniform paper is desirable in that it has the potential to improve printing properties. Finally, enzyme treatment of white water and pulp negatively impacted paper optical properties by significantly reducing brightness. It appeared that lignin oxidation and redeposition of polymerized phenolic substances onto the pulp during the enzyme treatments resulted in darker pulp and consequently paper with lower brightness. However, the application of a two stage hydrogen peroxide brightening sequence produced enzyme treated pulp with comparable ISO brightness values to the corresponding control pulps. Thus, it appears that the negative impact to paper optical properties resulting from enzyme addition can be easily overcome, further demonstrating the potential of this fungal and enzyme treatment strategy for DCS removal from pulp mill white water.

6.2 Future Work

The development of this novel treatment strategy in an attempt to provide mechanical pulp and paper mills with an efficient and cost effective solution to water system closure has been a challenging and enlightening process. Our previous work demonstrated the potential of using a fungal and enzyme treatment strategy to remove contaminants from different white waters. The work outlined in this thesis further evaluated this treatment strategy by investigating fungal growth and enzyme production,
enzyme thermostability and the resulting impacts to specific white water, fiber, and paper properties after enzyme treatments of white water and pulp. However, in order to bring this strategy to commercial fruition, further research and development is needed before actual implementation can occur.

The most obvious area requiring further investigation is that of fungal growth and enzyme production. Considering the huge volumes of water circulating within a mill, it seems likely that some form of a large continuous bioreactor will be required for enzyme production. To date, all of our fungal growth has been done in batch mode, with subsequent enzyme collection after a specified growth period and before addition to white water or pulp. It would be very useful to attempt fungal growth and enzyme production in a bioreactor with a continual input of fresh white water and release of culture filtrate. Initially, a smaller scale bioreactor could be used to optimize various factors such as flow rate, oxygen requirements, and temperature, before scale-up occurred. Furthermore, the decanted culture filtrate could be used to treat fresh white water in a model recirculating water system in order to further simulate treatment at a mill.

Another aspect warranting further research is the treatment schedule used in regards to enzyme addition and alum post treatment. It was clear that the addition of pulp to enzyme treated white water resulted in the redeposition of polymerized lignin-like material onto the fiber, which ultimately reduced handsheet brightness. Therefore, it may be useful to introduce an alum treatment step before pulp addition to remove these contaminants before deposition can occur. Doing so might result in handsheets with brightness values surpassing those of the controls, as it was apparent that alum post
treatment removed a significant amount of these colour producing contaminants. As well, other post treatment chemicals could be added in combination with alum to further induce floc formation and aid in particulate removal. Finally, enzyme addition directly to the mechanical pulp should be attempted, as this may result in enhanced fiber modification and improved paper strength properties.

Along with the work described, it is also desirable to continue some of the more fundamental research looking specifically at the changes made to both DCS and pulp as a result of enzymatic treatment. It would be valuable to elucidate the different mechanisms that lead to DCS removal, as well as the ultimate fate of the many different contaminants originally present in the white water. Changes to the fiber surfaces resulting from enzyme addition and redeposition should also be further quantified with more sensitive techniques such as ESCA and EDAX. It was clear from the research conducted that one of the results was increased fiber surface charge and therefore, different chemical additives commonly used should be included during papermaking to promote fiber crosslinking and paper strength.

If all of this future work is completed, the development of this fungal and enzyme treatment strategy for DCS removal from white water will indeed be greatly advanced. Once these additional points are addressed, incorporation into a pilot plant could be attempted, and finally full implementation into the water system at a mechanical pulp and paper mill.


