ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

DEGRADATION of PITCH COMPONENTS in RECYCLED CARDBOARD PRODUCTION by FUNGAL LIPASE

M.Sc. Thesis by
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Supervisors : Assoc. Prof. Dr. Candan TAMERLER BEHAR
Assist. Prof. Dr. Hakan BERMEK

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ABBREVIATIONS

CTMP : Chemo Thermo Mechanical Pulping
DAG : Diacylglycerol
ECF : Elementary Chlorine Free Bleaching
FPLC : Fast Performance Liquid Chromatography
FSOT : Fused Silica Open Tubular
FID : Flame Ionization Detector
GC : Gas Chromatography
GC-MS : Gas Chromatography Mass Spectrophotometry
HPLC : High Performance Liquid Chromatography
MAG : Monoacylglycerol
SCOT : Support-Coated Open Tubular
SPE : Solid-Phase Extraction
SSF : Solid State Fermentation
TAG : Triacylglycerol
TCF : Total Chlorine Free Bleaching
TLC : Thin Layer Chromatography
TMP : Thermo Mechanical Pulping
WCOT : Wall-Coated Open Tubular

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DEGRADATION of PITCH COMPONENTS in RECYCLED CARDBOARD PRODUCTION by FUNGAL LIPASE

SUMMARY

Biological pitch removal is a biotechnological method that has been developed for the paper and pulp industry. Accumulation of wood extractives in pulp and paper mills results in low-quality pulp and blockages that cause shutdowns of operations and important economic losses, reduces the quality of product and also create waste water toxicity. The wood extractives, so-called pitch is mainly composed of triacylglycerides, fatty acids, resin acids, waxes, sterols, sterol esters, mono and diacylglycerides The amount of problematic pitch can be reduced by biological and chemical processes during paper production. Pitch problems might increase when recycled paper is used since various new lipid compounds are introduced into the pulp, i.e. oil-based ink.

In the recent years, this problem was tried to be solved by utilizing wood-dwelling fungi which naturally degrade pitch components. It has been shown that the two most successful fungi for pitch degradation are *Fusarium oxysporum* and *Ophiostoma piliferum*. These fungi produce lipase that hydrolyzes triglycerides. The aim of this study is to analyze the pitch components found in recycled paper to develop a suitable degradation method.

In this study, acetone extractions on dried pulps were made for 6 hours. Samples were analyzed on a GC to detect the amount of triacylglycerides, free fatty acids, esters, hydrocarbons, mono and diacylglycerides. The enzymes used were *Fusarium oxysporum* A685 (IJFM) and *Ophiostoma piliferum* growth medium supernatants as well as commercial enzymes Lipozyme IM, Lipozyme TL IM, Novozym 435 from Novo Nordisk as controls. Lipase activity was assayed by using pH-stat. The amount of free fatty acids were also determined by acid value analysis in order to get a better quantification of the hydrolysis. Preliminary results demonstrated that *F. oxysporum* and *O. piliferum* growth medium supernatant can be efficiently used for pitch removal in recycled paper production.

GERİ DÖNÜŞÜMLÜ KARTON ÜRETİM İŞLEMİNDE PITCH BİLEŞİKLERİNİN KÜF MANTARI LİPAZI KULLANILARAK DEGRADE EDİLMESİ

ÖZET

Pitch maddelerinin biyolojik olarak arındırılması, kağıt endüstrisi için son yıllarda geliştirilmiş biyoteknolojik bir metoddur. Odun eksraktiflerinin kağıt makineleri üzerinde birikmesi, düşük kaliteli kağıt hamuru elde edilmesine ve blokajlara neden olur ki bu da üretimin durması, önemli ekonomik kayıplar, ürün kalitesinin düşmesi ve zehirli atık su oluşması gibi sonuçlar doğurur. Pitch denilen odun ekstraktifleri temel olarak triaçilgliseritler, yağ asitleri, reçine asitleri, mumlar, steroller, sterol esterleri, monogliseritler ve digliseritlerdir. Problemli pitch maddelerinin miktarı, kağıt üretimi esnasında biyolojik ve kimyasal işlemlerle azaltılabilir. Pitch maddelerinin oluşturduğu problemler, geri dönüşümlü kağıt kullanıldığında yeni yağ bileşiklerinin kağıt hamuruna ilave olması nedeniyle artabilir.

Son yıllarda, bu problem doğal olarak pitch bileşenlerini parçalayabilen ve odun üzerinde yaşayan küf mantarları kullanılarak aşılmaya çalışılmaktadır. Pitch parçalanmasında en başarılı olan küf mantarları *Fusarium oxysporum* ve *Ophiostoma piliferum*'dur. Bu küf mantarları triaçilgliseritleri hidrolize edebilen bir enzim olan lipaz üretir. Bu çalışmanın amacı, geri dönüşümlü kağıtta bulunan pitch bileşenlerini analiz ederek bunları parçalamak için uygun bir yöntem geliştirmektir.

Bu çalışmada, kurutulmuş kağıt hamurları aseton ile 6 saat süresince ektraksiyona tabi tutulmuştur. Örneklerdeki triaçilgliserit, serbest yağ asidi, ester, hidrokarbon, mono ve diaçilgliseritler; gaz kromatografi cihazında ölçülmüştür. Kullanılan enzimler, *Fusarium oxysporum* A685 (IJFM) ve *Ophiostoma piliferum* kültürlerinden elde edilen süpernatanlar ile kontrol olarak ticari enzimlerden Lipozyme IM, Lipozyme TL IM, Novozym 435 (Novo Nordisk). Lipaz aktivitesi pH-stat cihazı ile ölçülmüştür. Hidroliz miktarını daha iyi saptamak amacıyla serbest yağ asitlerinin miktarı asit değeri tayini ile belirlenmiştir. İlk sonuçlar göstermiştir ki, *Fusarium* ve *Ophiostoma* kültürlerinden elde edilen süpernatanları geri dönüşümlü kağıt üretiminde pitch maddelerinin yok edilmesinde etkilidir.

1. INTRODUCTION

1.1 Wood Structure

Wood is a complex polymeric material that contains 40-60 % cellulose, 17-32 % lignin and hemicellulose; and other components such as resins, lipids, starch, phenolic compounds, tannins, waxes, inorganic acids and bases in its structure (Başbakanlık Devlet Planlama Teşkilatı, 1990). The source of paper is cellulose, which is a carbohydrate, and not soluble in water. It is the primary cell wall component in most plants (Gökmen, 2000). The second important component of wood, lignin is a complex polymer made of C and O bridges of aromatic alcohols. It plays a crucial role in binding cellulose fibers in wood structure. Lignin is mostly found in cell membrane and middle lamella (Browning, 1963). Lipophilic compounds are a group of chemicals, that contain fatty acids and their derivatives, and elements that can chemically bind to them. Lipids are soluble in organic solvents such as ether, benzene and chloroform; but not soluble in water. The most important lipophilic compounds are fats, fatty acids, glycerides, resins, resin acids and waxes. Lipids are chemically non-homogenous mixtures that have triacylglycerols as main component and found in internal structure of plants and animals. Triacylglycerols are esters of glycerol and fatty acids. Fatty acids form nearly the 94-96 % of triacylglycerols as molecular weight (Civelekoğlu *et al.*, 1990).

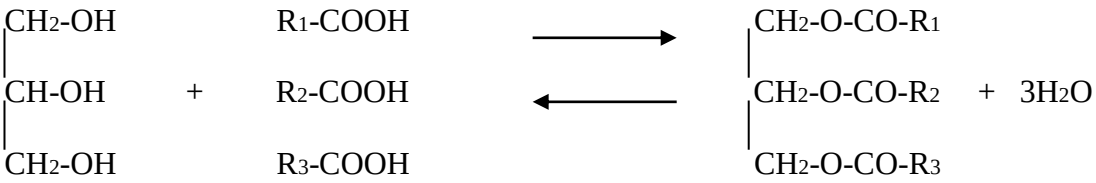

Figure 1.1: The ester bonds of fatty acids with glycerol form triacylglycerol molecule.

Glycerides are alcohols that can mix with water and ethyl alcohol in any proportion. Acylglycerols are esters of fatty acids with glycerin, which can be grouped into 3 by the number of fatty acid content, monoacylglycerols, diacylglycerols and triacylglycerols. Waxes are esters of complex fatty acids with alcohols. They are not

soluble in water, but soluble in organic solvents very well (Civelekoğlu *et al.*, 1990). Resins are one of the most important lipids in wood structure. They are not saponifiable and show differences in different tree types. Resin components are resin acids, free fatty acids and the esters of these with glycerol and other alcoholic compounds, sterols, alcohols, terpenes and waxes. Sterols are the most important part of resins. They are found in the lipids of animal products as free or esterified compounds while the sterols in wood structure are free (Browning, 1963).

1.2 Pulp and Paper Production

Paper pulp manufacturing is the first non-food industrial utilization of plant biomass. This industrial process consists of: (1) pulping the wood to separate fibers by chemical or mechanical means; and (2) bleaching the pulp by the sequential action of chemical reagents and alkaline extractions (Gutierrez *et al.*, 2001).

Paper is made from fibrous vegetable raw materials, in most cases contains non-fibrous inorganic additives. Pulp fibers mainly originate from various wood species and consist of organic lignocellulosic material. The production of paper consumes large amounts of natural resources, regardless of whether the paper products are based on chemically or mechanically defibrated pulps. In principle, pulp may be produced by chemical or mechanical pulping or by a combined method (Rousu *et al.*, 2002).

1.2.1 Pulping

The objective of pulping is to extract cellulose fibers from plant material, generally hard or softwood trees. Two approaches have been employed to pulp wood; mechanical pulping and chemical pulping. Reports show that a biological approach has potential for improving both the economics and environmental impact of pulp generation. Traditionally, a mechanical process that has been improved over time by modifications such as refiner mechanical pulping and thermomechanical pulping has accomplished less cellulose removal. Although efficient, these methods are extremely energy intensive. Chemical pulping dissolves lignin from the cellulose and hemicellulose fibers. A variety of chemical pulping processes has been developed, together with improved bleaching methods to meet growing demands for paper of increasing quality and in greater yields. The primary chemical pulping process

employed today is the kraft process, in which wood chips are cooked in a solution containing sodium hydroxide and sodium sulfide (Breen and Singleton, 1999) named 'white liquor'. Alkaline kraft pulping (sodium hydroxide and sulfide cooking at 155–180°C and 7–11 bar) is the dominant process for wood pulping because of the high quality of the pulps obtained after extensive removal of lignin (Gutierrez *et al.*, 2001). A subsequent bleaching step results in a high and reasonably permanent brightness of the pulp. Common bleaching chemicals are gaseous chlorine, chlorine dioxide and oxygen. Because of environmental reasons, gaseous chlorine is being replaced today either with chlorine dioxide (the so-called elementary chlorine free, ECF, bleaching) or with chlorine-free chemicals such as oxygen, hydrogen peroxide and ozone, (the so-called total chlorine free, TCF, bleaching) (del Rio *et al.*, 1998). In the chlorine-free effluents from TCF mills, resin acids, fatty acids and steroids released from wood during pulping have become the primary source of toxicity. The main differences between the two pulping processes are the yield, up to 99 % for mechanical pulps and ~50 % for chemical pulps, and the quality of the paper that has significantly higher mechanical properties in the case of chemical pulping. There is a degradation of cellulose together with lignin, which accounts for 20–30 % wood weight, a fraction of polysaccharides is solubilized during chemical pulping, which results in decreased pulp yield. By contrast, no significant modification or removal of wood constituents is produced during mechanical pulping and very high yields are obtained (Gutierrez *et al.*, 2001). In mechanical pulping, the raw material is utilized to make the end product with a high yield, while chemical pulping is not very yielding (Rousu *et al.*, 2002). The most important disadvantage of chemical pulping is the large amounts of potentially hazardous chemicals, which pose a danger to both mill workers and the environment (Breen and Singleton, 1999). Chemical pulp is produced from non-woody plant materials in some countries which do not have sufficient wood resources (Atchison, 1995). Generally, however, non-wood plant material pulping has resulted in overwhelming environmental problems (Runbin, 2000).

The office and kraft papers are typical papers and paperboard products based on chemical pulp. Mechanical processes, or combinations of chemical and mechanical processes, produce a different quality of pulp with a higher fiber yield than the chemical processes. The most prominent among the mechanical processes is TMP

pulping. An advantage of mechanical pulping processes is their high yield of pulp per ton of wood input (Byström and Lönnstedt, 2000).

Biopulping is a process involving the treatment of wood chips with white-rot fungi prior to pulping. White-rot fungi alter the wood cell wall, which softens the chips and substantially reduces the energy needed for pulping (Gadd, 2001). This biotechnological method provides a decrease of residual lignin levels in chemical pulps, improvement of paper strength properties, and waste reduction (Akhtar *et al.*, 1997; Messner, 1998). White-rot fungi capable of selective lignin degradation seem to be the best organisms for biopulping (Leatham *et al.*, 1990).

1.2.2 Pulp Bleaching

In the production of white paper from chemical pulps, a variety of oxidizing or reducing chemicals and alkaline extractions is needed. Bleaching agents, such as hydrogen peroxide and dithionite, are applied to mechanical pulps. Elemental chlorine has been traditionally used to bleach chemical pulps due to the high efficiency of this strong oxidant. However, it must be avoided because of the formation of chlorinated lignins and phenols that are discharged in wastewaters and cause toxicity. Environment friendly bleaching sequences involve the substitution of chlorine by chlorine dioxide, in the case of ECF bleaching, and the complete elimination of chlorinated reagents (to be substituted for example by hydrogen peroxide, oxygen, ozone or xylanases) in the case of TCF bleaching which are being introduced in the European pulp and paper industry to completely avoid chlorinated compounds in the mill effluents and final products, despite some difficulties in attaining high brightness degrees (Gutierrez *et al.*, 2001).

1.2.3 Recycled Paper

Pulp and paper industries are economically very important for many countries, because of their influence on world economy, and their products are consumed worldwide. Increased recycling of fibers has reduced the need for virgin fibers, but due to the increasing fast growth of paper consumption, the utilization of virgin fibers has continued to increase. Also not even electronic communication has been able to detract from the importance of paper products and their utilization (Rousu *et al.*, 2002).

Secondary fiber is a very important source of raw materials in the paper industry. Many important paper products are made from 100% recycled paper. With production at high levels, linerboard mills are increasing their capacity by adding recycled corrugated boxes to their product lines. However, the use of these raw materials is often accompanied by particular problems such as lower product quality or problems of machine functions (Hoekstra and May, 1990).

The closing up of mill water systems and the increased use of secondary fiber have certainly presented the papermaker with a new set of operating problems. Dirt, pitch stickies, etc. which previously had at least been partially removed through more open systems, now remain and cycle up in the paper making process. The increased use of secondary fiber has compounded the situation by introducing still higher levels of contaminants into the systems. The resultant build up of sticky deposits at wet end, on felts and throughout the dry end creates many problems for the papermaker. As a result, the various benefits and potential benefits of paper recycle and the closing up of mill water systems cannot be used because of these kinds of problems (Kenny and Engstrom, 1990).

The incorporation of secondary fiber in paper production affects the final product quality and the papermaking process. Basically, these changes are caused by the inferior fiber quality (length, flexibility) and the higher drainage resistance of recycled pulps. The first affects inter-fiber bonding and consequently fiber strength. The second makes sheet formation more difficult, decreases the paper machine runnability and increases energy consumption in dryers (Pommier, 1989). Several upgrading techniques are known, namely alkali treatment, refining/beating processes, additive usage or non-wood plant fiber incorporation. However, their advantages to paper manufacture are sometimes limited as they do not assure the simultaneous increase in paper resistance and pulp drainage ability (Allen, 1995). The use of enzymes is a promising alternative (Pala *et al.*, 2001).

Stickies is a term often used to describe certain deposits caused by organic materials used in paper and board converting operations and introduced into machine furnishes with the secondary fibers obtained from the trimmings, clippings, or other waste paper from these converting operations. The word derives from the fact that these deposits cause sticking on wires, felts, and other parts of paper machines (Hoekstra and May, 1990).

Sticky contaminants in waste paper originate from the adhesive backing for labels and tape, coated products (wax, asphalt, polyethylene, etc.) and plastics. In processing waste paper for reuse, these contaminants, individually or in combination, may cause serious papermaking and product quality problems. Stickies may agglomerate in the stock and affect final product appearance and end-use properties, e.g., at the printers. The contaminants may also form deposits impairing runnability and efficiency of process machinery and clothing (Krueger and Bowers, 1990). Surface charge plays an important role in sticky deposition. Charged particles of similar polarity are stabilized by repulsive electrostatic forces, which retard agglomeration. In the case of hydrophobic particles these forces are weak. If there is no energy barrier present, colloidal hydrophobic particles will rapidly agglomerate, producing secondary stickies (McKinney, 1990). Stickies commonly necessitate complete shut down of the manufacturing equipment in order to remove such deposition by some solvent washing techniques. This cleaning process is expensive due to downtime as well as solvent costs, and may present effluent problems (Miller, 1990).

Recycling and reuse have been key issues around the globe since 1980's. The trends towards increased material and energy efficiencies and increased recycling are similar across various industries. The recycling technology includes pulping, screening, refining, washing, drying and finishing. This system also includes primary treatment of wastewater. (Lopes *et al.*, 2003)

Waste paper pulping generally requires less energy per ton of pulp than pulping processes that use virgin fiber and saves the use of virgin fiber inputs. In contrast to chemical pulping of wood from virgin sources, waste paper pulping provides no woodwastes or dissolved chemicals for energy generation. As a consequence, increased waste paper utilization reduces the availability of biomass energy (Byström and Lönnstedt, 2000).

The waste paper cycle has been modelled by Byström and Lönnstedt in their model differ between 12 paper qualities: newsprint, SC paper, LWC, office paper (wood-free), coated paper (wood-free), tissue, white lined chipboard, 'return fiber chipboard', wrapping paper, white liner, kraft-liner and fluting. Each product uses its own recipe for how to mix fibers, filler and energy. Fibers could be virgin or recycled. Furthermore, different types of pulp exist. The two major qualities are

chemical and mechanical pulp. Chemical pulp is classified depending on the type of fiber, i.e. short or long fibers. Still another pulp quality is semichemical. For each pulp quality, the need for pulp wood (short and long fibers) and energy must be specified. At the site of the pulp mill, electricity can be produced from back-pressure power.

After end use, paper is recycled for the production of paper and board, recovered for energy and/or deposited as land-fill. If recycled, the paper is recovered, sorted, baled and transported to paper mills in the country where the consumption took place or exported to countries with waste paper deficits. If recovered for energy use or transported to a land-fill dump, the waste paper normally follows the waste-handling system (Byström and Lönnstedt, 2000).

1.2.4 Cardboard

There are the four main types of cardboard:

Chrome cardboard is used for packaging. The first layer of chrome cardboard is white. It is fixed suitable for printing with a proper filling material and face plastering process. The bottom and medium layers are grey that are made of enduring pulp (Özaslan, 2001).

Bristol cardboard is a high quality cardboard which has both faces white with bleached pulp. The first layer is very good plastered while bottom layer that has less filling material is not.

File holder cardboard is made of bleached kraft pulp in various colors. These are used for file holder and notebook cover production and in some packaging which is needed to be economic.

Grey cardboard is made of recycled cardboard by mechanical pulping. These are used in paperboard and bag production. Paperboard is a bulky material produced by attaching various cardboards together with sodium silica (Arıoğlu, 1992).

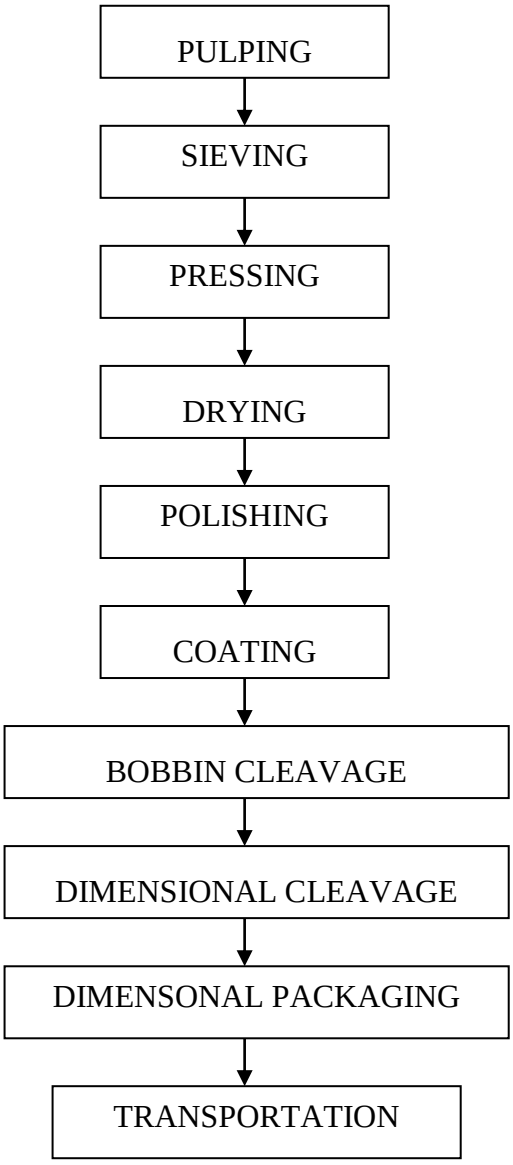

Figure 1.2: The flowchart of production process in Kartonsan Cardboard Industry and Commercial Inc.

1.3 Determination of Pitch

Wood extractives, a term that refers to a large number of wood compounds, which are soluble in organic solvents, are known to give sticky deposits on pulp and paper mills (del Rio *et al.*, 1999). Wood extractives that are extractable from wood with organic solvents cause production and environmental problems in pulp and paper manufacturing (Gutierrez *et al.*, 2001). Wood extractives amount to 2-6% of wood

dry weight. These compounds include fats, waxes, fatty acids and alcohols, free and esterified sterols, resin acids, terpenes, and phenolics (Fengel *et al.*, 1989).

Wood extractives that show lipophilic character consist of complex mixtures of many different compounds, from the low-molecular-mass resin and fatty acids to the high-molecular-mass waxes, sterol esters and triacylglycerols (Gutierrez *et al.*, 1998). These lipophilic compounds, which form the so-called wood resin, are the most problematic and they include free fatty acids, resin acids, waxes, fatty alcohols, sterols, sterol esters, glycerides, ketones and other oxidized compounds. There are differences in resin content and composition between different parts of the tree (including between heartwood and sapwood) and, depending on the age of tree, the growing conditions and other genetic and environmental factors. During wood pulping and refining of paper pulp, the lipophilic extractives in the parenchyma cells and softwood resin canals are released, forming colloidal pitch. These colloidal particles can turn into larger droplets that deposit in pulp or machinery forming 'pitch deposits', or remain suspended in the process waters (Gutierrez *et al.*, 2001).

The accumulation of pitch during pulping and papermaking cause significant technical and economic troubles in pulp and paper manufacture (Gutierrez *et al.*, 1998), results in low quality pulp and can cause the shutdown of mill operations. The pitch in pulp mills causes the loss of money as a result. Contaminated pulp is the source of the problems in paper machine operation, including the production of spots and holes in the paper, sheet breaks and technical shutdowns. Moreover, the increasing need for recirculating water in pulp mills is leading to an increase in pitch concentration, which results in higher deposition. In addition, some wood extractives could have an environmental impact when released into wastewaters (Gutierrez *et al.*, 2001). Pitch increases the toxicity of effluents (Tamerler *et al*., 1999).

Pitch can cause quality defects in the finished product. Wood triacylglycerols play a key role in the formation of pitch deposits, particularly in mills utilizing softwoods (Allen, 1988; Fischer, *et al.*, 1993). Waxes and sterol esters that are relatively abundant in hardwoods such as aspen (Populus tremuloides) and birch (Betula spp.), are also associated with pitch deposition (Breuil *et al.*, 1997; Rocheleau *et al.*, 1998). Furthermore, resin constituents extracted from the wood furnish during pulping have a major contribution to the aquatic toxicity displayed by forest industry effluents (Leach and Thakore, 1976; O'Connor *et al.*, 1992).

Table 1.1: The pitch components of softwoods compared with hardwoods (mg/g)

(Gutierrez, A., del Rio, J.C., Martinez, M.J. and Martinez, A.T., 2001)

	Softwoods		Hardwoods		
	Pinus sylvestris	*Picea abies*	*Betula verrucosa*	*Populus tremula*	*Eucalyptus globulus*
Free fatty acids	1.73	0.78	–	1.06	0.28
Resin acids	6.65	2.85	0.06	0.17	0
Hydrocarbons	0.74	0.19	0.40	1.14	0.17
Waxes or sterol esters	0.83	0.87	1.96	3.07	0.57
Monoglycerides	0.18	0.55	2.24	1.18	0.02
Diglycerides	0.32	0.55	1.72	0.58	0.02
Triglycerides	8.74	1.94	8.10	10.37	0.13
Higher alcohols or sterols	1.39	1.00	1.56	2.40	0.68
Oxidized compounds	0.43	1.36	2.94	1.53	0.22
Lipophilic compounds	22.9	10.4	20.3	22.7	2.60
Total acetone extract	31.0	22.2	34.6	45.3	15.2

The different classes of extractives have different chemical effects during and after pulping. In neutral to acidic processing of wood, the lipophilic extractives are difficult to remove, and resinous woods with a high content of triacylglycerols and resin acids pose more of a problem for pitch control. In alkaline processing, however, the total extractives content may not be as important as the composition of the extractives (Dunlop-Jones *et al.*, 1991). During alkaline kraft pulping, glycerol esters are completely saponified and fatty and resin acids dissolved, although some sterol esters and waxes are not completely hydrolyzed under the kraft pulping conditions (Chen *et al.*, 1995). These compounds, as well as free sterols, do not form soluble soaps and have a tendency to be deposited and cause pitch problems. Higher concentrations of these compounds in relation to the saponifiable extractives is the main cause for pitch problems in the kraft pulping of some hardwoods, such as aspen or eucalypt, that are commonly used in the pulp and paper industry (Swan, 1967; Allen *et al.*, 1991; Chen *et al.*, 1995; Leone and Breuil, 1998; del Rio *et al.*, 2000).

Pitch characteristics can be changed due to the degree of saponification of extractive components. The glycerol esters are completely saponified and the fatty acids are dissolved during kraft pulping, (Sjöström, 1993). However, sterol esters and waxes, which are present in significant amounts in hardwoods, are more resistant to saponification. These components can be only partially degraded, so there is always a risk of remaining in the pulp and being removed from it with process liquids. Moreover, sterols and sterol esters do not form soluble soaps under alkaline conditions used in kraft pulping, so they may deposit and cause pitch problems (Swan, 1967; Affleck and Ryan, 1969; Leone and Breuil, 1998). Wood extractives

released during the bleaching process are carried over to the pulp machine by the pulp and water (Gutierrez *et al.*, 2001).

Pitch problems are likely to become more severe in the future due to high production rates that can cause overloading of washing equipments resulting in dirtier, more pitch laden stock. Likewise, increasing reuse of mill waters and the trends towards complete closure of water circuits, is leading to an increase in pitch concentration, which results in higher deposition. The presence of impurities can severely impair product quality and is responsible for important economic losses in the pulp and paper industry. A break in production or defects in the final products may be very costly, and therefore a rapid characterization of the impurities occurring in pulp is important so that appropriate measures or treatments can be undertaken (del Rio *et al.*, 1999).

Pitch Deposition Problems

1. Dispersed wood resin: Work by Allen has shown that the concentration of dispersed wood resin in mill waters correlates well with pitch deposition problems provided dispersed wood resin is the source of the deposition. The work showed that the potential for pitch deposition and related problems increased linearly with increasing wood resin particle concentration (dispersed resin was the single cause of the deposition). Different mills can tolerate different concentrations of dispersed wood resin.

2. Metal soaps: The free fatty and resin acids of wood resin can react with metal ions to form metal soaps. Sodium soaps are soluble in water but soaps from multivalent metal ions such as Mg, Ca, Al and Ba are insoluble in aqueous environments and are very tacky (Back, 1960). Their tackiness results in sticky deposition in pulp and paper processes. These soaps are formed above pH 6 (Allen, 1998) where the acids are ionized. System closure will result in increased metal soaps deposition due to an increase in the concentration of multivalent metal ions and wood resin.

Deposition due to metal soaps will be particularly problematic in the bleach plant. Dissolved and colloidal substances such as wood resin and metals not removed in the washer prior to bleaching stages are more concentrated in a closed bleach plant than in conventional open bleach plant (Sithole and Allen, 2000).

3. Polymerization: Wood resin components are known to polymerize to form products that are difficult to dissolve in common solvents and in alkali. A report implicates polymerized wood resin as a major component of pitch deposition in a fine paper mill (Raymond *et al.*, 1998). As demonstrated in studies of polymerization of linseed oils in re-pulping processes, such polymerizations increase with time and temperature. System closure usually results in higher operating temperatures in process systems, it is conceivable that this will cause greater polymerization of wood resin components and subsequently result in pitch deposition problems (Sithole and Allen, 2000).

4. Foaming: Wood resin components are surface-active agents that often cause foaming problems in pulping and papermaking processes, especially in closed mills. Increased foaming causes the use of narrower water circuit pipes that result in higher pressures on pulp slurries and white waters, higher water temperatures, wood pulps with higher amounts of wood extractives (TMP and CTMP), use of recycled fibers, higher machine speeds, and twin wires on paper machines. The foaming can result in loss of vacuum, poor drainage, poor formation, holes and thin spots in the sheet, and overuse of de-foamers, which can cause deposit formation (Sithole and Allen, 2000).

1.4 The Methods of Pitch Control

1. Natural seasoning of pulpwood: Traditionally, pitch deposits in paper pulp manufacturing processes have been reduced by seasoning. Seasoning means storage in the wood yard. It is known that seasoning reduces the resin content of wood and changes the nature of the resin. Some extractives are lost through oxidative processes and hydrolysis by plant enzymes by the time of seasoning, as well as by the action of wood colonizing organisms. When the wood is stored in the form of chips, resin content decreases, because both chemical and microbial transformations proceed faster owing to the increased surface area (Gutierrez *et al.*, 2001). Seasoning considerably reduces the extractive content in wood; however, this requires storage of the wood for relatively long periods. Logs are usually seasoned for up to 12 months, while the seasoning of wood chips in piles may require two months (Allen *et al.*, 1991; Rocheleau *et al.*, 1998). Other disadvantages of wood seasoning include the high personnel and capital costs, and the appreciable loss of brightness and pulp yield (Dorado, 2000). Although this method is known to be a pitch reductor, wood

storage for a long time can result in decreased pulp yield and low pulp quality owing to the action of decay organisms (Gutierrez *et al.*, 2001). New approaches for pitch control need to be devised to overcome these difficulties. The cost of this procedure, in terms of time and storage space, directed the researches to develop fungal enzyme treatments, which is applicable during pulping processes (Fisher and Messner, 1992; Fisher *et al.*, 1993; Fujita *et al.*, 1992).

2. Chemical additives: Common solutions to minimize pitch deposition include the use of chemical additives in papermaking and wood seasoning. Chemical methods are mainly based on the flocculation of colloidal resin with alum (aluminum sulfate), adsorption of pitch on to talc, or dispersion of colloidal resin by addition of different types of dispersants and surfactants. These chemical additives can have undesirable side effects such as corrosion of pulp and paper mill equipment, decrease of paper strength and deterioration of other paper properties (Hillis and Sumimoto, 1989). Along with chemical methods, wood seasoning is often applied for pitch control (Gutierrez *et al.*, 2001).

1.5 Biological Pitch Control

Enzymes have played a central role in many manufacturing processes, since ancient times, such as in the production of wine, cheese, bread, modification of starch etc. The knowledge about the use of microorganisms, their metabolic products, and enzymes in a broad area of basic research and their potential industrial applications are growing day by day since 1950s. But only in the past 2 decades, microbial enzymes have been used commercially in the pulp and paper industry (Beg *et al.*, 2001).

In biological pitch control, microorganisms or enzymes are being used for the reduction of problematic pitch. In this manner different types of enzymes and various kinds of fungi can be selected for the degradation of pitch components. The biological treatment of wood with fungi to remove extractives before pulping has been suggested and tested in mill trials as an alternative to traditional methods for pitch control (Gutierrez *et al.*, 2001).

1.5.1 Pitch control at the pulping stage

Biotechnology provides environmentally sound and potentially economic solutions for the control of pitch based on the use of extractive-degrading fungi (Farrell *et al.*, 1993; Brush *et al.*, 1994; Gao *et al.*, 1994; Martinez-Inigo *et al.*, 1999) or enzymes (Fujita *et al.*, 1992; Fischer and Messner, 1992; Fischer *et al.*, 1993). The products commercialized with this purpose are based on enzymes such as lipase commercialized by Novo Nordisk as Resinase™ or organisms such as *Ophiostoma piliferum* strains commercialized by Clariant as Cartapip™, which predominantly hydrolyze triacylglycerols in pulp and wood. The latter compounds are saponified under kraft cooking conditions. With the aim of identifying those fungal strains to be used for direct *depitching* of eucalypt wood or as a source for enzymes acting on problematic eucalypt extractives, Martinez *et al.* screened 73 species of fungi from different taxonomic groups (Martinez *et al.*, 1999). Based on extractive degradation patterns, several fungal species can be selected for treating wood under solid-state fermentation (SSF) conditions because of their ability to remove those compounds involved in pitch deposit formation during kraft pulp manufacture (Gutierrez *et al.*, 1999). Weight loss constitutes one of the major drawbacks for the use of fungi in wood depitching. Thus, treatment duration must be taken into account in order to evaluate the industrial viability of the biological removal of wood extractives (Martinez-Inigo, 2000).

1.5.2 Pitch control at the paper production stage

Gutierrez *et al.* implied that the main part of the extractives in the deposits were sterols, sterol esters, steroid ketones and steroid hydrocarbons, similar to those found in the process waters and arising from the eucalypt wood extractives. They found that no major structural changes of sterols and sterol esters occurred during kraft cooking and TCF bleaching with hydrogen peroxide. In contrast, the bleaching with chlorine dioxide degrades unsaturated sterol structures, such as sitosterol and sitosterol esters, and only the saturated ones, such as stigmastanol, remain after this bleaching agent. (Gutierrez *et al.*, 2001)

Chlorine dioxide destroys free and esterified unsaturated sterols, which are the main lipophilic compounds in most wood extractives, but these compounds survive TCF bleaching with hydrogen peroxide (del Rio *et al.*, 1998, 2000). Thus, pitch problems

are dramatically enhanced when the elementary chlorine free bleaching sequences are substituted by TCF sequences for the manufacture of high-quality kraft pulps. In addition to this problem, complete closure of water circuits to meet environmental protection requirements is leading to an increase in pitch concentrations and the potential for deposition. In order to design a rational strategy for the efficient control of extractive-derived problems, different studies have been carried out. These have included the chemical analyses of *E. globulus* wood extractives (Gutierrez *et al.*, 1998, 1999) and pitch deposits formed during pulping and bleaching (del Rio *et al.*, 1998, 1999), with the aim of identifying those extractive compounds responsible for deposit formation, and the search for methods to remove or control the degree of deposition of these problematic compounds (Martinez-Inigo, 2000). Enzymatic treatment of mechanical pulp has been reported to solve pitch problems and reduce bleaching chemical requirements (Fujita *et al.* 1992; Hata *et al.*, 1996).

1.5.3 Used microorganisms

The ability of white-rot fungi to degrade all major components of the woody cell wall, i.e. lignin, cellulose and hemicellulose, is well characterized (Eriksson *et al.*, 1990; Blanchette, 1995). Dorado et al. have also studied the decomposition of wood extractives by white-rot fungi in 1998. The degree of lignin degradation selectivity by the various white-rot fungi was compared in order to evaluate their potential for application in biological pulping processes (Byström and Lönnstedt, 2000).

The biopulping strains *Ceriporiopsis subvermispora*, *Phanerochaete chrysosporium* and *Phlebiopsis gigantea* were reported to reduce the extractive content in wood (Fischer *et al.*, 1994, 1996; Behrendt and Blanchette, 1997). These findings suggest that the use of white-rot fungi could enable the development of a single biotechnological process for the depitching, detoxification and biopulping of wood chips. Such process could be very advantageous compared to recently introduce biological methods for pitch control, i.e. the deresination of wood chips by pretreatment with the sapstain fungus *Ophiostoma piliferum* (Farrell *et al.*, 1993; Brush *et al.*, 1994) and, the enzymatic degradation of the pulp triacylglycerols with lipase preparations (Fischer *et al.*, 1993; Mustranca *et al.*, 1995). Sapstain fungi are not able to attack the wood cell wall (Eaton and Hale, 1993) and, as a consequence, key benefits from biopulping such as refining energy savings and enhanced pulp

strength are not to be expected after this fungal pretreatment (Byström and Lönnstedt, 2000). Also the fungi *Schizophyllum commune*, *Trichaptum biforme* and *Phanerochaete gigantea* which are useful in reducing the pitch content of pulps and pulpwoods used in making cellulosic products (Blanchette *et al.*, 1995).

Ophiostoma piliferum has recently been used in biological control methods (Tamerler *et al.*, 1999). The colorless isolate of *O. piliferum*, Cartapip™, treatment results in a 60-80% decrease in the triacylglycerol content of wood extractives (Gao *et al.*, 1994). Indeed, a fungal product for pitch degradation has been commercially available since 1991 (Cartapip™). Tamerler *et al.* studied on the production of extracellular lipolytic enzymes of *O. piliferum* (Cartapip™) in order to identify the action of this fungus on the extractive compounds during wood treatment and to test its potential for direct treatment into the paper pulp.

Cartapip™ treatment removes up to 50% of eucalyptus extractives, but this does not decrease pitch during kraft pulping because the lipophilic compounds responsible for deposits (a minor fraction in eucalypt extractives) are not degraded efficiently. Triacylglycerols are the most problematic compounds during the manufacturing of mechanical and acidic sulfite pulps from pine and other softwoods. A 90% decrease of these compounds can be obtained with Cartapip™ treatment of pinewood, a percentage significantly higher than that obtained after seasoning. However, the decrease in content of resin acids, sterol ester and waxes after a two-week treatment of this wood with Cartapip™ is often similar to that obtained after natural seasoning under the same conditions (Gutierrez *et al.*, 2001).

1.6 Sapstaining fungi

Fungi contain cell walls and produce spores, among many other differences, and most describe species form a relatively tight phylogenetic cluster. Three major groups of fungi are recognized: the molds, the yeasts, and the mushrooms.

The habitats of fungi are quite diverse. Some are aquatic, living primarily in fresh water and a few marine fungi are also known. Most fungi, however, have terrestrial habitats, in soil or on dead plant matter, and these types often play crucial role in the mineralization of organic carbon in nature. A large number of fungi are parasites in terrestrial plants. Indeed, fungi cause the majority of economically significant

diseases of crop plants. A few fungi are parasitic on animals, including humans, although in general fungi are less significant as animal pathogens than are bacteria and viruses.

Cell Walls and Metabolism: Most fungi have non-cellulosic walls. Chitin, a polymer of the glucose derivative N-acetylglucosamine, is a common constituent of fungal cell walls. Fungal cell walls are generally 80-90% polysaccharide, with proteins, lipids, polyphosphates, and inorganic ions making up the wall-cementing matrix. An understanding of fungal cell wall chemistry is important because of the extensive biotechnological uses of fungi and because the chemical nature of the fungal cell wall has been useful in classifying fungi for research and industrial purposes.

Fungi are chemoorganotrophs and generally have simple nutritional requirements. Many species can grow at environmental extremes of low pH or high temperature and this coupled with the ubiquitous nature of fungal spores, makes these organisms common contaminants of food products, microbial culture media, and most surfaces. Molds and yeasts are not, however, classified on physiological grounds but instead by their diverse array of life cycle patterns including the formation of a variety of different sexual spores.

Filamentous Fungi (Molds): Molds are filamentous fungi. They are widespread in nature and are commonly seen on stale bread, cheese, or fruit. Each filament grows mainly at the tip, by extension of the terminal cell. A single filament is called a hyphae. Hyphae usually grow together across a surface and form compact tufts, collectively called a mycelium, which can be seen easily without a microscope. The mycelium arises because the individual hyphae form branches as they grow, and these branches intertwine, resulting in a compact mat. In most cases, the vegetative cell of a fungal hyphae contains more than one nucleus-often hundreds of nuclei are present. Thus, a typical hyphae is nucleated tube containing cytoplasm.

From the fungal mycelium, other hyphal branches may reach up into the air above the surface, and on these aerial branches spores called conidia are formed. Conidia are asexual spores (their formation does not involve the fusion of gametes), often highly pigmented and resistant to drying, and function in the dispersal of the fungus to new habitats. When conidia form, the white color of the mycelium changes, taking

on the color of the conidia, which may be black, blue-green, red, yellow, or brown. The presence of these spores gives the mycelial mat a rather dusty appearance.

Some molds also produce sexual spores, formed as a result of sexual reproduction. The latter occur from the fusion either of unicellular gametes or of specialized hyphae called gametangia. Alternatively, sexual spores can originate from the fusion of two haploid cells to yield a diploid cell, which then undergoes meiosis and mitosis to yield individual spores. Depending on the group to which a particular fungus belongs, different types of sexual spores are produced. Spores formed within an enclosed sac (ascus) are called ascospores, and those produced on the ends of a club-shaped structure (basidium) are basidiospores. Zygospores, produced by zygomycetous fungi like the common bread mold Rhizopus, are macroscopically visible structures and the result of the fusion of hyphae and genetic exchange. Eventually the zygospore matures and produces asexual spores that are dispersed by air and germinate to form fungal mycelia.

Sexual spores of fungi are usually resistant to drying, heating freezing, and some chemical agents. However, fungal sexual spores are not resistant to heat as are bacterial endospores. Either an asexual or sexual spore of a fungus can germinate and develop into a new hyphae and mycelium.

A major ecological activity of many fungi, especially members of the Basidiomycetes, is the decomposition of wood, paper, cloth, and other products derived from natural sources. Basidiomycetes that attack these products are able to use cellulose or lignin from the product as carbon and energy sources. Lignin is a complex polymer in which the building blocks are phenolic compounds. The decomposition of lignin in nature occurs almost exclusively through the action of certain Basidiomycetes called wood-rotting fungi. Two types of wood rots are known: brown rot, in which the cellulose is attacked preferencially and the lignin left unchanged and white rot, in which both cellulose and lignin are decomposed. The white rot fungi are of considerable ecological interest because they play such an important role in decomposing woody material in forests (Madigan *et al*., 2003).

It has been shown that the treatment of wood chips with sapstain fungi that degrade resinous compounds prior to pulping is an alternative method for pitch control (Brush *et al.*, 1994; Farrell *et al.*, 1993). Sapstaining fungi include a diverse group of

non-decay fungi that cause discoloration of timber. Most sapstainers are microfungi members of the *Ascomycotina* and *Deuteromycotina*. Sapstaining fungi are ideally suited for wood pretreatment because they are capable of assimilating the easily available carbon and nitrogen nutrients present in parenchyma cells, resin ducts and other woody tissues. These fungi can rapidly colonize on non-sterile wood chips. Research on the biotechnological application of sapstain fungi for wood depitching has been mainly concerned with fungal strains belonging to the species *Ophiostoma piliferum*. These research efforts have led to the commercialization of an albino strain of *O. piliferum* (Cartapip™) for pitch reduction in wood chip piles. The effectiveness of this strain to degrade problem-causing triacylglycerols has been demonstrated on both softwood (Brush *et al.*, 1994) and hardwood species (Rocheleau *et al.*, 1998). However, Cartapip™ does not degrade effectively other pitch forming compounds such as sterols, steryl esters and waxes (Leone and Breuil, 1998; Chen *et al.*, 1994) nor toxic extractives such as resin acids (Gao and Breuil, 1995; Farrell *et al.*, 1993). The identification of other sapstain strains may propose novel methods for the biological control of pitch-related problems (Dorado, 2000).

Wood decay fungi are of considerable biotechnological interest in paper pulp manufacturing. Conidial fungi from the genera *Penicillium*, *Trichoderma* and *Gliocladium*, were used in the first patent on pitch biocontrol, no information on their efficiency in removing extractives is given. Another patent describes wood treatment with sapstain ascomycetes from the genera *Ophiostoma* and *Ceratocystis*, as well as other ascomycete-type fungi and was filed in 1990. The removal of extractives cause a decrease in pulp brightness, because of the production of dark pigments by wild type sapstain fungi. To overcome this problem, non-pigmented strains of *O. piliferum* were obtained using classical genetics techniques and patented and marketed by Clariant Corporation under the trade name Cartapip™. Wood treatment with this commercial fungus also results in the biocontrol of other organisms, including wild sapstain fungi (Gutierrez *et al.*, 2001).

For pitch control, it is important to analyze the removal not only of the total extractives but also of the individual lipid classes. Despite some limitations, Cartapip™ provides efficient control for pitch deposits in mechanical and acidic sulfite pulping. With this product, the field of microbial control of pitch has been opened and new products must be developed to solve other pitch problems. A

screening has shown some sapstain fungi with better performances than Cartapip™ for softwood depitching (Gutierrez *et al.*, 2001).

1.7 Fusarium species : *Fusarium oxysporum*

All strains of *Fusarium oxysporum* exist saprophytically and most of them are pathogen of many plants. Some are well-known for inducing wilt or root rots on plants whereas others are considered as nonpathogenic. Fravel *et al.* pointed that some of the nonpathogenic strains of *F. oxysporum* can be used in biocontrol. It produces sickle-cell shaped spores and grows well on several media including malt, potato dextrose and yeast agar (National Centre for Biotechnology Education, 2004).

Like various other plant pathogens, *Fusarium oxysporum* has several specialized forms, known as formae specialis that infect a variety of hosts causing various diseases (Raabe *et al.*, 1981). The distribution of *Fusarium oxysporum* is known to be cosmopolitan. However, the different special forms of *F. oxysporum* often have varying degrees of distribution.

In solid media culture, such as potato dextrose agar (PDA), the different special forms of *F. oxysporum* can have varying appearances. In general, the aerial mycelium first appears white, and then may change to a variety of colors ranging from violet to dark purple, according to the strain (or special form) of *F. oxysporum* (Smith *et al.*, 1988).

F. oxysporum produces three types of asexual spores: microconidia, macroconidia, and chlamydospores (Agrios, 1988). Microconidia are one or two celled, and are the type of spore most abundantly and freqeuntly produced by the fungus under all conditions. It is also the type of spore most frequently produced within the vessels of infected plants. Macroconidia are three to five celled, gradually pointed and curved toward the ends. These spores are commonly found on the surface of plants killed by this pathogen. Chlamydospores are round, thick-walled spores, produced either terminally or intercalary on older mycelium or in macroconidia. These spores are either one or two celled. The fungus can survive either as mycelium, or as any of its three different spore types (Agrios, 1988).

If the soil contaminated with the fungus, healthy plants can become infected by *F. oxysporum*. The fungus can invade a plant either with its sporangial germ tube or

mycelium by invading the plant's roots (Agrios, 1988). Once inside the plant, the mycelium grows through the root cortex intercellulary. When the mycelium reaches the xylem, it invades the vessels through the xylem's pits. At this point, the mycelium remains in the vessels, where it usually advances upwards toward the stem and crown of the plant. As it grows the mycelium branches and produces microconidia, which are carried upward within the vessel by way of the plant's sap stream. When the microconidia germinate, the mycelium can penetrate the upper wall of the xylem vessel, enabling more microconidia to be produced in the next vessel. The fungus can also advance laterally as the mycelium penetrates the adjacent xylem vessels through the xylem pits (Agrios, 1988).

When the fungus grow within the plant's vascular tissue, the plant's water supply is greatly affected. This lack of water induces the leaves' stomata to close, the leaves wilt, and the plant eventually dies. It is at this point that the fungus invades the plant's parenchymatous tissue, until it finally reaches the surface of the dead tissue, where it sporulates abundantly (Agrios, 1988). The resulting spores can then be used as new inoculum for further spread of the fungus.

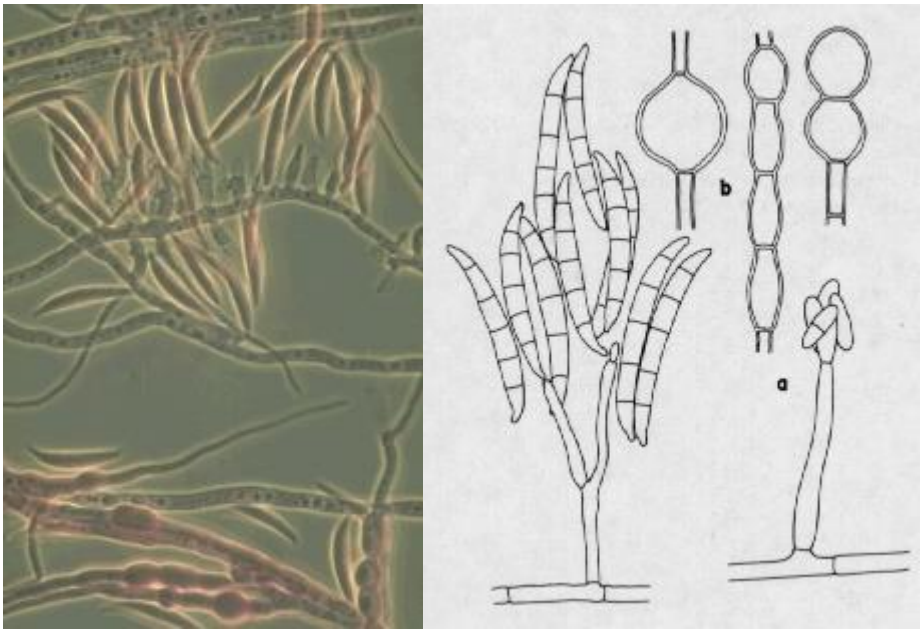

Figure 1.3: The spore types and hyphal swelling of *F. oxysporum*

(http://www.botany.utoronto.ca/ResearchLabs/MallochLab/Malloch/Moulds/Fusarium.html)

Most characteristic are the colourless spores (conidia), which are canoe-shaped, have a distinct "foot cell" at the lower end, and are divided by several cross-walls. Two other spore forms may occur, microconidia (a) resembling spores and phialides of

Acremonium, and chlamydospores (b), thick-walled swellings along the filaments. Cultures may be brightly coloured (Booth 1971, 1977; Burgess, *et al.*, 1988; Gerlach and Nirenberg, 1982; Nelson, *et al.*, 1983).

Figure 1.4: The steps of *F. oxysporum* growth in the culture, from spore to hyphae (http://www.botany.utoronto.ca/ResearchLabs/MallochLab/Malloch/Moulds/Fusarium.html)

1.8 Lipases

Lipases are used extensively in biotechnological fields such as food technology, clinical and industrial chemistry (Arnold *et al.* 1975; Macrae 1983; Brockman 1984; Zaks and Klibanov 1985). Many industrially used lipases are prepared from fungi (Iwai and Tsujisaka, 1974; Wisdom *et al.*, 1987; Derewenda *et al.*, 1994).

Lipases (triacylglycerol hydrolases EC 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerols at the oil-water interface. Lipases act more rapidly on triacylglycerols (TAG) and less on monoacylglycerols (MAG) and diacylglycerols (DAG) (Striby *et al.*, 1999). It is specific for lysis of triacylglycerols of small molecular mass and lower fatty acids liberated are used as precursors for production of methyl ketones and secondary alcohols (Petrovic, *et al.* 1990). Lipases cleave ester bonds in glyceride and related molecules using a nucleophilic mechanism via an activated serine. They are inactive under aqueous conditions and their catalytic activation occurs within the lipid-water interface (Wooley and Petersen, 1994). They are produced by animals, plants and microorganisms (Sztajer *et al.*, 1998; Aires-

Barros *et al.*, 1994; Ionita *et al.*, 1997). Microbial lipases have a great potential for commercial applications due to their stability, selectivity and broad substrate specificity (Jaeger *et al.*, 1994). To date, a large number of lipases from filamentous fungi have been extensively studied. The most productive species belong to the genera *Geotrichum*, *Penicillium*, *Aspergillus* and *Rhizomucor* (Stöcklein *et al.*, 1993; Miura *et al.*, 1997). There are also a certain number of lipases produced by yeasts, most of them belonging to the Candida genus that have been used for biotechnological purposes (Tomizuka *et al.*, 1966; Lotti *et al.*, 1993). Lipases from unicellular bacteria, mainly those produced by various species of the genus Pseudomonas, have also proved to be useful both in organic reactions and in the detergent industry. Many of them have been purified, characterized and their encoding genes cloned (Jaeger *et al.*, 1996). The actinomycetes have not been deeply identified for lipase studies, and there is little information available concerning lipase producing actinomycetes (Cruz *et al.*, 1994).

Among the high number of lipases described in the literature, only the enzymes belonging to a few species have been demonstrated to have adequate stability and biosynthetic capabilities to allow routine use in organic reactions, and hence their consideration as industrially relevant enzymes (Margolin, 1993; Azerad, 1995). The nature of the reaction medium, involving parameters such as water activity and log P, and the use of optically active solvents, is important for the enhancement of reaction yields, and the selectivity of the lipases (Bell, 1995; Zaks and Klibanov, 1988; Arroyo and Sinisterra; 1995). Cardenas *et al.* studied the selection by screening techniques of fourteen lipase-producing microorganisms for the selection of the most beneficial lipase for the application to industrial processes.

Lipids are insoluble in water and have to be hydrolyzed extracellularly to their components, prior to absorption, if they are to function as nutrients for the cell. Like the enzymes that degrade biologic polymers, lipase activity is sometimes found to be cell-bound either only for a certain period on its way into the culture medium or throughout the cultivation. The enzyme activity hydrolyzing lipids becomes maximal only when the lipases are adsorbed to the oil- water interface. The majority of lipases exhibit a high activity toward lipids with fatty acid residues of C_8 to C_{18} chain length. Some enzymes that are highly active toward short-chain, water-soluble glycerol esters are more properly classified as esterases (EC 3.1.1.1). Many microbial lipases

are only produced in the presence of an inducer. This may be a triacylglycerol, a fatty acid, or another lipid (Tsujisaka *et al.*, 1973; Muderhwa *et al.*, 1985; Shimada *et al.*, 1992). Lipids or fatty acids do not appear to be required for the production of lipase activity in a second group of microorganisms, but incorporation of these compounds increased the level of lipase activity produced (Yoshida *et al.*, 1968; Chen *et al.*, 1992). In a third group of microorganisms, the lipases are only produced constitutively (Chander and Klostermeyer, 1983). Although data showing how lipids and fatty acids induce the formation of lipase activity are scarce, much less is known about the effect of glucose or glycerol on production of lipase activity. Therefore, besides the induction of formation of lipase activity and its localization during fungal growth, the repression of formation of lipase activity was studied. For this purpose *Fusarium oxysporum* f. sp. *vasinfectum* was chosen, which was found to be a good producer of lipase activity (Rapp and Backhaus, 1992).

Lipase activity can be determined either by pH-stat or a spectrophotometer:

The pH-stat assay: The triacylglycerols are treated with lipase solutions in a constant temperature. The decrease in the pH implies the liberation of fatty acids and this decrease is compensated by automatic titration of a basic solution (NaOH), maintaining the original pH value. Triolein (1 g), Triton – X (30 ml) and NaCl (0.9 %, 200 ml) are used as emulsification solution (pH 7.5 with 1 M. NaOH). The mixture in the vessel is held at the optimum temperature for the specific lipase and titrated with 10 mM NaOH. One unit of enzyme liberates 1 μmol fatty acid min^{-1} which is equivalent to 0.1 ml of NaOH consumed (Vorderwulbecke, *et al.* 1992). Tributyrin can also be used as substrate in another procedure together with gum Arabic, sodium taurocholate, $CaCl_2$ and NaCl. The activity of *Fusarium oxysporum* lipase was determined by emulsifying 10% (v/v) olive oil and 1% (m/v) gum Arabic in distilled water, in a sonicator for 2 min and mixing with enzyme and assay buffer (1 mM Tris-glycine, 0.1 M NaCl and 5 mM CaCl2, pH 5.5). The reaction was stopped by addition of Cu^{2+} reagent (2.5 ml) and chloroform (5.0 ml). Also, triolein can be used as substrate and the emulsifying solution (gum Arabic, deoxycholic acid and NaCl, pH: 8.0) is titrated with 0.02 M. NaOH (Woolley and Petersen, 1994).

Spectrophotometric assay: In this system ρ-nitrophenol release from ρ-nitrophenol palmitate is determined. 10 mM p-nitrophenyl palmitate dissolved in acetone and emulsified in a solution containing sodium deoxycholate, gum Arabic and Tris-HCl.

Lipase addition causes ρ-nitrophenol release from ρ-nitrophenol palmitate and the reaction is stopped by Na_2CO_3, and then assayed at 410 nm spectrophotometrically (Tamerler *et al.* 1999). The solution mentioned is mixed with Triton-X-100 (2 g), gum arabic (0.5 g) dissolved in 450 ml buffer (Tris-HCl 50 mM, pH 8.0) (Vorderwulbecke, *et al.* 1992).

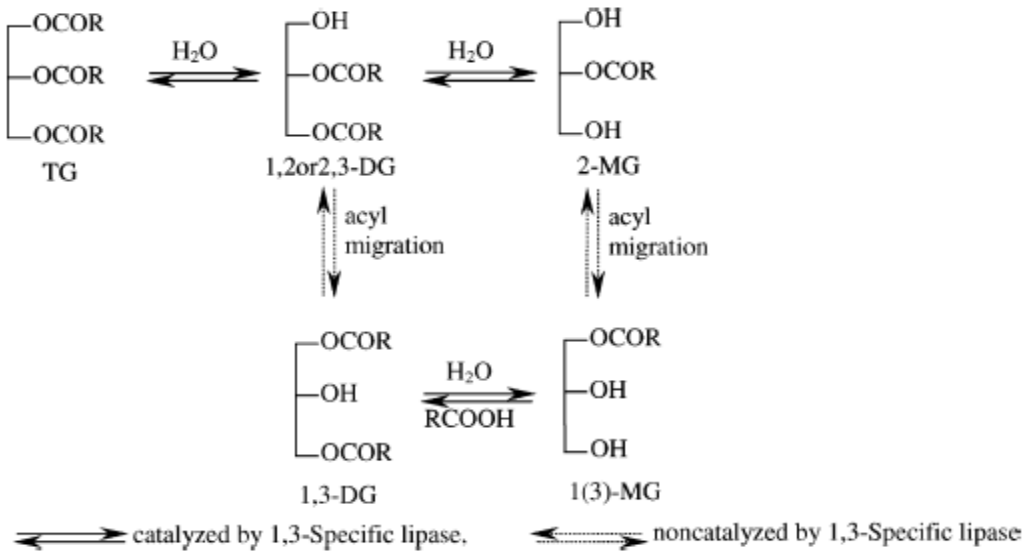

Figure 1.5: The hydrolysis mechanism of lipase

(Tan, T. *et al.*, 2002)

1.9 Chromatography Techniques

Chromatographic techniques can be grouped into two main classes, column and thin-layer chromatography. The principle of column chromatography is the attachment of the stationary phase to a matrix, which is packed into the column, where the mobile phase passes through by gravitational force or by a help of a pumping system or a gas pressure. In the thin-layer chromatography, the stationary phase is attached to a matrix, which is coated onto a glass, plastic or metal plate. The mobile phase which is liquid passes the thin-layer plate horizontally or vertically by capillary action.

Today the most favorite chromatography type is column chromatography. Different devices may be used due to the purpose, such as GC, HPLC, FPLC, and TLC.

Parrish and Ackman implied that the Iatroscan, a type of TLC-FID system, combines the efficiency of TLC and the sensitivity of FID; it also has a wide spectrum of

application fields especially in the distribution of lipid classes (Parrish and Ackman, 1983).

The principle of gas chromatography depends on the vaporization of the sample which is injected onto the head of the chromatographic column. The mobile phase is an inert gas that causes the transportation of sample by its flow. The column itself contains a liquid stationary phase which is adsorbed onto the surface of an inert solid.

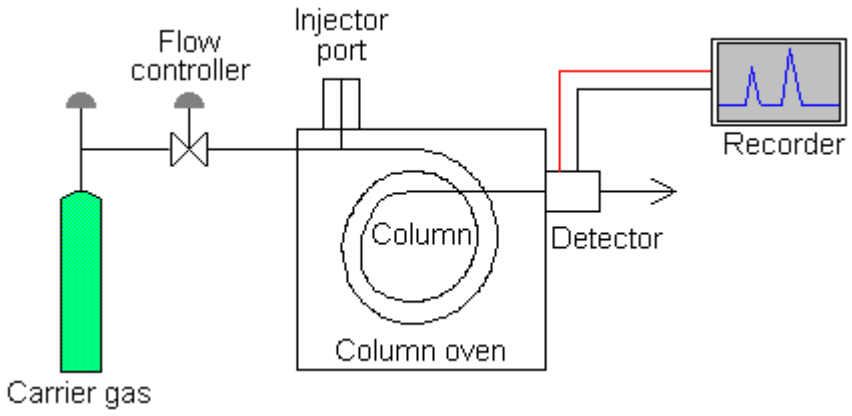

Figure 1.6: Schematic diagram of a gas chromatograph

(http://www.shu.ac.uk/schools/sci/chem/tutorials/chrom/gaschrm.htm)

Carrier gas: The carrier gas must be chemically inert. Commonly used gases include nitrogen, helium, argon, and carbon dioxide. The carrier gas changes upon the type of detector which is used. The carrier gas system also contains a molecular sieve to remove water and other impurities.

Sample injection port: The most common injection method is where a microsyringe is used to inject sample through a rubber septum into a flash vaporizer port at the head of the column. The temperature of the sample port is usually about 50°C higher than the boiling point of the least volatile component of the sample. For packed columns, sample size ranges from tenths of a microliter up to 20 microliters. Capillary columns, on the other hand, need much less sample, typically around 10-1 mL. For capillary GC, split/splitless injection is used.

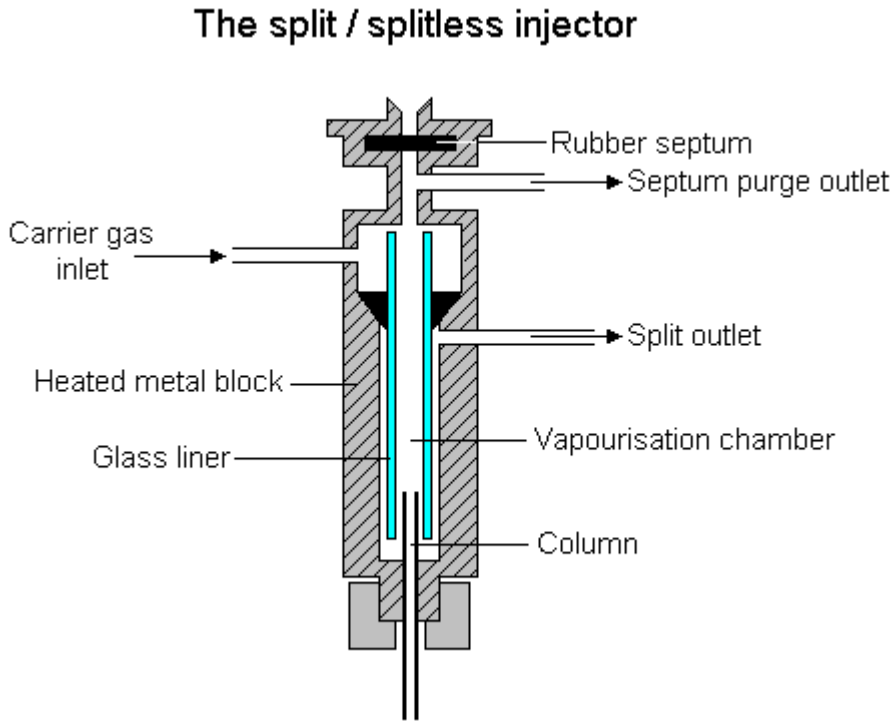

Figure 1.7: Schematic diagram of split/splitless injector

http://www.shu.ac.uk/schools/sci/chem/tutorials/chrom/gaschrm.htm

There are two modes for the injection; split or splitless. The injector contains a heated chamber containing a glass liner into which the sample is injected through the septum. The carrier gas enters the chamber and can leave by three routes (when the injector is in split mode). The sample vaporizes and forms a mixture of carrier gas, vaporized solvent and vaporized solutes. A proportion of this mixture passes onto the column, but most exits through the split outlet. The septum purge outlet prevents septum bleed components from entering the column.

Columns: There are two general types of column, packed (open) and capillary (tubular). Packed columns contain a finely divided, inert, solid support material coated with liquid stationary phase. Most packed columns are 1.5 – 10m in length and have an internal diameter of 2 - 4mm. Capillary columns can be one of two types; wall-coated open tubular (WCOT) or support-coated open tubular (SCOT). Wall-coated columns consist of a capillary tube whose walls are coated with liquid stationary phase. In support-coated columns, the inner wall of the capillary is lined with a thin layer of support material, onto which the stationary phase has been adsorbed. SCOT columns are generally less efficient than WCOT columns. Both types of capillary column are more efficient than packed columns. The Fused Silica

Open Tubular (FSOT) column; is another type of WCOT column which have much thinner walls than the glass capillary columns. They have the advantages of physical strength, flexibility and low reactivity.

Column temperature: The optimum column temperature depends upon the boiling point of the sample. As a rule of thumb, a temperature slightly above the average boiling point of the sample results in an elution time of 2 - 30 minutes. Minimal temperatures give good resolution, but increase elution times. If a sample has a wide boiling range, then temperature programming can be useful. The column temperature is increased as separation proceeds.

Detectors: As the detector change selectivity differs. There are two types of detectors, concentration dependant and mass flow dependant. The signal from a concentration dependant detector is related to the concentration of solute in the detector. Dilution of with make-up gas will lower the detectors response. Mass flow dependant detectors usually destroy the sample, and the signal is related to the rate at which solute molecules enter the detector. The response of a mass flow dependant detector is unaffected by make-up gas.

Sandström *et al.* succeeded the separation of fatty acids from resin acids by TLC; while the method for the separation of lipid classes in acetone extracts by solid-phase extraction (SPE) which has been described by Chen *et al.*, cannot. This modern method is also beneficial for the separation of neutral extractives into sterols, TGs and steryl esters. (Sandström *et al.*, 1996)

Gutierrez *et al.* showed that short-length, high-temperature, capillary columns are a good choice for the rapid separation of lipophilic wood extractives without fractionation, derivatization steps (Gutierrez *et al.*, 1998).

J.C. del Rio *et al.* characterized organic deposition produced in the kraft pulping of Eucalyptus globulus wood. The acetone extracts were analyzed by GC using high temperature short capillary columns, which enables elution and separation in a wide range of molecular masses from fatty acids to sterol esters and triacylglycerols. Results showed that the composition of the extractives from E. globulus wood is mainly of hydrocarbons, fatty acids, waxes, steroids (hydrocarbons, alcohols, ketones and esters) and triacylglycerols. The main compounds of pitch deposits produced fter oxygen prebleaching were found to be waxes, sterols and sterol esters.

Triacylglycerols were hydrolyzed and fatty acids dissolved during the kraft cooking. So that after kraft cooking and oxygen prebleaching, there was a wax accumulation in the range from C_{20} to C_{40} which is consisted of a complex mixture of many different compounds (del Rio, 1998).

J.C. delRio *et al.* worked on the different pitch deposits in *E. globulus* wood chips. After 24 hours of extraction with acetone samples were analyzed by GC and GC-MS. They found that steroid compounds (hydrocarbons, alcohols, ketones and fatty acid esters) were the major lipophilic components producing pitch in the eucalypt wood survived the cooking and bleaching processes. Sitosterol was found to be the dominant sterol in all deposits (del Rio, 2000).

1.10 Aim of the study

Today, the deposition problems in mills are tried to be solved by various methods including conventional filtration, membrane filtration including reverse osmosis, flotation, biological treatment, precipitation and evaporation. It has been shown that biotechnology is the best choice on the resolving this problem. The characteristics of the pitch components in the given mill must be well known before deciding on the combination of the method. The objective of this study is to obtain knowledge in the presence and composition of pitch compounds derived from process pulps during manufacturing of cardboard from recycled papers and the degradation of those compounds responsible for pitch deposition in the different phases of the industrial processes by the help of enzymes. By this way, a method will be developed for the removal of pitch having minimal impact on the environment. In this manner, different enzymes were selected for constitute a comparison area. Beside the commercial enzymes, fungi *Fusarium oxysporum* and *Ophiostoma piliferum*, which were grown in laboratory conditions, were used. Lipase activities were assayed by the pH-stat using tributyrin as substrate. The extracellular lipases of these fungi and also the commercial ones were added to the paper pulps for the hydrolysis of pitch components. The detection of hydrolysis was obtained by using GC.

The scope of this work is to hydrolyze the problematic components so-called pitch in the cardboard production process by fungal lipase. To reach the aim the first step was classification of the pitch components in paper pulp samples taken from Kartonsan; a cardboard factory in Izmit. The factory consists of four main production lines:

Line 10 and 11: They produce the same product; also their production mechanism is the same. Only one of them works at a time. The fiber source for production is only cellulose; any recycled paper does not enter this process.

Line 12: Cellulose and recycled paper are processed together in this line.

Line 13: Cellulose is not used in this line, only recycled paper processed. This means that the pollution in this line is greater than the other lines.

Line 14: Only recycled paper is used as paper source and has a strong pitch problem.

In Kartonsan there are not any chemical cleaning steps, but some filters make elimination. Waste papers are pressed and became compact; enter the pulper and paper pulps are formed. After filtering, pulps enter the mill and cardboard is produced. In this study the samples were taken from the different stages of the production process from different lines. The samples were taken from:

1. Line 10 or 11 First layer pulper
2. Line 12 Protection layer pulper
3. Line 13 Bottom layer pulper
4. Line 14 Medium line pulper
5. Line 12 Disk filter outflow
6. Line 12 Sorter outflow
7. Line 13 Sieve outflow
8. Line 14 Disk filter outflow
9. Line 14 Mixing tank
10. Top layer machine storage tank
11. Protection layer machine storage tank

The lines that were predicted to have high pitch contents are lines 13 and 14. In lines 10, 11 and 12, pure cellulose is the only source and pitch content is less than the lines 13 and 14 that contain recycled paper.

The difference between the samples is not only the lines but also the part of the process they were taken. If the pitch problem can be solved in the main pulper, which

is the first step of the process, there will not be any further need for the repetition of pitch reduction process.

The first step of the sample analysis was detection of the pitch amount. For this reason the pulp samples, which did not contain any enzyme, were analyzed and pitch amount in the samples were determined. All samples were filtered for dehydration, because pulp has sludge-like watery structure. Then they were dried at room temperature and crumbled into fibers to increase the surface area. This step is very important, because it makes extraction easier and increases the enzyme activation surface in the samples. The most important and main reason for drying the pulps is that wood extractives contain only the 2-6% of the wood dry weight. The samples contain little pitch, therefore the excess water content which cause extra weight and volume must be removed. Also the thimbles of the Soxhlet extractor that contains the sample can take 35 g of wet pulp while more extractives were obtained from more dried pulp. The extractives were split into 6 main groups; hydrocarbons, esters, free fatty acids, triacylglycerols, mono and diacylglycerols. The percentage of the extractives changed according to the type of wood paper was made from. Recycled papers contained any kind of wood, so their extractives showed a wide range of variability in their percentage of amount.

In Kartonsan, there are 4 main types of paper sources:

1) First Quality Paper: All writing and typing papers enter this production line. Bales are pressed and entered the process. Extra grade first quality paper pulp contains less ink than back sized paper pulp.

2) Newspaper: In this production line, newspapers are recycled. Magazines cause a leakage (loss) because they have less fiber than other types of paper.

3) Mixed Clean Old Paper: This production line contains every kind of paper, even the own cardboard produced in Kartonsan.

4) Corrugated Cardboard: All kind of corrugated cardboard enters into the process in this production line.

First quality office paper composes the second layer in the cardboard and processed in a separate line.

The cardboard produced in Kartonsan does not undergo any chemical cleaning processes and it has a gray color because of the ink content.

Production lines in Kartonsan:

Line 10 and 11: Contains 100% cellulose. When both of the lines are working, luxury triplex cardboard, of which, both sides are white is produced.

Line 12: First quality paper pulp line

Line 13: Produces the bottom line of the cardboard. 80% newspaper, 20% corrugated cardboard

Line 14: Produces the medium line of the cardboard. 40% newspaper, 40% mixed clean old paper and 20% corrugated cardboard

In the lines 13 and 14, there is not any ink cleaning only staplers and clasps are taken out in the cleaning step.

In the line 12, there is ink cleaning step with flotation. There is a reject outflow in this production line, which elutes the materials that have to be cleaned out of the chipboard except fiber in the time of pulping. The density of the paper pulp must be under 1% for the clean pulp. There are also sieves in the production lines, which filters the paper pulp.

Machine storage tank is a kind of stock tank used for the storage of the cleaned paper pulp that is ready for the production.

Line 12 produces the first layer of the cardboard. The pH of the pulper tank is 8.5-9. An industrial calcium soap is used for flotation of the ink that works at 50°C. Paper turns into pulp in 20 minutes. A 2.5-mm-sieve holds the big particles.

Line 14 produces the second layer of the cardboard. This layer is the thickest of all. In the line 14 pollution rate is very high when compared to other production lines.

Line 13 produces the bottom layer of the cardboard. Recycled water is used in this line. Sorter is used for crude elimination of solid waste. Sorter is the first equipment after pulper. The principle in the sorter is the precipitation of solid wastes that have a high density. Fiber sorter is the 8 mm filter works in the production lines 12, 13 and 14. There are also low density cleaners in these lines which reduces the pulp density to 1% by vortex vibration. Densening sieve openings dehydrate and concentrate the

pulp in the line 13. Disk filters play the same role in line 12 and 14. Disperser makes small stains (≤200μ) invisible at high temperatures. At 95°C fibers swell and easily disperse. After filtering and dispersing, deinking starts. Bubbles occur by rotation that arrest ink and run over (overflow) from the deinking tank.

In Kartonsan, cleaning and servicing is made every 6 weeks. At this time production stops. Cleaning is made with city water and slimes that accumulate on the mill are cleaned. There are not any visible quality changes in the product before and after stopping. But the slime accumulation cause problems if cleaning is not done every 6 weeks. The most important problem is in the lines 13 and 14, because the pollution is greater than other two lines and pollution occurs in the blind spots of the mill. Although the cardboard quality does not change, the pulp quality cannot be the same every time because of the variability of the paper types in the paper recycling.

2. MATERIALS and METHODS

2.1 Fungal strains

The sapstaining fungi used in this study was *Fusarium oxysporum* A685 (IJFM), which was naturally, isolated soil, samples. It has kindly provided from Professor Tajalli Keshavarz, University of Westminster, School of Bioscience, Department of Biotechnology, London, U.K.

The other fungus used in this study was *Ophiostoma piliferum*, which was a mutant of stress conditions by directed evolution, was kindly provided by Berna Toktay, Istanbul Technical University, Department of Molecular Biology and Genetics, Istanbul, Turkey.

2.2 Media and Buffers

Potato Dextrose Agar (PDA) per liter

Potato Extract	4.0g
Glucose	20.0g
Agar	15.0g

Liquid Medium for Growth and Production of *F. oxysporum* per liter

NaH_2PO_4	12 g
KH_2PO_4	2 g
$CaCl_2 \cdot 2H_2O$	330mg
$MgSO_4 . 7H_2O$	300 g
$ZnSO_4 \cdot 7H_2O$	30mg
$MnSO_4 \cdot 4H_2O$	12mg
$FeSO_4 \cdot 7H_2O$	500µg
Glucose	20 g

Olive oil	12.5 ml
Yeast extract	20.5 g

The pH of the medium was adjusted to 7.5 with 1 M NaOH and autoclaved at 121°C for 15 min.

Buffer for the enzymatic treatment of pulp:

25mM Tris-HCl buffer (pH: 7) kept at + 4°C.

Lipase emulsifier for pH-stat analysis

Gum Arabic	1 %
Tributyrin	10%

Tributyrin was emulsified in the presence of gum Arabic getting use of ultrasonication. 3 ml of the emulsified substrate was added to 45 ml of buffer placed in the reaction chamber of the pH-stat system.

2.3 Solid media and growth

O. piliferum mutated in the stress conditions by directed evalution and *F. oxysporum* A685 (IJFM) strains were stored at potato dextrose agar at 4˚C.

F. oxysporum spores were maintained in potato dextrose agar (PDA) using sterile glass beads.

Petris were incubated at 27°C for 9-10 days then kept in the fridge (4°C) to be used only for one week.

2.4 Liquid media and growth

F. oxysporum was preserved on slant agars. Spores maintained on potato dextrose agar (PDA) slants were scrapped off using sterile glass beads. The sterilization duration was 20 minutes at 121ºC for glass beads and 15 minutes at 121ºC for all media and glassware. The glass beads were placed in 100 ml shake flasks and autoclaved. After cooling, sufficient numbers of glass beads were poured onto the PDA slants and were moved up and down the surface of the slants. The spores gaining mobility or sticking the beads were collected adding 15 ml of Tween 80 and transferred to another sterile shake flask. This stock was used as the initial spore

solution to prepare spore solution. The initial spore solution was inoculated into PDA containing petri dishes. Slopes were prepared by pouring 25mL of autoclaved medium (PDA) into sterile Petri dishes aseptically. The PDA containing petri dishes were inoculated spreading 400 µl of the *initial spore solution.* Inoculum was incubated at 27°C for 9-10 days then kept in the fridge (4°C) to be used when needed.

2.5 Determination of lipase activity with pH-stat

A pH-stat system is an automatic titrator that adjusts the pH of its reaction chamber to a set value. At first, the reaction chamber is prepared within a set value and the reaction is started for a defined period of time. Then the pH changes because of the given NaOH into the reaction chamber for titration. The reaction yield is measured by terms of the titrated base in order to catch again back the set pH. Emulsified triacylglycerols can be hydrolyzed into fatty acids after addition of lipase when the pH and temperature of the reaction chamber is controlled, by an appropriate pH buffer (NaOH), thus pH of the system decreased. The decrease in the pH stops when the lipase exhausted all of the substrate. Then automatic titrator starts and does titration until the pH before reaction is caught. This operation calculates the volume of a standardized NaOH solution that eliminates the effect of added lipase sample. The lipase activity is defined by the means of titrated base volume. The base solution (0.01 M NaOH) volume in the pH-stat equipment used was 1 µl.

The pH-stat device

The dark colored base tank (carrying 0.01 M NaOH) was placed on the base provided on the digital unit. The stirrer and reaction chamber were placed on the magnetic stirrer unit. The appropriate electrodes were placed in the reaction chamber to ensure pH and temperature control. The reaction chamber was connected to digital unit, base tank and water reservoir equipped with heating and cooling units (in order to ensure water temperature accuracy). The reaction chamber was a jacketed one, so heat transfer between the reservoir and chamber was provided by means of the continuously flowing water. Remote control keyboard was connected to the digital unit. pH electrodes were calibrated and keyboard set values were specified. After the steps mentioned, system was ready to be used. The optimum pH of lipase of *F. oxysporum* was determined as 7.5 after reaction and titration operations were carried

out for a 7-day liquid culture filtrate. The each experimented value was tried as the set pH of the system. The titrated amounts were recorded each time. When the experimented values were compared, the highest titration amount determined pH optimum. For many authors, the pH-stat system is the most suitable method for the determination of lipase activity.

Lipase assay

Samples for observing lipase production were taken on every 24 hours. The lipase activities were measured in terms of 0.01 M NaOH titrated after one minute of reaction at set pH 7.5 and constant temperature (30°C). The reaction chamber contained 45 ml. Tris-glycine buffer [1 mM Tris-glycine (pH 7.5), 0.1 M NaCl and 5 mM $CaCl_2$] and 1.5 ml emulsified substrate [10% tributyrin (the triacylglycerol to be emulsified), 1% gum Arabic (emulsifier) solution in distilled water]. The above mentioned substrate solution was emulsified by the help of a sonicator. After the addition of emulsified substrate onto the buffer solution, the pH was set to the 7.5, the pH optimum. 1 ml sample taken from different liquid media was added each time to the system and titration with NaOH was started 1 minute later. The buffer and substrate was renewed each time to ensure that accumulation of samples or fatty acids did not create inhibition of enzyme activity.

2.6 Pulp Samples

The paper pulp samples taken from the different process levels of Kartonsan Cardboard Industry and Commercial Inc. were used in this study and they were identified as:

1. Line 10 or 11 First layer pulper sample: This sample is made up of 80 % long fiber and 20 % short fiber cellulose. It does not contain any recycled paper fiber and cleaning process.
2. Line 12 Protection layer pulper sample: This is the first quality paper pulp that was treated with a deinking process. Office papers, documents, etc. are types of first quality papers which do not include foreign bodies such as ink and glue.
3. Line 13 Bottom layer pulper sample: This production line contains only waste papers as cardboard source. 80 % of this source is newspaper while 20 % is corrugated cardboard.

4. Line 14 Medium line pulper sample: This production line is the most polluted one in which all kinds of recycled paper was used. These paper pulps were treated with some cleaning steps.

All other chemicals used in this study were in analytical purity.

2.7 Moisture determination analysis

The pulp samples taken from Kartonsan were filtered under vacuum pressure and dried at room temperature (~27°C) for 48 hours and their moisture determination analysis were made.

2.8 Soxhlet extraction method

The first part of the project involved extracting the compound of interest from the pulp samples. To accomplish this task, a Soxhlet extraction was performed on the pulps. Soxhlet Extraction is a method to extract a soluble fraction from a solid medium.

Extra pure acetone was used to perform the Soxhlet extraction as a solvent. Acetone was preferred as solvent because it is organic and slightly polar. Our compounds of interest also probably have polar components, making it soluble in polar solvents. Also, acetone is a solvent that is generally used in extractions because of its ability to extract a wide variety of compounds especially in paper and pulp extractions.

200 ml extra pure acetone was used to extract lipophilic compounds from pulp samples. The only concern was to make sure that enough solvent was used so that there would be a substantial amount still present as a liquid in the bottom of the flask even when the Soxhlet chamber was completely filled with distilled solvent. The fact that solvent would inevitably evaporate out of the Soxhlet extraction apparatus also had to be taken into consideration, especially when dealing with the highly volatile acetone. The colors of the extracts were very different from each other, because all samples were taken from a different production line.

The Soxhlet extraction systems are used to extract soluble components from a solid sample into an organic solvent. The thimble is placed with dried sample in soxhlet and the flask containing the solvent is heated. Solvent vapors rise (passing through the extraction thimble), enter the water-cooled condenser, and reliquefy. When the

liquid level in the extractor reaches the top of the siphon tube, siphoning action returns the extract-enriched solvent to the flask. Soxhlets are made of low extractable borosilicate glass and come complete with extractor, condenser, and flask. Extractors have ground glass joints. An extraction thimble is required to support the sample within the extractor.

Pulps taken from Kartonsan were dried at room temperature. There are 11 production lines that results 11 different samples. All of the samples were Soxhlet extracted with acetone for 6 hours.

Pulp samples from 11 production lines of the factory were Soxhlet exracted for 6 hours. After the acetone was fully evaporated in a rotary evaporator, pulp extracts were dissolved in 2 ml chloroform and centrifuged at 500 rpm for 5 min followed by at 5000 rpm for 3 min to get rid of waste particles. Recycled pulps contain lots of waste compounds, to get rid of them samples were centrifuged and supernatant which has a dark color were disposed. After centrifugation, chloroform was evaporated under nitrogen stream until it seems like droplets. All of the pulp extractives are totally soluble in chloroform.

2.9 Acid value analysis

The acid value is defined as the milligrams of potassium hydroxide necessary to neutralize the free acids in 1 gram of sample. With samples that contain virtually no free acids other than fatty acids, the acid value may be directly converted by means of a suitable factor to percent free fatty acids (AOCS Official Method, 1992-1993).

Acid value analyses were made for the determination of the amount of free fatty acids before and after enzyme treatment. Acid value was measured according to standard acid value analysis method. Samples were analyzed before and after fungal supernatant treatment.

In this analysis system, pulp samples were incubated with fungal enzymes for 3 hours at 37 °C with Tris-HCl buffer (pH:7.00) by the addition of *F. oxysporum* growth medium supernatants. The same volume was obtained by the addition of only Tris-HCl buffer into the controls. After the incubation period, samples were vacuum filtered and filtrates were obtained. This filtrate was treated with n-

hexane which can extract the fatty acids found in the pulp sample filtrates. After a rapid shaking of filtrate with n-hexane in a funnel, bottom phase was disposed and top phase was taken into a clean flask. 5 drops of phenol phtalein were added into this phase and titrated with 0.005 N KOH. Acid value was determined by calculating consumed KOH amount.

Calculation:

The acid value, mg KOH of sample = [(A - B) x N x 56.1]/W

Where

A = mL of standard alkali used in the titration

B = mL of standard alkali used in titrating the blank

N = normality of standard alkali

W = grams of sample

2.10 GC analysis

After the Soxhlet Extraction of the pulps using acetone solvent was completed, all of the extracts were filtered using hydrophilic polypropylene membrane filters with diameter pores in order to get rid of all particulate matter present in the extracts.

The GC analyses were performed in a 6890N network GC system (Agilent Technologies, USA) with a flame ionization detector. Pulp extracts dissolved in 2 ml chloroform were filtered through 0.22 micrometer syringe filters (Millipore, USA). 1 µl of this filtrate was run on a fused-silica capillary column (DB-5HT, 15 m X 0.25 mm I.D., 0.1 µm film thickness; J & W, USA).

The run conditions were set as follows: Total run time was 27.6 min. Oven temperature was inreased from 100°C (1 min) to 350°C at a range of 15°C/min and held at that temperature for 10 min. Helium was the mobile phase and the injection was performed in splitless mode.The injector is the tapered type of microsyringe from Agilent Technologies (5µl), which can be used in split–splitless mode. Standards (mixture of palmitic acid, stigmasta-3,5-diene, sitosterol, cholesteryl oleate and triheptadecanoin; Sigma chemical Co, St. Louis, MO, USA) with a concentration range between 0.1 and 1.0 mg/ ml, were used for the quantitation of wood extractives. The correlation coefficient was higher than 0.99 in all the cases. All peaks were quantified by peak area. Squalene was quantified by using the calibration

curve for stigmasta-3,5-diene, while steroid ketones were quantified by using the sitosterol curve and the waxes by using the sterol esters curve.

Several authors have used short GC conventional capillary columns, which allow the elution and separation of high-molecular-mass lipids, for the analysis of wood lipophilic extractives, although they do not allow the best resolution. In the present study, using a high-temperature capillary column has extended the range of temperature of the analysis. On the other hand, capillary columns with thin films, which are necessary for an optimal analysis of high-molecular-mass lipids such as waxes, sterol esters and triacylglycerols, were preferred for this study. Therefore, the column finally selected for the chromatographic analyses was a DB5-HT capillary column of 0.25mm I.D. and with a film thickness of 0.1 μm.

3. RESULTS and DISCUSSION

3.1 Moisture determination analysis

The moisture determination analysis results showed that all of the pulp samples used in this study contained trace amount of moisture.

3.2 Extraction of pulp samples

The extraction of dried pulp samples were done in the Soxhlet extractor apparatus. In this system the solvent used was acetone and the extraction period was 6 hours. The organic solvent was evaporated by a Rotary evaporator. After the solvent evaporation and amount determination, the lipophilic components of pulp samples were determined by the method that is shown in the figure 3.1 (Şen, 2003).

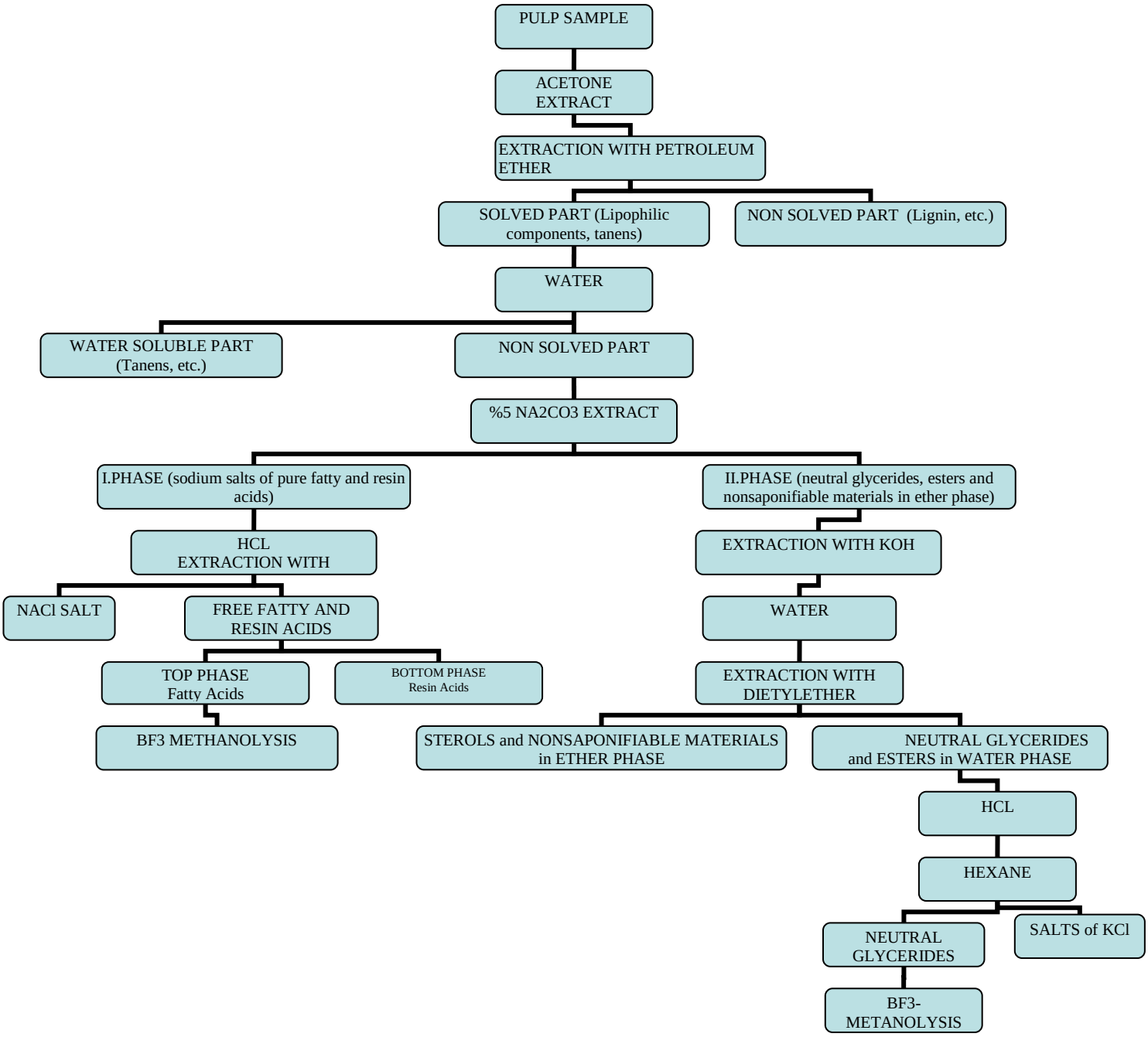

Figure 3.1: The process for the determination of lipophilic components of the paper pulp extracts.

3.3 The Lipophilic Components of Pulp Samples

All of the pulp samples taken from Kartonsan were analyzed to determine the amounts of their lipophilic component contents.

Table 3.1: The lipophilic components of pulp samples as in the means of weight percentages

Fraction	sample I	sample II	sample III	sample IV
Soluble in Petrol Ether	95,71	26,4	66	62,13
Insoluble in Petrol Ether	4,29	73,6	34	37,84
Neutral glyceride	42,56	6,6	11,72	16,7
Free Fatty and Resin Acids	30,63	27,4	10,2	16,9
Sterols and nonsaponifiable materials	23,96	64	79,2	69,7

In the table 3.1 the fraction which is soluble in petrol ether shows the lipophilic components while the fraction which is insoluble in petrol ether shows the percentage of lignin. Neutral glycerides are the main components of sample I, which was made up of 80 % long fiber, 20 % short fiber cellulose, had an extractive weight of 0,191 g as acetone extracts from 25,85 g dry pulp. The production line which sample I was taken from is not a problematic line, because it does not contain any deposits. The production line which sample II was taken from has a deinking process, preventing the possible problems. The dry weight of sample II was 63,483 g and the acetone extract was 1,17 g. The third production line that was represented with sample III composed of 80 % corrugated cardboard, 20 % newspaper. This sample was 65,97 g as dry weight and its acetone extract amount was 1,12 g. This production line has various cleaning and filtering processes to get rid of the pollutant materials such as ink, glue and stamps. The sample IV was taken from the last production line that produces the medium layer of the cardboard in Kartonsan. The dry weight of sample IV was 63,621 g and the acetone extract amount of this sample was 0,779 g.

3.4 Determination of enzyme activity

In the scope of this work, the pitch content of the recycled paper pulps were studied to be reduced by fungal lipase. The methods explained in detail in the materials and methods part of the thesis, were used to study the effects of different enzymes, sapstaining fungi *Fusarium oxysporum*, *Ophiostoma piliferum* and commercial enzymes Lipozyme TL IM, Novozym 435, Lipozyme IM. Different methods were carried out to study the detection and reduction of the pitch components with respect to the controls. The fungus was first grown on solid medium, potato dextrose agar (PDA) for activation and preparation of slopes. *F. oxysporum* was grown in liquid medium, to study the production of an extracellular enzyme, lipase. Presence of lipase activity within liquid cultures was denoted by the pH-stat. The maximum lipase activity was reached at the 6th day and a decrease was observed after 10 days.

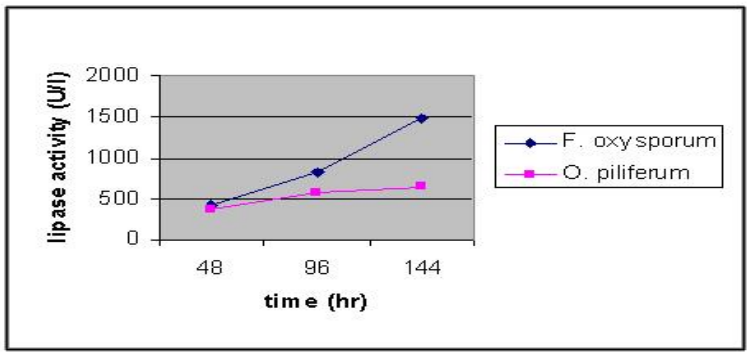

Figure 3.2: The comparison of lipase produced by the fungi used

F. oxysporum was grown in PDA plates and stocks were obtained from the spores harvested from the plates. These spores were added to liquid media and media were sampled every 24 hours to determine the extracellular lipase activity. Figure 3.2 showed that *F. oxysporum* growth medium supernatants activity was higher than that of the *O. piliferum* after 6 days of growth period. That was because of the stress conditions by directed mutagenesis that caused a decrease on the enzyme activity of this mutant strain of *O. piliferum.*

O. piliferum is a favorite fungus for the pulp and paper industry for pitch control. It has been preferred because of its extracellular lipase specificity on the problematic lipophilic compounds. Also, it has been commercialized although the lipase used in this study was obtained from an *O. piliferum* strain, which was mutated in stress

conditions by directed evolution. It has been understood from the pH-stat results that the lipase of *O. piliferum* is not very active because of the stress factors and its highest activity was 650 U/l on the 7th day. Equal units of the enzymes were used throughout this study.

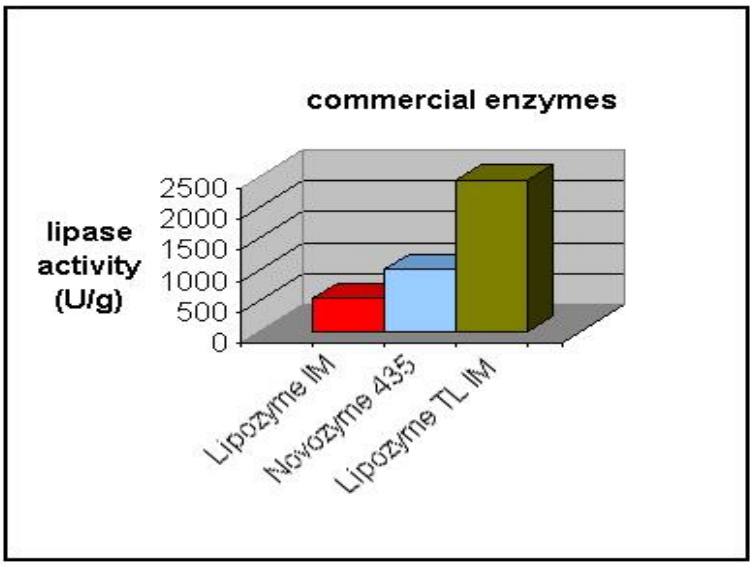

Figure 3.3: Activities of commercial enzymes

Figure 3.3 indicated the activities of different commercial enzymes from Novo Nordisk. Lipozyme TL IM had the highest activity while Lipozyme TL had the lowest. After these results Lipozyme TL IM was used preferentially because of its high activity.

It has not been reported that Lipozyme TL IM, which is a commercial lipase of Novo Nordisk, was used for depitching in the paper industry. Lipozyme TL IM that is an immobilized enzyme has a high activity of 2400 U/g. So it was expected that Lipozyme TL IM has a high degradation capacity on TAGs. This commercial enzyme was used for the comparison of growth medium supernatants obtained from fungi grown in laboratory conditions.

Although lipases change their specificity from microorganism to microorganism, it has been known that all lipases act on TAG preferentially when compared with DAG, if both of them are in the same media.

Figure 3.4: *F. oxysporum* at the growing stage in PDA

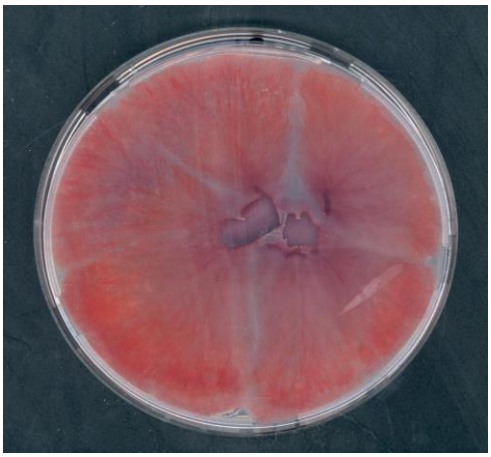

Figure 3.5: *F. oxysporum* in PDA, covered the plate

F. oxysporum was first grown in PDA, the purple color occurs by the activity of its secondary metabolism. These pictures were taken at different stages of growth.

3.5 Acid value analysis

The acid value analysis was done by standard lipid determination methods to demonstrate the FFA increase by the lipase activity (Cocks, L. V. and von Rede, C., 1966). In this analysis system, 7 g. of pulp sample from the production lines were taken and their acid values were determined and taken as controls of *F. oxysporum* growth medium supernatants added ones. The amount of pulp was increased while enzyme units were constant to show the increasing FFA quantity. Also, lipase amounts were increased to test the hydrolysis of TAG and DAG, when the sample weight was constant.

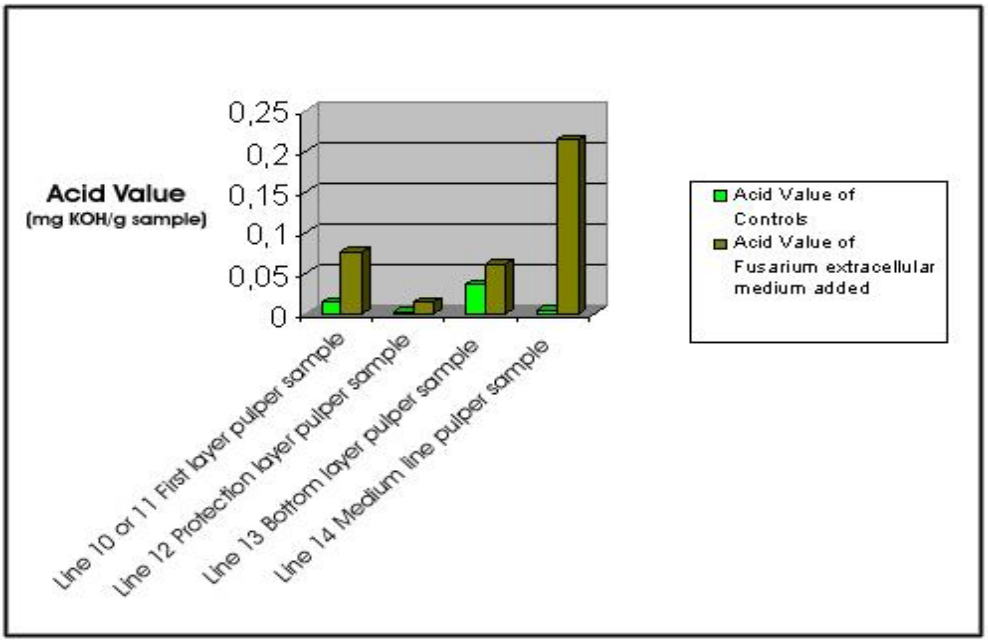

Figure 3.6: Acid value was determined for the growth medium supernatant added pulp samples taken from the four main production lines and controls, respectively.

The acid value results from the Figure 3.6 showed that the addition of *F. oxysporum* growth medium supernatants increased the acid value of all of the samples. The activity of this enzyme reached the highest to 600 U/L on the 144th hour of incubation in liquid media. In this experiment 60 U of enzyme were used for 7 g. of paper pulp. The acid value increase, after the addition of *F. oxysporum* growth medium supernatants were several folds than the acid value of controls that did not contain any enzyme. The FFA increase was obviously determined.

3.5.1 Effect of pulp dry weight

The sample taken from the most problematic production line was used in this part. The *F. oxysporum* growth medium supernatant amount was constant while 10, 30 and 50 grams of pulp were used. The enzyme activity was 90 U.

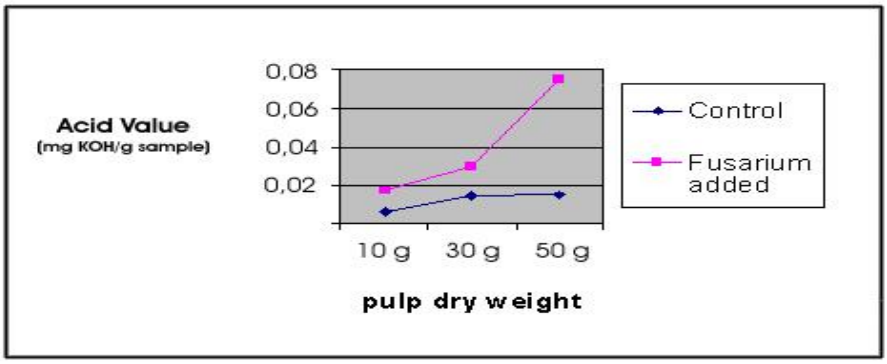

Figure 3.7: The effect of pulp weight between controls and *F. oxysporum* growth medium supernatants added samples.

In the figure 3.7, FFA ascended nearly 4 times as the pulp weight was increased from 10 g to 50 g. The hydrolysis capacity of *F. oxysporum* growth medium supernatant was detected with this experiment. As the pulp weight increases, the acid value of the *F. oxysporum* growth medium supernatant added ones increase. The pitch content of the pulp samples became higher when the weight of the pulps was increased. These results indicated that fungal growth medium supernatants hydrolyzed the increasing amount of pitch without increasing the enzyme amount.

3.5.2 Effect of enzyme amount

20 g of pulp sample was used in this experiment from the production line 14. The enzyme activity was increased from 24 to 120 units and results were compared with control.

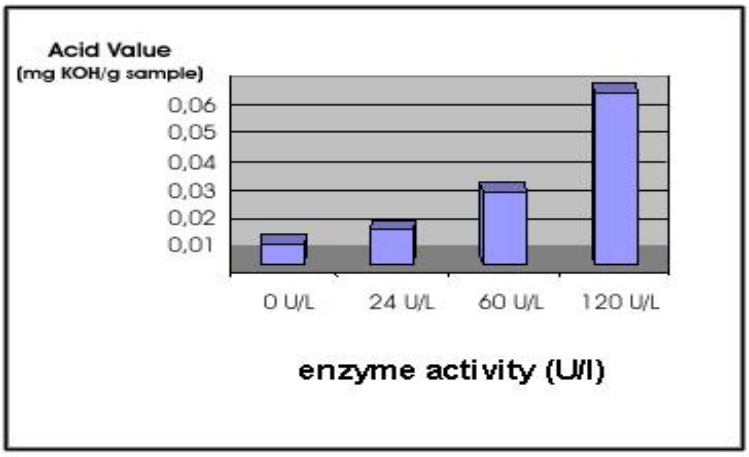

Figure 3.8: The effect of increasing enzyme activity on the sample 4.

Sample 4 was taken from the production line 14 which was the most problematic line of all. The control of this sample provided the determination of acid value increase in *F. oxysporum* growth medium supernatants added ones. The acid value of control was 0,007 which increased from 0,012 to 0,059 due to the enzyme activity. These results clearly showed that the addition of *F. oxysporum* growth medium supernatants caused an increase in the free fatty acid amount

3.6 Gas chromatography analysis

GC was used to demonstrate the FFA increase when fungal lipase was added to samples. Also, the change in the amounts of other pitch components was analyzed in this system. The GC method was fixed on a 27.7 minute run which had a temperature range from 100°C to 350°C. The detector temperature duration was changed from 3 min to 10 min for the cleaning of the capillary column. The internal standards: palmitic acid, stigmasterol, β-sitosterol, cholesteryl-oleate, triheptadecanoin were used for the calibration of fatty acids, waxes and steroid hydrocarbons, sitosterols and sterol esters, steroid ketons and triacylglycerols, respectively. The lipophilic compounds in the paper pulp samples were analyzed by this system.

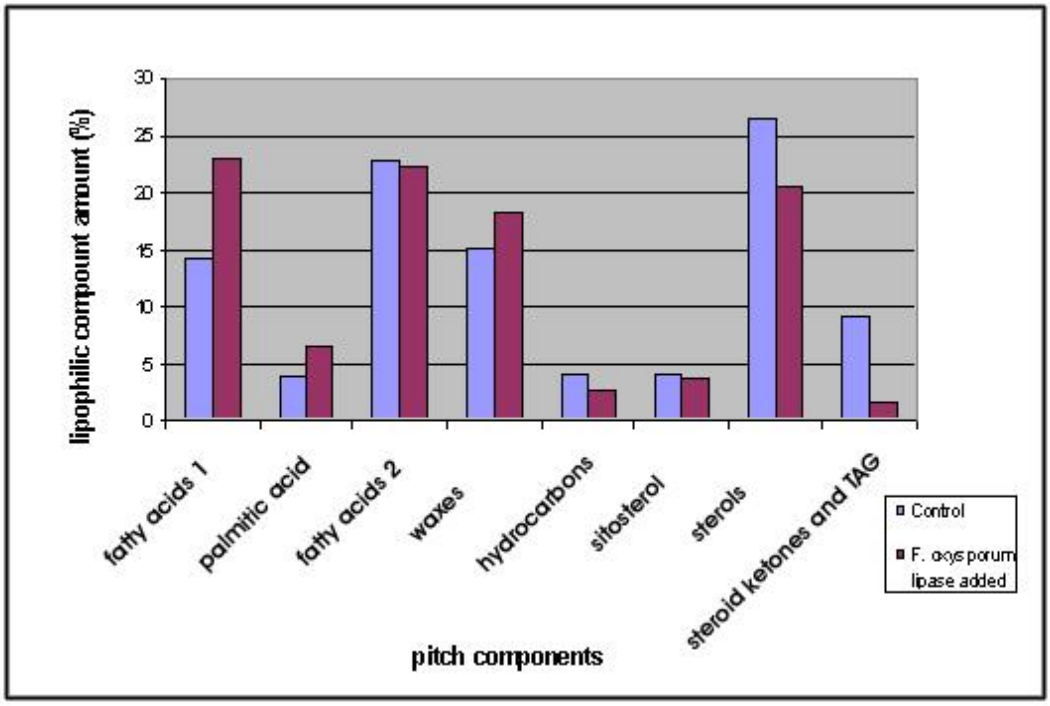

Figure 3.9: GC results of the pitch content of sample 1.

The GC analysis of the sample taken from the production line 10 showed that free fatty acids were increased while sterols, steroid hydrocarbons, sitosterols, steroid ketones and triacylglycerols were decreased when *F. oxysporum* growth medium supernatants were added. These results proved that this fungal enzyme is successful on the degradation of problematic pitch compounds when compared with the control.

4. CONCLUSION and RECOMMENDATIONS

As a result of the study, the hydrolysis of the problematic pitch compounds in the paper pulp samples taken from Kartonsan Cardboard Industry and Commercial Inc. was investigated and reported in detail. The study contained investigations of the lipophilic extractives of the problematic production lines and their pitch content. The experiments were designed by applying *F. oxysporum* and *O. piliferum* growth medium supernatants and commercial lipases for the evaluation of the hydrolysis when compared with the controls which did not contain any enzyme.

First step of the experiment was the fungal growth which was sampled every 24 hours to determine the highest lipase activity. It was found that *F. oxysporum* reached the highest activity at the 6^{th} day of the growth while *O. piliferum* reached at the 7^{th} day. The activity of these fungal enzymes sharply decreased after 10^{th} day in the liquid culture. As a result of the first step, fungal growth medium supernatants were obtained successfully.

The second step of this study was the addition of the enzymes into the pulps. The enzyme amount and pulp dry weight changed to detect the change in pitch content, respectively. After the incubation of pulp samples with the fungal and commercial enzymes, Soxhlet extraction was made for 6 hours for the extraction of pitch components, before sampling into the gas chromatograph.

Acid value analyses were made for the comparison of fatty acid contents of *F. oxysporum* growth medium supernatant added ones with controls. These results implied that the enzymes act on the lipophilic components and changed the pitch content. Especially, *F. oxysporum* growth medium supernatants reached a high degree of hydrolysis that was understood from FFA increase. In some production lines, *F. oxysporum* growth medium supernatants hydrolyzed DAG also, that gives us the information about its specificity. The acid value analysis results implied that fungal growth medium supernatants of *F. oxysporum* and *O. piliferum* were effective in degradation of some pitch components.

Acid value analysis strongly proved that *F. oxysporum* growth medium supernatant caused an increase in acid value of all the samples used which means that this enzyme was effective in degradation of triacylglycerols and diacylglycerols.

Finally GC analysis showed that *F. oxysporum* growth medium supernatants degraded pitch components that free fatty acids were increased while steroid hydrocarbons, sitosterols, steroid ketones and triacylglycerols were decreased. The sample taken from the production line 10 was used for this experiment. The results showed the efficiency of fungal enzyme, clearly.

As a consequence, *F. oxysporum* and *O. piliferum* growth medium supernatants were successful for the degradation of pitch in the samples of Kartonsan Cardboard Industry and Commercial Inc. This was proved by the acid value and GC results.

The next step in this study will be the application of these fungal enzymes into the industry. The analysis and degradation of the problematic compounds in this study will help the salvation of the pitch problem in paper pulp industry. Obtaining these enzymes in higher activities and the purification of these fungal enzymes will help the application in industrial scale.

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