Heterologous Expression and Application of Lipolytic Enzymes for Mitigation of Lipophilic Compounds in *Eucalyptus* Species

by

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PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, South Africa, under the supervision of Dr. R. Govinden and the co-supervision of Prof. B. Sithole. The research was financially supported by the National Research Foundation (NRF) and the Centre for Scientific and Industrial Research (CSIR).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Signed: Dr. R. Govinden Dr. B. Sithole

Date: 15 December 2017

DECLARATION 1: PLAGIARISM

I, Lucretia Ramnath, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

(iv) this dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them has been referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

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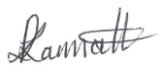

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Signed: Lucretia Ramnath

Date: 15th December 2017

DECLARATION 2: PUBLICATIONS

My role in each paper and presentation is indicated. The * indicates corresponding author.

Chapter 2

1. Ramnath, L., Sithole, B. and Govinden, R*. 2017. Classification of lipolytic enzymes and their biotechnological applications in the pulping industry. *Canadian Journal of Microbiology*, **63**: 179-192.
2. Ramnath, L., Sithole, B. and Govinden, R*. Application of esterases and lipases for the control of pitch deposits in the pulping industry. Submitted to the *Nordic Pulp & Paper Research Journal*. Date of submission: 10th April 2017.

These papers are a review of the literature search conducted and written by myself.

Chapter 3

1. Ramnath, L., Sithole, B*. and Govinden, R. Chemical composition of four *Eucalyptus* wood species used in pulp and paper industries. Submitted to the *Canadian Journal of Forest Research*. Date of submission: 13th August 2017.

This work is an analysis of wood materials used in this study. I analysed the data and wrote the paper.

Chapter 4

1. Ramnath, L., Sithole, B. and Govinden, R*. 2018. The effects of wood storage on the chemical composition and indigenous microflora of *Eucalyptus* spp. used in the pulping industry. *BioResources* **13**: 86-103.

2. Ramnath, L., Sithole, B. and Govinden, R*. Effects of storage on the lipophilic extractives of major *Eucalyptus* species used in the South African pulping industry. South African Society for Microbiology 2016 Biennial Congress, Durban, South Africa, 17th to 20th January 2016. Presented by L. Ramnath.

This work is an analysis of wood materials stored during the study. I analysed the data and wrote the paper.

Chapter 5

1. Ramnath, L., Sithole, B. and Govinden, R*. 2017. Identification of lipolytic enzymes isolated from bacteria indigenous to *Eucalyptus* wood species for application in the pulping industry. *Biotechnology Reports*, **15**: 114-124.

This paper is based on the characterization of lipolytic enzymes isolated from isolates in the laboratory culture collection. I conducted the experiments, analysed the data and wrote the paper.

Chapter 6

1. Ramnath, L., Sithole, B. and Govinden, R*. Heterologous expression of esterases from bacteria indigenous to *Eucalyptus* spp. wood chips for application in biodepitching. Submitted to *Microbiology SGM*. Date of submission: 4th September 2017.
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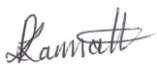
The research reported is based on the cloning and characterization of esterases. I conducted the experiments, collected and analysed the data and wrote the paper.

Chapter 7

1. Ramnath, L., Sithole, B*. and Govinden, R. Optimization of lipolytic treatment for the control of pitch components in *Eucalyptus* spp. using response surface methodology. Submitted to *Process Biochemistry*. Date of submission: 29th August 2017.

This work is an analysis of data from the pulp treatment experiments. Prof. Sithole, Dr. Govinden and myself, designed the experiments. I conducted the experiments, analysed the data and wrote the paper.



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ABSTRACT

The formation of pitch deposits is a major problem in the pulp and paper industry as it results in reduced production levels, higher operating costs, increase in equipment maintenance costs and reduced quality of product. Lipophilic compounds responsible for pitch problems are fatty acids, fatty alcohols, resin acids, hydrocarbons, steroids, triterpenoids and triglycerides. Different lipophilic extractives may cause pitch problems along the entire pulping and bleaching process depending on their chemical nature and the pulping process used. Traditional methods of controlling pitch have included seasoning of logs (storage of logs in the mill before pulping) and addition of chemicals such as alum, talc, ionic dispersants and polymers for the adsorption or dispersion of pitch particles. The drawbacks of applying these techniques are decreased pulp brightness and yield due to uncontrolled action of microbes during prolonged storage of logs, greater environmental pollution due to the addition of chemicals and higher cost. The application of enzymes offers an environmentally friendly and efficient alternative to chemical reagents and has shorter treatment times and higher specificity in the removal of wood components compared to microbial inoculation, thus providing an excellent prospective solution to alleviate the risk of pitch formation.

Evaluation of the chemical composition of four *Eucalyptus* wood species (*E. dunnii*, *E. grandis*, *E. nitens* and *E. smithii*) indicated substantial variation among them. *E. grandis* and *E. smithii* would have less severe problems related to the presence of wood resin, as they contained smaller amounts of fatty acids and sterols than *E. dunnii* and *E. nitens* species. *E. nitens* contained high lipophilic content, indicating a greater potential for wood resin problems during pulping and subsequent processing of the pulps. In addition, *E. nitens* had the highest amounts of klason lignin (6.6%) and acid-insoluble lignin (5.6%). Highest pulp yield (54.1%) was obtained for *E. grandis* and the lowest (50.3%) for *E. smithii*. The low pulp yield for *E. smithii* correlates with the low viscosity (547.17 ml/g) and glucose concentration (87.7%) obtained for this species. However, *E. smithii* pulp had the lowest kappa number of 8.25, implying that less chemicals would be required during bleaching. Of the four wood species evaluated, *E. grandis* would be ideal for the production of dissolving wood pulp based on high viscosity (570.37 ml/g), pulp yield (54.1%) and glucose concentration (89.8%), and low klason lignin (4.5%), acid-soluble lignin (4.4%) and high carbohydrate concentration (90.1%).

As a matter of interest, the chemical characterization of wood from three popular eucalypt species [*E. nitens*, *E. grandis*, and *E. dunnii* (of different site qualities)] and their pulps was performed. The effects of storage at -20°C (for 6 months) of wood was investigated by examining their chemical composition and indigenous microflora, as storage at this temperature is believed to halt any chemical and microbiological changes. Fatty acids were the main lipophilic compounds among *E. dunnii* (SQ3 and SQ4) and *E. grandis* wood extractives. The wood of *E. nitens* displayed the lowest amounts of these pitch-forming compounds, and therefore poses the lowest risk for pitch deposit formation, making it the most suitable *Eucalyptus* sp. for pulping. Storage of wood chips at -20°C had a similar effect as the traditional method of seasoning (storage of wood outdoors prior to pulping) used for the reduction of lipophilic extractives. Additionally, site quality of the *Eucalyptus* plantation had considerable influence over chemical composition of the wood material. The total extractive content was higher for *E. dunnii* (SQ3) (13.2%) compared to *E. dunnii* (SQ4) (7.7%), however, cellulose and acid-insoluble lignin contents of *E. dunnii* (SQ4) were higher at 44.8% and 27.5%, respectively. This indicates that greater pulp yield may be achieved with *E. dunnii* (SQ4) but at a greater risk of pitch formation and retention of insoluble lignin. Variations in bacterial and fungal communities were observed after storage, and should be taken into consideration when conducting laboratory scale trials, as degradation of wood components during storage would influence the outcome of experiments. It is therefore recommended that if storage of wood chips is necessary, they should be retained for a maximum of 3 months at -20°C under laboratory conditions. The findings from this study have potential to greatly influence storage practices in the pulping industry, thus improving current measures employed in dealing with wood resin components and pitch formation in the pulp and paper industry.

This study also aimed to assess lipolytic enzymes produced by microflora indigenous to *Eucalyptus* wood by screening bacteria for lipases and esterases, and fungi for laccases. Phenol red agar plates supplemented with 1% olive oil or tributyrin were ascertained to be the most favourable method of screening for lipolytic activity. Maximum lipolytic activity of the various enzymes was 45-61 U/ml at temperature and pH optima of 30-35°C and pH 4.0-5.0, respectively. pH influenced the substrate specificity of the enzymes tested. The majority of enzymes examined showed activity towards long aliphatic acyl chain substrates such as dodecanoate (C_{12}), myristate (C_{14}), palmitate (C_{16}) and stearate (C_{18}), indicating that they could be characterised as potential lipases. Prospective esterases with specificity towards acetate (C_2), butyrate (C_4) and valerate (C_5) were also detected. Enzymes retained up to 95% activity at the

optimal pH and temperature for 2-3 h. Fungal species isolated from the same *Eucalyptus* wood chip piles, were screened for laccase activity, reported to assist in the degradation of wood resin components and pitch formation. Laccase activity of up to 3.1 U/ml was observed in *Paecilomyces formosus* and *Phialophora alba*. These fungal isolates also demonstrated high substrate specificity towards dodecanoate at 35°C and 30°C, respectively.

Furthermore, the genomic DNA of two isolates that were positive for lipolytic activity were used as templates for amplification of esterase genes. Enzymes were heterologously expressed and evaluated for their ability to degrade lipophilic compounds in pulp. Activity assays using partially purified enzymes from isolates and clones, and *p*-nitrophenyl esters as substrates, yielded high activities ranging from 60-148 Units/ml for a broad range of esters. Enzyme activity varied with substrates at different pH and temperature, and were generally higher than those reported in the literature. This study highlights the importance of determining substrate specificity of lipolytic enzymes at various pH and temperatures, as part of the characterization process. The influence of pH and temperature in relation to substrate specificity of enzymes is significant and is generally overlooked. Optimal enzyme activity was observed an acidic pH of 4.0-5.0, moderate temperatures of 30-35°C, stability up to 3 h, and lipophilic compound reductions of 63% and 78% with recombinant acetyl esterase and carboxylesterase, respectively. This indicates that the enzymes are excellent candidates for treatment of wood resin components and pitch during pulping under the acidic conditions of acid-bisulphite pulping used to produce dissolving pulp.

Response surface methodology (RSM) was used to determine the optimum combination of enzymes and various parameters such as dosage, pH, and reaction times for significant reduction in lipophilic content of pulp samples. Pyrolysis gas chromatography with mass spectrometry (Py-GC-MS) was conducted on the treated and untreated pulp samples to determine the profile of lipophilic extractives. Based on the RSM optimization, large laboratory scale pulping trials were performed and the quality of the treated pulp was evaluated. The application of the characterized enzymes to dissolved wood pulp resulted in dramatic differences in the lipophilic and lignin content of the pulp. The results showed that lower pH and dosage produced the greatest response in the reduction of lipophilic content. However, the reaction time did not affect the response. Reduction in lipophilic content was optimal in *E. dunnii* pulp at an enzyme dosage of 3 U/ml treated for 4 h at pH 4.0, whereas in *E. grandis* pulp, optimal treatment was at pH 5.0, for 4 h with an enzyme dosage of 6 U/ml. The enzymatic

pretreatment of pulp resulted in 8.4% reduction in kappa number of the pulp, showing significant delignification with the enzyme treatment applied. Increases in pulp viscosity were also observed, contributing to improvement of the pulp properties. This is the first report describing the combined application of lipases, esterases and laccases in the treatment of dissolving pulp for the reduction of wood resin components that contribute to the formation of pitch. Use of lipolytic-xylano-lignolytic combinations for future applications in the pulping industry will assist in making the process eco-friendly and economical. This treatment strategy could potentially improve the quality of pulp produced and mitigate the amount of chemicals currently being added to the dissolving pulp process to reduce wood resin components and the risk of pitch deposit formation and improve brightness of the pulp.

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There is no passion to be found playing small- in settling for a life that is less than the one you are capable of living.

– Nelson Mandela –

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CHAPTER ONE

INTRODUCTION

1.1 Introduction

The forestry and forest products sector plays an important role in the South African economy providing a R3.8 billion turnover and livelihood support to 652 000 people of the country's rural population (Sappi, 2017). Paper Manufacturers Association of South Africa (PAMSA) reported a contribution of R28 billion value-added to the economy annually for 2015 and a R5 billion direct contribution to the balance of trade. The forestry-paper contribution to total South African GDP (Gross Domestic Product) was 0.5%, whilst the forestry-paper contribution to manufacturing GDP was 4.2% and the forestry-paper contribution to agricultural GDP was 23.3%) (PAMSA, 2016). Hence, pulp and paper production is an important industry in South Africa and manufacture of dissolving wood pulp (DWP) is a major enterprise of this country. Indeed, South Africa is the world's largest producer of dissolving pulp, producing 1.13 million tons per annum (tpa) and is expected to increase capacity by 100 000 tpa in 2017 (Sappi, 2017). Dissolving pulp is sold globally for use in textiles such as viscose fibres or rayon, and has a sustainable future as it originates from wood fibre grown in sustainably managed forests, compared to the fibres produced from limited sources such as fossil fuels. Products from dissolving pulp can be used in a wide range of applications such as; cigarette filters, fillers in fat-free yoghurt, tablets and washing powders, cellophane wrap, rheological modifiers in lipsticks, micro crystalline cellulose and ethers (Christov *et al*., 1998; Sappi, 2017). The continually growing DWP market means that there is an increasing demand for dissolving pulp, and global production is expected to double in the next 20 years (Lê *et al*., 2016).

Since DWP is used in high end products, its manufacture requires extensive cleaning operations to ensure high purity of the product. As a result, efficient operation of mills is essential as any downtime in mill operations severely impedes production, resulting in millions of rands in revenue being lost every year. A major challenge in this industry is the formation of pitch caused by the release of lipophilic extractives (resins) from wood material during pulping. The resins can coalesce to form sticky black spots in the pulp (reducing quality) and accumulate in

machinery, causing interruptions and increased maintenance (del Rio *et al*., 2000; Gutiérrez *et al*., 2009).

In addition, mills need to improve on the sustainability of the pulping process. Chemical pulping is a highly water intensive process, and the recycling of wastewater would be beneficial, especially since Eastern and Central South Africa are currently facing the worst drought since 1992 (South African Sugar Industry Directory, 2017). Based on the sustainability report from 2016, 88% of water utilized is returned to the environment or reused in the process. However, wood resins contaminate process waters and require removal before being fed back into the system so as not to compound the pitch problem (Sappi, 2016). By addressing this issue, the reuse of process water may be increased to 100%, creating a closed system with no wastewater generated and would reduce the amount of fresh water drawn by this industry.

Lipophilic compounds responsible for pitch problems in the paper and pulp industry are fatty acids, fatty alcohols, resin acids, hydrocarbons, steroids, triterpenoids and triglycerides (Gutiérrez *et al*., 2010). The predominant compounds found in *Eucalyptus* dissolving pulp are sitosterol, ketositosterol and steroid ketone. Different lipophilic extractives may cause pitch problems along the entire pulping and bleaching process depending on factors such as type of wood material, chemical nature of the pitch and the pulping process used (Ekman and Holmbom, 2000; Gutiérrez *et al*., 2001). Currently, there are two processes implemented for the production of dissolving pulp, the sulphite and prehydrolysis kraft processes (Sixta, 2006), as illustrated in Figure 1.1. Both methods generate low pulp yield as the intent is to produce a high purity DWP that is comprised of mainly cellulose (>95% cellulose). Acid-sulphite pulping is used in up to 70% of the total world production of dissolving pulp. This pulping process allows for high recovery rates of inorganic cooking chemicals, higher reactivity of pulp, better bleachability and total chlorine free bleaching. However, this is at the cost of varied molecular weight distribution of cellulose and inflexibility of type of raw material used compared to kraft pulping (Christoffersson, 2005; Sixta, 2006). In addition, the prehydrolysis kraft process does not utilize the hemicellulosic by-products (Håkansson *et al*., 2005; Mendes *et al*., 2009), hence the sulphite method is preferred. The use of total chlorine-free methods are known to increase the severity of pitch problems due to the low reactivity of the bleaching agent with pulp lipids (Freire *et al*., 2006). Pitch deposit formation reduces production levels, increases operating and equipment maintenance costs and reduces quality of product (Bajpai, 1999).

Although wood resin occurs in low concentrations in wood, its adverse effects however, are quite extensive (Back and Allen, 2000). As a result, considerable amount of time, effort and money are spent to reduce the impact of the resin components and pitch deposits. Traditional methods of controlling pitch include seasoning of logs (storage of logs in the mill before pulping) and addition of substances such as alum (Hubbe, 2000), talc (Allen *et al*., 1993), ionic dispersants (McLean *et al*., 2011) and polymers (Maher *et al*., 2005) for the adsorption or dispersion of pitch particles. The drawbacks of applying these techniques are decreased pulp brightness and lower yield due to uncontrolled action of microbes during prolonged storage of logs, greater environmental pollution due to the addition of chemicals and higher cost (Bajpai, 1999a). The application of enzymes offers an environmentally friendly and efficient alternative to chemical reagents and has moderate reaction conditions, non-destructive alterations to the polymer surface, and higher specificity in the removal of wood components responsible for pitch formation compared to microbial inoculation (Pallesen, 1996; Mai *et al*., 2004).

Currently, a number of biocatalysts such as lipases, esterases, laccases, ligninases, and xylanases from both bacteria and fungi are being used in the pulp and paper industry to enhance specific biological reactions (McMillen, 1998; Periyasamy *et al*., 2017). McMillen (1998) patented a multi-enzymatic microbial biostimulant that overcame environmental limits on enzyme activity and improved the rate of activity, directing biological treatment to minimal stable levels, thus lowering the rate of sludge build-up, and removing filamentous bulking and unpleasant odours from effluent treatment plants (McMillen, 1998). However, there have been no investigations into identifying lipolytic enzymes from microorganisms indigenous to the wood material being pulped for use in control or reduction of wood resin components and pitch deposits, as well as the combined effect of such enzymes on *Eucalyptus* pulp, which is the major wood material used in the South African pulping industry (Meadows, 1999). Enzymatic hydrolysis is usually identified as a limitation factor in the biocatalytic process due to the excessive cost of commercial enzymes and/or low efficiencies. To address these issues three approaches are generally employed. To reduce the cost of hydrolysis, enzymes may be manufactured on-site, and used as crude extracts compared to purified commercial extracts (Falkoski *et al*., 2013). Genetic modification of microorganisms to heterologously produce specific enzymes is another approach frequently used to improve enzyme production. The third technique involves developing a more complete enzyme cocktail by combining enzyme extracts with complementing activities to achieve a more comprehensive hydrolysis selection for degradation of hemicelluloses, lignin and lipophilic extractive fractions (Hu *et al*., 2011).

An alternative option which has been overlooked would be the combination of crude enzyme extracts. This method has huge potential as no activities are eliminated during purification or concentration preparations. Combinations of crude enzyme extracts provides enzyme synergy, resulting in higher enzyme activities and consequently enhanced hydrolysis efficiencies (Gottschalk *et al*., 2010). This concept of enzyme synergy is exceptionally complex and potentially reliant on various factors such as substrate effect and experimental factors (Kostylev and Wilson, 2011; Visser *et al*., 2013). Combining enzyme extracts is usually developed by combining those which complement each other. In biomass processing there is a variation of this method known as the minimal enzyme cocktail concept which involves finding the minimal number, the minimal levels, and the optimal combination of the top performing key enzymes. There are two hypotheses behind this concept: i) the crude cellulolytic and/or xylan degrading enzyme preparations are not optimal for degradation of cellulose in pre-treated lignocellulosic biomass nor for degradation of heteroxylans in hemicellulose-rich product streams; ii) it would be viable to substitute crude multienzyme preparations with designed combinations of the minimal number of essential enzyme activities for biomass processing (Meyer *et al*., 2009). In the present study enzyme cocktails were developed by combining minimal amounts of crude cellulase-free enzyme extracts targeting lipophilic extracts as well as hemicelluloses and lignin fractions of *Eucalyptus* wood pulp.

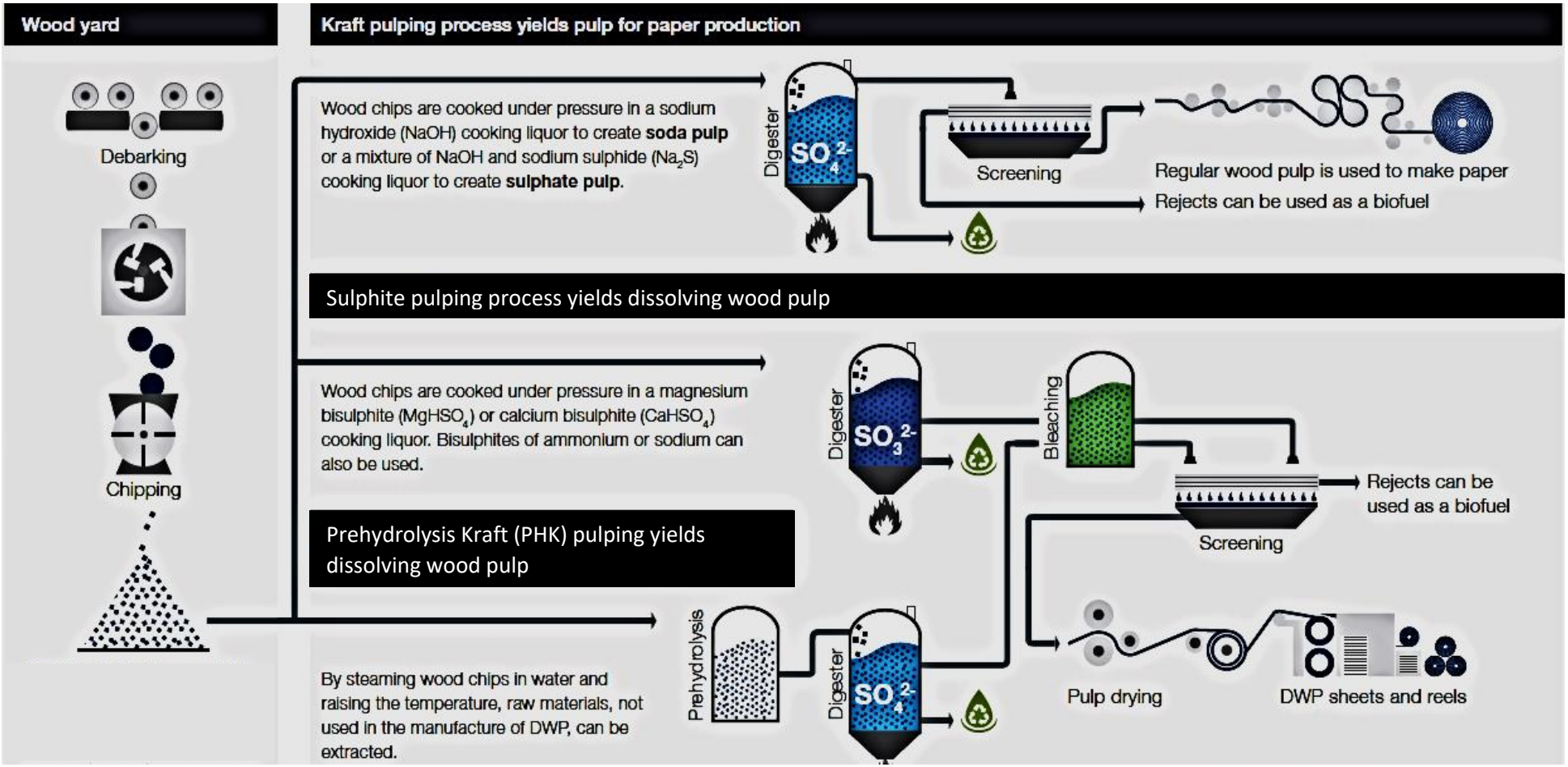

Figure 1.1: A comparison of the pulping processes used for the production of dissolving pulp. Reproduced from Sappi (2017). https://cdn-s3.sappi.com/s3fs-public/Sappi-FAQs-Dissolving-wood-pulp.pdf.

1.2 Problem Statement

Reduced production levels, higher operating and equipment maintenance costs and reduced product quality are some of the negative effects of pitch deposit formation in the pulp industry (Back and Allen, 2000; Gutiérrez *et al*., 2010). Due to the negative aspects of traditional methods (Bajpai, 2012a,b), the application of enzymes offers an environmentally friendly and efficient alternative to chemical reagents currently being used. In addition, enzymes have shorter treatment times and higher specificity in the removal of wood components compared to microbial inoculation. Previous studies have focussed on the application of individual enzymes for treatment of pitch (Farrell *et al*., 1993; Farrell *et al*., 1997; Bajpai, 1999a; Dubé, 2008; Gutiérrez *et al*., 2010; Bajpai, 2012). However, due to the complex nature of these deposits, this approach is not very effective. For example, lipase will only target glycerides and not other pitch causing components such as sterols, fatty acids, etc. A combination of lipolytic enzymes would be a more practical approach as it would target most of the pitch causing components that have diverse chemical structures. This is a novel aspect of the dissertation. The effective treatment of pitch problems is only possible if the nature of the wood resin components that cause pitch deposits is understood. Profiling the lipophilic content of various *Eucalyptus* species will assist in the development of strategies for the efficient removal or control of pitch components. The results will be used to develop a combination of lipolytic enzymes to target the compounds.

1.3 General Aim of the Study

This study aimed to investigate the potential of microflora, indigenous to *Eucalyptus* species, to produce enzymes that would assist in the removal of lipophilic extractives in DWP. The variation of lipophilic content in *Eucalyptus* species is an important consideration with regard to targeted treatment of wood resins and pitch deposits, as tree species and type of pulping process are known to influence the nature of the pitch deposits. Therefore, in this project, profiles of the lipophilic content of four major *Eucalyptus* species in South Africa were studied, as well as the chemical composition of their respective pulps. In addition, enzyme cocktails were developed using response surface methodology (RSM) for the efficient removal of pitch causing compounds. The effects of storage on *Eucalyptus* species wood material were also investigated.

Metagenomics was initially used as a primary tool to determine the spectrum of lipophilic enzymes present in the bacterial metagenome isolated from the wood chips of the *Eucalyptus*

species. Ideally, identification of substrate specific lipophilic enzymes would have enabled the development of a recombinant bacterial strain capable of producing enzymes that target pitch-causing compounds. The direct application of these enzymes to the pulping process, would circumvent the issue of pitch deposit formation. This study encompassed techniques currently applied in this area of research in chemistry, however, the microbiological aspect of evaluating indigenous microflora of *Eucalyptus* wood species appears to be novel. This study also aims to identify key lipases and esterases involved in the degradation of wood resin components and pitch deposits.

1.3.1 Specific objectives and aims

The following specific objectives were set to achieve the general aim of the study:

1. To mine a bacterial metagenome and laboratory culture collection, both obtained from commercial *Eucalyptus* wood chip piles, for lipolytic enzymes using selected gene sequences and screening techniques, respectively.

- The BLAST program (NCBI) was used to search for applicable enzymes and the metagenome sequenced from the wood chip piles was mined for specific lipolytic enzymes using CLC-Bio. SEQ MATCH TOOL was used to compare sequences with the Ribosomal Data Base Project and sequences in GenBank.

2. To clone enzyme genes into appropriate hosts for expression and to characterize recombinant enzymes.

- Enzyme specific primers were designed and synthesized based on sequences obtained. Primers were used to amplify genes encoding for the specific enzymes from the metagenome and pure isolates selected from culture collection. Amplified genes were cloned and expressed in an appropriate host.

3. To determine baseline lipophilic profiles of four major *Eucalyptus* species and identify the major contributors to pitch formation.

- Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) was used evaluate lipophilic content. The effects of storage on lipophilic content of the wood was also investigated.

4. To determine the effects of various combinations of enzymes on the lipophilic content of pulp using Py-GC-MS and Response Surface Methodology (RSM).

- Statistical analyses of the model were assessed and optimized conditions were then applied for large scale trials to validate the model.

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CHAPTER TWO

LITERATURE REVIEW

2.1. *Eucalyptus* species in South Africa and Internationally

Eucalypts are an essential supply of material for the South African pulp and paper industry as well as other countries such as Brazil, South-Western Europe, Japan and South America. *Eucalyptus* is the most valuable and most extensively planted variety of plantation forest tree species in the world (>20 million ha) due to its extremely fast growth rate (average annual growth rates up to 100 m^3/ha/year), broad adaptability, good form and excellent wood and fibre properties. Eucalypts are also recognized as fast-growing, short-rotation, renewable biomass crops for energy production (Myburg *et al*., 2006). The predominant *Eucalyptus* species are: *E. dunnii, E. nitens, E. fastigata, E. grandis, E. microcorys, E. macarthurii, E. saligna, E. smithii,* and *E. viminalis* as well as a number of hybrids such as *E. urophylla, E. tereticornis,* and *E. camaldulensis*. The introduction of *Eucalyptus* species into South Africa did not occur in vast numbers as it did in Brazil and Europe. However, the breeding of eucalypts for industrial plantations advanced quickly in South Africa as well as Brazil, Portugal and Chile (Grattapaglia and Kirst, 2008). *E. grandis, E. dunnii, E. saligna, E. smithii, E. tereticornis, E. nitens* and *E. europhylla* species are commonly utilized by the South African pulp and paper industry. Virtually all pulping mills utilize a mixture of wood species, despite generally available advanced forest technology in the country (Foelkel, 2008). The genetic differences amongst these *Eucalyptus* species gives rise to variations in the resistance and hardiness of these trees. *E. grandis* and its hybrids, tailored to areas lower than 1400 metres altitude, are the greatest valued genetic material in the South-African silviculture. Above this altitude species more tolerant of cold or frost such as *E. dunnii, E. nitens,* and *E. macarthurii*, are grown (Foelkel, 2008).

2.2. Wood Characteristics of *Eucalyptus* Species

The major hardwood species grown in South African plantation forests include: *E. dunnii, E. grandis, E. nitens, E. smithii* and a range of hybrids, as well as wattle (*Acacia mearnsii*). Recently, *E. dunnii* has become more popular globally because of its high wood density, adaptability to a range of site conditions, naturally good form, and tolerance to pests and

diseases in temperate planting regions (Sappi, 2012). This species is characterized as mildly drought tolerant, but susceptible to wind, frost and snow damage. It is suited to sites in the summer rainfall regions of South Africa with average temperatures higher than 15.5°C and average annual rainfall in the range 822-925 mm for optimum growth. Sappi, the largest pulp manufacturer in South Africa, has a breeding programme with probably one of the most extensive collections of *E. dunnii* material, comprising more than 700 individual families from 20 different regions in Australia. This priceless resource gives Sappi breeders the genetic freedom to determine the most appropriate material for end-uses of pulp in terms of growth and fibre properties. This gene pool also permits for some protection against pests and diseases that could challenge the species in the future (Sappi, 2012). Species of *Eucalyptus* are selected for pulping based on the pulping process to be implemented and the end products generated (Foelkel, 2007).

2.3. Pulping Process: Kraft vs. Acid-Bisulphite Pulping

Wood materials used in the production of pulp are comprised of four key components: cellulose fibres (essential for paper production); hemicelluloses (shorter branched carbohydrate polymers); lignin (glues cellulose fibres together); and wood resin (lipophilic extractives) or pitch deposits. Dissolving pulp is considered to be a low-yield chemical pulp with low hemicellulose and lignin content and high cellulose content (Kumar and Christopher, 2017). It is used in the manufacturing of cellulosic materials such as acetates, cellophane, rayon, cellulose ethers and esters, graft and cross-linked derivatives (Kumar and Christopher, 2017; Sappi, 2017). The manufacturing entails derivatization and solubilization of highly purified cellulose. Dissolving wood pulp (DWP) is produced via acid pulping of wood; however, the process is plagued with problems due to residual hemicellulose and wood resin compounds in pulp. Thus residual hemicelluloses and wood resin compounds are problematic issues in the manufacture of DWP. These problems can be addressed by pulping under alkaline conditions as in the pre-hydrolysis kraft process (Sithole and Allen, 2002), during which, hemicelluloses are removed by pre-treatment of the wood chips with water at high-temperatures. The wood chips are then subjected to kraft pulping under alkaline conditions for removal of lignin and wood resin components. The resulting pulp is then bleached to produce relatively pure cellulose similar to DWP. During downstream processing of dissolving pulp, hemicelluloses such as xylans, can accumulate on the cellulose micro-fibrils so causing operational and quality issues, and thus need to be removed in the pulping process (Sixta, 2006). Also, hemicellulosic

contaminants trigger colour and haze in the product. High caustic loadings and specific pulping conditions are used for their removal, the latter is restricted to sulphite pulping and the former to acid-pretreated kraft pulping. Hemicelluloses removed from this process are a beneficial source of hexose and pentose sugars (Amidon *et al*., 2008; Chambost *et al*., 2008; Amidon *et al*., 2009; Canilha *et al*., 2012), that can be converted into valuable products such as polymers, ethanol, and other chemicals (van Heiningen *et al*., 2005). This pre-hydrolysis kraft dissolving pulp production process therefore naturally fits into the forest bio-refinery concept.

Paice and Jurasek (1984) first proposed the use of xylanase for purifying cellulose. It was reported that complete enzymatic hydrolysis of hemicelluloses within the pulp is complex. Even with very high enzyme loading, only a moderately small amount of xylan could be removed (Verardi *et al*., 2012; Álvarez *et al*., 2016). However, in the last 30 years hemicellulose removal from lignocellulosic material has greatly improved (Valls *et al*., 2010; Bajpai, 2011; Martín-Sampedro *et al*., 2012; Leu and Zhu, 2013; Hutterer *et al*., 2017; Zhao *et al*., 2017; Cebreiros *et al*., 2017). Even with these advances, complete removal of residual hemicelluloses seems impossible, possibly due to the inaccessible sites of the substrate. Nonetheless, xylanase treatment may facilitate xylanase extraction from kraft pulps or reduce the chemical loading required during the caustic extraction.

2.4. Pitch Composition and Formation

Pitch, also known as wood resin or lipophilic extractives, accounts for only about 0.5-5% of wood material, and varies with wood species. However, pitch causes production and runnability problems, the impact of which far exceeds their concentrations in the wood (Back and Allen, 2000). Additionally, the extractives also contribute to environmental pollution and complicated waste remediation (Kontkanen *et al*., 2004; Wang and Jiang, 2006). These extractives are a collection of diverse substances with unpredictable chemical behaviour which, in theory, may be arranged into two main groups; lipophilic and hydrophilic, based on their hydrophobicity and solubility in mill process waters (Nguyen, 2006). Several compounds have been identified as the major contributors of pitch, including triglycerides, waxes, steryl esters and fatty acids (Gutiérrez *et al*., 1999; del Río *et al*., 2000; Gutiérrez *et al*., 2001; Freire *et al*., 2005; Valto, 2011) with triglycerides being the most problematic (Back and Allen, 2000). Pitch is released during the pulping process. As wood is pulped, the lipophilic extractives in the parenchyma cells and softwood resin canals are released, forming colloidal pitch (Sjöström,

1993). These colloidal particles can coalesce into larger droplets that deposit on pulp or equipment, forming the so-called pitch deposits, or may remain suspended in the process waters (Hubbe *et al*., 2006).

Various softwood, hardwood and non-woody species are used as raw material in the pulp and paper industry. Wood extractive composition may differ depending on tree species and age, season of harvesting and other genetic and environmental elements (Back and Allen, 2000). The resin components of softwoods, such as Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*), are typically represented by triglycerides, resin acids, fatty acids and steroids which are frequently linked to pitch formation (Table 2.1) (Ekman and Holmbom, 2000, Kontkanen, 2006). Lipophilic profiles of soft- and hardwoods vary considerably, with different initiators of pitch deposits (Allen, 1988; Breuil *et al*., 1997; Sithole *et al*., 2013).

Due to the increasing use of fast-growing *Eucalyptus* species by the paper and pulp industry, recent information on the composition of lipophilic extractives has come from this hardwood. Table 2.1 highlights the variable nature of pitch amongst *Eucalyptus* species. Careful consideration needs to be taken when pulping various species of *Eucalyptus* to avoid combining those with high lipophilic extractive content which could lead to pitch formation. Pitch is generated from pulp fibres at different points of the process particularly when there is a change of pH and/or temperature (Bajpai, 1999; Dai and Ni, 2010). Tackiness of pitch deposits is generally attributed to chemical and physical characteristics associated with the pitch compounds. These qualities are influenced by pH (Strand *et al*., 2011), temperature, calcium, magnesium, resins and oils (Berglund, 2012).

Table 2.1: Composition (mg/g) of lipophilic extractives from pine, spruce, birch, poplar and various eucalypt wood (Gutiérrez and del Rio, 2001; Rencoret *et al*., 2007).

	Softwoods		**Hardwoods**							
	Pinus sylvestris	***Picea abies***	***Betula verrucosa***	***Populus tremula***	***Eucalyptus globulus***	***Eucalyptus globules***	***Eucalyptus nitens***	***Eucalyptus maidenii***	***Eucalyptus grandis***	***Eucalyptus dunnii***
Free fatty acids	1.73	0.78	-	1.06	0.28	0.33	0.45	0.21	0.32	0.44
Resin acids	6.65	2.85	0	0	0	0	0	0	0	0
Hydrocarbons	0.74	0.19	0.40	1.14	0.17	31.3	30.5	22.2	19.4	26.6
Waxes or sterol esters	0.83	0.87	1.96	3.07	0.57	0.25	0.83	0.43	0.21	0.29
Monoglycerides	0.18	0.55	2.24	1.18	0.02	0.12	0.12	0.05	0.059	0.067
Diglycerides	0.32	0.55	1.72	0.58	0.02	-	-	-	-	-
Triglycerides	8.74	1.94	8.10	10.37	0.13	11.2	21.0	38.1	15.7	28.0
Higher alcohols or sterols	1.39	1.00	1.56	2.40	0.68	0.27	0.54	0.36	0.36	0.27
Oxidized compounds	0.43	1.36	2.94	1.53	0.22	-	-	-	-	-

The technique used in this study for the characterization of the extractive content of wood and pulp was Pyrolysis-Gas Chromatography and Mass Spectoscopy (py-GC-MS). The use of py-GC-MS is very useful in tracing the origin of deposits. A detailed review on Py-GC/MS and its applications in analysis and characterization of pitch components is available (Sithole, 2000). Several studies have successfully implemented the use of py-GC-MS to evaluate the nature of pitch deposits in pulp (del Río *et al*., 2000; Gutiérrez and del Río, 2005; Sithole and Watanabe, 2013; Melo *et al*., 2017).

2.5. Traditional Methods to Control Pitch

Traditionally, logs were debarked (or chipped) and stored for prolonged periods of outdoor storage before pulping (known as seasoning), to naturally reduce wood resins and pitch deposits (Allen and Ouellet, 2007). Storage reduces the extractives content due to their hydrolytic or oxidative conversion by plant enzymes and the activity of indigenous microflora. These natural reactions have been researched in many wood species such as eucalypt, pine, spruce, aspen and birch (Ekman, 2000; Silvério *et al*., 2008). Drawbacks of seasoning include high expense, reduced pulp brightness and yield caused by uncontrolled growth and action of indigenous microorganisms. As a result, commercial mills may not incorporate or extend a period of seasoning. Chemical approaches became popular in the early 1990's and involved adsorption or distribution of pitch elements by the addition of talc, alum, cationic polymers, ionic or non-ionic dispersants, and other varieties of additives (Rojas and Hubbe, 2004).

2.6. Biological Control Methods

As an alternative to conventional natural methods, biological man-made strategies can be used to expedite and control wood seasoning. Accumulation of wood extractives can be managed with enzymes or microorganisms to reduce the risk of pitch formation to a satisfactory level (Fischer and Messner, 1992; Fujita *et al*., 1992; Bajpai, 1999; Ramos *et al*., 1999; Gutiérrez *et al*., 2006; Bajpai, 2011). This strategy reduces defects on paper as well as the frequency of cleaning pitch deposits in the paper machine. At the same time, it also offers other advantages, such as an eco-friendly and nontoxic technology, improved pulp and paper quality, reduction in bleaching chemical consumption, reduction of effluent load, and space and cost saving in a mill wood yard by using unseasoned logs. By reducing the outside storage time of logs, this method reduces wood discoloration, wood yield loss, and the natural wood degradation which occurs over longer storage time (Bajpai, 2011).

2.6.1 Microorganisms for removal of pitch components

Several studies have examined the deresination of wood by treatment of wood chips with a fungus capable of metabolizing lipophilic wood extractives (Blanchette *et al*., 1992; Brush *et al*., 1994; Su *et al*., 2011; Bajpai, 2012). This technique reduces extractive levels and shortens the seasoning period for fresh wood species such as, southern yellow pine (Brush *et al*., 1994), lodgepole pine (Chen *et al*., 1994), and aspen (Rocheleau *et al*., 1999). *Ophiostoma* species are reportedly useful in metabolizing wood extractives. This fungus is a saprophyte that

proliferates through parenchyma cells, tracheids, and resin ducts, metabolizing available nutrients such as sugars and lipophilic extractives. Cartapip 97® is a colourless strain of *Ophiostoma piliferum*, which was developed for the treatment of aspen wood chips to control seasoning (Hoffman *et al*., 1992). It degrades extractives and inhibits the growth of other microorganisms on the wood chips if applied to fresh chips (Josefsson *et al*., 2006). Treatment with Cartapip® was successful in deresinating fresh aspen chips (Rocheleau *et al*., 1999). Concerns were raised as to the ability of Cartapip® to grow on wood chips prepared from aged logs, as wood sometimes remains at the felling site for longer periods of time before reaching the pulping mill. Even though it is generally used for pines, Cartapip® is capable of reducing resins in wood with high and low extractive content. Up to 50% of eucalypt extractives may be eliminated by using Cartapip, however, it does not reduce the amount of pitch as the compounds responsible for the pitch (a fraction of the extractives) are not effectively degraded (Gutiérrez *et al*., 2001). Other fungi have shown potential to degrade considerable amounts of pitch (Gutiérrez *et al*., 2011). Fischer and colleagues proposed *C. subvermispora* to be the best biopulping fungus which removed as much as 30% of the extractives compared to the control (Fischer *et al*., 1994). Concurrently Farrell and colleagues treated wood chips with fungal spores to promote the seasoning of wood; theirs was the first report of wood pre-treatment to control pitch (Farrell *et al*., 1997). Application of microorganisms may be a better strategy than conventional seasoning of wood, but it does have its disadvantages, such as; decrease in yield and brightness, lower efficiency in winter (temperature too low for growth of organism), long storage time, large area required for pre-treatment and the potential health threat due to airborne fungal spores (Farrell *et al*., 1997).

2.6.2 Enzymes for pitch removal

Enzyme catalyzed pitch reduction is an exceptionally effective biotechnological method (Irie and Hata, 1990; Fujita *et al*., 1992; Jegannathan and Nielsen, 2013). Numerous benefits arising from this include: (i) the reduction of paper defects, (ii) eco-friendly and non-toxic technology, (iii) reduction in chemicals used in bleaching, (iv) enhanced pulp and paper quality, (v) reduction of effluent load, and (vi) economic use of space and expenses in a commercial wood yard by utilizing logs that have not been seasoned. Reduction in outdoor storage of logs reduces wood yield loss, wood discoloration, and wood degradation by indigenous microorganisms occurring over longer storage periods (Bajpai, 2011).

In the past, the application of enzymes was not thought to be technically or economically practical in the pulp and paper industry. This was mainly because there were no appropriate enzymes easily accessible, except for restricted application in starch modification for paper coatings (Cui, 2005). A focussed effort by scientific institutions and enzyme manufacturers, targeting the development of industrially relevant enzymes, has meant that the application of enzymes to promote bioprocessing in pulp and paper manufacturing has developed exponentially since the mid 1980's (Bajpai, 1999; Lin, 2014).

Consideration of enzyme application for the control of wood resins and pitch in pulp and paper manufacturing began in Japan in the 1980's. Red pine wood was an essential material for groundwood pulp in Japan. It was used in the production of newsprint and light-weight papers. However, this type of pulp had a high amount of pitch. To gain a better understanding of pitch, research was carried out by Irie and Hata (1990) who discovered that pitch was triggered by triglycerides in the resinous material of the wood material. They then went on to develop the first lipase enzyme from *Candida cylindracea* for the control of pitch. By the early 1990's this technique was routinely applied in commercial pulping processes, and was the first case in history to apply an enzyme directly to the pulping process (Fujita *et al*., 1992; Fujita *et al*., 1991).

Nippon Paper Industries (Japan) established a pitch management technique that utilizes a lipase from *Candida rugosa* capable of hydrolysing 90% of the wood triglycerides (Sharma *et al*., 2001). Research continued in supplementary mill trials with the application of an enhanced lipase formula marketed by Novozymes (Bagsvaaerd, Denmark, previously known as Novo Nordisk) under the trademark Resinase®, a lipase (recombinant) expressed in *Aspergillus oryzae* (Fujita *et al*., 1992; Fujita *et al*., 1991; Sarmiento *et al*., 2015). Resinase® applied to pine (*Pinus densiflora*) mechanical pulp, hydrolysed approximately 95% of the triglycerides present. Furthermore, Resinase® decreased the amount of pitch deposits, the quantity of holes and spots in paper, facilitated decreases in the dosage of talc to manage pitch and enabled the use of greater quantities of fresh wood (Gutiérrez *et al*., 2001).

The potential application of a characterized sterol esterase from the saprophytic fungus *Ophiostoma piceae* (Calero-Rueda *et al*., 2002b) in pitch control during pulp manufacturing has been examined and patented for its ability to hydrolyse triglycerides and sterol esters

(Calero-Rueda *et al*., 2002a; 2004). The activity of sterol esterases is restricted to substrates such as sterol esters and glycerides, and they are incapable of degrading other extractives that make up pitch (Gutiérrez *et al*., 2001). Resinase A 2X, manufactured by Novozymes, is a commercially available recombinant lipase expressed in *Aspergillus oryzae* (Gutiérrez *et al*., 2001). It is capable of hydrolysing approximately 95% of the triglycerides in the mechanical pulp of pine wood (*Pinus densiflora*). A variant of Resinase A 2X called Resinase HT has an optimum temperature range of 70-85°C, and is employed in mills in China, Japan, the USA, Canada and other countries in the Far East (Gutiérrez *et al*., 2009).

In the following years, numerous commercial pulp and paper mills in China and Japan have established lipase-based pitch management methods in mechanical pulping while pilot-scale experiments for pitch management in softwood sulphite pulp utilizing Resinase® in Europe have yielded favourable results (Hata *et al*., 1996; Sarmiento *et al*., 2015). More recently other commercial lipases, for instance Lipidase 10000 (American Lab. Inc.), *Candida* and *Aspergillus* lipases, have been explored for their potential in pitch management (Romo-Sanchez *et al*., 2010; Singh and Mukhopadhyay, 2012). These lipases were able to hydrolyse triglycerides efficiently. However, only partial hydrolysis of steryl esters was achieved (Viikari and Lantto, 2002; Vaquero *et al*., 2015a).

While application of lipase enzymes in the reduction of pitch may be valuable, the activity of these enzymes is limited to triglycerides and they do not assist in the breakdown of other extractives that contribute to pitch formation (Dubé *et al*., 2008). Lipases hydrolyse triglycerides to free fatty acids and glycerol. Glycerol is water-soluble and therefore poses no threat. Free fatty acids however, precipitate at temperatures below their melting points, causing deposits on paper and blockages of pipes (Sithole and Allen, 2002). Fatty acids are also dependent on pH as well as chain length and number of double bonds. At high pH levels, they are surface active and act as soaps (Strand *et al*., 2011).

Hence, enzymes operating on a wider variety of substrates are being considered, and assorted populations of organisms are being screened for novel enzymes. Sterol esterases are a less popular group of hydrolases, and have potential application in pitch management as sterol esters are frequently at the source of pitch deposits, due to their elevated tackiness and challenges in kraft pulping (Calero-Rueda *et al*., 2002a; Kontkanen, 2006). Esterases are also

used in the control of ‘stickies’, which are formed during the processing of recycled paper. The glue, adhesives, binders and coatings in the paper aggregate, causing paper tears and holes which reduces the quality of the final product and causes obstruction of machinery (Patrick, 2004; Sarja, 2007; Bajpai, 2011). Traditional removal of pitch deposits involves chemical and mechanical cleaning of machinery which requires electricity, steam and solvent expenditures. A major component of ‘stickies’ is polyvinyl acetate (PVAc). Application of an esterase would assist in controlling ‘stickies’ by hydrolyzing the PVAc to a less adhesive alcohol form which is simpler to suspend in water (Hubbe *et al*., 2006). This would reduce energy utilized during recurring production breaks and solvent use, thus saving on energy and solvents (Jones, 2005; Skals *et al*., 2008).

On the other hand, the application of esterases alone is not sufficient to reduce wood resins or control pitch deposits. Esterases enhance the level of carboxylic acid groups in the tacky substance, therefore, esterases combined with highly charged cationic polymers may assist in achieving the desired results (Hubbe *et al*., 2006). Other considerations for the application of enzymes include the properties of the enzyme (e.g. pH and temperature range) and deciding at which stage of the pulping process they should be added for greatest effect. Every enzyme has a specific range of pH and temperature requirements for maximal activity. Therefore the parameters of a particular stage in a process needs to be considered when selecting enzymes for use in pulp and paper production.

There is a range of pulp and papermaking processes currently being used worldwide. The main ones involve mechanical, chemical, and recycled pulping. Mechanical pulping entails mechanical treatment of wood to effect fibre separation. Thermomechanical pulping used to be a popular choice in the pulping industry and involved producing wood pulp from pre-steaming wood chips at temperatures of about 125°C. However, further research revealed that refining at this temperature exceeded the glass transition point of lignin (above this point lignin fibres become hardened and resistant to further breakdown during subsequent refining) which had negative effects on the quality of pulp (Roffael *et al*., 2001). In addition, mechanical pulping is was reported to only slightly affect the composition of extractives (Gutiérrez *et al*., 2009).

Chemical pulping on the other hand is able to saponify triglycerides, whereas sterol and triterpenol esters are much less affected (Gutiérrez *et al*., 2001). Chemical pulping can be done

by sulphate (kraft) and sulphite pulping. Sulphite pulping is carried out at 130-140°C (retention time of 6-8 h) under acidic conditions (pH range of 1.5 to 5.0), which is the cause of their higher extractives content compared to kraft pulps. The predominant lipophilic extractives in sulphite processes are fatty acids, glycerides and sterols (Sithole *et al*., 2010). Although some triglycerides are hydrolyzed to glycerol and free fatty acids, no saponification occurs as with kraft pulp because of the very weak acidic conditions in the process. The unchanged fatty and resin acids are insoluble under acidic conditions. Since many parenchyma cells remain intact during the process, more triglycerides and sterol esters are passed along in the pulp with the potential to cause serious pitch problems (Young and Akhtar, 1998). Kraft pulping is an alkaline process and is performed at 170°C (retention time of 3-4 h) and pH of 13.0-14.0 (Colodette *et al*., 2002). Mild chemical cooking can also be done in the neutral semi-sulphite chemical pulping (NSSC) process that occurs under neutral conditions. During kraft pulping the polymerized aliphatic hydrocarbons found are primarily unsaturated fatty acids and alcohols that undergo condensation reactions. A study found that the more double bonds the hydrocarbons had, the faster the condensation reactions took place (Ohtani and Shigemoto, 1991). Fatty acids were also polymerised faster than fatty alcohols. Such extensive polymerisation of aliphatic hydrocarbons are not soluble in organic solvents (Scheepers, 2000). Generally, sodium salts of wood resin are harmless in kraft systems due to their high solubility, however, high ionic concentrations of these salts radically reduces their solubility. Studies have shown that 0.75 mmol/L of NaCl results in the insolubility of sodium soaps of oleic and abietic acids, resulting a new variety of deposition (Sithole and Allen, 2002). Recycled pulps are produced by re-pulping of mechanical or chemical papers. Mechanical, sulphite, and recycled (mechanical) pulps contain significant amounts of lipophilic extractives as they are made under non-alkaline conditions that do not facilitate removal of the extractives (Marques *et al*., 2010).

Enzymes that would be considered for application in these pulping processes would be either acidophilic or alkalophilic and thermophilic. Mirza and colleagues (2006) evaluated the effect of a lipase (Buzyme 2517) on extractive composition in a Themo-mechanical pulping process (TMP). The lipase was applied at the removal of the disc filter moving into the TMP storage tank where it remained for 8-12 h at a temperature of 65°C. A 45-53% reduction in triglycerides in the TMP mainline was observed, however, reduction in triglyceride levels was only detected 12 h after the enzyme treatment began (Mirza *et al*., 2006).

Noteworthy bacterial lipase producers include *Bacillus*, *Pseudomonas*, and *Burkholderia*, whilst *Aspergillus*, *Penicillium*, *Rhizopus* and *Candida* represent the fungal producers (Singh and Mukhopadhyay, 2012; Gupta *et al*., 2004). Bacterial lipases usually have an optimum temperature range of 30 to 60°C (Canet *et al*., 2016), however, there have been reports of lipases with both lower and higher optima (Latip *et al*., 2016). Amongst bacteria, thermophilic *Pseudomonas* spp. produce lipases which are stable at 70°C (Royter *et al*., 2009; Devi *et al*., 2015). Other bacteria producing thermostable lipases include *Bacillus pumilus*, *Burkholderia multivorans*, *Geobacillus* sp., *B. stearothermophilus*, and *Thermoanaerobacter thermohydrosulfuricus* and *Chromobacterium viscosum* (Li and Zhang, 2005; Gupta *et al*., 2007; Antranikian, 2008; Ahmed *et al*., 2010; Chaiyaso *et al*., 2012). Lipases produced by *Aspergillus niger* and *Rhizopus japonicas* and are stable at 50°C and the thermotolerant fungus *Humicola lanuginosa* secretes a lipase stable at 60°C (Kreilgaard *et al*., 1999; Osman *et al*., 2014). The production of thermophilic lipolytic enzymes from extremophiles such as *Bacillus thermoleovorans* (Castro-Ochoa *et al*., 2005), *Thermus thermophiles* (Dominguez *et al*., 2005) and *Geobacillus* sp. (Li and Zhang, 2005) is therefore not surprising. Ikeda and Clark (1998) cloned the *Pyrococcus furiosus* esterase gene in *E. coli* and the expressed enzyme was found to be thermostable with a half-life of 50 min at 126°C and thermoactive with an optimum temperature of 100°C. Generally lipases have a broad pH range of 4.0-11.0, with a high activity range of 5.0-9.0 and maximum activity between pH 6.0 and 8.0 (Gupta *et al*., 2004). Lipases from *A. niger*, *R. japonicas* and *C. viscosum* however, are active at low pH levels and *P. nitroreductans* and *Pseudomonas* spp. produce lipases functional at pH 11.0 (Mehta *et al*., 2017).

In the past decade, the application of laccases together with compounds operating as redox mediators has become popular in bleaching of various wood pulps and in recent times has been successfully applied for the reduction of lipophilic compounds at different steps of the pulping process, irrespective of the type of process, wood material or nature of the extractives present (Gutiérrez *et al*., 2006; Grönqvist, 2014; Fillat *et al*., 2017). Lignin-derived phenols can also function as laccase mediators in the reduction of lipophilic extractives in paper pulp. Gutiérrez *et al*. (2006) treated unbleached kraft pulp with laccases and obtained over 90% removal of conjugated and free sitosterol with syringaldehyde as laccase mediator. *Pycnoporus cinnabarinus* was reported to be effective in eliminating conjugated and free sterols from

Eucalypt kraft pulp, as well as sterols, resin acids and triglycerides from spruce (Molina *et al*., 2008).

For efficient reduction of pitch deposits, the lipophilic extractives at the source of pitch formation need to be determined. Once this has been established, targeted degradation of the lipophilic compounds may be achieved with specific enzymes. Therefore, for the development of targeted approaches to prevent pitch formation, a detailed understanding of enzyme substrate specificity is required. Lipases and esterases are the two key lipolytic enzymes used for the treatment of wood resin components and pitch deposits (Toktay, 2004). In the next section various lipolytic families are reviewed and their differences highlighted, followed by an examination of their functioning and potential applications of lipases and esterases.

2.7. Lipolytic Enzyme Families

The three-dimensional configuration of esterases and lipases displays the characteristic α/β-hydrolase fold (Martínez-Martínez *et al*., 2013) and a stable order of α-helices and β-sheets. Bacterial lipolytic enzymes are classified into eight families (Families I – VIII) based on variances in their amino-acid sequences and biological characteristics (Cavazzini *et al*., 2017). Of the eight different lipolytic families, Family I ('true' lipases- interfacial activation and presence of a lid is characteristic of this family) is the largest and is further divided into six subfamilies. Carboxyl esterases along with various other lipases are grouped into the other seven families, e.g. Family II (also called GDSL [sequence motif] family), Family III, Family IV (also called HSL [hormone sensitive lipase] family), Family V, Family VI, Family VII, and Family VIII (Lee, 2016).

2.7.1 Lipolytic Family I

Lipolytic family I is the most prevalent family and is divided into seven subfamilies, with an estimated total of 27 members. Lipases of this family share a Gly-Xaa-Ser-Xaa-Gly consensus sequence (Messaoudi *et al*., 2010). Family I include the 'true' lipases, e.g. lipases produced by Gram-negative *Pseudomonas* and Gram-positive bacteria, such as *Bacillus, Propionibacterium, Staphylococcus* and *Streptomyces* (Arpigny and Jeager, 1999). The expression of active lipases belonging to subfamilies I.1 and I.2 is dependent on a chaperone protein called lipase-specific foldase (Lif). Two aspartic residues are implicated in the Ca-binding site found at homologous positions in all sequences and the two cysteine residues

forming a disulphide bridge are conserved in most sequences. These four residues are believed to be important in stabilizing the active site of these enzymes (Kim *et al*. 1994). An example of such a lipase (LipC12) (Ogierman *et al*. 1997) belongs to family I.1, which has a chaperone-independent folding, is calcium ion dependent and has no disulphide bridges. LipC12 is stable between pH 6.0 to 11.0 and is active from pH 4.5 to 10.0, with higher activity in the alkaline pH range. Stability is observed up to 3.7 M NaCl environments and temperatures ranging from 20 to 50°C, with highest activity at 30°C over a 1-hour incubation period. The purified enzyme had specific activities of 1767 U/mg and 1722 U/mg with pig fat and olive oil, respectively (Glogauer *et al*., 2011).

Bacillus lipases are generally classified under two subfamilies, I.4 and I.5 (Messaoudi *et al*., 2010). Several *Bacillus* lipases are known to have a common alanine residue that replaces the first glycine in the conserved pentapeptide: Ala-Xaa-Ser-Xaa-Gly. However, *Bacillus pumilus*, *Bacillus subtilis* and *Bacillus licheniformis* lipases from the I.4 subfamily are the smallest true lipases (approx. 19-20 kDa) and they share minimal similarity at the amino acid level (approx. 15%) with the other *Bacillus* and *Staphylococcus* lipases (Arpigny and Jaeger, 1999). Temperatures above 45ºC result in decreased activities for subfamily I.4 lipases, however, they display high activity at basic pH (between 9.5 and 12.0) (Guncheva and Zhiryakova, 2011). Some exceptions include the lipases of *Bacillus thermocatenulatus* which have a molecular mass of about 43 kDa and display maximal activity at approx. 65°C and pH 9.0 (Carrasco-Lopez *et al*., 2009). Another *Bacillus* sp. known as *B. stearothermophilus*, also produces a lipase with a molecular mass of 67 kDa and optimal activity at pH 11.0 and 55ºC (Bacha *et al*. 2015). Massadeh and Sabra (2011) reported maximal lipase activity of 90.57 U/ml from *B. stearothermophilus* at 45°C and pH 8.0. Other examples of lipases in this family are produced by staphylococcal isolates. They produce larger lipases than *Bacillus* sp. (approx. 75 kDa) that are secreted as precursors and cleaved in the extracellular medium by a specific protease, generating a protein of approximately 400 residues. The pro-peptide (207±267 residues) seemingly operates as an intramolecular chaperone and assists in translocation of the lipase across the cell membrane (Priji *et al*., 2016).

2.7.2 Lipolytic Family II

Lipolytic family II has five members that have been characterized and reported. Enzymes categorized as Family II do not demonstrate the usual pentapeptide (Gly-Xaa-Ser-Xaa-Gly) but

exhibit a Gly-Asp-Ser-(Leu) [GDS(L)] motif comprising the active-site serine residue (Sangeetha *et al*., 2011). This residue is situated closer to the N-terminus in these proteins than in other lipolytic enzymes (López-López *et al*., 2014). Unlike conventional lipases, GDSL enzymes do not possess a nucleophile elbow, which is a region containing a beta-beta-alpha structural motif and holds the nucleophilic and the oxyanion hole amino acid residues that form the catalytic site in a variety of enzymes. The nucleophile (Ser, Asp or Cys) appears as a distinct turn, hence proposed as the nucleophile elbow. They are known to have flexible active sites that are able to modify their structure in the presence of specific substrates, thus increasing the substrate specificity range (Anobom *et al*., 2014). Included in this family are esterases of *Salmonella typhimurium*, *Streptomyces scabies*, *Pseudomonas aeruginosa*, *Photorhabdus luminescens*, *Vibrio mimicus*, *Escherichia coli*, *Aeromonas hydrophila* (Montella *et al*., 2012; Nakamura *et al*., 2017) and lipases of *A. hydrophilia*, *Vibrio parahemolyticus*, *Xenorhabdus luminescens* and *Streptomyces rimosus* (Borrelli and Trono, 2015). An example of an extracellular lipase from this family was isolated from *P. aeruginosa* with a molecular weight of 30 kDa. The lipase was stable up to 45°C and maintained activity in the alkaline pH range (Sharon *et al*., 1998). The enzyme was found to be highly stable in the presence of methanol and ethanol, and cationic surfactants, such as Triton X-100 and Tween-80, substantially increased activity. The presence of a calcium-binding site in this *Pseudomonas* lipase is predicted as its activity was stabilized considerably by Ca^{2+} and the inhibitory effects of EDTA was suppressed by subsequent $CaCl_2$ treatment (Sharon *et al*., 1998).

2.7.3 Lipolytic Family III

Family III is comprised of three characterized members so far. These enzymes possess the conserved consensus sequence Gly-Xaa-Ser-Xaa-Gly. Members of this family are very closely related (Lee, 2016). They exhibit the canonical fold of α/β-hydrolase, including the characteristic catalytic triad. High activities at low temperature (less than 15°C) were believed to originate from conserved sequence motifs of these enzymes (Rauwerdink and Kazlauskas, 2015). However, distinct sequence similarity between esterases from psychrophilic (*Moraxella* sp. *Psychrobacter immobilis*), mesophilic (*Alcaligenes eutrophus*, *E. coli*) and thermophilic (*Alicyclobacillus acidocaldarius*, *Archeoglobus fulgidus*) microorganisms suggest that temperature variation is not responsible for such considerable sequence conservation (Arpigny and Jaeger, 1999). A comparative study of these enzymes would be beneficial in resolving the unique properties of this hydrolase family. An example of an enzyme from this family is the

extracellular lipase from *Serratia marcescens*, ECU1010, with a molecular mass of 65 kDa, pH and temperature optima of 8.0 and 45°C, respectively, and a pI of 4.2 (Zhao *et al*., 2008).

2.7.4 Lipolytic Family IV

Family IV, otherwise known as the hormone-sensitive lipase (HSL) lipolytic family, due to their high sequence similarity to the mammalian HSL (Hausmann and Jaeger, 2010), has six characterized members. The hydrolysis reaction of triacylglycerols in adipose tissue is catalyzed by HSL, in addition to being a rate-limiting enzyme in the exclusion of fatty acids from stored lipids (Lass *et al*., 2011). This family has two highly conserved consensus motifs, the common GXSXG and HGG, which plays a role in the oxyanion hole formation (Mohamed *et al*., 2013). Enzymes are known to exhibit an α/β-hydrolase fold (Ngo *et al*., 2013). Enzymes from this family have been identified in *Pseudomonas* sp., *A. eutrophus*, *Moraxella* sp. *A. acidocaldarius*, *E. coli* and *A. fulgidus* (Manco *et al*. 2000; Manco *et al*., 2001; Feller *et al*., 2009). An esterase from *Pyrobaculum calidifontis* is considered as a member of the HSL family. Specific activities reported for this enzyme are similar if not higher than previous reports with 1050 U/mg at 30ºC and 6410 U/mg at 90ºC (Manco *et al*., 2000; Rashid *et al*., 2001; Hotta *et al*., 2002).

2.7.5 Lipolytic Family V

Six characterized enzymes make up this family to date. Some of them originate from mesophilic (*Acetobacter pasteuriannus*, *Pseudomonas oleororans*, *Haemophilus influenza*), psychrophilic (*Sulfolobus acidocaldarius*) and thermophilic microorganisms (Arpigny and Jaeger, 1999). Typically, this family possesses the conserved motif GXSXG, as well as the other common motif PTL (Nacke *et al*., 2011). A lipolytic enzyme (EstV) from *Helicobacter pylori* has been isolated, cloned, purified and classified as a Family V hydrolase. This enzyme was predominantly active with short-chain substrates (*p*-nitrophenol acetate, *p*-NP butyrate, *p*-NP valerate) and did not display interfacial activation, but was stable and had a maximum activity at 50°C and pH 10.0 (Ruiz *et al*., 2007).

2.7.6 Lipolytic Family VI

There are five well characterized members that are classified as family VI lipolytic enzymes based on their size. This family is comprised of the smallest esterases, with a molecular mass of 23±26 kDa. The subunit has the α/β-hydrolase fold and a traditional Ser-Asp-His catalytic

triad. A carboxylesterase from *Pseudomonas fluorescens* is an example of such an esterase (Pesaresi *et al*., 2005). This enzyme hydrolyses a wide range of small ester-containing compounds and demonstrates no activity towards long-chain triglycerides (Lu *et al*., 2010a). In another example, an extracellular alkaline lipase produced by *P. fluorescens* AK102 was stable between pH 4.0 and 10.0 with an optimal pH between 8.0 and 10.0 and an optimum temperature of 55°C (Kojima *et al*., 1994). This enzyme could have a potential application in the pulping industry for the reduction of wood resins and pitch deposits. There is limited information on other enzymes in this family. Interestingly, esterases from this family show 40% homology at the amino acid level to eukaryotic lysophospholipases (Bornscheuer, 2002), which are responsible for the liberation of fatty acids from lysophospholipids (Quach *et al*., 2014).

2.7.7 Lipolytic Family VII

Family VII is a small family with only three well known members. They have an estimated size of ±55 kDa, and share substantial amino acid sequence homology (30% identity, 40% similarity) with eukaryotic acetylcholine esterases and intestine/liver carboxylesterases (Arpigny and Jaeger, 1999). This family has the classical triad G-X-S-X-G pentapeptide. The esterase from *Arthrobacter oxydans* is classified under this family and is specifically active against phenylcarbamate herbicides by hydrolysing the central carbamate bond (Hayatsu *et al*., 2001). The esterase is plasmid-encoded and thus has the potential to be more easily transferred to other strains or species. Interestingly, the esterase of *B. subtilis* is capable of hydrolyzing *p*-nitrobenzyl esters, and may be applied in the final removal of *p*-nitrobenzyl ester utilized as a protecting group in the synthesis of β-lactam antibiotics (Ding *et al*., 2015). Another esterase isolated from a *Bacillus* strain was found to be thermostable and had maximum activity at 60°C and maintained 100% activity at 75°C for 30 min (Andualema and Gessesse, 2012). These characteristics would be appropriate for application in the reduction of pitch during the pulping process (high process temperatures) where glyceride lipids may be targeted.

2.7.8 Lipolytic Family VIII

Family VIII consists of three characterized members. Theses enzymes are approximately 380 amino acids in length with similarity to many class C β-lactamases. A 150 amino acid fragment (from positions 50 to 200) showed 45% similarity at the amino acid level to an *Enterobacter cloacae ampC* gene product (Galleni *et al*., 1988). *AmpC* codes for resistance to cefazolin,

cefoxitin, cephalothin, most penicillins, and beta-lactamase inhibitor-beta-lactam combinations (Jacoby, 2009). This trait indicates that the active site (Ser-Xaa-Xaa-Lys) conserved in the N-terminal, belongs to class C β-lactamases (Patel and Richter, 2015). Contrary to this, the esterase/lipase consensus sequence (Gly-Xaa-Ser-Xaa-Gly) of the *P. fluorescens* esterase was proposed to be involved in the active site of β-lactamases (Kim *et al*., 1994; Arpigny and Jaeger, 1999). Esterases from *Streptococcus chrysomallus* also possess this motif; but, it is not conserved in the *Arthrobacter globiformis* esterase. Also, the motif is situated in close proximity to the C-terminus of the *P. fluorescens* and *S. chrysomallus* enzymes with an absence of the histidine attachment (amino acid used in the synthesis of proteins). This demonstrates the unconventional nature of these enzymes as the Ser-Asp-His residue sequence is conserved throughout the entire superfamily of lipases and esterases. Site-directed mutagenesis studies have demonstrated that the Gly-Xaa-Ser-Xaa-Gly motif does not play a significant role in enzyme functioning of an esterase (EstB) from *Burkholderia gladioli* (Petersen *et al*., 2001).

A number of genera, such as *Pseudomonas* and *Streptomyces*, are known to produce hydrolases which are classified into different families (Figure 2.1). Arpigny and Jaeger (1999) first classified bacterial lipolytic enzymes into the eight families according to their amino acid sequences and biological properties. This became the reference point for classification of novel lipolytic enzymes to a family. However, unique families are being discovered through the use of metagenomics (Lee *et al*., 2006; Kim *et al*., 2009; Fu *et al*., 2011). Table 2.2 summarizes the different classes of lipolytic enzymes currently described.

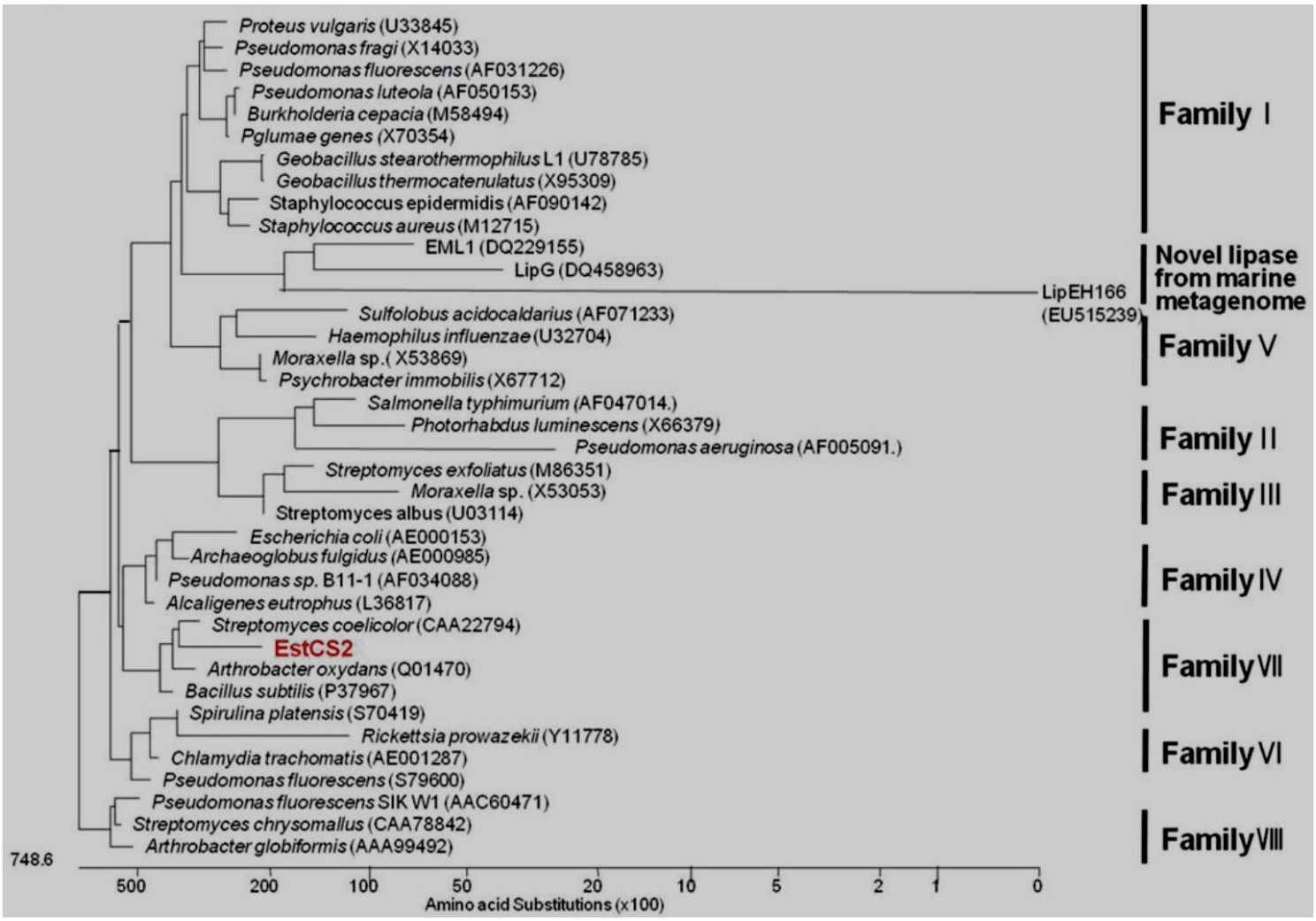

Figure 2.1: Phylogenetic tree based on a novel amino acid sequence of EstCS2 and closely related proteins. Protein sequences for previously identified families of bacterial lipolytic enzymes retrieved from GenBank (http://www.ncbi.nlm.nih.gov). The units at the bottom of the tree indicate the number of substitution events (adapted from Kang *et al*. 2011).

Table 2.2: Current classification of bacterial lipolytic enzymes. Description of bacterial lipolytic families I-VIII in the Arpigny and Jaeger classification, and new families and subfamily discovered by functional metagenomics (*) (adapted from Arpigny and Jaeger, 1999; Lee *et al*., 2006; Kim *et al*., 2009; Fu *et al*., 2011).

Family	Description
I	Group of true lipases subdivided into six subfamilies: *Pseudomonas* lipases and relatives (subfamilies I.1, I.2 and I.3), *Bacillus* and *Staphylococcus* lipases and relatives (I.4 and I.5) and other lipases (I.6).
II	Modified pentapeptide motif around the active serine: Gly-Asp-Ser-(Leu) [GDS(L)]. Secreted and membrane-bound esterases
III	Extracellular lipases and esterases.
EstA*	Related to family III but different conserved motifs (pentapeptide GHSMG). Discovered from surface seawater.
IV	Many members of this family show sequence similarity to mammalian hormone-sensitive lipase (HSL). Typical motif HGG. Lipolytic enzymes from psychrophilic, mesophilic and thermophilic origins.
EstB*:	New subfamily in family IV with second active site glutamate (conserved sequence EXLLD) instead of the aspartate. (DPLXD) of the representative members of family IV. It was discovered in surface sea water.
V	Conserved motif HGGG upstream of the pentapeptide motif GDSAG. Sequence similarity with non-lipolytic enzymes: epoxide hydrolases, dehalogenases and haloperoxidases. Esterases from psychrophilic, mesophilic and thermophilic origins.
EstF*	Related to family V but with a modified pentapeptide, GTSXG, and different flanking regions around the HG motif and their own unique conserved sequence motifs. Isolated from deep sea sediments.
VI	The smallest esterases known (23–26 kDa). Sequence similarity to eukaryotic lysophospholipases.
VII	Large bacterial esterases (55 kDa). Sequence homology with eukaryotic acetylcholine esterases and intestine\ liver carboxylesterases.
VIII	Similarity to several class C β-lactamases.
LipG*	Presence of an Arg-Gly sequence in oxyanion hole instead of His-Gly, a signature sequence distinctive of filamentous fungal lipases. Isolated from tidal flat sediments.
LipEH166*	Comprise newly discovered lipase LipEH166 of psychrophilic origin, and three putative open reading frames. Isolated from intertidal flat sediments.
EstY*	Derived from pathogenic bacteria. First possible lipolytic virulence factors that do not belong to the GDSL family. Isolated from surface river water.

2.8. Lipases

Lipases (e.g. triacylglycerol hydrolase, EC 3.1.1.3) hydrolyse long-chain acyl groups (C_7-C_{10}) to acylglycerols and fatty acids (Ollis *et al*., 1992; Verger, 1997; Bornscheuer, 2002; Canet *et al*., 2016). Lipases can be differentiated from esterases by the occurrence of interfacial activation, which is only observed in lipases, whilst esterases obey classical Michaelis-Menten kinetics. Structural analysis showed that interfacial activation is a result of the hydrophobic domain (lid) covering the active site of lipases – only in the presence of a minimum substrate concentration, i.e. only in the presence of triglycerides or hydrophobic organic solvents, does the lid move apart, making the active site accessible (Rehm *et al*., 2010; Stauch *et al*., 2015). Therefore, lipases have altered properties from esterases, which have an acyl binding pocket (Bornscheuer, 2008).

Microbial lipases specifically have unlimited potential in commercial applications, such as additives in fine chemicals, wastewater treatment, food processing, cosmetics, detergents, pharmaceuticals, degreasing formulations, paper manufacture and accelerated degradation of fatty wastes and polyurethane (Li *et al*., 2012; Liu *et al*., 2012; Brabcova *et al*., 2013; Lailaja and Chandrasekaran, 2013; Nerurkar *et al*., 2013; Whangsuk *et al*., 2013; Zhang *et al*., 2013; Adulkar and Rathod, 2014; Gerits *et al*., 2014; Li *et al*., 2014b; Saranya *et al*., 2014; Fulton *et al*., 2015; Rodrigues *et al*., 2016; Speranza *et al*., 2016; Shu *et al*., 2016). Even though a large number of lipases have been described in the literature, a limited number of enzymes belonging to a few species have proved their stability and biosynthetic activity amenable to use in organic solvents, and thus their consideration as industrially applicable enzymes (Kumar *et al*., 2016). Their biotechnological potential is dependent on their capacity to catalyze not only the hydrolysis of triglycerides, but also their synthesis from glycerol and fatty acids (Mehta *et al*., 2017). Lipases are known to hydrolyse up to 90% of triglycerides in pitch to fatty acids and glycerol/monoglycerides, which are considerably less sticky and more hydrophilic (simple to remove) than the triglycerides (Andualema and Gessesse, 2012).

2.8.1 Classification of lipases

Classification of lipolytic enzymes as ‘true’ lipases requires the fulfilment of two criteria: (i) they should be activated by the presence of an interface, i.e. their activities should dramatically increase as soon as the triglyceride substrate forms an emulsion. This occurrence is termed “interfacial activation”; (ii) they should also contain a “lid”, which is a surface loop of the

protein covering the active site of the enzyme that moves away on contact with the interface (Cheng *et al*., 2012). The 'true' lipase family (Family I) covers the 6 subfamilies which predominantly catalyze the hydrolytic reactions of substrates with long acyl chains (Messaoudi *et al*., 2011).

2.8.2 Mechanism of action of lipases

Lipases take effect on ester bonds occurring in acylglycerols to release free fatty acids and glycerol in a liquid medium (Borrelli and Trono, 2015). In limited liquid environments, these enzymes are capable of reversing this reaction (esterification), *via* acidolysis, interesterification, and alcoholysis (Rajendran *et al*., 2009). Elucidation of their structures showed that the interfacial activation observed is attributable to a hydrophobic domain (lid) covering the active site of lipases, and that high levels of activity was observed in minimum substrate concentration only (Khan *et al*., 2017).

2.8.3 Microorganisms producing lipases

Lipases have originated from animals, plants and microorganisms. However, bacterial lipases are the most versatile, stable and reactive in organic medium (Andualema and Gessesse, 2012). Numerous microorganisms are known to produce lipases when incubated with lipid substrates (Basheer *et al*., 2011; Sethi *et al*., 2013; Veerapagu *et al*., 2013). The majority of bacterial lipases originate from Gram-negative bacteria, the most valuable being *Pseudomonas* which includes at least seven lipase-producing species, which are *P. aeruginosa* (Tielen *et al*., 2013; Prasad, 2014), *P. cepacia* (Badgujar *et al*., 2016; Cao *et al*., 2016; Sasso *et al*., 2016), *P. alcaligenes* (Chen *et al*., 2014a; Patel *et al*., 2014), *P. glumae* (Knapp *et al*., 2016), *P. fluorescens* (Xun *et al*., 2012; Guldhe *et al*., 2015; Lima *et al*., 2015), *P. fragi* (Santarossa *et al*., 2005; Dey *et al*., 2014) and *P. putida* (Fatima *et al*., 2014).

2.8.4 Heterologous production of lipases

Lipases from *Pseudomonas* species require the functional assistance of about 30 different cellular proteins prior to recovery from the culture supernatant in an enzymatically active state, demonstrating that folding and secretion are highly specific processes that generally do not function properly in heterologous hosts (Rosenau *et al*., 2004; Maffei *et al*., 2017). Extracellular enzymes, such as lipases, must be translocated through the bacterial membrane to a suitable location to fulfil their function. Gram-positive bacteria secrete enzymes which

cross a single cytoplasmic membrane. These proteins generally contain a signal sequence, directing translocation *via* the secretion machinery (Fekkes and Driessen, 1999). The TAT pathway is a second translocation pathway found in lipase secreting Gram-negative and Gram-positive bacteria (Heravi *et al*., 2009; Shruthi *et al*., 2010). Proteins utilizing this pathway contain a unique twin arginine translocation motif in their signal sequence (Berks, 2015). In general, active expression of lipases from *Pseudomonas* and *Burkholderia* requires the presence of a chaperone protein known as the lipase-specific foldase (Lif), for precise folding of the lipase (Quyen *et al*., 2012; Wu *et al*., 2012). A text book example would be that of the cold-active lipase gene isolated from *Psychrobacter* sp. which was expressed in *E. coli* BL21 yielding a specific activity of 66.51 U/mg. When the recombinant plasmid was co-expressed with a ‘‘chaperone team’’ the lipase displayed a specific activity of 108.77 U/mg (Cui *et al*., 2011). Other expression hosts, such as *Bacillus* species, have also been explored. A lipase isolated from *P. vulgaris* was expressed in *B. subtilis* WB800, with a high lipase activity of 356.8 U/ml after a 72-hour induction with sucrose (Lu *et al*., 2010b). Lipolytic genes have also been isolated from metagenomes and expressed in different host strains (Liaw *et al*., 2010). A lipase from a metagenome has been cloned in *Streptomyces lividans* with maximal activity of 4287 U/mg towards *p*-nitrophenyl butyrate at 60°C and pH 8.5 (Cote and Shareck, 2010).

2.9. Esterases

Esterases retain significant applications in various biotechnological processes due to their stability in organic solvents, extensive substrate specificity, stereoselectivity, regioselectivity, and lack of requirement for cofactors (Fazary and Ju, 2008). Esterases (EC 3.1.1.1) hydrolyse the ester bonds of water-soluble fattyacid esters with short-chain acyl groups (C_2-C_8) (Ollis *et al*., 1992; Verger, 1997). Several methods have been developed to screen and isolate novel esterases (Elend *et al*., 2006; Kim *et al*., 2006; Kumar *et al*., 2012; Seo *et al*., 2014; Gu *et al*., 2015), including metagenomic techniques (Gao *et al*., 2016; Popovic *et al*., 2017; Pereira *et al*., 2017). A range of esterase characteristics have been described, primarily in molecular biology, targeted synthesis, purification, quantitation, production, and distribution (Faulds, 2010; Biely, 2012; Montella *et al*., 2012; Martínez-Martínez *et al*., 2013; López-López *et al*., 2014).

2.9.1 Mechanism of action of esterases

Esterases boast a diversity of substrate specificities; however, they typically possess a catalytic triad composed of Ser, His, and Asp/Glu in the polypeptide chain (Ollis *et al*., 1992; Verger, 1997; Bornscheuer, 2002). The active site Ser residue is integrated at the centre of the conserved pentapeptide sequence motif, Gly-Xaa-Ser-Xaa-Gly (Ollis *et al*., 1992). The motif is usually positioned in the sharp turn between a β-strand and α-helix, known as the nucleophilic elbow (Verger, 1997). Ester bond hydrolysis is mediated by the nucleophilic attack of the active Ser on the carbonyl of the substrate in a charge-relay system with the two other amino acid residues (His and Asp/Glu) (Ollis *et al*., 1992). Ester formation or hydrolysis is fundamentally identical for lipases and esterases and involves four phases. Initially, the substrate is attached to the active serine, generating a tetrahedral intermediate which is stabilized by the catalytic His and Asp residues. Next, the alcohol is liberated and an acyl- enzyme complex is produced. A nucleophile is then attacked and forms a tetrahedral intermediate again, which following resolution generates the product (an acid or an ester) and free enzyme (Sayali *et al*., 2013). In some cases, esterases may appear to function *in vitro* as esterases, however they may end up functioning as transferases *in vivo*. An ideal example is that of the *O*-acetyl peptidoglycan esterase 2 (Ape2) protein in *Neisseria gonorrhoeae*, which was thought to be an *O*-acetylPG esterase but instead functioned as a PG *O*-acetyltransferase (Moynihan and Clarke, 2010). This may prove to be a major obstacle in the implementation of enzymes for biotechnological applications.

2.9.2 Microorganisms producing esterases

Esterases are produced by an array of organisms such as; *Streptomyces* sp. (Uraji *et al*., 2014), *Pseudomonas* sp. (Prim *et al*., 2006; Tserovska *et al*., 2006), *Bacillus* sp. (Metin *et al*., 2006; Ding *et al*., 2014), *Lactobacillus* sp. (Xu *et al*., 2017), *Thermoanaerobacterium* sp. (Moriyoshi *et al*., 2013), *Micrococcus* sp. (Morales *et al*., 2010), *Ophistoma* sp. (Calero-Rueda *et al*. 2002b), *Pencillium* sp. (Atta *et al*., 2011), *Aspergillus* sp. (Damásio *et al*., 2013), *Humicola* sp. (Htzakis *et al*., 2003), *Myceliophthora* sp. (Katsimpouras *et al*., 2014), *Saccharomyces* sp. (Kwolek-Mirek *et al*., 2011), *Candida* sp. (Ge *et al*., 2011), plant (Vanholme *et al*., 2013) and animals (Finer *et al*., 2004) and may be applied in valuable biological processes. The efficient hydrolysis of both triglycerides and sterol esters using sterol esterase from *O. piceae* has been successfully applied for pitch control in the pulp and paper industry (Calero-Rueda *et al*., 2002b; Gutiérrez-Fernández *et al*., 2014; Coloma *et al*., 2015). Steryl and cholesteryl esterases

from *Pseudomonas* sp. (Vaquero *et al*., 2016; Wicka *et al*., 2016), *C. viscosum* (Kontkanen *et al*., 2004) and *C. rugosa* (Barriuso *et al*., 2016) are also reported to provide valuable assistance in the reduction of pitch deposits during pulp and paper manufacturing.

2.9.3 Heterologous production of esterases

Numerous studies have reported on the cloning and expression of microbial esterases (Ro *et al*., 2004; Brod *et al*., 2010; Huang *et al*., 2010; Terahara *et al*., 2010; Kim *et al*., 2012; Liu *et al*., 2013). When considering heterologous expression one needs to investigate the type of expression system to be applied as this could have a significant effect on the level enzymes produced. For instance, *O. piceae* produces a sterol esterase with high activity in the hydrolysis of triglycerides and sterol esters, however, once expressed in *Pichia pastoris*, greater activity was observed due to higher solubility (Cedillo *et al*., 2012). This is due to the alteration in the N-terminal sequence of the protein expressed in *P. pastoris*, which included 4-8 additional amino acids which ultimately modified its aggregation performance (Vaquero *et al*., 2015b).

In 2004, Choi and colleagues discovered a novel esterase gene (*est*I). The amino acid sequence revealed that it may be classified as a novel member of the GHSMG family of lipolytic enzymes. *E. coli* BL21 (DE3)/pLysS containing the *est*I gene expressed a unique 67.5 kDa protein linking EstI in an N-terminal fusion with the S-tag peptide. The optimal pH and temperature of the purified enzyme were 7.0 and 37°C, respectively. The highest specificity was towards *p*-nitrophenyl-caprylate (C8) with Km and kcat values of approximately 14 µM and 1,245 s^{-1}, respectively (Choi *et al*., 2004).

Moukouli *et al*. (2008) successfully cloned a Type C feruloyl esterase (FAE) in *S. cerevisiae*, transcriptionally controlled by the alcohol oxidase (AOX1) promoter and integrated into *P. pastoris* X-33 to validate FAE activity. A recombinant protein with a molecular weight of 62 kDa and a pI of 6.8 was produced. When incubated together with the xylanase from *Trichoderma longibrachiatum* in de-starched wheat bran, ferulic acid (FA) was effectively liberated. The esterase showed stability over a wide pH range rendering it applicable for alkaline pulp treatments (Moukouli *et al*., 2008).

The *axe* gene which codes for an acetylxylan esterase from *Thermobifida fusca* NTU22, has been cloned, sequenced and expressed in *E. coli*. The optimum pH and temperature of the

purified esterase was 7.5 and 60°C, respectively. A significant increase in xylooligosaccharide production was observed when oat-spelt xylan was treated with a combination of the recombinant xylanase and acetylxylan esterase compared to independent treatment with xylanase or acetylxylan esterase (Huang *et al*., 2010). A sterol esterase from *O. piceae* was expressed in two hosts; *S. cerevisiae* and *P. pastoris*. The highest activity of 42 U/ml was produced by *P. pastoris* at 28°C, however low activity was observed in *S. cerevisiae*. The heterologous expression of a functional fungal esterase in yeast is quite an accomplishment and opens up an opportunity to develop more robust enzymes (Vaquero *et al*., 2015a).

2.10 Prospecting for Novel Enzymes

Over the past four decades, enzymes have been successfully developed for the production of specialty chemicals, complex drug intermediates and chemicals in the food, pharmaceutical and chemical. Advancements in high-throughput technologies, cloning technologies, proteomics and genomics have promoted the generation of innovative enzymes and bioprocesses. In the past decade, directed evolution has been an effective tool in the improvement of enzyme properties, even without information on the enzyme structure and mechanisms (Valetti and Gilardi, 2013). The practice of directed evolution has been examined on a number of occasions by various researchers (Kaur and Sharma, 2006; McLachlan *et al*., 2009; Martínez and Schwaneberg, 2013; Currin *et al*., 2015; Porter *et al*., 2015). In lipase exploration, directed evolution has been engaged for the construction of enantioselective catalysts for organic synthesis. This was initially achieved with a bacterial lipase from *P. aeruginosa* (Jaeger *et al*., 2001). The lipase evolved towards a model substrate, 2-methyldecanoic acid p-NP ester, to produce a lipase mutant with >90% enantiomeric excess, in contrast with 2% for the wild-type lipase (Liebeton *et al*., 2000). This group also exploited a *B. subtilis* lipase as a catalyst in the asymmetric hydrolysis of meso-1,4-diacetoxy-2-cyclopentene, with the creation of chiral alcohols (Jaeger *et al*., 2001). Numerous studies have also been conducted on the cloning, over-expression and characterization of lipases and esterases from various *Bacillus* strains (Ewis *et al*., 2004; Soliman *et al*., 2007; Abdel-Fattah and Gaballa, 2008; Chis *et al*., 2013). The majority of the studies mentioned above involved isolation of a lipase/esterase genes from thermophilic *Bacillus* spp. and over-expression in *E.coli*. The isolation of novel lipolytic genes from extreme microorganisms will assist in a number of bioprocessing areas due to the inherent ability of the encoded proteins to withstand extreme environments.

Principally there are two different strategies to identify novel enzyme genes, viz., sequence-based screening used in public or commercial DNA-databases such as the National Center for Biotechnology Information (NCBI) or the ERGOTM bioinformatics suite provided by Integrated Genomics Inc. These databases constitute a steadily expanding source of gene sequences easily screened by computer tools. The one major drawback of this strategy is that it is only possible to identify genes homologous to already known sequences. However, activity-based screening in culture collections and the metagenome is possible. Enormous libraries of wild type strains or recombinant expression strains are screened for desired enzyme activities. Genetic selection is the most sophisticated and powerful way to identify the 'unique' one (Drepper *et al*., 2006).

2.10.1 Metagenomic approach

Microbial diversity is a vital resource for biotechnological processes and products. The biosphere is dominated by microbes, however, the majority of microorganisms in nature have not yet been investigated (<1% identified) due to the limiting method of isolating pure cultures (Stewart, 2012). An alternative method is to exploit the genetic diversity of microbes in a particular environment in its entirety (known as the "metagenome") to encounter original or enhanced genes and gene products for biotechnological targets (Chen *et al*., 2014b; Culligan *et al*., 2014; Morris and Marchesi, 2016). Sequencing of sizeable metagenomic DNA fragments has led to the discovery of a multitude of open reading frames, several of them coding for enzymes such as lipase, esterase, xylanase, chitinase, DNase, amylase, protease, etc. (Yeh *et al*., 2011; Bayer *et al*., 2013; Grunwald, 2014). Henne *et al*. (2000) screened environmental DNA libraries from diverse soil samples for genes encoding lipolytic activity and identified four clones possessing esterase and lipase activities. In 2002, Bell *et al*. illustrated a PCR method suitable for the identification of lipase genes directly from environmental DNA, using primers constructed from lipase consensus sequences (Bell *et al*., 2002).

In silico searches for novel lipase and sterol esterase sequences from the metagenomes of environmental fungi have been performed (Barriuso *et al*., 2013). Six putative enzymes were elected and their substrate specificity and kinetic properties and were considered based on their similarity with formerly classified sterol sterases/lipases with recognized structures. This strategy combined examination of conserved motifs, sequence homology, phylogenetic and protein model analyses, allowing the identity of six candidate sequences (Barriuso *et al*., 2013).

The search for novel lipases is relentless as demonstrated by the recognition of new families of microbial lipases generally by metagenomic approaches (Nagarajan, 2012). Among the multitude of sequences coding for lipases discovered through metagenomic studies, it is remarkable that unique sequences are often reported. A novel alkali-stable lipase from a metagenome assembled from marine sediments was found to impart a characteristic and pleasant flavour and aroma in milk fat flavour manufacturing (Peng *et al*., 2014). Lee *et al*. (2006) isolated and characterized a novel family of bacterial lipase from tidal flat sediments. Hårdeman and Sjöling (2007) also isolated a unique low temperature active lipase from uncultured bacteria of marine sediment, of which the conserved regions, in addition to the putative active site and catalytic triad, were found to be comparable to the culturable lipases.

In a study by Selvin *et al*. (2012) functional screening of a marine sponge fosmid metagenomic library revealed a novel halotolerant lipase. The activity and stability over a broad salinity, temperature and pH range, and presence of metal ions and organic solvents indicates the potential application of this enzyme in a selection of manufacturing processes. Lipases have been isolated and characterized by Glogauer *et al*. (2011), Chow *et al*. (2012), Fu *et al*. (2013), and Ngo *et al*. (2013) from numerous metagenomic libraries showing unique features, such as activity at low temperatures, thermal stability, organic solvent tolerance and alkaline stability; making them prospective candidates for industrial use (Bashir *et al*., 2014). A novel esterase was cloned from the thermophilic fungus *Thermomyces lanuginosus* DSM 10635 and heterologously expressed in *Escherichia coli*. Highest activity was observed with *p*-nitrophenyl butyrate (C4) and displayed maximum activity at 60°C and pH 8.5 (Li *et al*., 2014a).

Protein engineering methods are being applied to enhance activity of lipolytic enzymes in diverse industrial applications, in a coordinated process for the search of novel enzymes. Characteristics that require improvement are substrate specificity, activity at higher temperatures and pH, and stability. Improving enzymatic activity at high temperatures is vital in mechanical pulping as the pulp is treated with the enzyme at a temperature of ~80°C (Calero-Rueda *et al*., 2002a). Similarly, enzymes functioning at elevated pH and temperature levels would be attractive as a biocontrol agent in pitch management during chemical pulping processes.

2.11 Applications

Enzymes are currently being applied in multiple industries. The flexibility of enzyme properties enables application in a number of degradation and synthesizing processes such as the detergent, food, textile, beverage, animal feed, pulp and paper, organic synthesis and leather industries (Table 2.3). A global platform for industrial biotechnology has provided a niche for the development of innovative enzymes, particularly in the production of biofuels and bioenergy. Based on current projections, global crude oil reserves will be depleted in less than 50 years, therefore an alternative to crude oil is desperately needed (Tanksale, 2017). Biofuels produced from biomass is considered an ideal substitute. The production of second and third generation bioethanol from plant residues appears to be the way forward, as it provides a dual function of providing biofuels and eliminating waste generated from crops (Ozdingis and Kocar, 2017). However, there are limitations of using second and third generation biofuels that need to be overcome, such as; complexity of biomass, production process, harvesting, transporting less-dense biomass to biorefineries, environmental and technological issues (Thomas *et al*., 2017). As a result, enzymes need to be designed based on the limiting parameters of processes, and their stability and effectiveness in catalyzing specific reactions.

Lipases in biofuel production, as well as various industries have enormous potential due to their availability, enantioselectivity toward the substrate and stability in organic media (Sharma and Kanwar, 2014; Choudhury and Bhunia, 2015; Contesini *et al*., 2017). Application of lipases in medicine and the dairy industry has become invaluable in esterification and transesterification processes (Gopinath *et al*., 2013; Anbu *et al*., 2017). Renewable lipases have become popular in the food industry for processing and synthesis of aroma ester acetate to improve food flavours (Memarpoor-Yazdi *et al*., 2017). Less popular esterases, on the other hand, also show great potential in industry applications and processes. Feruloyl esterases have multiple applications in the synthesis of bioactive compounds (Fazary and Ju, 2008; Christakopoulos *et al*., 2014; Antonopoulou, 2017; Kang *et al*., 2017), improvement of feed digestibility and hydrolysis of ester bonds in complex crop fibre (Silva *et al*., 2017). Esterases are also known to hydrolyse ester bonds between lignin and hemicelluloses to promote access to cellulose and hemicelluloses (Silva, 2017).

As mentioned previously, the application of lipases in the pulp and paper making industry is vital in the control of pitch formation. Esterases on the other hand are used to break down the

polyvinyl acetate in glues present in recycled paper processing (Jegannathan and Nielsen, 2013). In the recycling of newspaper, the paper needs to be deinked prior to production of newsprint and white paper (Bajpai, 2013). When the ink is composed of vegetable oil, lipases are often used to break down the lipophilic components of the ink (Hasan *et al*., 2006; Zedong *et al*., 2017). Yang and his colleagues (1994) patented a specific combination of parameters and ratio of cellulases, xylanases and lipases for the removal of ink from various paper samples whilst preserving the quality of the salvaged pulp.

Recent advances in metagenomics and proteomics have supported the discovery of novel enzymes and genetic engineering of microbes (Adrio and Demain, 2014). Future applications of enzymes from microbial, plant and animal resources will ensure a more feasible approach to bioprocessing, as well as reducing the amount of waste generated and overall impact on the environment.

Table 2.3: Enzymes used in various industrial processes and their application (adapted from Vijayalakshmi *et al*., 2011; Imran *et al*., 2012; Sarrouh *et al*., 2012; Gurung *et al*., 2013; Hasunuma *et al*., 2013; Nigam, 2013; Elleuche *et al*., 2014; Choi *et al*., 2015; Anbu *et al*., 2017; Mishra *et al*., 2017).

Industry	Enzyme	Application
Detergent	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, colour clarification
	Mannanase	Reappearing stains
Starch and fuel	Amylase	Starch liquefaction and saccharification
	Amyliglucosidase	Saccharification
	Pullulanase	Saccharification
	Glucose isomerase	Glucose to fructose conversion
	Cyclodextrin-glycosyltransferase	Cyclodextrin production
	Xylanase	Viscosity reduction
	Protease	Protease (yeast nutrition – fuel)
	Lipase	Synthesis of lipase-catalyzed biodiesel
Food	Protease	Milk clotting, flavour
	Lipase	Improvement of food texture
	Lactase	Lactose removal (milk)
	Pectin methyl esterase	Firming fruit-based products
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties
Baking	Amylase	Bread softness and volume
	Xylanase	Dough conditioning
	Lipase	Dough stability and conditioning
	Phospholipase	Dough stability and conditioning
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Dough strengthening, bread whitening
	Protease	Biscuits, cookies
	Transglutaminase	Laminated dough strengths
Animal feed	Phytase	Phytate digestibility
	Xylanase	Digestibility
	B-Glucanase	Digestibility
Beverage	Pectinase	Depectinization, mashing
	Amylase	Juice treatment, low calorie beer
	B-Glucanase	Mashing
	Acetolactate decarboxylase	Maturation (beer)
	Laccase	Clarification (juice), flavour (beer)
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	Desizing
	Pectate lyase	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Pulp and paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal

	Amylase	Starch coating, deinking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	Deinking, drainage improvement, fibre modification
Fats and oils	Lipase	Transesterification
	Phospholipase	Degumming, lysolecithin production
Organic synthesis	Lipase	Resolution of chiral alcohols and amides
	Acylase	Synthesis of semisynthetic penicillin
	Nitrilase	Synthesis of enantiopure carboxylic acids
Leather	Protease	Unhearing, bating
	Lipase	Depickling
Personal care	Amyloglucosidase	Antimicrobial
	Glucose oxidase	Bleaching, antimicrobial
	Peroxidase	Antimicrobial
Environmental application	Lipase	Degradation of lipid wastes
		Removal of solid and water pollution by hydrocarbons, oils and lipids

Since structural adaptations for the endurance of lipases and esterases at high temperatures or extreme pH levels are not well established, random mutagenesis approaches could be combined with site-directed mutagenesis to generate lipolytic enzymes with enhanced abilities for application in the pulp and paper industry. Thus, new generations of designer lipolytic enzymes with improved adaptability to inconsistent pitch problems in various paper and pulp manufacturing processes could be accessible in the near future. Mining of genetically untouched resources for lipases and esterases of specific characteristics using culture-independent metagenomic techniques has demonstrated its potential for biotechnological evolution.

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CHAPTER THREE

CHEMICAL COMPOSITION OF FOUR *Eucalyptus* WOOD SPECIES USED IN PULP AND PAPER INDUSTRIES

3.1 Abstract

Eucalyptus wood species are an invaluable forest crop in the pulp and paper industry for the production of high-grade cellulose pulp. The presence of wood resin (lipophilic extractives) and pitch deposit formation during pulping is a challenge faced by many pulp mills, especially so for dissolving wood pulp mills. This highlights the need to better understand the chemistry of the *Eucalyptus* wood species used in pulping. In this study, the wood chemistry of four *Eucalyptus* wood species typically used in South African mills (*E. dunnii*, *E. grandis*, *E. nitens* and *E. smithii*) and their acid-bisulphite pulps were investigated. Substantial variation in extractive content was observed among the wood species. *E. grandis* and *E. smithii* contained lower levels of fatty acids and sterols than *E. dunnii* and *E. nitens* implying that the problems related to the presence of wood resin would be less severe. *E. nitens* contained high lipophilic content, indicating a greater potential for problems during pulping and subsequent processing of the pulps. In addition, *E. nitens* had the highest amounts of klason lignin (6.6%) and acid-insoluble lignin (5.6%). Highest pulp yield of 54.1% was obtained for *E. grandis* and the lowest (50.3%) for *E. smithii*. The low pulp yield for *E. smithii* correlates with the low viscosity (547.17 ml/g) and glucose concentration (87.7%) obtained for this species. However, *E. smithii* pulp also had the lowest kappa number of 8.25, thus would require smaller amounts of chemicals during bleaching. Of the four wood species evaluated, *E. grandis* would be ideal for the production of dissolving pulp based on high viscosity (570.37 ml/g), pulp yield (54.1%) and glucose concentration (89.8%), and low klason lignin (4.5%), acid-soluble lignin (4.4%) and carbohydrate concentration (90.1%). This information would be invaluable in the commercial pulping industry as it would help circumvent potential pitch deposit problems.

Key words: *Eucalyptus, chemistry of wood and pulp, dissolving pulp*

3.2 Introduction

Of the 700 species of identified eucalypts worldwide, only a select few are suitable for use in the pulping industry (Rockwood *et al*., 2008). *Eucalyptus globulus* is considered to be the ideal raw material for kraft pulp manufacturing, producing especially high pulp yields. The composition of *E. globulus* wood has been comprehensively described (Gutiérrez *et al*., 1998; Freire *et al*., 2002; del Río *et al*., 2005; Miranda *et al*., 2013), however other eucalypt species such as *E. nitens*, *E. maidenii*, *E. smithii* and *E. dunnii* used in the pulping industry, have not been as extensively characterized.

Profiling of organic extractives in wood and the dissolving pulping process has been explored, however, research was based on only two *Eucalyptus* species, viz., *E. grandis* and *E. dunnii* (Kilulya *et al*., 2014). More than 15 *Eucalyptus* species (including hybrids) are currently used in commercial pulping mills worldwide (ENCE, 2009). Lipophilic extractives, naturally present in wood materials, have the potential to form pitch deposits during pulping, which disrupts operation of machinery and reduces pulp quality (Back and Allen, 2000; Gutiérrez *et al*., 2001). This highlights the importance of identifying the chemical composition of the wood materials prior to pulping.

In addition, a South African study reported an increased lipophilic extractive content of *Eucalyptus* trees in sites with elevated compositions of clay soil and organic matter (Kilulya *et al*., 2014). Knowledge of the chemical composition of wood materials enables the evaluation of potential wood species for pulp and paper manufacturing. Therefore, investigations into *Eucalyptus* species grown on plantations in South Africa would provide a more accurate representation of the chemical composition (particularly lipophilic extractive content) and pulping characteristics of *Eucalyptus* wood species currently being used in local and international mills for the production of dissolving pulp.

Three key features define the potential of wood in the dissolving wood pulp (DWP) industry; pulp yield, kappa number and viscosity. In this study, the chemical composition profile, lipophilic extractive content and the key pulping characteristics of four popular *Eucalyptus* wood species, *E. dunnii*, *E. grandis*, *E. nitens* and *E. smithii* used in South Africa and internationally, were evaluated. These results will provide a baseline profile and comparison of and potential ranking of the acid-bisulphite pulping potential (for the production of dissolving pulp) of these species.

3.3 Materials and Methods

3.3.1 Wood material

Wood was obtained from the following *Eucalyptus* species trees: *E. dunnii*, *E. grandis*, *E. nitens* and *E. smithii* from a plantation in KwaZulu-Natal (South Africa). Logs from approximately 10-year old trees were chipped to an average dimension of 12 mm × 5 mm × 2 mm. Chip samples were collected and milled to coarse sawdust using a Brabender Wiley-mill. The sawdust was then passed through a 0.40 mm (40 mesh) screen and used for analyses according to standard methods (T257 cm-12). The remainder of the wood chips were dried at room temperature for 2 weeks, pulped according to the acid-bisulphite process and then washed for chemical analyses.

3.3.2 Water-soluble extractives (hot water extraction)

Hot water (water-soluble) extractives were determined by weighing out five grams of sawdust (moisture content noted) in a 400 ml conical flask and slowly adding 100 ml of hot deionised water. The contents of the conical flasks were stirred to prevent the sawdust from floating. The conical flask was placed on a pre-heated hot plate and left to stand for 3 h. The initial level of the contents was kept constant by the addition of hot deionised water. A pre-weighed number 3 crucible (Pyrex) and a vacuum pump were used to filter the saw dust which was washed with 200 ml of hot deionised water and dried at 60ºC overnight (T207 cm-08).

3.3.3 Extractives (solvent extraction)

The Soxhlet extraction method was used to determine solvent (water-insoluble) extractives in the sawdust samples. Four grams of hot water extracted sawdust (moisture content noted) was weighed into an extraction thimble and placed in a Soxhlet apparatus that was attached to a pre-weighed 500 ml round-bottomed flask containing 300 ml toluene-ethanol (2:1). The solvent was allowed to cycle through the system six times over 4 h. The extractives were rotary evaporated to dryness, cooled in a desiccator and weighed (T204 cm-07).

3.3.4 Pulping and analyses

Pulping was performed according to the acid-bisulphite process currently implemented at Dissolving Wood Pulp (DWP) mills. Pulp quality was determined by assessing viscosity (T203 om-94), final pulp yield, kappa number (T236 om-60), alpha cellulose, and acid-insoluble lignin (T222 om-02). The carbohydrate content of pulp was characterized by High Performance Liquid Chromatography (HPLC) for quantification of cellulose and hemicelluloses (glucose,

mannose, arabinose, xylose, and galactose) (T249 cm-85; Wallis *et al*., 1996; Wright and Wallis, 1996).

3.3.5 Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) analysis

Py-GC-MS was employed for characterisation of lipophilic compounds in the sawdust and pulp samples. Samples of approximately 0.2 mg were weighed out and five microliters of tetramethylammonium hydroxide (TMAH) (Sigma Aldrich, USA) was added directly to the sample as a derivatizing agent to increase detection of fatty acid compounds (Fukushima *et al*., 2009). A multi-shot pyrolyzer, EGA/PY-3030 D, (Frontier Lab, Japan) attached to an ultra-alloy capillary column (30 m x 0.25 mm, 0.25 μm) was used. The samples were pyrolyzed at 550°C for 20 s and the interface temperature was fixed at 350°C. The chromatographic separation of the volatile components released by pyrolysis was performed using an ultra-alloy column. The injection temperature was set to 280°C and the column flow rate was set to 1.0 mL/min with helium used as a carrier gas. The temperature was programmed as follows: 50°C for 2 min; rate 3°C/min up to 200°C and held for a further 4 min. The ion source and interface temperatures were set to 200°C and 300°C, respectively. The scan range used for mass selective detector was 40-650 m/z. The pyrolysis products were identified by searching the NIST mass spectrum library (modified from Sithole and Watanabe, 2013).

3.4 Results and Discussion

The extractive contents of the *Eucalyptus* species were evaluated to assess the suitability of the species for production of dissolving pulp (Tables 3.1). *E. dunnii* and *E. grandis* had the higher amounts of hot water extractives, 3.3 and 3.7%, respectively, compared to *E. nitens* and *E. smithii*. Other studies have reported similar or slightly higher amounts of extractives (Klash *et al*., 2010; Rencoret *et al*., 2012; Neiva *et al*., 2014). The toluene-ethanol extracts (lipophilic extractives) ranged from 0.70-1.9% compared to 2.7-3.7% for the water-soluble extracts, indicating that the greater fraction of extractive content, viz. the hydrophilic extractives (water-soluble) would be removed during the washing step of the pulping process, which would be beneficial to downstream processing of the pulp. The solvent extractives (lipophilic extractives) therefore pose a greater threat as these compounds will remain during and after pulping, thus contributing to pitch deposition. *E. nitens* wood exhibited the lowest lipophilic content (0.8%), which in theory marks it as most suitable for pulping. In contrast, *E. smithii* showed the highest amount of lipophilic extractives (1.9%), more than double than that of *E. nitens* (0.8%). The smallest increase in lipophilic extractive content exacerbates the risk of wood resin and pitch

deposit formation (Richardson *et al*., 2012). Based on the results in Table 3.1, *E. smithii* would not be considered suitable for pulping, particularly in combination with other *Eucalyptus* species of similar or higher lipophilic content. However, the nature of the lipophilic compounds present also needs to be considered as only certain types of compounds contribute to pitch deposit formation.

Table 3.1: Water and toluene-ethanol-soluble extractives of fresh wood chips.

	Water-Soluble Extractives		Solvent Extractives	
Sample	**Moisture Content (%) ±SD**	**Extractives (%) ±SD**	**Moisture Content (%) ±SD**	**Extractives (%) ±SD**
E. dunnii	36.81±0.18	3.26±0.15[a]	14.64±0.24	1.49±0.27 [a]
E. grandis	37.64±0.12	3.65±0.20 [a]	49.22±0.14	1.36±0.31 [b]
E. nitens	45.75±0.24	2.76±0.17 [a]	35.81±0.23	0.79±0.19 [a]
E. smithii	46.82±0.21	2.87±0.32 [a]	27.80±0.33	1.89±0.16 [a]

*Analyses conducted in duplicate. SD – standard deviation. p-value: a <0.05; b >0.05

Lipophilic profiles of the different *Eucalyptus* spp. and the effect of pulping on the lipophilic extractives were generated by Py-GC-MS analysis of the wood chips and their respective pulps. Typical pyrograms of the total wood lipophilic extracts as represented by *E. nitens* in Figure 3.1A (additional chromatograms for the other species in Appendix 3A) and the major wood resin components identified are listed in Table 3.2. In hardwoods steroids, sterols, fatty alcohols and fatty acids are the main contributors of pitch formation (Gutiérrez and del Río, 2001; Freire *et al*., 2006; Rencoret *et al*., 2007).

E. dunnii and *E. nitens* had similar lipophilic extractive profiles, but varied with respect to the ratios of the various components (Figure 3.1). Fatty acids and fatty alcohols were the predominant groups present in wood extractives followed by sterols, and smaller amounts of long-chain hydrocarbons, steroid hydrocarbons and ketones (Table 3.3). The main fatty acids were represented by hexadecanoic, 9,12-octadecadienoic, 9-octadecenoic and octadecanoic acids. The variations in the amounts and types of lipophilic extractives can be explained by the differences in tree physiology (due to genetic variability among species) and soil composition (higher organic matter content implies higher fertility and clay soil affects water availability). Sterols were the second main group dominated by sitosterol and stigmastanol, with small amounts of campesterol and stigmasterol. Other components that were detected in small quantities were steroid hydrocarbons (mainly stigmasta-3,5-diene) and steroid ketones (mainly

stigmasta-3,5-dien-7-one and stigmast-4-en-3-one). The main fatty alcohol components were 1-octadecanol and 1-hexadecanol, followed by 1-nonadecanol, 1-eicosanol and 1-tricosanol.

The types and amounts of lipophilic extractives was higher in *E. nitens* than in *E. dunnii*, *E. grandis* and *E. smithii* species. Reports in literature show varying amounts of lipophilic extractives obtained from total extractive content of wood and pulp. This implies that the amount of solvent extractives detected is not reflective of the amount of lipophilic compounds contributing to the risk of pitch formation (Silvério *et al*., 2007). Kilulya *et al*. (2014) ascertained that a *Eucalyptus* species found to have the lowest total acetone extractives, in fact contained the highest amount of lipophilic extractives.

E. grandis and *E. smithii* had the lowest free and conjugated sterol levels, while *E. nitens* had the highest amount of these unfavourable lipophilic compounds (704.5 mg/kg). Although this species contained the lowest amount of solvent extractives compared to the other *Eucalyptus* species, the composition of the extractives was predominantly sterols. Nevertheless, *E. nitens* will continue as a major feedstock for the pulp industry as they are a hardy species known for their high tolerance to snow and cold environmental conditions (Swain *et al*., 2013). The drawback associated with wood resin content in pulps may be alleviated by pulping a combination of potentially "high risk" species such as *E. nitens* (704.5 mg/kg) with lower sterol and fatty acid containing species such as *E. smithii* (365.2 mg/kg) or *E. grandis* (485.5 mg/kg).

Table 3.2: Names and structures of a selection of extractives identified in the *Eucalyptus* pulp samples.

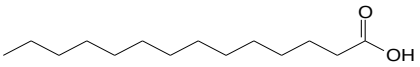
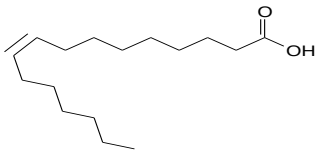
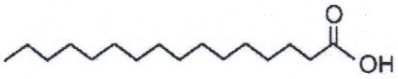
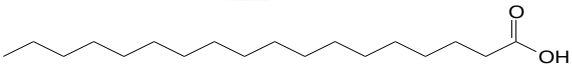
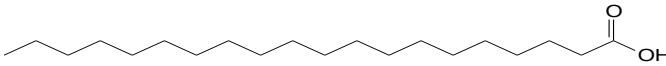
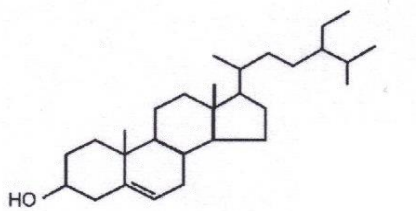
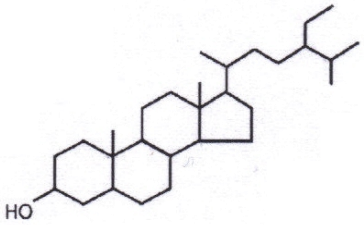
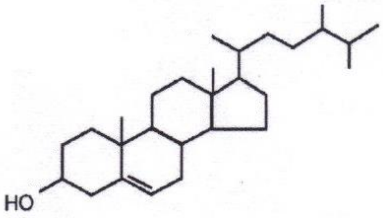
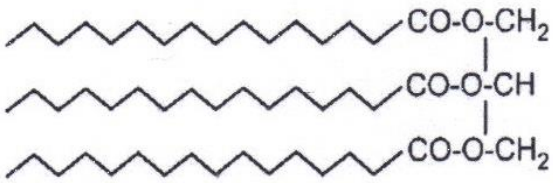
Name	Trivial Name	Structure
	Fatty acids	
Tetradecanoic acid $CH_3(CH_2)_{12}COOH$	Myristic acid	
9-Hexadecanoic acid $(CH_3(CH_2)_5CH=(CH_2)_7COOH$	Palmitelaidic acid	
Hexadecanoic acid $CH_3(CH_2)_{14}COOH$	Palmitic acid	
Octadecanoic acid $CH_3(CH_2)_{16}COOH$	Stearic acid	
Eicosanoic acid $CH_3(CH_2)_{18}COOH$	Arachidic acid	
	Sterols	
β-sitosterol ($C_{29}H_{500}$)		
Stigmastanol		
Campesterol		
	Triglycerides	
Tripalmitin		

Table 3.3: A profile of extractives in *E. nitens* wood.

Compound name	Types
Oxalic acid	Acid
Acetic acid	Acid
2-Piperidinecarboxylic acid	Acid
Glutaric acid, nonyl 2,4,6-trichlorobenzyl ester	Acid
Kaura-9(11),16-dien-18-oic acid, (4.alpha)-	Acid
Dehydroabietic acid	Acid
1,2,3,5-Cyclohexanetetrol, (1.alpha.,2.beta.,3.alpha.,5.beta.)-	Acid
Pimaric acid	Acid
3-(4,7-Dimethoxy-2H-1,3-benzodioxol-5-yl)prop-2-enoic acid	Acid
4-(2,6,6-Trimethyl-cyclohex-1-enyl)-butyric acid	Acid
Dehydroabietic acid	Acid
3.beta.-Acetoxy-5-cholenic acid	Acid
L-Erythro-Hexonic acid, 2,4,6-trideoxy-3-O-.beta.-D-glucopyranosyl-, ethyl ester, pentaacetate	Acid
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Acid
4-(2,6,6-Trimethyl-cyclohex-1-enyl)-butyric acid	Acid
Citronellyl tiglate	Acid
Ergosta-4,6,22-trien-3.alpha.-ol	Acid
5.alpha.-Cholestan-22(26)-epoxy-3,16-dibenzoxy	Acid
Ethanol	Alcohol
Androsta-3,5-diene-3,17-diol, 17-acetate 3-(heptafluorobutanoate), (17.beta.)-	Alcohol
Nonanal	Aldehyde
2-Undecenal	Aldehyde
(E)-Tetradec-2-enal	Aldehyde
Tetradecanal	Aldehyde
7-Oxabicyclo[2.2.1]heptane	Alkanes
2-Dimethylsilyloxytridecane	Alkanes
erythro-9,10-Dibromopentacosane	Alkanes
Heneicosane	Alkanes
erythro-9,10-Dibromopentacosane	Alkanes
Dotriacontane, 1,32-dibromo-	Alkanes
Dotriacontane, 1,32-dibromo-	Alkanes
1-Nonadecene	Alkene
Pregnane-3,17,20-triol, (3.alpha.,5.beta.,20S)-	Alkene
Metharbital	Amide
8-Methyl-6-nonenamide	Amide
5.alpha.Androst-16-ol, 17-ethylidene-3,5-dedihydro-6-methoxy-, pivalate	Amides
Oxazolidine, 2,2-diethyl-3-methyl-	Amine
5.alpha.-Cholestan-6.beta.-amine, N,N-dimethyl-	Amine
o-Cymene	Aromatic
Benzoic acid	Aromatic
Ethanone, 1-(7-amino-5-phenyl-[1,2,5]oxadiazolo[3,4-b]pyridin-6-yl)-	Aromatic
Benzaldehyde, 3,4,5-trimethoxy-	Aromatic
Benzaldehyde, 2,4,5-trimethoxy-	Aromatic
1,4-Methanonaphthalene,1,4-dihydro-9-((1-methylethylidene)-	Aromatic
p-Phenylenediurethane	Aromatic

5-(Phenyl-p-tolyl-methyl)-3H-[1,3,4]oxadiazol-2-one	Aromatic
5-(Phenyl-p-tolyl-methyl)-3H-[1,3,4]oxadiazol-2-one	Aromatic
5-(Phenyl-p-tolyl-methyl)-3H-[1,3,4]oxadiazol-2-one	Aromatic
5-(Phenyl-p-tolyl-methyl)-3H-[1,3,4]oxadiazol-2-one	Aromatic
2,4,6-Triisopropylphenetole	Aromatic
5-(Phenyl-p-tolyl-methyl)-3H-[1,3,4]oxadiazol-2-one	Aromatic
2,3-Diazabicyclo[2.2.1]hept-2-ene, 4-methyl-1-(pent-4-en-1-yl)-	Cycloheptene
1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-	Cycloheptene
Cycloheptasiloxane, tetradecamethyl-	Cycloheptene
1H-Cycloprop[e]azulene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.beta.,7b.alpha.)]-	Cycloheptene
alpha.-Phellandrene	Cyclohexene
1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	Cyclohexene
beta.-Phellandrene	Cyclohexene
1,3-Cyclopentadiene	Cyclopentene
Diethylmalonic acid, di(2-(3,3-dimethyl-2,4-oxacyclopentyl)ethyl) ester	Ester
Hexanoic acid, 2-ethyl-2-propyl-, methyl ester	Ester
l-Phenylalanine, N-butoxycarbonyl-, undec-10-enyl ester	Ester
Succinic acid, 2-methylpent-3-yl dec-4-en-1-yl ester	Ester
Tripalmitin	Ester
9-Octadecenoic acid (Z)-, hexadecyl ester	Ester
4-Fluoro-2-methoxyphenol, 3-methylbutyl ether	Ether
1-Triethylsilyloxyheptadecane	Ether
Furan, 3-methyl-	Furans
Furan, 2,5-dimethyl-	Furans
2,5-Furandione, 3-methyl-	Furans
2-Furancarboxaldehyde, 5-methyl-	Furans
2-Furancarboxylic acid, 2,2-dimethylpropyl ester	Furans
5-Hydroxymethylfurfural	Furans
Furfural	Furfural
Chloromethane	HD Aliphatic
Acetone	Ketone
2-Propanone, 1-hydroxy-	Ketone
1-Nitro-2-propanone	Ketone
4-Cyclopentene-1,3-dione	Ketone
1,2-Cyclopentanedione	Ketone
7-Tridecanone	Ketone
Cyclohexanone, 2,3,3-trimethyl-2-(3-methylbutyl)-	Ketone
Propan-2-one, 1-cyclododecyl-	Ketone
2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)-	Ketone
2,6-Dimethoxybenzoquinone	Ketone
Cholest-7-en-3-one, 4,4-dimethyl-, (5.alpha.)-	Ketone
Cyclopentasiloxane, decamethyl-	Other
Cyclohexasiloxane, dodecamethyl-	Other
5.alpha.-Androstane, 17-ethyl-1,3-dihydroxy-, (1.beta.,3.beta.)-	Other
Cholest-2-eno[2,3-b]indole, 5'-methoxy-	Other
Cholestan-3-ol, acetate, (3.beta.,5.alpha.)-	Other
1-Phenanthrenemethanol, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, 1-acetate, (1R,4aS,10aR)-	Other

Colchicine, N-desacetyl-N-[(2',2',5',5'-tetramethyl-N'-oxy-pyrrolid-3'-yl)carbonyl]-	Other
Phenol	Phenol
Phenol, 2-methoxy-	Phenol
Creosol	Phenol
Catechol	Phenol
3-[3-Bromophenyl]-7-chloro-3,4-dihydro-10-hydroxy-1,9(2H,10H)-acridinedione	Phenol
1,2-Benzenediol, 3-methoxy-	Phenol
4-Hydroxy-2-methylacetophenone	Phenol
Phenol, 2,6-dimethoxy-	Phenol
Phenol, 2,6-dimethoxy-	Phenol
Benzaldehyde, 3-hydroxy-4-methoxy-	Phenol
4-Hydroxy-2-methoxybenaldehyde	Phenol
1,4-benzenediol, 2,5-dimethoxy-	Phenol
3,5-Dimethoxy-4-hydroxytoluene	Phenol
3,5-Dimethoxy-4-hydroxytoluene	Phenol
3,4,5-Trimethoxyphenol	Phenol
Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	Phenol
Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-	Phenol
Desaspidinol	Phenol
stigmast-4-en-3-one	Phenol
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Pyran
erythro-9,10-Dibromopentacosane	Solvent
Stigmasta-3,5-diene	Sterols
beta.-Sitosterol acetate	Sterols
3,5-Cyclo-6,8(14),22-ergostatriene	Sterols
beta.-Sitosterol acetate	Sterols
21-Hydroxyprogesterone, trimethylsilyl ether, bis(O-methyloxime)	Sterols
Ergosta-4,6,22-trien-3.alpha.-ol	Sterols
3Beta,21alpha-diacetoxy-18,22,22-trimethyl-17,27,29,30-tetranor-c-homoolean-14-ene	Sterols
3-Heptafluorobutyriloxypregna-3,5,16-trien-20-one	Sterols
Cholestan-3,22,26-triol, 16-[2-thiohydroxyethyl]-, 3,26-diacetate	Sterols
1-Androsten-3,17-dione, di-trimethylsilyl	Sterols
Cholestan-3,22,26-triol, 16-[2-thiohydroxyethyl]-, 3,26-diacetate	Sterols
Dedihydroxypseduosarsasapogenin, 3,27-diiodo-	Sterols
4-Campestene-3-one	Sterols
Stigmastan-7-one	Sterols
Stigmasta-3,5-diene	Sterols
Pregnane-3,20-diol, bis(trifluoroacetate), (3.beta.,5.alpha.)-	Sterols
Stigmasta-3,5-diene	Sterols
beta.-Sitosterol acetate	Sterols
1-Phenanthrenemethanol, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, 1-acetate, (1R,4aS,10aR)-	Sterols
(3S,8S,9S,10R,13R,14S,17R)-17-((2R,5R)-5-Ethyl-6-methylheptan-2-yl)-3-methoxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,1	Sterols
Stigmast-5-en-3-ol, oleate	Sterols
Stigmasta-3,5-dien-7-one	Sterols
Stigmastan-7-one	Sterols
3Beta,21alpha-diacetoxy-18,22,22-trimethyl-17,27,29,30-tetranor-c-homoolean-14-ene	Sterols
Stigmast-4-en-3-one	Sterols

Stigmast-4-en-3-one	Sterols
Stigmasta-3,5-dien-7-one	Sterols
Stigmasta-3,5-dien-7-one	Sterols
Thiositosterol disulphide	Sterols
Stigmast-5-en-3-ol, oleate	Sterols
Stigmast-5-en-3-ol, oleate	Sterols
gamma.-Sitostenone	Sterols
Stigmast-4-en-3-one	Sterols
4,25-Secoobscurinervan-4-ol, 22-ethyl-15,16-dimethoxy-, diacetate (ester), (4.beta.,22.alpha.)-	Sterols
Cholest-7-en-3-ol, 2,2-dimethyl-, (3.beta.,5.alpha.)-	Sterols
24-Norursa-3,12-diene	Sterols
(3S,8S,9S,10R,13R,14S,17R)-17-((2R,5R)-5-Ethyl-6-methylheptan-2-yl)-3-methoxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,1	Sterols
Stigmasta-5,22-dien-3-ol, acetate, (3.beta.)-	Sterols
Digitoxose	Sugar
D-Allose	Sugar
1,6-Anhydro-.alpha.-d-galactofuranose	Sugar
gamma.-Terpinene	Terpine

Figure 3.1: Chromatograms of the total lipids extract from *Eucalyptus nitens*. A- wood; B- pulp.

A summary of the lipophilic extractives in the different *Eucalyptus* species is shown in Table 3.4. Sterols were present in highest concentrations in all the *Eucalyptus* spp. examined. They ranged from 704.5 mg/kg (*E. nitens*) to 365.2 mg/kg (*E. smithii*) in the wood samples and were notably lowered in the pulp to 325.2 mg/kg in *E. nitens* and 188.4 mg/kg in *E. smithii*. Acid-bisulphite pulping drastically reduced the fatty acid, fatty alcohol and steroid ketone content for all *Eucalyptus* species. Similar results were seen in a study by Kilulya *et al*. (2012) where the lipophilic fraction was almost completely removed towards the end of the bleaching process. Fatty acid esters are generally saponified during the sulphite pulping process, whilst terpenes, terpenoids and flavonoids are partly dehydrogenated, and resin components are sulfonated, thus increasing their hydrophilicity rendering them soluble in the liquor. Fractions of the resins are consequently removed during sulphite cooking (Sjöström, 1993).

Table 3.4: Composition of lipophilic extractives from different *Eucalyptus* species and their pulps (mg/kg).

Compounds	***E. dunnii***		***E. grandis***		***E. nitens***		***E. smithii***	
	Wood ±SD	*Pulp* ±SD	*Wood* ±SD	*Pulp* ±SD	*Wood* ±SD	*Pulp* ±SD	*Wood* ±SD	*Pulp* ±SD
Fatty acids	43.5±0.12 [a]	13.9±0.43 [a]	50.2±0.28 [a]	13.1±0.81 [a]	38.4±0.63 [b]	15.0±0.18 [a]	45.7±0.23 [a]	14.9±0.17 [a]
Fatty alcohols	15.4±0.23	3.2±0.24	12.7±0.31	4.1±0.25	16.2±0.33	7.8±0.39	15.8±0.41	3.6±0.30
Sterols	658.2±0.28 [a]	320.1±0.31 [a]	485.3±0.22 [a]	210.7±0.36 [b]	704.5±0.24 [a]	325.2±0.60 [a]	365.2±0.21 [a]	188.4±0.41 [a]
Steroid ketones	121.4±0.19	12.3±0.14	98.7±0.30	7.8±0.47	114.5±0.30	9.2±0.74	101.3±0.25	11.7±0.37

*Analyses conducted in duplicate. SD – standard deviation. p-value: a <0.05; b >0.05

Properties of unbleached pulp

Results for *E. dunnii*, *E. grandis*, *E. nitens* and *E. smithii* (yield, viscosity, kappa number, lignin content, cellulose and hemicelluloses) are shown in Tables 3.5 and 3.6. Pulp viscosity was highest for *E. grandis* (570.37 ml/g), followed by *E. nitens* (568.92 ml/g), *E. dunnii* (548.63 ml/g) and *E. smithii* (547.17 ml/g) (Table 3.5). Viscosity of pulp indicates the average degree of polymerization of cellulose, indirectly providing information on the degradation of cellulose resulting from the pulping and/or the bleaching process (Bodhlyera *et al*., 2015). The viscosity of *Eucalyptus* pulp produced on a lab-scale is generally in the range of 400-550 ml/g (Vehmaa, 2013). The degree of delignification of the pulp can be surmised from the Kappa number (Danielewicz and Surma-Ślusarska, 2006). *E. dunnii* and *E. smithii* pulps had low kappa numbers, demonstrating improved pulp brightness implying that reduced amounts of chemicals would be required during bleaching. Unbleached pulp has an initial kappa range of 8-14 that is

reduced during bleaching stages to 2.8 after the oxygen stage, 0.9 after ECF bleaching, with a final kappa number of <0.5 (Vehmaa, 2013). Klason lignin contents were at desirable levels of 4-6% for all *Eucalyptus* species. This indicates bleaching of these pulps will be easier and that milder bleaching conditions (lower temperatures and chemical dosages) will be adequate to attain suitable kappa numbers (Dutt and Tyagi, 2011). Dissolving pulp yields obtained were within the range for conventional pulping, viz., 43.5-51.5% (Clarke, 1995; Gardner, 2001). However, it is important to note that following elemental chlorine-free (ECF) bleaching, pulp yield is expected to decrease to 35-39% (Flickinger *et al*., 2011; Gominho *et al*., 2015). Glucose levels recorded were high ranging from 87.7-89.8 (Table 3.6), indicating high cellulose content of the pulp (cellulose content values as high as 96% are desirable).

Maximum removal of hemicelluloses is desirable in the manufacture of DWP. The presence of sugar monomers xylose, mannose and galactose is indicative of hemicellulose content. The results in Table 3.6 indicate that *E. grandis* is ideal for DWP due to its lowest content of hemicellulose sugars among the four *Eucalyptus* species studied.

Table 3.5: Characteristics of pulp produced from *Eucalyptus* wood chips.

	Pulp Yield (%) ±SD	**Viscosity (ml/g)** ±SD	**Kappa number** ±SD	**Klason lignin (%)** ±SD	**Acid-Soluble lignin (%)** ±SD
E. dunnii	52.4±0.28	548.63±0.24 [a]	9.95±0.16 [a]	6.12±0.27 [a]	5.15±0.82
E. grandis	54.1±0.47	570.37±0.2 [a]	11.54±0.24 [b]	4.48±0.58 [a]	4.36±0.57
E. nitens	51.1±0.12	568.92±0.31 [a]	10.49±0.19 [b]	6.62±0.23 [a]	5.56±0.09
E. smithii	50.3±0.14	547.17±0.17 [a]	8.25±0.30 [a]	5.68±0.22 [a]	4.35±0.15

*Analyses conducted in duplicate. SD – standard deviation. p-value: a <0.05; b >0.05

Table 3.6: Quantification of pulp cellulose and hemicelluloses using HPLC (%).

	Arabinose ±SD	**Galactose** ±SD	**Glucose** ±SD	**Mannose** ±SD	**Xylose** ±SD	**Sum** ±SD
E. dunnii	0.15±0.19	0.33±0.34	89.34±0.54	0.52±0.36	4.37±0.14	94.51±0.04
E. grandis	0.04±0.17 [a]	0.40±0.28 [a]	89.76±0.41 [a]	0.35±0.21 [a]	0.81±0.10 [a]	90.06±0.14 [a]
E. nitens	0.05±0.21	0.32±0.30	88.52±0.59	0.76±0.11	1.86±0.31	91.51±0.19
E. smithii	0.03±0.39	0.22±0.11	87.69±0.28	0.61±0.24	2.59±0.24	90.14±0.27

*Analyses conducted in duplicate. SD – standard deviation. p-value: a <0.05; b >0.05

3.5 Conclusions

The eucalypt species in this study had similar types of lipophilic extractives but the amounts of various compounds differed. The variations observed appeared to be influenced by tree species.

Amongst the *Eucalyptus* wood species tested, *E. nitens* contained the highest concentrations of lipophilic compounds, signifying that DWP from this species are more likely to result in pitch formation during pulping. Py-GC-MS analyses revealed that free sterols, sterol esters and fatty alcohols were present at highest concentrations. By pulping a combination of *Eucalyptus* spp. with low and high lipophilic contents, this would assist in reducing the overall concentration of lipophilic extractives in the pulp, thus reducing the risk of pitch deposit formation. Comparison of pulp properties for the four *Eucalyptus* species revealed *E. grandis* to be a superior wood species producing the highest amount (pulp yield- 54.1%) and best quality (viscosity- 570.37 ml/g; glucose concentration- 89.8%) of pulp. In addition, the total lignin content was lowest for *E. grandis* (8.8%), signifying lower chemical requirements during bleaching. Pulp of *E. smithii* was of the lowest quality and yield, however, the kappa number was the lowest compared to the other *Eucalyptus* species. Xylose was the major component in the hydrolysates of the carbohydrate fraction, with arabinose, mannose and galactose present in small amounts. The information on the chemistry of eucalypt wood provided by this study contributes towards the knowledge of this valuable crop and will assist in optimizing selection of eucalypt species for pulping.

3.6 Acknowledgements

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CHAPTER FOUR

THE EFFECTS OF WOOD STORAGE ON THE CHEMICAL COMPOSITION AND INDIGENOUS MICROFLORA OF *EUCALYPTUS* SPECIES USED IN THE PULPING INDUSTRY

4.1 Abstract

Lipophilic extractives naturally occurring in wood tend to coalesce during pulping to form pitch deposits, which have distinctively undesirable effects on the pulping process and quality of pulp produced. The production of dissolving pulp (high-grade cellulose pulp), in particular, employs an acidic pulping process which aggravates the formation of these pitch deposits. A baseline profile of the lipophilic extractive content of popular *Eucalyptus* species [*E. nitens, E. grandis,* and *E. dunnii* (of different site qualities)] used in the pulping industry would assist in management strategies to reduce pitch formation. This study determined the effects of storage of wood chips at -20°C (for 6 months), by the examining the wood chemical composition and indigenous microflora. The lipophilic extractive profiles of these *Eucalyptus* species were studied using pyrolysis gas chromatography-mass spectrometry. Fatty acids were the main lipophilic compounds among *E. dunnii* (SQ3 and SQ4) and *E. grandis* wood extractives. Unexpectedly, *E. nitens* wood displayed the lowest and *E. grandis* the highest amount of these pitch-forming compounds. This may be due to the handling of the wood logs prior to chipping. Storage of wood chips at -20°C had a similar effect as the traditional seasoning methods (storage of wood outdoors prior to pulping) used for the reduction of lipophilic extractives. The site quality of the *Eucalyptus* plantation had considerable influence on chemical composition of the wood material. The total extractive content was higher for *E. dunnii* (SQ3) (13.2%) compared to *E. dunnii* (SQ4) (7.7%), however, cellulose and acid-insoluble lignin contents of *E. dunnii* (SQ4) were higher at 44.8% and 27.5%, respectively. This indicates that greater pulp yield may be achieved with *E. dunnii* (SQ4) but at a greater risk of pitch formation and high insoluble lignin content. After storage, variations in bacterial and fungal communities were observed, and need to be taken into consideration when conducting lab scale trials, as degradation of wood components during storage would influence the outcome of experiments. It is therefore recommended that if storage of wood chips is necessary, they should be retained for a maximum of 3 months at -20°C under laboratory conditions.

Key words: *storage, wood chemistry, microbial populations, Eucalyptus*

4.2 Introduction

The South African pulp and paper industry relies heavily on *Eucalyptus* wood material as a resource for the production of pulp and paper (Sappi, 2017). The major hardwood species grown in South African plantation forests include several *Eucalyptus* spp. including: *E. dunnii*, *E. grandis*, *E. nitens*, *E. smithii* and a range of hybrids (Sappi, 2012). The *Eucalyptus* species are used in the production of various grades of pulp such as dissolving pulp. Dissolving pulp is almost pure cellulose (91-98%), as it contains minimal amounts of lignin and hemicelluloses, and is used for the production of cellulose derivatives such as microcrystalline cellulose, rayon, cellulose acetates and cellulose nitrates (Kumar and Christopher, 2017; Sappi, 2017).

All plant materials are made up of three major components, cellulose, hemicelluloses and lignin and a small component of lipophilic extractives (wood resin) (McKendry, 2002). Cellulose molecules link together to form microfibrils which form the framework of the cell wall and are responsible for the strength of wood. Hemicelluloses are a group of compounds made up of different sugars and combine with lignin, which provides rigidity, to form the glue that holds the cells together. Extractives make up the minor fraction of wood but have the greatest potential to cause problems (Leskinen *et al*., 2015). The extractive content of wood is highly variable amongst species (Moodley, 2011; Yang and Jaakkola, 2011), therefore careful selection of *Eucalyptus* species for pulping is required and currently not taken into consideration in commercial pulping operations (Personal communication, 2016).

The lipophilic fraction of wood extractives are responsible for the formation of pitch deposits during pulp production (Sjöström, 1993; Kontkanen *et al*., 2004; Wang and Jiang, 2006), resulting in poor pulp quality and gumming up of machinery (Hillis and Sumimoto, 1989; Patrick, 2004; Sarja, 2007). The extractives in the different wood species and pulping process employed are directly related to the composition of the wood resin components in pitch deposits (Back and Allen, 2000; Holmbom, 2000). Traditionally, wood resin components are reduced by seasoning logs and wood chips (outdoor storage of wood prior to processing) (Allen *et al*., 1991; Back and Allen, 2000). Storage reduces the extractive content by hydrolytic or oxidative conversion by plant enzymes and the activity of indigenous microflora. The reduction of extractive content occurs much faster in woodchips rather than log form, as the oxidation

processes proceed more easily and rapidly (Gutiérrez *et al*., 1998; Burnes *et al*., 2000). Temperature is also known to have an effect on the degradation of lipophilic compounds (Silvério *et al*., 2008). It has been reported that during winter the hydrolysis of wood esters, which forms part of the pitch, is drastically reduced compared to the summer months (Almeida and Silva, 2001). Thus, the ester fraction of the pulp is greater in winter, leading to an increase in the hydrophobicity and viscosity of pitch (Olm, 1984). In industry, seasoning wood at ambient temperatures usually significantly reduces the lipophilic content in the wood. However, care has to be taken as prolonged seasoning could lead to uncontrolled action of microorganisms and insects (Bajpai, 1999; Gutiérrez *et al*., 2001). The activity of such organisms could lead to cellulose and hemicellulose loss in the wood, which would ultimately result in the loss of pulp quality (Burnes, 2000). Similarly, care has to be taken when storing wood for scientific investigations. Under laboratory conditions, wood chips are usually stored at -20°C to prohibit any natural degradation of the lipophilic extractives, contamination by foreign organisms and to preserve the original state of the wood (Venn, 1980; Petäistö, 2006).

The lipophilic extractives of *E. globulus* and certain hybrids have been extensively characterized (del Río *et al*., 1998; Gutiérrez *et al*., 1998; Gutiérrez *et al*., 1999; Gutiérrez *et al*., 2001; Freire *et al*., 2002; Freire *et al*., 2006; Prinsen *et al*., 2012), however, there is a lack of information on the extractive content of *E. grandis*, *E. urograndis* (Freire *et al*., 2006), *E. urophylla*, *E. camaldulensis* (Silvério *et al*., 2007), *E. nitens*, and *E. dunnii* (Rencoret *et al*., 2007). In addition, only limited information on the effect that site quality plays on extractive content of *Eucalyptus* species is available. Incorporating the knowledge on the lipophilic content of different *Eucalyptus* species when selecting wood for pulping, will go a long way towards reducing the overall lipophilic content of pulp, consequently reducing the risk of pitch formation. In the present study, the chemical composition of the wood and pulp of three *Eucalyptus* species, *E. grandis*, *E. nitens*, *E. dunnii* and two site qualities for *E. dunnii* [*E. dunnii* SQ3 and *E. dunnii* SQ4] were evaluated. Furthermore, the effects of storage at -20°C on the chemical composition of different *Eucalyptus* spp. and their indigenous microflora was investigated.

4.3 Materials and Methods

4.3.1 Samples

E. grandis, *E. nitens*, *E. dunnii* (SQ3) and *E. dunnii* (SQ4) were obtained from a commercial pulping mill in Umkomaas, located on the south coast of South Africa. Wood chips from individual wood species were collected as they were being chipped in the wood yard. Samples were transported back to the laboratory and stored at -20°C. It is important to note that the wood material obtained here may not have been as fresh as the material in chapter three. The logs spent an indeterminate period in the woodyard prior to chipping. Chip samples of the different wood species were milled to coarse sawdust using the Brabender® Wiley-mill (Triad Scientific, New Jersey, USA). The saw dust was then passed through a 0.40 mm (40 mesh) screen and used for chemical analyses (T257 cm-12). The following analyses were conducted on the milled wood chips before and after storage at -20°C for 6 months. Wood chips were also pulped and evaluated for changes in quality.

4.3.2 Hot water extraction

Hot water (water-soluble) extractives (waxes, fats, some resins, photosterols, non-volatile hydrocarbons, low molecular weight carbohydrates, salts, and other water-soluble substances) were evaluated by placing 5 g of sawdust (moisture content recorded) in a 400 ml conical flask and slowly adding 100 ml of hot deionised water. The contents of the conical flasks were well stirred to prevent the sawdust from floating. The conical flask was placed on a pre-heated hot plate for 3 h. The initial level of the contents was noted and kept constant by the addition of hot deionised water. A pre-weighed number 3 crucible (Pyrex, Massachusetts, USA) and a vacuum pump were used to filter the sawdust, which was washed with 200 ml of hot deionised water and dried at 60ºC overnight (T207 cm-08). The experiments were performed in triplicate.

4.3.3 Solvent extraction

The Soxhlet extraction method was used to evaluate the solvent (water-insoluble) extractives in the wood samples. Four grams of hot water extracted sawdust (moisture content recorded) were weighed into an extraction thimble, which was placed in a Soxhlet apparatus that was attached to a pre-weighed 500 ml round bottomed flask containing 300 ml toluene-ethanol (2:1). The heating mantle was adjusted such that the solvent cycled through the system six times over 4 h. The extractives were rotor evaporated to dryness, cooled in a desiccator, and weighed (T204 om-88). The experiments were performed in triplicate.

4.3.4 Chemical analyses

The chemical characteristics of the wood chips were evaluated by acid-insoluble lignin (T222 om-88), extractive analyses such as near infra-red reflectance analyser (NIRA) for the rapid quantification of wood chemical components and high performance liquid chromatography (HPLC) for quantification of cellulose and hemicelluloses (glucose, mannose, arabinose, xylose and galactose) (T249 cm-85; Wallis *et al*., 1996; Wright and Wallis, 1996).

4.3.5 Pyrolysis-gas chromatography-mass spectrometry (GC-MS)

Pyrolysis GC-MS was utilised to profile lipophilic extractives. A multi-shot pyrolyzer, EGA/PY-3030 D, (Frontier Lab, Fukushima, Japan) attached to an ultra-alloy capillary column (30 m x 0.25 mm, 0.25 μm) was used for analysis. The samples were pyrolysed at 550°C for 20 s and the interface temperature was set at 350°C. The chromatographic separation of the volatile components released by pyrolysis was performed using an ultra-alloy column. The injection temperature was set to 280°C, and the column flow rate was set to 1.0 mL/min, with helium used as a carrier gas. The temperature was programmed as follows: 50°C for 2 min; rate 3°C/min up to 200°C, and hold for 4 min. The ion source and interface temperatures were set to 200°C and 300°C, respectively. The scan range used for mass selective detector was 40 to 650 m/z. Pyrolysis products were identified by comparing their mass spectra with the mass spectrum NIST library attached to the instrument (modified from Sithole and Watanabe, 2013).

4.3.6 Pulping

Pulping was performed according to the acid-bisulphite process currently implemented in dissolving wood pulp (DWP) mills. Pulp quality was determined by assessing viscosity (T203 om-99), screened pulp yield, kappa number (T236 om-06), copper number, alpha cellulose, S8, S10 (T235 om-60) and hemicelluloses (Forestry and Forest Products Research Centre, CSIR, Personal Communication, 2014).

4.3.7 DNA isolation and polymerase chain reaction (PCR)

DNA was extracted from 0.2 g of milled chips using a kit as per the manufacturer's specifications (Soil DNA Extraction Kit, Zymo Research, California, USA). Ribosomal genes were amplified from microbial genomic DNA using 16S and ITS region of 18S rRNA genes with the universal primer sets: 63F/1387R (Marchesi *et al*., 1998) and ITS5F/ITS4R (White *et al*., 1990), respectively. Each amplification reaction (50 μl) contained 1.25 mM MgCl2, 0.125

μM forward and reverse primers, 0.2 mM deoxynucleoside triphosphate (dNTPs), 0.25 U SuperTherm *Taq* DNA polymerase (Southern Cross Biotech, Cape Town, South Africa), and 20 to 200 ng of template DNA. PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems, California, USA). For amplification of 16S rRNA, PCR conditions were as follows: initial denaturation at 95°C for 5 min, 30 cycles of denaturation annealing and extension at 95°C for 1 min, 55°C for 1 min, 72°C for 1.5 min and a final extension at 72°C for 5 min. The 18S rRNA amplification conditions were: initial denaturation at 95°C for 2 min, 25 cycles of 95°C for 30 sec, 53°C for 45 sec, 72°C for 1 min and a final extension at 72°C for 8 min. The amplicons were analyzed by electrophoresis on 1% agarose (SeaKem) gels in 1× TAE running buffer at 90 V for 45 min. After electrophoresis, the gels were stained in 0.5 μg/ml ethidium bromide and visualized using the Chemi-Genius 2 BioImaging System (Syngene, Cambridge, UK). Upon confirmation of 16S and 18S amplicons, the products were purified using a GeneJET™ PCR purification kit (Fermentas, Massachusetts, USA) and re-amplified in a touchdown thermal profile program using nested PCR and primers with GC clamps. PCR for 16S rRNA genes were initially performed using two primer sets: 338F-GC with a GC-clamp: 5´-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3´ and 518R 211 (237-bp fragment) (Handschur *et al*., 2005); 933F-GC with a GC-clamp: 5´-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3´ and 1387R (500-bp fragment) (Ji *et al*., 2004). The composition of the reaction mixtures was the same as that used for the first PCR. For amplification of 16S rRNA, PCR conditions were as follows: initial denaturation at 95°C for 5 min, 30 cycles of denaturation annealing and extension at 95°C for 1 min, 55°C for 1 min, 72°C for 1.5 min and a final extension at 72°C for 5 min. The 18S rRNA amplification conditions were: initial denaturation at 95°C for 2 min, 25 cycles of 95°C for 30 sec, 53°C for 45 sec, 72°C for 1 min and a final extension at 72°C for 8 min. (Schabereiter-Gurtner *et al*., 2001).

4.3.8 Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using the D-Code Universal Mutation Detection System (BioRad, California, USA), and an optimized method (Ramnath *et al*. 2013) modified from Muyzer and Smalla (1998). PCR samples were loaded onto vertical perpendicular polyacrylamide gels (6% acrylamidebisacrylamide [37.5:1]) in 1× TAE buffer prepared using 30% and 60% denaturant (100% denaturant corresponds to 7 M urea and 40% formamide). Gradients of 30% and 60% were optimal for bacterial amplicons (~237 bp) and 25% and 50% gradients were used for

fungal amplicons (~316 bp). A pre-run was performed at a constant voltage of 150 V at 60°C for 30 min, following which, DGGE profiles were generated at a constant voltage of 60 V in 1× TAE buffer at 60°C for 16 h. After electrophoresis, gels were stained in 0.5 µg/ml ethidium bromide for 60 min, destained in the same volume of 1× TAE buffer for 30 min and visualized using the Chemi-Genius 2 BioImaging System (Syngene).

4.4 Results & Discussion

A comparison of the samples before and after storage at -20°C revealed a general decrease in hot water and solvent extractives (Figure 4.1). Hot water extraction is an efficient technique for the removal of hemicelluloses from wood chips prior to preparing dissolving pulp (Li *et al*., 2010). The highest hot water extractive content was observed for *E. dunnii* SQ3 (11.2%). Extractive contents vary from tree to tree and considerably from species to species (Hillis, 2014). Surprisingly, *E. nitens* did not follow the trend observed in the previous chapter, as it displayed the lowest amount of lipophilic extractives and *E. grandis* the highest. This variation may be due to the storage of *E. nitens* logs in the woodyard for a longer period than *E. grandis* logs, which may have influenced the lipophilic content of the wood in the process referred to as seasoning. Alternatively, *E. nitens* may exhibit a better response to seasoning in terms of reduction of lipophilic content than the other *Eucalyptus* species. *E. dunnii* SQ3 exhibited higher hot water extractive (11.2%) and solvent extractive (2%) content compared to SQ4 (6% and 1.7%, respectively) – a striking variation in chemical characteristics (more especially the hot water extractives) amongst *Eucalyptus* wood of the same species but different site qualities. This result is supported by Kilulya *et al*. (2014) who also observed that variations in lipophilic content are influenced by tree species and site qualities. It is also worth mentioning that storage may also affect the analysis of the extractives. It has be reported that some components undergo oxidation and radical reactions during storage, which result in modified components that are either more difficult to extract or identify (Bialczak *et al*., 2011).

The study of Kilulya *et al*. (2014) also validated the present finding of higher amounts of total lipophilic extractives in *E. dunnii* compared to *E. grandis*. Cohen and Mackney (1951) observed that wood chips with hot-water-soluble contents exceeding 7% retained higher levels of lignin in the pulp. They postulated that the delignification process was not optimal due to competition between extractives and lignin for the active cooking chemicals during pulping. Consequently, higher amounts of active alkali were required to achieve efficient delignification

with the negative outcome of increased cost and quantity of waste generated (Cohen and Mackney, 1951). A similar result was observed in this study, however, the trend was only true for *E. dunnii* (SQ3 and SQ4) where only 7.6% and 9.2% reduction, was observed, respectively. The hot water extractive content for *E. grandis* (9%) and *E. nitens* (7%) was in the range reported to be inhibitory to delignification (≥ 7%), yet total lignin was reduced by 33% and 40%, respectively during pulping. Extractives may cause liquor decomposition, also resulting in higher chemical requirements per ton of pulp produced (Hillis, 2014). A comparison of the two site qualities revealed that cellulose and acid-insoluble lignin contents of *E. dunnii* (SQ4) were higher at 44.8% and 27.5%, respectively. This indicates that greater pulp yield may be achieved with *E. dunnii* (SQ4) but at a greater risk of pitch formation and insoluble lignin. Overall, *E. grandis* had the highest amount of cellulose, followed by *E. nitens*, before storage.

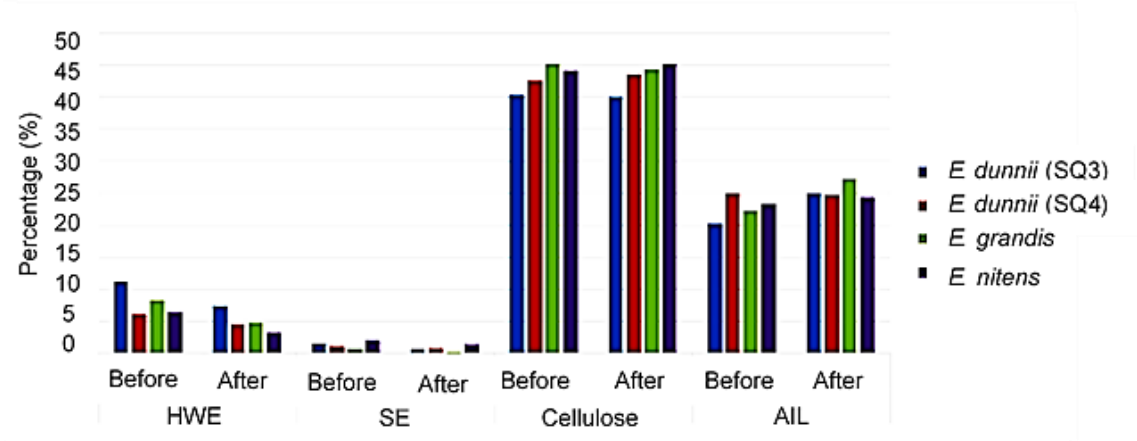


Figure 4.1: Wet chemistry of wood chips of different *Eucalyptus* spp. before and after storage at -20°C. HWE – hot water extractives, SE – solvent extractives, AIL – acid-insoluble lignin.

High performance liquid chromatography (HPLC) was used to quantify hemicelluloses (glucose, mannose, arabinose, xylose and galactose) in the wood chips before and after storage. There was an increase in xylose content after storage for all *Eucalyptus* species, attributed to the natural degradation of glucuronoxylan during storage (Table 4.1). Pentosans, abundant in hardwoods, are mostly glucuronoxylan. The bonds between xylose units in glucuronoxylan are easily hydrolysed (Testova *et al*., 2009), highlighting the potential of natural degradation during storage. Galactose and arabinose levels decreased for all *Eucalyptus* spp. after storage, with the greatest decrease observed in *E. nitens*. This decrease during storage may be attributed to microbial degradation. Previous studies have shown that during the initial stages of fungal degradation, the carbohydrates most modified were sugars derived from the primary walls and middle lamella (galactose and arabinose) (Skyba *et al*., 2013; Rytioja *et al*., 2014). These

molecules are the building blocks of hemicelluloses and/or arabinogalactan proteins in the primary wall (Skyba *et al*., 2013). The effects reported here are contrary to most reports that maintain that storage of wood chips at -20°C retards seasoning (Promberger et al., 2004; Hildén and Persson, 2007).

The mannose content of the wood material decreased by 24%, 21% and 9% for *E. dunnii* (SQ3), *E. grandis* and *E. nitens*, respectively, after storage. This decrease in mannose content may be caused by the activity of hemicelluloytic enzymes produced by microorganisms indigenous to the wood, particularly in *E. dunnii* (SQ3) and *E. grandis.* This activity may have occurred during the freeze thaw cycles or during sample preparation. Wood treated with specific enzymes enable the extraction of high molecular weight materials, whilst non-specific enzymes were found to degrade hemicelluloses to a large extent (Azhar, 2015). Thus, the increase in sugars observed in this study was attributed to non-specific hemicelluloytic activity of indigenous microflora found in the wood material.

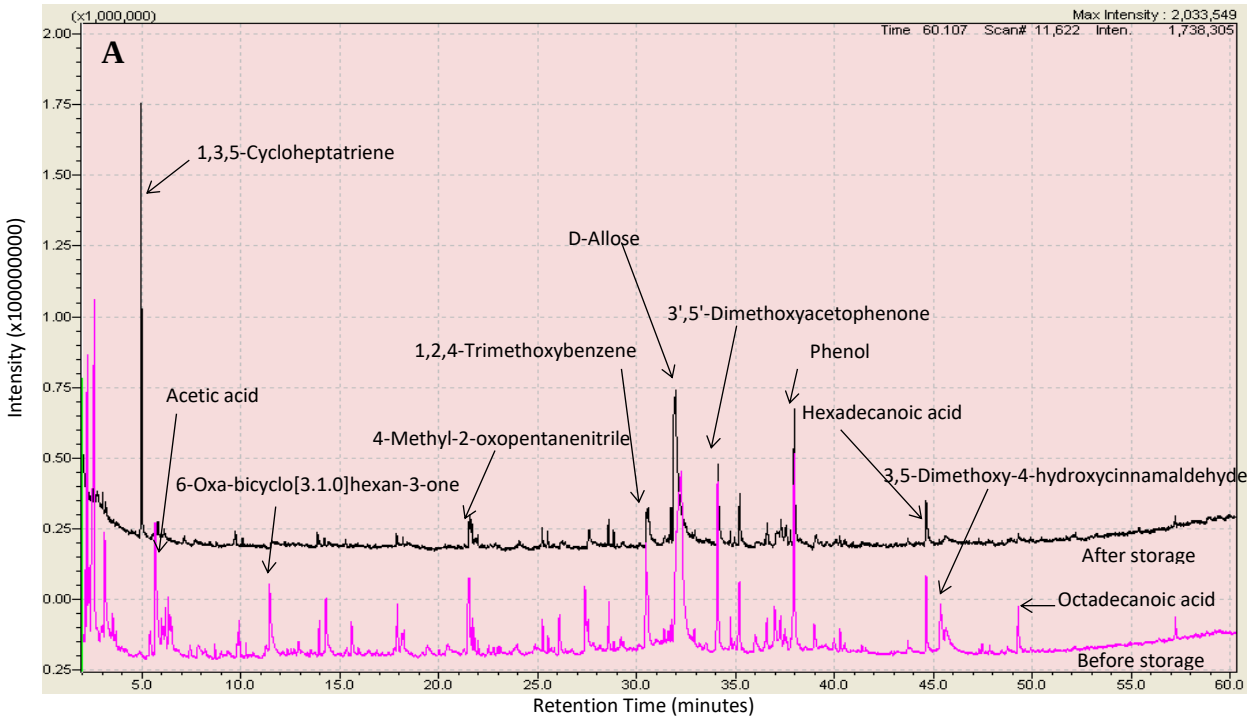
Table 4.1: Carbohydrate content of the wood chips before and after storage at -20°C.

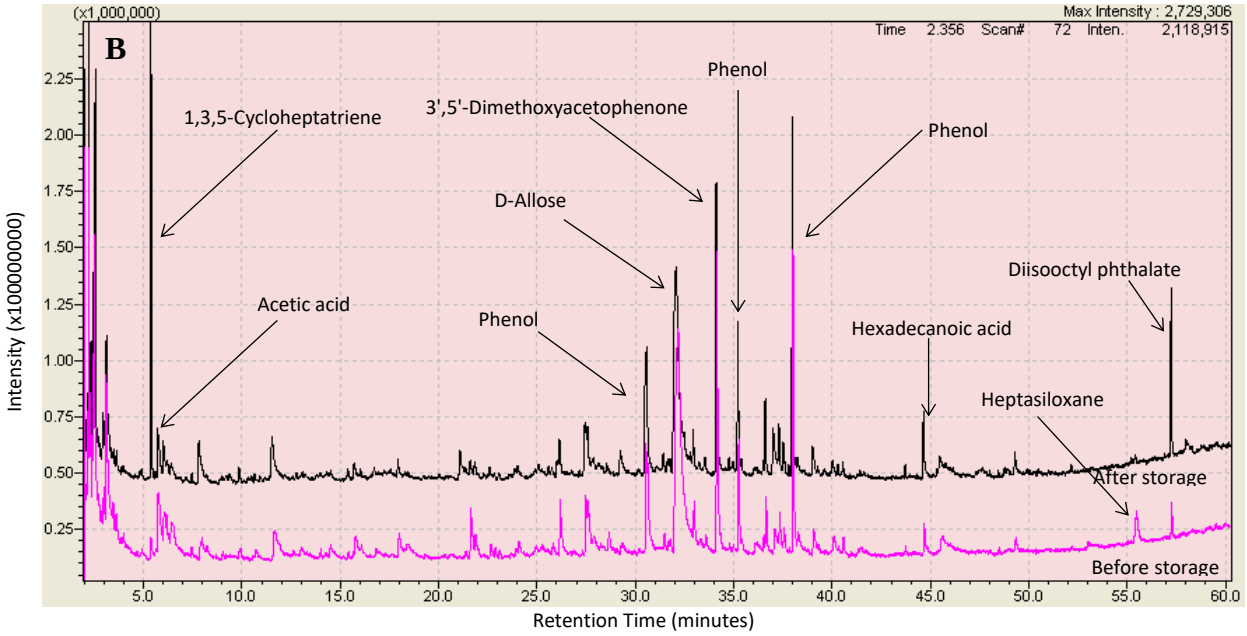
	Arabinose % ±SD		**Galactose % ±SD**		**Glucose % ±SD**		**Xylose % ±SD**		**Mannose % ±SD**	
	Before	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*
E. dunnii (SQ3)	0.41±0.11	0.32±0.23 [a]	1.43±0.30	1.15±0.25 [a]	49.40±0.12	48.09±0.47	9.05±0.39	10.68±0.26	1.31±0.14	1±0.19 [a]
E. dunnii (SQ4)	0.27±0.69	0.19±0.04 [a]	0.95±0.48	0.70±0.18 [a]	49.18±0.36	49.84±0.20	10.82±0.21	11.24±0.45	1.44±0.28	1.51±0.36 [b]
E. grandis	0.27±0.32	0.16±0.21 [a]	1.04±0.63	0.79±0.32 [a]	50.62±0.21	49.24±0.44	7.83±0.4	9.97±0.33	1.74±0.20	1.37±0.47 [a]
E. nitens	0.16±0.41	0.09±0.33[a]	0.65±0.08	0.37±0.24[a]	50.37±0.11	51.5±0.17	9.87±0.31	11.35±0.69	1.69±0.48	1.54±0.14 [a]

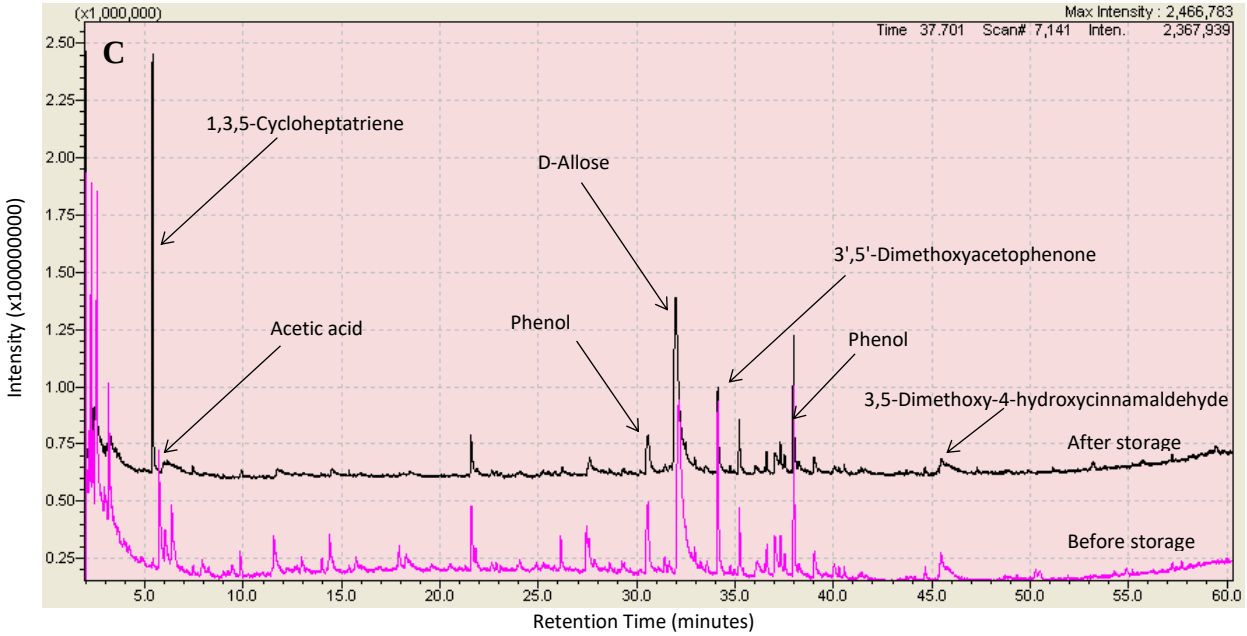
*Analyses conducted in duplicate. SD – standard deviation. p-value: a <0.05; b >0.05

Py-GC-MS analyses provided insight into the organic content of the samples. The Py-GC-MS profiles in Figure 4.2 show that the samples contained mainly lignin (3',5'-dimethoxyacetophenone) and lipophilic (octadecanoic acid and hexadecanoic acid) degradation products due to pyrolysis. Storage at -20°C had a discernible effect on the lipophilic content of the wood. The hexadecanoic and octadecanoic fatty acids observed are an indication of polymerised lipids. Polymerisation is the cause of incomplete removal of lipids by extraction (Tao *et al*., 2010). A reduction in these fatty acids was observed for *E. dunnii* (SQ4), *E. nitens* and *E. grandis*. This reduction may lead to a decrease in pitch deposit

formation and is considered a positive effect. These results confirm that storage at -20°C resulted in degradation of pitch components, as previously reported in Aspen wood chips (Allen *et al*., 1991). However, if the wood chips are being stored for future experimental use, any changes from the original state of the wood is not favourable. *E. grandis* (Figure 4.2D) contained significant amounts of higher molecular weight lipophilic extractives (hexadecanoic and octadecanoic fatty acids), compared to the other wood species. These compounds were present in the other samples but at much lower levels, reflecting the inherently greater lipophilic content of *E. grandis*. Extractives were slightly lower than what has been reported for *Eucalyptus* species, which may indicate that samples were not fresh and that extractives may have transformed into higher molecular weight lipids (Kilulya *et al*., 2014). The phthalate and siloxane observed in *E. dunnii* (SQ3) are contaminants and are not part of the wood. Traces of phthalates and siloxane are frequently found in most industrial samples and inevitably form part of the surface composition (Fardim and Durán, 2003).




B
(x1,000,000)
Max Intensity : 2,729,306
Time 2.356 Scan# 72 Inten. 2,118,915
1,3,5-Cycloheptatriene
3',5'-Dimethoxyacetophenone
Phenol
Phenol
D-Allose
Diisooctyl phthalate
Acetic acid
Phenol
Hexadecanoic acid
Heptasiloxane
After storage
Before storage
Intensity (x100000000)
Retention Time (minutes)


C
(x1,000,000)
Max Intensity : 2,466,783
Time 37.701 Scan# 7,141 Inten. 2,367,939
1,3,5-Cycloheptatriene
D-Allose
3',5'-Dimethoxyacetophenone
Acetic acid
Phenol
Phenol
3,5-Dimethoxy-4-hydroxycinnamaldehyde
After storage
Before storage
Intensity (x100000000)
Retention Time (minutes)

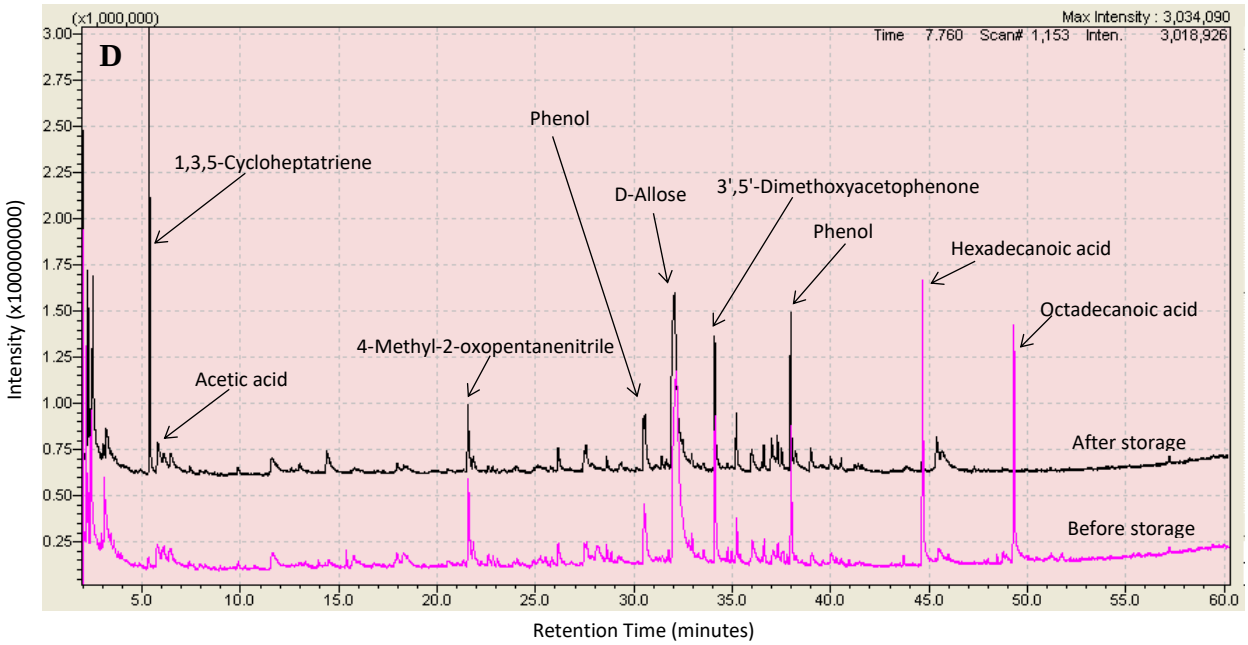


Figure 4.2: Lipophilic profiles of different *Eucalyptus* species, before and after storage at -20°C. A- *E. dunnii*, B- *E. dunnii* (FFP), C- *E. nitens*, D- *E. grandis*.

The pulp produced from the wood chips before and after storage was evaluated by measuring basic pulp chemistry: kappa number, viscosity, pulp yield, alpha cellulose, S10 and S18, and copper number. The kappa number provides an estimate of the amount of chemicals required during bleaching of wood pulp to achieve a pulp with a specified degree of whiteness. Therefore, the amount of bleach required is correlated to the lignin content of the pulp. The kappa number can be used to determine the effectiveness of the lignin-extraction phase of the pulping process. It is approximately relative to the residual lignin in the pulp (Costa and Colodette, 2007). The total lignin content of the pulp produced from wood chips before storage was reduced for all the *Eucalyptus* species after storage (Table 4.3). Greater lignin removal may have been possible due to the opening up of the wood structure just before or after storage by enzymes produced by the indigenous microflora present. The pulp produced after storage had a lower kappa number than the starting material, but had a relatively low pulp viscosity (Table 4.2). Since hemicelluloses shield the cellulose chain from alkaline hydrolysis (Lindström and Teder, 1995), the low pulp viscosities after storage are possibly due to the removal of protective hemicelluloses (Azhar, 2015). Pulp viscosity (degree of polymerization of cellulose fibres) reveals the relative consistency of the cellulose fibres after pulping. The pulp viscosity varied between the different *Eucalyptus* species from 28.01 to 131.7 cP. A general decrease in the viscosity was observed for all samples after 6 months of storage.

Viscosity values for *E. dunnii* (SQ3) were very low and may be explained by the degradation of cellulose fibres by cellulase-producing microorganisms in this species (Ramnath *et al*., 2013). A considerable decrease in viscosity of 41% and 66% in *E. grandis* and *E. nitens* pulp, respectively, was observed after storage. This may be attributed to endoglucanase activity, which causes random cleavage of cellulose chains leading to lower degrees of polymerization and therefore lower viscosity. The retention of long cellulose fibres is essential for producing dissolving pulp (high-grade cellulose pulp). Cellulase activity results in cellulose degradation, thus these enzymes need to be eliminated or minimized from crude enzyme cocktails.

After storage, glucose levels varied between 80.9 and 86.2% and xylose from 5.3% to 6.4% (Table 4.3). This result provides an approximate indication of the cellulose to hemicellulose ratio in the different species (Neiva *et al*., 2014). *E. dunnii* (SQ3) and *E. dunnii* (SQ4) displayed the lowest and highest ratio of glucose/xylose, respectively. These results support the notion that site quality has an effect on the chemical composition of the wood. The hemicelluloses content in *E. dunnii* (SQ4) pulp was higher than in the other *Eucalyptus* species examined. A general increase in xylose content was observed for all *Eucalyptus* pulp samples (Table 4.3). Fišerová *et al*. (2013) found that xylose content of hydrolysed beech and oak wood samples increased with increasing wood weight loss. Hence the increase in xylose was attributed to wood weight loss during storage, which may be caused by wood decaying fungi that became activated once thawed. Wood decaying fungi have acquired a range of hydrolytic enzymes including cellulases, hemicellulases and oxidative enzymes that breakdown of lignocellulose into free monomers such as xylose, mannose, glucose, galactose and arabinose (Álvarez *et al*., 2016). In another study, greater xylose levels were observed when *Eucalyptus* and rice straw materials were pretreated with a culture supernatant of *Trichoderma reesei* than with traditional steam explosion as a pretreatment step (Álvarez *et al*., 2016). This emphasizes the xylan-hydrolyzing potential of *T. reesei* to release xylose oligomers and other soluble sugars (Jørgensen *et al*., 2007), and may explain the higher xylose concentrations documented in this study. In addition, xylanases may assist in the pulp bleaching process, by removing xylan. This effect facilitates bleaching of the cellulose fibres, therefore consuming less bleach and energy (Garg *et al*., 2011). This partial degradation of xylan could also assist in the degradation of lignin by other microorganisms present, which is essential because lignin also impedes pulp bleaching (Subramaniyan and Prema, 2002).

Prior to storage, pulping of *E. nitens* wood resulted in the highest pulp yield of 50.8%. However, after storage the yield was reduced to 44.8%, the lowest level amongst the *Eucalyptus* spp. examined. After storage, *E. dunnii* (SQ3) generated the highest pulp yield of 48.7%. Considering the pulping characteristics of the different species without the effect of storage, *E. nitens* is the most suitable for pulping as it generated the greatest pulp yield (50.8%) and highest pulp viscosity (131.7%). Integrating the effects of storage, *E. dunnii* (SQ4) is the most suitable for pulping as minimal effects on pulp yield and viscosity were observed for this species. Although the handling of the material may have varied from the previous chapter, the effects of storage on the wood chips can be seen with an increase in simple sugars and decrease in lipophilic extractives indicating degradation of the material.

Table 4.2: Characteristics of the pulp produced from wood chips before and after storage.

	SPY (%) ±SD		Viscosity (cP) ±SD		K-number ±SD		S10 ±SD		S18 ±SD		Alpha cellulose (%) ±SD		Copper number ±SD	
	Before	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*
E. dunnii (SQ3)	49.68±0.28	48.65±0.52 [a]	28.01±0.41	27.98±0.12 [a]	4.06±0.47	3.76±0.36	9.77±0.48	9.78±0.25	7.08±0.17	6.93±0.11	91.56±0.21	91.57±0.12 [a]	3.15±0.31	3.15±0.04
E. dunnii (SQ4)	49.96±0.36	48.02±0.15 [a]	90.26±0.85	81.56±0.23 [a]	4.39±0.15	3.34±0.01	10.04±0.62	10.54±0.52	7.95±0.32	7.23±0.32	91.29±0.99	90.08±0.26 [a]	2.93±0.32	3.04±0.10
E. grandis	49.46±0.25	47.54±0.18 [a]	74.04±0.25	43.73 ±0.32 [a]	4.58±0.62	3.85±0.15	11.04±0.21	9.85±0.22	8.20±0.26	6.29±0.15	90.38±0.51	91.67±0.52 [a]	3.06±0.26	2.61±0.32
E. nitens	50.78±0.48	44.77±0.11 [a]	131.7±0.42	44.77±0.30 [a]	5.04±0.65	2.31±0.28	10.06±0.55	11.36±0.56	7.36±0.12	6.09±0.22	91.01±0.22	90.81±0.21 [a]	2.47±0.35	2.53±0.11

SPY – screened pulp yield. Analyses conducted in duplicate. SD – standard deviation. p-value: a <0.05; b >0.05

Table 4.3: Quantification of lignin and hemicelluloses of the pulp produced from wood chips before and after storage.

	Glucose (%) ±SD		Xylose (%) ±SD		Mannose (%) ±SD		Klason lignin (%) ±SD		Acid-insoluble lignin (%) ±SD		Total lignin (%) ±SD	
	Before	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*
E. dunnii SQ3	90.76±0.56	84.69±0.36 [a]	2.82±0.21	5.27±0.41 [b]	1.49±0.45	0.83±0.18	2.24±0.22	2.02±0.32	1.69±0.42	1.61±0.32	3.93±0.33	3.63±0.15 [a]
E. dunnii SQ4	91.07±0.25	86.19±0.24 [a]	5.37±0.22	6.44±0.15 [a]	1.15±0.52	0.77±0.28	2.63±0.14	2.14±0.12	1.82±0.15	1.91±0.21	4.46±0.10	4.05±0.11 [a]
E. grandis	90.35±0.21	81.02±0.14 [a]	3.63±0.48	5.72±0.01 [a]	2.55±0.5	1.47±0.63	1.96±0.30	1.68±0.15	1.78±0.54	1.28±0.14	4.91±0.25	2.96±0.14 [a]
E. nitens	90.94±0.11	80.85±0.32 [a]	4.97±0.35	5.93±0.32 [a]	1.35±0.21	0.95±0.85	1.25±0.08	1.04±0.52	1.82±0.22	2.02±0.11	4.59±0.23	3.06±0.10 [a]

Analyses conducted in duplicate. SD – standard deviation. p-value: a <0.05; b >0.05

Following successful DNA isolation and PCR, DGGE analysis of the bacterial communities revealed that there was a significant increase in the diversity and abundance of bacteria present in the wood samples after 6 months of storage (Figure 4.3). The microbial community of harvested crops is transformed during storage and is particularly dependent on the water content of the samples (Barry Kay *et al*., 2009). The microorganisms were identified using a molecular weight marker of known microbial species previously isolated from *Eucalyptus* wood chips. *Bacillus thuringiensis* and *B. cereus* were prevalent in all of the samples after storage, except for *E. dunnii* (SQ4). The bacterial community detected in *E. dunnii* (SQ4) after storage was minimal compared with the other *Eucalyptus* species. Following storage of *E. grandis*, DGGE analysis revealed the presence of *Bacillus thuringiensis*, *Klebsiella* sp., *Pantoea ananatis*, *Micrococcus luteus* and *Inquilinus limosus*, while *Prauserella* and *Saccharomonospora* spp. were lost. *E. nitens* lost the *Inquilinus limosus* population and gained *Lecleria* sp., *Saccharomonospora* sp. and *B. cereus* populations.

Fungal diversity amongst the different *Eucalyptus* species was evident. For all *Eucalyptus* species, various fungal species observed prior to storage showed an increase in the intensity of their populations after storage. This was observed for the following fungal species; *Phialophora alba* (*E. dunnii* SQ3 and *E. grandis*), *Brachyalara straminea*, *Loddermayces elongisporus* (*E. dunnii* SQ4), *Aspergillus fumigatus* (*E. grandis*) and *Basidiomycota* sp. (*E. nitens*). In addition, fungal species such as *Paecilomyces variotii*, *Curvularia* sp. and *Pichia scolytii* were discovered only after storage in *E. dunnii* (SQ3), *E. dunnii* (SQ4) and *E. grandis*, respectively. This may seem anomalous, as at temperatures of -10°C to -12°C, microbial growth is minimal. However, some microorganisms remain inactivated during storage and then continue growing once thawed, leading to microbial degradation of the thawed product (Hui and Sherkat, 2005). Freezer storage of material may eliminate the risk of microbial activity during the storage period, but wood material is still at risk during cooling and thawing processes (Hansen, 1990). Petäistö (2006) found that the growth of grey mould, such as *Botrytis cinerea* which is commonly found in soil, on Norway spruce progressed at the beginning and/or thawing phase of cold storage at -3°C. Temperatures above zero before and after the cold-storage phase may intensify the threat of grey mould damage although the optimum temperature for *B. cinerea* is approximately 20°C (Petäistö, 2006).

Low concentrations of extractives in wood are associated with low resistance to decay, as extractives may function as a fungicide (Haupt *et al*., 2003; Latorraca *et al*., 2011; Severo *et al*., 2016). The increase in microbial populations and diversity may have been facilitated by the lower extractives content observed in the tested samples. The microorganisms identified here have been described in other studies on hardwood chips, including *Paecilomyces* sp., *Phialophora* sp., *Bacillus* sp., *Pseudomonas* sp. and *Micrococcus* sp. (Adair *et al*., 2002; Rajala *et al*., 2010; Kropacz and Fojutowski, 2014; Zhang *et al*., 2014; Szulc *et al*., 2017). The microorganisms detected in the wood chips of different *Eucalyptus* species have the potential to produce cellulolytic, hemicellulolytic and xylanolytic enzymes (Schmidt, 2006; Seo *et al*., 2013; Nandimath *et al*., 2016), which would affect the chemical composition of the wood material. *E. dunnii* (SQ4) exhibited the lowest diversity of indigenous microorganisms, which supports the idea of minimal degradation of cellulose fibres as evidenced by the high pulp yield and viscosity levels obtained for this species, making it the most suitable species for pulping.

Some of the bands on the gels were not associated with known species, so the microbial diversity may be underrepresented. However, the principle of DGGE that one band represents one genus/species is not always correct as a single point mutation occasionally results in two bands (Muyzer and Smalla, 1998; Miller *et al*., 1999; Adil, 2015). Therefore, there is the possibility of multiple bands for a single species due to single base pair mutations in their DNA.

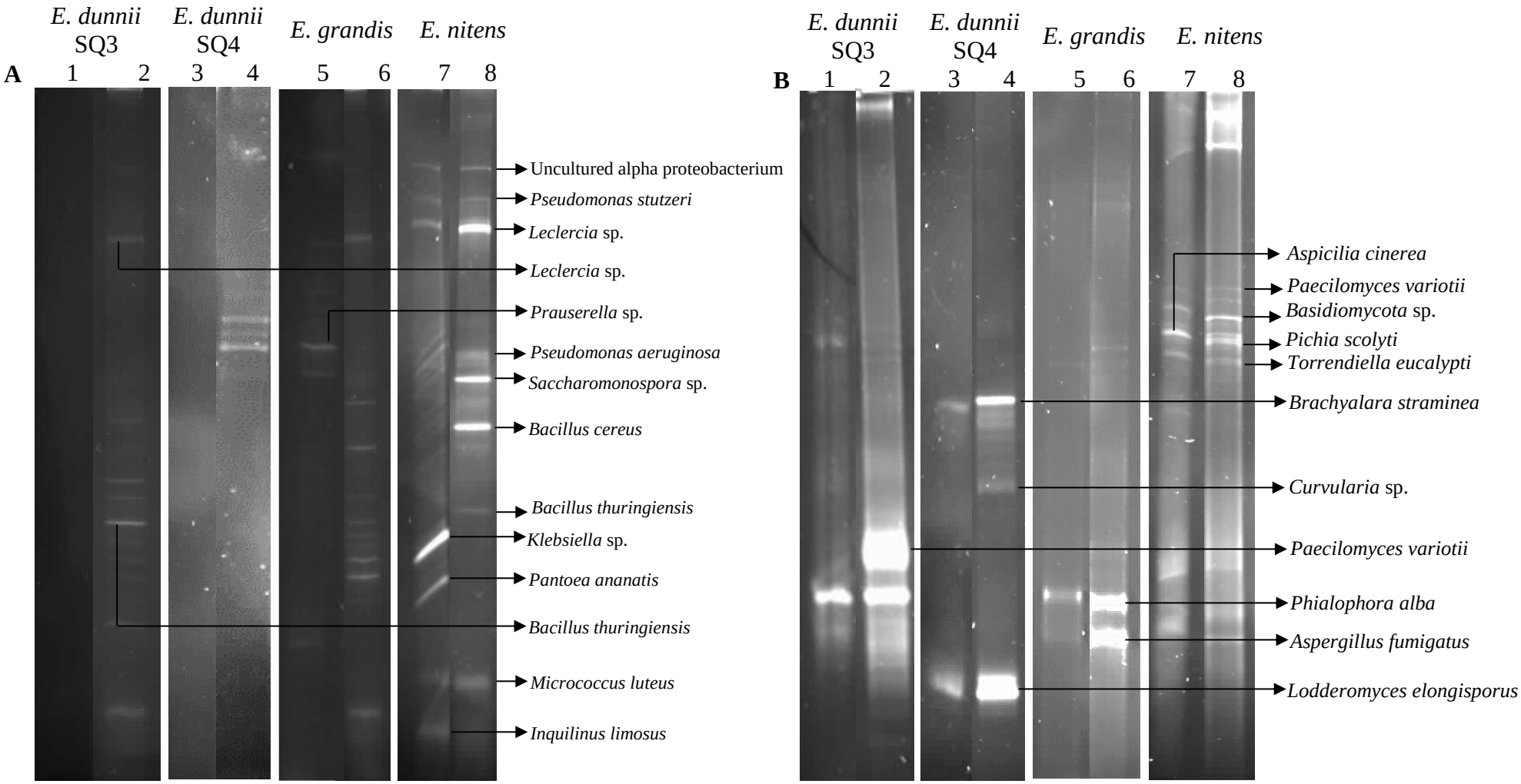


Figure 4.3: DGGE profiles of bacterial (A) and fungal (B) communities present in different *Eucalyptus* species before and after storage at -20°C. Lanes: 1- time zero *E. dunnii* (SQ3); 2- after storage *E. dunnii* (SQ3); 3- time zero *E. dunnii* (SQ4); 4- after storage *E. dunnii* (SQ4); 5- time zero *E. grandis*; 6- after storage *E. grandis*; 7- time zero *E. nitens*; 8- after storage *E. nitens*.

4.5 Conclusions

Storage of wood chip samples at -20°C influenced the chemical nature of *Eucalyptus* wood, particularly the lipophilic extractives. This effect was similar to that seen with traditional methods of seasoning used for the reduction of lipophilic extractives. In addition, changes in bacterial and fungal communities were observed after storage, which should be taken into consideration when conducting lab scale trials. It is therefore recommended that if storage is necessary under laboratory conditions, it should be not be for more than a period of 3 months at -20°C.

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CHAPTER FIVE

IDENTIFICATION OF LIPOLYTIC ENZYMES ISOLATED FROM BACTERIA INDIGENOUS TO *Eucalyptus* WOOD SPECIES FOR APPLICATION IN THE PULPING INDUSTRY

5.1 Abstract

This study highlights the importance of determining substrate specificity at variable experimental conditions. Lipases and esterases were isolated from microorganisms cultivated from *Eucalyptus* wood species, then concentrated (cellulases removed) and characterized. Phenol red agar plates supplemented with 1% olive oil or tributyrin was ascertained to be the most favourable method of screening for lipolytic activity. Lipolytic activity of the various enzymes were highest at 45-61 U/ml at the optimum temperature and pH of between 30 and 35°C and pH 4-5, respectively. Change in pH influenced the substrate specificity of the enzymes tested. The majority of enzymes tested displayed a propensity for longer aliphatic acyl chains such as *p*-nitrophenyl dodecanoate (C_{12}), myristate (C_{14}), palmitate (C_{16}) and stearate (C_{18}) indicating that they could be characterised as potential lipases. Prospective esterases were also detected with specificity towards acetate (C_2), butyrate (C_4) and valerate (C_5). Enzymes maintained up to 95% activity at the optimal pH and temperature for 2-3 h. It is essential to test substrates at various pH and temperature when determining optimum activity of lipolytic enzymes, a method rarely employed. The stability of the enzymes at acidic pH and moderate temperatures makes them excellent candidates for application in the treatment of pitch during acid-bisulphite pulping, which would greatly benefit the pulp and paper industry.

Key words: *Lipase, esterase, substrate specificity, pitch, pulp and paper*

5.2 Introduction

Lipase and esterase are two major classes of hydrolase enzymes (Kulkarni *et al*., 2013). Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyse the hydrolysis of long chain triacylglycerol substrates (>C_8) (Verger, 1997), whereas esterases (EC 3.1.1.x) catalyse the hydrolysis of glycerolesters with short acyl chains (<C_8) (Bornscheuer, 2002). The three-dimensional (3D) structures of both enzymes exhibit the characteristic α/β-hydrolase fold (Ollis *et al*., 1992) a definite order of α-helices and β-sheets. The catalytic triad is comprised of Ser-Asp-His (Glu instead of Asp for some lipases) and typically also a consensus sequence (Gly-x-Ser-x-Gly) is found around the active site serine (Kazlauskas, 1994; Dodsen and Wlodawer, 1998). These lipolytic enzymes have been isolated from plants, animals, and microorganisms (Kulkarni *et al*., 2013; Sharma and Kanwar, 2014), however, microbial lipolytic enzymes are reported to be more robust in nature than plant or animal enzymes (Ramakrishnan *et al*., 2013; Thomas and Kavitha, 2015). They are also appealing due to ease of manipulation and their low production cost (Kulkarni *et al*., 2013). Some microbial species reported to produce these enzymes include *Bacillus* sp. (Tehreema *et al*., 2011; Lailaja and Chandrasekaran, 2013; Nerurkar *et al*., 2013); *Pseudomonas* sp. (Kiran *et al*., 2008; Padhiar *et al*., 2011; Dey *et al*., 2014; Guldhe *et al*., 2015); *Burkholderia* sp. (Lau *et al*., 2011; Knapp *et al*., 2016); *Candida* sp. (Padhiar *et al*., 2011; Mouad *et al*., 2016); *Aspergillus flavus* (Padhiar *et al*., 2011); *Thermomyces lanuginosus* (Fernández-Lafuente, 2010) and *Rhizopus oryzae* (Rodrigues *et al*., 2016). Due to the versatility of lipases and esterases, they have various applications in industries such as detergents, starch and fuels, food, baking, pulp and paper, fats and oils, organic synthesis, leather and environmental application (Kirk *et al*., 2002; Margesin *et al*., 2002; Chang *et al*., 2004; Cavicchioli and Siddiqui, 2004; Ramteke *et al*., 2005; Vijayalakshmi *et al*., 2011; Imran *et al*., 2012; Gurung *et al*., 2013, Nigam, 2013).

In the pulp and paper industry, the presence of wood extractives plays a vital role. During pulping, pitch particles (composed of extractives such as triglycerides, fatty acid esters, glycosides, free and conjugated sterols) (Back and Allen, 2000) tend to coalesce to form black pitch deposits in the pulp and on machinery which has a negative impact on the process and quality of pulp (Gutiérrez *et al*., 2009a; 2010). Sulphite pulps (acidic) in particular retain greater amounts of extractives in relation to kraft pulps (alkaline), as the alkaline method disbands and dissolves the wood resin (Sithole *et al*., 2010). The production of dissolving pulp, which is a high-grade cellulose pulp, is generated using the acid-bisulphite method.

Traditional methods for the control of pitch include seasoning and the addition of chemicals such as alum, talc, ionic or non-ionic dispersants and cationic polymers (Allen *et al*., 1993; Maher *et al*., 2005; McLean *et al*., 2011). The biotechnological approach of using enzymes for pitch control is an alternative choice, especially for removal of glycerides. The treatment of pulp with lipases has been effective in reducing triglycerides (TG), however, steryl esters (SE) are frequently at the source of pitch formation (Gutiérrez *et al*., 2009b). As seen in Figure 5.1 the removal of fatty acids, resin acids and sterols surrounding the TG and SE compounds, would be necessary to help destabilize the pitch particle for degradation. Nonylphenol ethoxylates (NPEs) are the best chemicals for removing pitch components in chemical pulping. NPE is an amphipathic compound, meaning they have both hydrophilic and hydrophobic properties, allowing them to surround non-polar substances such as pitch compounds, thus isolating them from water and allowing easy removal. Unfortunately, their use is frowned upon due to their estrogen mimicking effects. Indeed, their use has been banned in North American and European chemical pulp mills as pulp handlers in European markets are reluctant to handle pulps treated with NPEs (Sithole and Pimentel, 2009; Sithole *et al*., 2010). Also, the residual NPE in sulphite pulps are undesirable since the pulps are commonly used in pharmaceutical and food applications. Based on a mill study conducted by Sitholé *et al*. (2010) it was suggested that the inclusion of an enzyme to target residual steryl esters could deliver a strategic solution to removing the extractives present in sulphite pulps.

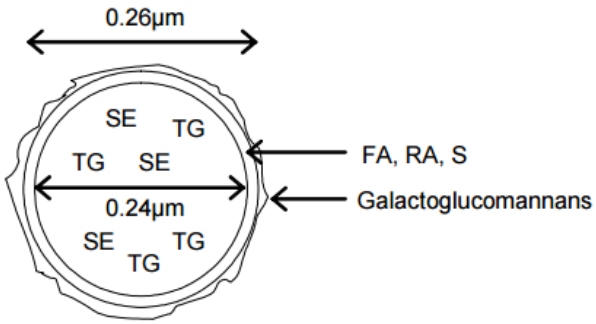

Figure 5.1: Sterically stabilized pitch particle. SE; steryl esters, TG; triglycerides. FA; fatty acids, RA; resin acids, S; sterols (Adapted from Sundberg *et al*., 1996; Qin *et al*., 2003).

Oxidative enzymes such as laccases have also been implemented in the degradation of various lipophilic extractives such as triglycerides, free and conjugated sterols, fatty acids and resin acids (Bajpai, 1999). Laccases are typical for white-rot fungi and have been described as prime lignin degraders. Treatment of wood or pulp with these enzymes could offer a dual advantage

in the company of redox mediators (Paice, 2005; Dube *et al*., 2009). Redox mediators facilitate laccase removal of residual lignin, in conjunction with extensive degradation of extractives (Gutiérrez *et al*., 2006). A decrease in kappa number and improved pulp brightness can also observed (Gutiérrez *et al*., 2006; Gutiérrez *et al*., 2009b; Gutiérrez *et al*., 2011).

The enzymes characterized in this study are for application in the pulp and paper industry, for reduction or elimination of pitch deposit formation during pulping. Previous studies have reported the incomplete degradation of pitch by lipases (Gutiérrez *et al*., 2009b; Sitholé *et al*., 2010), hence we are confident that the inclusion of esterases will assist in targeting the side groups that are theoretically present once the longer chain acyl chains (triacylglycerides) have been degraded by lipases. Lipases, esterases and laccases were included as part of this study and were selected based on their stability and activity at temperatures and pH levels employed during the acid-bisulphite pulping of *Eucalyptus* wood species. To our knowledge, the lipolytic enzymes produced by microorganisms indigenous to *Eucalyptus* sp. wood have not been previously investigated. The results of the present study will provide more information on the characteristics of these enzymes and their potential for reduction of pitch components in pulps. For this study it was important to include different types of enzymes that could benefit the pulping process. Therefore purification of the enzymes of interest was not necessary, as a cocktail of enzymes (excluding cellulases) is required and ideal in this study for the removal or degradation of all unwanted compounds (excluding cellulose). Combinations of hemicellulases, ligninases and other accessory enzymes are known to be essential for hydrolysis of plant biomass (Robl *et al*., 2016). It was also important to test the effects of various conditions on substrate specificity as most researchers focus only on the pH and temperature optima of the enzyme and thereafter test substrate specificity at optimum conditions. Neglecting to investigate the effects of pH and temperature on substrate specificity of enzymes could have drastic implications for its efficiency and effectiveness. Therefore, the aim of this study was to screen indigenous microflora from *Eucalyptus* species for lipolytic activity and to determine the effects of pH and temperature on the hydrolysis of different substrates of these lipolytic enzymes (lipases, esterases and laccases).

5.3 Materials and Methods

5.3.1 Isolation and identification of bacterial and fungal cultures

Five grams of wood chips from a commercial wood chip pile (composed of *E. dunnii*, *E. nitens*, *E. grandis*, *E. smithii*, *E. globulus*, *E. macarthurii* and a few hybrids) and individual *Eucalyptus* spp. were thoroughly washed by vortexing with 5 ml of phosphate buffer (pH 8.0) for 5 min. The washings were serially diluted and spread onto nutrient agar (NA) and potato dextrose agar (PDA) (Merck, South Africa) and incubated at 37°C and 40°C for 1 and 5 days, for the growth of bacteria and fungi, respectively. Colonies were selected based on morphological features; size, shape, pigmentation, margin, consistency and elevation, and sub-cultured till pure isolates were obtained (Ramnath *et al*., 2014). DNA was extracted from isolates and 16S rRNA and 18S rRNA for bacteria and fungi, respectively, were amplified according to Ramnath *et al*. (2014). Following PCR, the amplicons were sequenced (Inqaba Biotech, South Africa), and the sequences edited and entered in the Basic Alignment Search Tool (BLAST) algorithm (Altschul *et al*., 1990) for identification of microorganisms.

5.3.2 Optimization of plate screening assays for lipolytic activity

There are a number of methods currently available for the screening of lipases and esterases. However, they vary in sensitivity, cost and ease of preparation. In this study a few methods were tested and evaluated (Table 5.1).

Table 5.1: Screening methods implemented for the detection of lipases and esterases.

Screening Method	Enzyme Activity	Principle	Reference
Basal media agar supplemented with tributyrin	Esterase	Degradation of tributyrin is visualized by zones of hydrolysis represented by clear halos around the point of inoculation.	Kaiser *et al*. (2006)
Rhodamine B agar supplemented with olive oil	Lipase	Enzyme activity detected by the presence of luminous orange halos around the point of inoculation under UV at 350 nm. Fluorescence is due to complexes of rhodamine B with the free fatty acids released from the olive oil by lipases.	Kouker and Jaeger (1987)
Phenol red agar supplemented with olive oil or tributyrin	Lipase/esterase	Drop in pH of the media from 7.3 to a more acidic pH results in a colour change from red to orange/yellow. Increase in acidity is due to release of fatty acids following lipolysis.	Singh *et al*. (2006)
Tween 20 or Tween 80 agar	Lipase/esterase	Hydrolysis of tween releases fatty acids which binds with the calcium in the medium and precipitates as calcium salt, which is visualized as insoluble crystals around the point of inoculation.	Kumar *et al*. (2012a)

All strains were pre-cultivated in Luria-Bertani (LB) medium and malt extract broth for bacteria and fungi, respectively. For detection of esterase activity, a basal medium containing 0.5% (w/v) peptone, 0.3% (w/v) yeast extract and 2% bacteriological agar (pH 7.0) supplemented with 1%, 2% and 5% tributyrin was used. Five millimetre wells were bored into the agar plates and inoculated with 50 µl of pure bacterial cultures. Plates were incubated at 37°C for 48 h. After incubation the isolates were observed for zones of hydrolysis (clear halos) around the colonies. Lipase activity was screened for on olive oil/rhodamine B agar plates. Rhodamine B (1 mg/ml; Sigma Chemical Co., Munich, Germany) was dissolved in distilled water and filter-sterilized. The agar plates contained 8 g nutrient broth, 4 g sodium chloride, 10 g agar (per litre) (pH 7.0). After autoclaving the medium was cooled to 60°C, 31.25 ml olive oil and 10 ml of Rhodamine B solution (0.001% [wt/vol]) was added and stirred vigorously for 1 min. The medium was allowed to stand for 10 min to reduce foaming before pouring into sterile petri dishes. Lipase production was detected by irradiating plates with UV light at 350 nm (Kouker and Jaeger, 1987). Due to difficulty encountered with reading the screening plates using the above mentioned methods, two additional screening methods were tested, viz., assay with phenol red and tween agar plate screenings. Phenol red olive oil/tributyrin agar plates were prepared as follows (g/L); 0.01% (w/v) phenol red, 0.1% (w/v) $CaCl_2$, 1% (v/v) substrate, 2% (w/v) agar and pH adjusted to 7.3-7.4 with 0.1 N NaOH (Salihu *et al*., 2011). Organisms were inoculated onto the phenol red agar plates supplemented with 1% substrate and incubated at 37°C for 2-4 days. The principle behind this assay is that a slight drop in pH from 7.3 (end point of the phenol red dye) to a more acidic pH will result in a change of colour from red to orange. The increase in acidity is due to the release of fatty acids following lipolysis (Rai *et al*., 2014). A precipitation test using Tween 20 and Tween 80 agar plates was carried out to confirm lipolytic activity. Tween substrate plates were prepared as follows (g/L); 10 g peptone, 5 g $NaCl_2$, 0.1 g $CaCl_2.2H_2O$, 20 g agar and 10 ml (v/v) Tween 20/80 (Kumar *et al*., 2012a). This method is based on the principle of calcium salt precipitation. The hydrolysis of Tween releases fatty acids which bind with the calcium in the medium to form insoluble crystals around the point of inoculation. Tween 80 is used for the detection of lipases as it contains esters of oleic acid, whilst Tween 20 is used for esterases as it contains esters of lower chain fatty acids (Kumar *et al*., 2012a). The organisms were inoculated onto the plates and incubated at 37ºC for 2-4 days. A white precipitation around the boundary of the colony was indicative of lipase activity (Rai *et al*., 2014). To establish cellulase activity, substrates specific for the detection of exoglucanase (1% (w/v) avicel) and endoglucanase (1% (w/v) carboxymethyl

cellulose (CMC)) were used to screen isolates on NA and PDA agar plates for bacteria and fungi, respectively. All screening assays were performed in duplicate.

Fungal isolates were screened for laccase activity on PDA plates supplemented with 0.2% bromophenol blue (Singh and Singh, 2010) (Merck, South Africa). Plates were incubated at 40°C for 5 days, and then visually examined to evaluate the decolourizing ability of the fungal enzymes. To establish cellulase activity, substrates specific for the detection of exoglucanase (1% (w/v) avicel) and endoglucanase (1% (w/v) carboxymethyl cellulose (CMC)) were used to screen isolates on NA and PDA agar plates, for bacteria and fungi, respectively. All screening assays were performed in duplicate.

5.3.3 Enzyme assays

Lipolytic activity was determined spectrophotometrically by measuring the release of *p*-nitrophenol. *p*-Nitrophenyl (*p*-NP) esters with various lengths of aliphatic acyl chains were used to determine esterase: *p*-NP acetate (C_2), *p*-NP butyrate (C_4), *p*-NP valerate (C_5) and lipase: *p*-NP octanoate (C_8), *p*-NP dodecanoate (C_{12}), *p*-NP myristate (C_{14}), *p*-NP palmitate (C_{16}), and *p*-NP stearate (C_{18}) activity. The substrate mixture consisted of 0.5 mM *p*-NP substrate in methanol, 50 mM Tris-HCl buffer (pH 8.0) and 0.1% Triton X-100. The standard assay mixture contained 200 µl of substrate mixture and 20 µl of the crude supernatants, which were incubated at 37°C for 1 hour. The enzyme activity was determined by measuring the release of *p*-NP at an absorbance of 405 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 nM of *p*-NP per min under the assay conditions. Lipase/esterase and laccase activity was calculated from the formula derived from the Beer-Lambert Law:

$$Enzyme\ activity\ (U.ml^{-1}) = \frac{\Delta AV}{\varepsilon tv}$$

where, U is the unit of enzyme activity; the ΔA is the change in absorbance over time; V is the total volume of reaction mixture (ml); ε is the molar extinction coefficient in $nM^{-1}.cm^{-1}$; t is the incubation time in minutes, and v is the volume of the enzyme in the assay mixture (ml) (Desai *et al*., 2011). The appropriate extinction coefficient for each substrate under these assay conditions was used to calculate activity (Hu *et al*., 2010).

Laccase activity was determined based on the oxidation of syringaldazine substrate according to a protocol from Sigma-Aldrich (USA) (Ride, 1980). The assay mixture (1 ml) contained 733 µL of acetate buffer (100 mM, pH 4.0/5.0) and 167 µL of laccase enzyme extract. The reaction vessels were equilibrated to 37°C and absorbance monitored at 530 nm until constant. Thereafter, 100 µL of 0.216 mM syringaldazine was added to the assay (to begin the reaction), followed by immediate mixing by inversion. The assays were incubated for 10 min and the increased absorbance was recorded using a UV-1800 Shimadzu UV Spectrophotometer (Japan). Production of the corresponding quinone was monitored at 530 nm (ε530 = 65 000 M-1.cm-1). One enzyme unit is defined as the amount of enzyme that will oxidise 1 µmol of syringaldazine per min, under the assay conditions (Wang *et al*., 2010). The dinitrosalicylic acid (DNS) assay was used to determine cellulase activity by detecting reducing sugars which are liberated by the hydrolytic action of endo and exo-glucanase on different cellulose substrates (avicel and carboxymethylcellulose) (Bailey *et al*., 1992).

5.3.4 Effects of temperature and pH on lipase/esterase activity and stability

The effect of temperature on enzyme activity was determined by conducting assays at incubation temperatures ranging from 25 to 50ºC (with 5°C increments) and various *p*-NP esters as substrates (Bülow and Mosbach, 1987). Temperature stability of partially purified enzyme was determined by incubating the enzyme at various temperatures (25 to 50ºC) and estimating residual enzyme activities after incubation for 30 min, 1, 1.5, 2, 2.5, and 3 h. The effect of pH on enzyme activity was determined by assaying enzyme activity over a pH range of 3.0-12.0 using *p*-NP esters as substrates (Bülow and Mosbach, 1987). Citrate–phosphate buffer (pH 3.0 to 6.0), Tris–HCl buffer (pH 7.0 and 8.0), Carbonate–bicarbonate buffer (pH 9.0 and 10.0) and sodium-bicarbonate and sodium-phosphite buffer (pH 11.0 and 12.0) were used as buffer systems. Stability of the purified enzyme over a range of pH was also determined by measuring the residual activity after incubating 200 µl of the enzyme in 1800 µl of the above mentioned buffer systems (pH 3.0 to 12.0) for 3 h at the optimum temperature. Absorbance was read at 405 nm (Lailaja and Chandrasekaran, 2013).

5.3.5 Production of crude enzyme extracts

The selected bacterial isolates were grown in basal medium containing 0.5% (w/v) peptone and 0.3% (w/v) yeast extract supplemented with 1% tributyrin. Flasks were incubated at 37°C for 24 h at 180 rpm. Cells were harvested by centrifugation at 7850 x *g* for 10 min. The cell pellet

was then resuspended in lysis buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) and disrupted by ultrasonic treatment for 10 min in 10 second intervals. The cell lysate was centrifuged at 7850 x *g* for 10 min at 4°C, and the supernatant was recovered to test intracellular activity. To test extracellular activity the cell-free supernatant was collected and concentrated 10-fold by ultrafiltration with an Amicon system (Millipore, Massachusetts, USA) using first a 3 kDa cut-off membrane, after which a 50 kDa cut-off membrane was used on the concentrated sample to remove proteins larger than 50 kDa.

5.3.6 Native-PAGE and SDS-PAGE

Protein sizes were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as outlined by Judd (1996). Samples were electrophoresed by Native-PAGE (no SDS included) and SDS-PAGE in 12% polyacrylamide gels according to the method of Laemmli (1970). Protein concentration was determined using the Bradford assay (Bradford, 1976).

Native SDS-PAGE was utilized to confirm removal of potential cellulases. To identify endo and exo-glucanases, 12% native-PAGE gels containing 1% avicel and carboxymethylcellulose, respectively, (prepared in 50 mM phosphate buffer pH 7.0) were prepared. Following electrophoresis at 100 V for approximately 90 min at room temperature, the gel slab was cut in two halves; one half was stained using 0.5% Coomassie Brilliant Blue R250 (Sigma-Aldrich, Germany) to determine the size of the proteins and the other portion was used to detect enzyme activity. The gel for activity staining was washed with 50 mM phosphate buffer (pH 7.0) for 5 min, followed by staining in Congo-Red solution (0.1%, [w/v]) for 15 min. The gel was then destained with 1 M NaCl to visualise the clearing zone of hydrolysis, and then fixed with 0.5% (v/v) acetic acid (Govender *et al*., 2009).

5.3.7 Statistical analysis

Results shown here are the means of three independent determinations. Standard deviations for each of the experimental results were calculated using Microsoft Excel software and represented as error bars.

5.4 Results and Discussion

Identification of isolated bacteria and fungi

A total of ten different bacterial strains were isolated using the traditional culture and identification method using 16S rRNA sequencing: three *Bacillus* spp., three different *P. aeruginosa* isolates, *Inquilinus* sp., *Micrococcus* sp., *Pantoea* sp., *Klebsiella*, *Streptomyces* sp. and *Cellulosimicrobium* sp. (Table 5.2) (all with a similarity index of more than 97%). *Bacillus* spp. were the predominant bacterial species (33%). Some of these genera such as *Bacillus, Pantoea, Klebsiella* and *Pseudomonas* have previously been identified in other woods (Bagley *et al*., 1978; Li *et al*., 1992; Clausen, 1996; Van Zyl, 1999; Castro *et al*., 2014; Miguel *et al*., 2016), whilst others such as *Inquilinus* and *Mucilaginibacter* have not been observed in woods. The two fungal isolates described in this study were identified as *Paecilomyces formosus* (F4) and *Phialophora alba* (X) using 18S rRNA sequencing. Both these fungal isolates had not previously been identified in *Eucalyptus* spp. wood.

Optimization of plate screening assays for lipase and esterase activity

From the different concentrations tested, one percent tributyrin (esterase activity) was optimal for bacteria isolated from the mixed wood sample (Table 5.2), however, 2% was optimal for bacteria from individual wood species (Table 5.3). Slight halos were observed for a few of the bacterial isolates in 5% tributyrin plates. Plate screening assays for lipase activity revealed minimum lipase activity for isolates from mixed wood species; however, for bacteria isolated from individual *Eucalyptus* species, 1% substrate concentration was optimal. Sixty-seven percent, 28% and 28% of the isolates displayed activity on 1%, 2% and 5% tributyrin plates, respectively. *B. firmus* was capable of hydrolysing all three concentrations of tributyrin, but largest halos were observed at 1% substrate concentration. *Micrococcus luteus*, *P. aeruginosa*, and *C. cellulans* were also identified as esterase producers. Eight percent, 63% and 22% of the isolates displayed activity on 1%, 2% and 5% tributyrin plates, respectively. *Curtobacterium flaccumfaciens, B. thuringiensis, B. cereus, Pantoea agglomerans* and *P. vagans* produced the greatest zones of hydrolysis indicating esterase activity, with a halo zone of 2-5 mm (Figure 5.2). Other studies have also had some degree of success with the use of tributyrin and olive oil/rhodamine B as substrates and methods for screening for lipolytic activity (Shu *et al*., 2009; Sirisha *et al*., 2010; Reyes-Duarte *et al*., 2012; Kumar *et al*., 2012b; Niyonzima and More, 2013; Veerapagu *et al*., 2013).

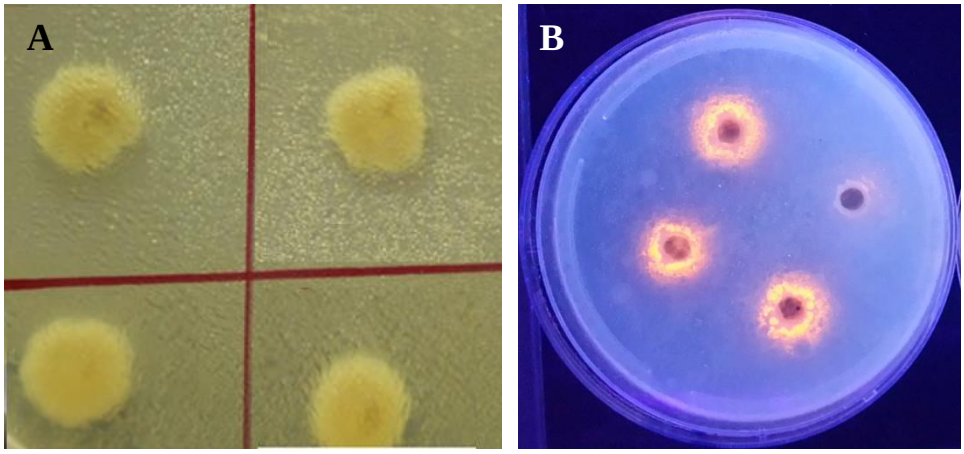

Figure 5.2: Plate screening assays for the detection of lipolytic activity using tributyrin and olive oil. **A-** tributyrin (1%) plate screening assays for the detection of esterase activity of pure bacterial isolates from *Eucalyptus* wood species, **B-** olive oil/rhodamine B (1%) plate screening assays for the detection of lipase activity of pure bacterial isolates from *Eucalyptus* wood species.

Due to difficulty encountered with visualization and of the clearing zones, additional assays such as phenol red and tween agar plate screenings were also performed to validate the results obtained. Both assays confirmed the results, however, the phenol red agar plate assay was more sensitive than the other assays. Distinct clearings for the phenol red plates and precipitation zones for the tween plates were observed (Figure 5.3). The phenol red screening plates were used to quantify activity (Tables 5.2 and 5.3).

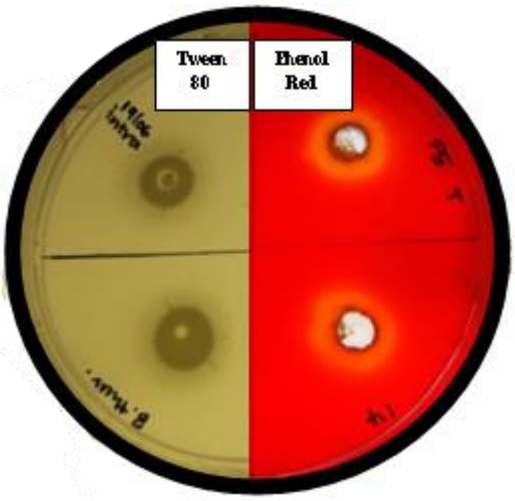

Figure 5.3: Plate screening assays for the detection of lipolytic activity using Tween 80 and tributyrin. Tween 80 (1%) agar plates (left) and tributyrin phenol red (1%) agar plates (right) for the detection of lipase and esterase activity, respectively, of pure bacterial isolates from *Eucalyptus* wood species.

Table 5.2: Lipase and esterase activity of bacteria isolated from a mixed *Eucalyptus* wood chip pile.

	Species	Accession Number	Esterase 1% Trb	Esterase 2% Trb	Esterase 5% Trb	Lipase 1% Oil	Lipase 2.5% Oil
B1	*Pseudomonas aeruginosa*	JX945659	+	+	-	-	-
B2	*Pseudomonas aeruginosa*	JX945660	++	-	-	-	-
B4	*Bacilllus firmus*	JX945657	+	+	-	+	+
B5	*Micrococcus luteus*	JX945661	+	+	-	+	-
B6	*Bacillus* sp.	JX945662	++	-	-	+	-
B7	*Inquilinus limosus*	JX945663	+++	-	+	-	-
B9	*Pantoea* sp.	JX945664	++	-	-	+	-
B10	*Klebsiella* sp.	JX945665	+	-	-	-	-
B12	*Bacillus ginsengihumi*	JX945658	++	++	+	+	-
B14	*Streptomyces costaricanus*	JX945666	-	-	-	-	-
B15	*Pseudomonas aeruginosa*	JX945667	-	-	+	-	-
B16	*Cellulosimicrobium cellulans*	JX945668	-	-	-	+	+

Key: + = slight halos (1-2 mm), ++ = medium halos (2-5 mm), +++ = large halos (>5 mm), Trb= tributyrin, Oil= olive oil,− = no halos

Table 5.3: Lipase and esterase activity of bacteria isolated from different *Eucalyptus* spp.

	Species	GenBank Number	Esterase 1% Trb	Esterase 2% Trb	Esterase 5% Trb	Lipase 1% Oil	Lipase 2.5% Oil
	E. dunnii						
DF1	*Mucilaginibacter* sp.	JF999998.1	-	-	+	-	-
DF2	Unidentified	-	-	++	+	-	+
DF3	*Curtobacterium flaccumfaciens*	HE613377.1	-	++	+	-	-
DF5	*Pantoea vagans*	CP002206.1	-	-	+	+	-
DF6	Unidentified	-	-	++	+	+	-
DF7	*Bacillus thuringiensis*	FN667913.1	-	++	+	+	+
DF8	Unidentified	-	-	+	+	-	-
	E. grandis						
G1	*Pantoea agglomerans*	FJ11844.1	-	++	+	-	-
G2	*Curtobacterium flaccumfaciens*	JF706511.1	-	++	-	-	-
G3	*Pantoea vagans*	CP002206.1	-	++	+	+	-
G4	Unidentified	-	-	-	+	-	-
	E. nitens						
N1	*Bacillus cereus*	JF758862.1	++	++	+	-	-
N2	*Pantoea* sp.	JN853250.1	-	-	+	-	+
N3	*Curtobacterium* sp.	HQ219967.1	-	+++	+	-	-
N4	*Bacillus cereus*	JQ308572.1	-	-	+	-	+
N5	*Bacillus cereus*	EU621383.1	-	-	+	-	-
N6	*Bacillus* sp.	EU162013.1	-	++	+	-	+
N7	*Bacillus thuringiensis*	FN667913.1	-	++	+	-	-

Key: + = slight halos (1-2 mm), ++ = medium halos (2-5 mm), +++ = large halos (>5 mm), Trb= tributyrin, Oil= olive oil,− = no halos

Lipases and esterases have been identified by screening microorganisms on various types of agar plates such as phenol red, rhodamine B, Tween 20/80 and Nile blue (Lanka and Latha, 2015). However varying degrees of success have been reported with the different methods of screening. An extracellular lipase isolated from a psychrotrophic *Pseudomonas* strain was discovered by screening on olive oil agar plates. Some researchers have found success with the rhodamine B dye method developed by Kouker and Jaeger (1987) (Lee *et al*., 1999; Kumar *et al*., 2012a; Robbani *et al*., 2013; Bakir and Metin, 2015; Laptip *et al*., 2016). However, others encountered difficulties in preparing the media, as well as visualizing activity of weaker lipases (Thomson *et al*., 2006). Based on the results from this study, the recommended method of screening for lipolytic activity would therefore be, phenol red agar plates supplemented with 1% olive oil or tributyrin.

In addition, isolates were also screened for cellulase activity. In the pulp and paper industry, the presence of cellulases has undesirable effects on the quality of pulp generated, particularly in the production of dissolving pulp (high grade cellulose pulp, >98% cellulose content). Potential cellulases would hydrolyse the cellulose fibres resulting in a decrease in alpha cellulose, thus impacting yield (Christov *et al*., 1999; Sindhu *et al*., 2006). Consequently, the detection and elimination of cellulase activity is important. Both the qualitative (screening plates) and quantitative (DNS assay) revealed negligible cellulase activity except for *C. flaccumfaciens* (Table 5.4). This was addressed by using spin columns with specific cut-off sizes to eliminate the larger proteins (>50 kDa) which were potential cellulases.

Native and SDS-PAGE

Native PAGE gels supplemented with carboxymethylcellulose and avicel were used to ensure that the minimal endoglucanase and exoglucanase activity observed was eliminated. Samples concentrated with the 3 kDa spin column were thereafter passed through a 50 kDa spin column to remove the larger proteins, presumably thought to be cellulases (Figure 5.4 and 5.5). However, it is important to note that most fungal laccases are also larger than 50 kDa. Nevertheless, it was imperative that the enzyme extracts characterized here, contain no cellulase activity that may degrade the cellulose fibers. All other accessory enzymes such as xylanases, laccases, and ligninases that may be present will positively contribute to the production of high quality cellulose pulp. Bacterial lipases and esterases generally have an expected protein size of between 15 and 45 kDa (Sharma *et al*., 2001). Proteins larger than 50

kDa were regarded as potential cellulases. Cellulases have a negative impact on the final pulp by reducing cellulose chains. An esterase as small as 1.57 kDa from *Bacillus stearothermophilius* has been described by Simoes *et al*. (1997). *Bacillus thuringiensis* has been reported to produce a 38 kDa phospholipase (Kupke *et al*., 1989).

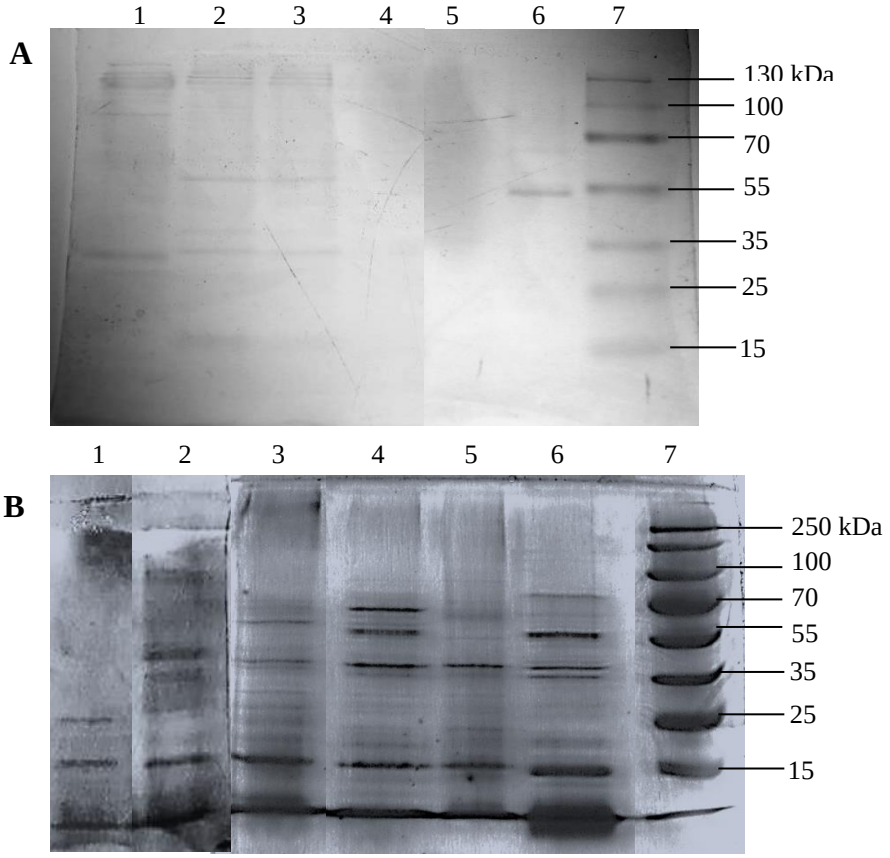

Figure 5.4: Concentration of isolates and removal of non-essential proteins. A (before concentration): 1-BT (*Bacillus thuringiensis*), 2-DF7 (*Bacillus thuringiensis*), 3-DF3 (*Curtobacterium flaccumfaciens*), 4-B9 (*Pantoea* sp.), 5-X (*Phialophora alba*), 6-F4 (*Paecilomyces formosus*), 7-marker. B (after concentration): 1-X, 2-F4, 3-BT, 4-DF7, 5-B9, 6-DF3, 7-marker.

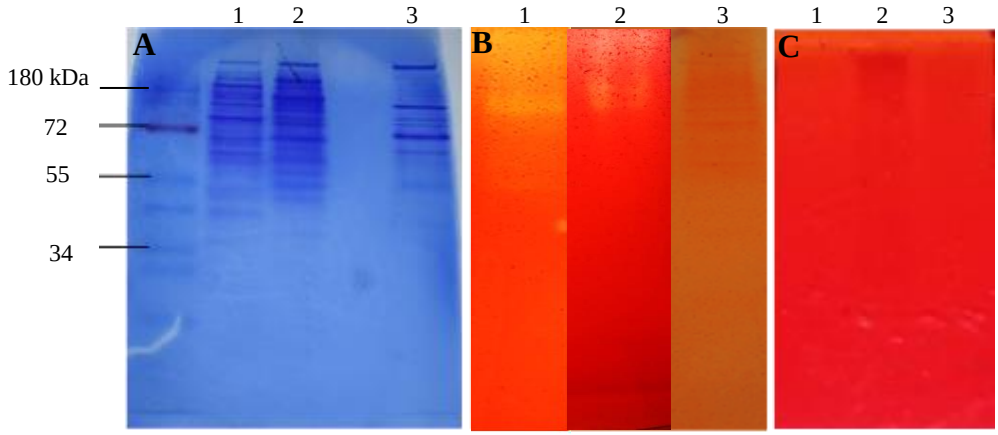

Figure 5.5: Native PAGE gels supplemented with carboxylmethylcellulose (CMC) to confirm removal of any potential endoglucanases. A: SDS-PAGE of crude enzymes, 1- DF7 (*Bacillus thuringiensis*), 2- DF3 (*Curtobacterium flaccumfaciens*), 3-BT (*Bacillus thuringiensis*). B: Native PAGE of crude enzymes, 1- BT, 2-DF3, 3- DF7. C: Native page of crude enzymes after partial purification, 1- BT, 2- DF3, 3- DF7.

Lipase and esterase activity

Upon evaluation of the preliminary screenings, the following isolates were selected for further study, DF3- *C. flaccumfaciens*, DF7- *B. thuringiensis*, B9- *Pantoea* sp. and BT- *Bacillus thuringiensis*. In addition to the bacterial isolates selected, two fungal isolates F4- *Paecilomyces formosus* and X- *Phialophora alba* were chosen based on similar preliminary plate screenings (data not shown) as well as previous studies on laccase activity (Gokul, 2014). The effect of initial pH on the extracellular and intracellular lipase/esterase activity of the selected isolates was investigated at pH 8.0 and 37°C with acetate and butyrate as substrates (generally selected for initial investigations). The results in Table 5.4 show a higher enzyme activity in the extracellular fractions of BT, DF7, and DF3, whilst B9 demonstrated higher activity in its intracellular fraction. Therefore, the appropriate fractions were used for further characterization of these enzymes. Fungi are known to produce extracellular enzymes to degrade polymers that cannot be absorbed (Sunesson, 1995), therefore it was not unexpected that the intracellular fraction yielded no enzyme activity.

Table 5.4: Lipase/esterase and cellulase activity (endoglucanase and exoglucanase activity using the DNS assay) and protein concentrations of the intracellular and extracellular fractions from the different isolates.

		Acetate (U/ml) ±SD		Butyrate (U/ml) ±SD		Protein Conc. (µg/ml) ±SD		Endoglucanase Activity (U/ml) ±SD	Exoglucanase Activity (U/ml) ±SD
		Ext.	Int.	Ext.	Int.	Ext.	Int.		
BT	*Bacillus thuringiensis*	5.55±0.32	5.24±0.01	9.75±0.41	5.78±0.45	212.9±0.24	1.57±0.28	0.057±0.50	0.043±0.32
DF7	*Bacillus thuringiensis*	10.71±0.15 [a]	5.16±0.11	10.98±0.85 [a]	4.34±0.41	414.3±0.11	1.84±0.35	0.021±0.32	0.013±0.22
B9	*Pantoea sp.*	5.12±0.35	6.75±0.52 [a]	2.82±0.54	5.27±0.25 [a]	1.69±0.18	25±0.54	0.012±0.24	0.015±0.11
DF3	*Curtobacterium flaccumfaciens*	10.35±0.14[a]	4.09±0.48	10.70±0.33 [a]	3.44±0.22	62.86±0.21	1.88±0.22	0.203±0.14	0.121±0.01
F4	*Paecilomyces formosus*	7.78±0.21 [a]	–	18.89±0.12 [a]	–	51.43±0.51	–	0.019±0.47	0.029±0.33
X	*Phialophora alba*	2.18±0.22 [b]	–	30.11±0.24 [a]	–	98.57±0.47	–	0.034±0.41	0.041±0.20

Ext – extracellular, Int – intracellular. Analyses conducted in duplicate. SD – standard deviation. p-value: a <0.05; b >0.05

Effects of temperature and pH on enzyme activity

Specificity of lipases are directed by a variety of properties such as type of substrate, position of esters fatty acids, stereospecificity and a combination of all four (Table 5.5). These include factors that alter the binding of the enzyme to the substrate, the molecular properties of the enzyme, and structure of the substrate (Jensen *et al*., 1983). Therefore, in the work reported here, it was vital to institute an experimental design to test the effects of pH and temperature on the hydrolysis of a range of substrates. A majority of reported studies elect to determine pH and temperature optima and then test the substrate specificity of the optimal expressed enzyme (Jung *et al*., 2002; Borkar *et al*., 2009; Gökbulut and Arslanoğle, 2013; Prasad, 2014); less detailed studies have demonstrated some effect of pH on substrate specificity of lipases and esterases (Ghatora *et al*., 2006; Ertuğrul *et al*., 2007). Ertuğrul and colleagues found that at pH 6.0, lipases from a *Bacillus* strain demonstrated highest activity towards the long chain triglyceride trimyristin (C_{14}), however, at pH 9.0, the shorter chain triglycerides such as tributyrin (C4) and triacetin (C2) provided higher esterase activity compared to the longer chain triglycerides (C_8-C_{14}) (Ertuğrul *et al*., 2007). This behaviour has also been reported for acetyl esterases from *Thermomyces lanuginosus* where no activity was observed against pNP-acetate at pH 9.0, however, activity at pH 4.0 was recorded (Ghatora *et al*., 2006). This reveals the varying degrees of lipase and esterase activity depending on the pH of the medium, which may be attributed to the presence of isoenzymes. Results of our study indicate that substrate specificity is affected by changes in pH and temperature.

Table 5.5: Types of specificity (Jensen *et al*., 1983).

Types of Specificity			
1. Substrate	Different rates of lipolysis of TG, DG and MG by the same enzyme	Separate enzymes from the same source for TG, DG and MG	
2. Positional	Primary esters	Secondary esters	All three esters or nonspecific hydrolysis
3. Fatty acid	Preference for similar fatty acids		
4. Stereospecificity	Faster hydrolysis of one primary ester as compared to the other		
5. Combinations of 1 to 4			

Key: TG- triacylglycerols, DG- diacylglycerols and MG – monoacylglycerols

The enzymes in this study show a preference for acidic conditions which is fairly uncommon amongst bacterial lipases. The majority of lipases are known to display their highest activities at a neutral or alkaline pH (Watanabe *et al*., 1977; Lesuisse *et al*., 1993; Wang *et al*., 1995; Lin *et al*., 1996; Choo *et al*., 1998; Abramić *et al*., 1999; Fojan *et al*., 2000; Kumar *et al*., 2005; Amoozegar *et al*., 2008; Sirisha *et al*., 2010; Guncheva and Zhiryakova, 2011). However, there are reports of the production of acidic lipases from bacteria although with varying amounts of activity. Ramani *et al*. (2010) described the production of an acidic lipase by *Pseudomonas gessardii* which had a maximum activity of 156 U/ml at a pH of 3.5. On the lower end of the scale, an acidic lipase produced by *Aeromonas* sp. demonstrated optimal activity of 0.7 U/ml at a pH of 6.0 (Liu *et al*., 2007).

The highest hydrolysis rates were obtained with potential lipases isolated from *B. thuringiensis* (BT and DF7) on *p*-NP-valerate (C_5), *p*-NP-octanoate (C_8), *p*-NP-dodecanoate (C_{12}), and *p*-NP-myristate (C_{14}), indicating the enzymes' propensity for longer acyl chain lengths (Figure 5.7). The *p*-NP esters of palmitic and stearic acids were also good substrates, however the shorter acyl chain esters such as acetate, butyrate and valerate were hydrolysed at a lower rate but with relatively comparable activity to the longer chain acyl chain substrates. This suggests that the enzymes from both *B. thuringiensis* isolates could potentially produce both lipases and esterases. Lipases from *Bacillus* species such as *Bacillus stearothermophilus* have been reported to hydrolyse synthetic substrates with acyl group chain lengths between C_8 and C_{12}

with optimal activity on C_{10} *p*-NP-caprate (Sinchaikul *et al*., 2001). On the other hand, a lipase isolated from *B. stearothermophilus* had a wide substrate specificity towards triglycerides with C_4 to C_{18} (Kambourova *et al*., 2003).

Initially, when the enzymes were tested at pH 8.0, greater activity was observed with *p*-NP acetate and *p*-NP butyrate (data not shown). However, at the optimal pH of 4.0 and 5.0, greater activity towards dodecanoate, myristate and palmitate was noted (Table 5.6). This suggests that changes in pH have an influence on the substrate specificity of the enzyme. These findings may be explained by the phenomenon of induced fit model. This model claims that the substrate may cause substantial transformation in the three-dimensional link of the amino acids at the active site and these modifications in protein structure initiated by a substrate will bring the catalytic groups into a suitable orientation for reaction (Koshland, 1958). Post and Ray (1995) showed that conformational changes can enhance the specificity of an enzyme with suboptimal catalytic efficiency.

The enzymes isolated from the other microorganisms (DF3, F4, X) showed a preference for dodecanoate, palmitate, myristate, octanoate and stearate substrates. The enzymes' specificity in relation to lipids with fatty acid residues of C_8-C_{18} chain length compellingly suggests that the enzymes described in this study could be true lipases. Enzymes isolated from *Pantoea* sp. (B9) could potentially be classified as esterases due to their specificity towards butyrate and valerate. The criteria used to differentiate esterases from lipases, is that esterases do not hydrolyse esters containing an acyl chain length of longer than 10 carbon atoms (Rhee *et al*., 2005). It is unusual for isolate B9 to prefer pNP-butyrate over pNP-acetate, such specificity is uncommon in nature, however, novel esterases from *Lactobacillus casei* and *E. coli* have previously demonstrated such catalytic preference (Choi *et al.*, 2004; Rhee *et al*., 2005; Ghatora *et al*., 2006). *C. flaccumfaciens* (DF3) displayed highest activity of 60 U/ml at 30°C with substrate specificity towards palmitate. *C. flaccumfaciens*- an endophytic bacteria associated with crops such as rice, potato, yam, tobacco, and cucumber- is capable of producing lipases (Araújo *et al*., 2008). This could be the first report of a characterized lipase from *C. flaccumfaciens* isolated from *Eucalyptus* wood.

Table 5.6: Optimized reaction conditions for lipolytic enzymes from bacteria and fungi

Isolate	Optimum pH	Optimum Temperature	Substrate Specificity
BT	5.0	30°C	Dodecanoate, Myristate, Octanoate, Acetate
DF7	4.0	35°C	Dodecanoate, Octanoate, Valerate, Butyrate
B9	4.0	35°C	Valerate, Dodecanoate, Butyrate, Octanoate
DF3	4.0	30°C	Palmitate, Dodecanoate, Myristate, Octanoate
F4	4.0	35°C	Dodecanoate, Palmitate, Octanoate, Myristate
X	5.0	30°C	Dodecanoate, Stearate, Myristate, Octanoate

Low activities were obtained for laccases (Figure 5.6), and this is expected as extracellular laccases from basidiomycete fungi are known to be produced in low amounts (Octavio *et al*., 2006). It is recognized that when fungi are grown in a medium of pH 5.0, laccases will be produced in excess, however most studies show that a pH range of 3.6 to 5.2 is suitable for enzyme production (Thurston, 1994; Ghatora *et al*., 2006; Madhavi and Lele, 2009). Optimal temperatures for laccase activity can vary significantly amongst organisms. There are reports of activities in the range of 25 to 80°C, with most enzymes having an optimum at 50 to 70°C (Snajdr and Baldrian, 2007). In this study the optimum temperatures of the lipases and esterases were 30 and 35°C, respectively. Therefore, laccase activity and stability were tested at these temperatures as the final application of this study would be to create an enzyme cocktail to treat pulp for effective removal of lipophilic extractives. Nevertheless, there was minimal variation in activity from the optimal pH and temperature of isolates F4 and X. Isolate F4 displayed 6.8% and 9.7% more activity at the optimal conditions of 40°C and pH 5.5, respectively. Isolate X showed 15.3% more activity at 50°C, whilst the optimal pH remained the same. Our results are comparable to another study where the maximum production of laccase from *Trichoderma harzianum* was observed at 35°C and pH 5.0 after 6 days (Abd El Monssef *et al*., 2016).

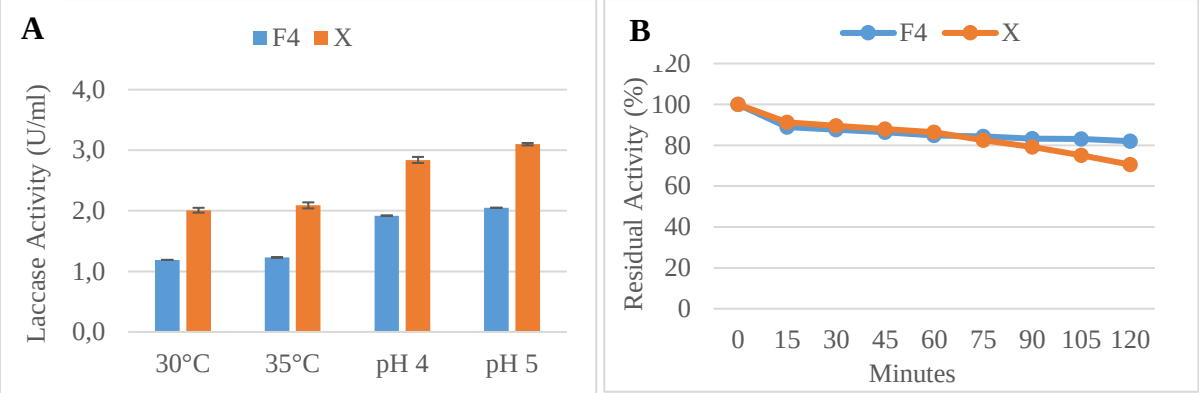

Figure 5.6: Activity and stability of laccases from fungal isolates F4 and X. A: activity at 30°C, 35°C, pH 4.0 and pH 5.0; B: enzyme stability at 35°C and pH 4.0 for F4 and 30°C and pH 5.0 for X.

In addition to demonstrating laccase activity (up to 3.1 U/ml) (Figure 5.6), *P. formosus* (F4) and *P. alba* (X) also demonstrated high substrate specificity towards dodecanoate at 35 and 30°C, respectively. Limited information has been published on the enzymes produced by *P. alba*, however, previous work indicate that xylanases from this microorganism were characterized with activity of up to 420 IU/ml (Mosina, 2013). The presence of enzymes from this microorganism could greatly assist in the reduction of pitch formation as well as the breakdown of xylan which will reduce the amount of chemicals used in the downstream processing of pulp (Gübitz *et al*., 1997; Dhiman *et al*., 2008; Gallardo *et al*., 2010; Brodeur *et al*., 2011). Laccases also have the ability to degrade both phenolic and non-phenolic compounds. Plant phenols released by hardwoods during pulping may have an inhibitory effect on enzyme activity (Upadhyay *et al*., 2016), therefore the inclusion of fungal laccases in this study could mitigate the inhibitory effects of phenolic compounds.

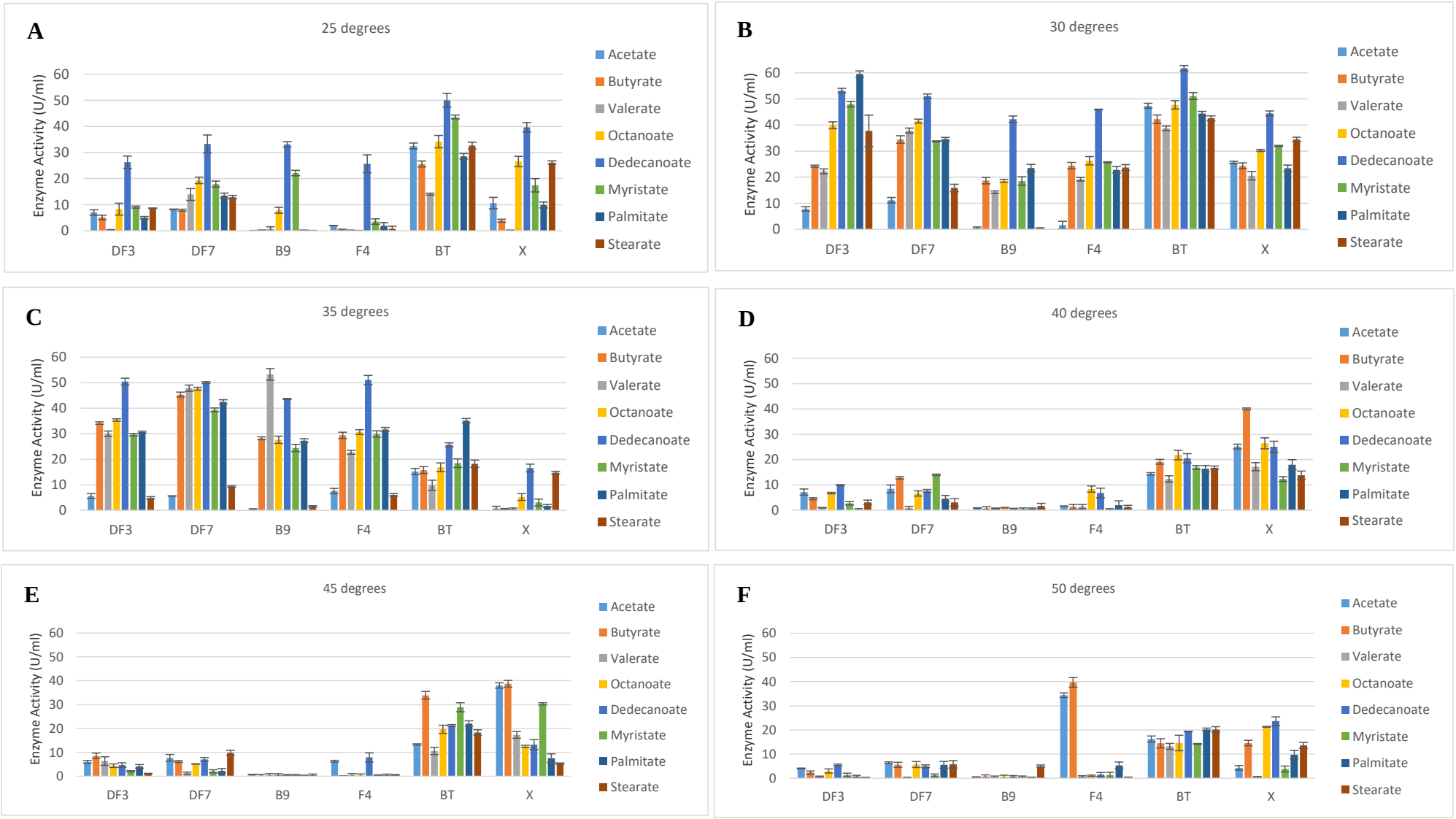

Figure 5.7: Effect of temperature (at optimum pH 4.0 or 5.0) on the activity of esterases/lipases from isolates DF3 (pH 4.0), DF7 (pH 4.0), B9 (pH 4.0), F4 (pH 4.0), BT (pH 5.0) and X (pH 5.0) on *p*-NP esters (C_2-C_{18}).

Effects of temperature and pH on stability of enzymes

In the pulp and paper industry, the enzyme pre-treatment of pulp is a tricky affair. When considering the addition of enzymes to pulp, a number of variables such as dosage, incubation period, temperature, pH and combination of enzymes needs to be taken into account. Time is money, so a minimal amount of time for enzyme pre-treatment would be optimal. Therefore, when determining enzyme stability, a shorter range for the incubation period was selected. Stability was however tested at 18 h to establish a broader range for incubation time. However, in industry, pre-treatment times of up to 18 h are not economically feasible.

The enzymes from the various microorganisms appear to be relatively stable over a period of 18 h at their optimal temperature. Enzymes from DF3, DF7, and X maintained their lipolytic activity over a period of 3 h with minimal loss in activity and retained at least 60% activity after 18 h (Figure 5.8). Enzymes isolated from BT, X, F4, DF3 and DF7 were fairly stable up to 2 h and thereafter a 30-40% decrease in activity was observed. More than 90% of the original activity was retained after 18 h for DF3 with dodecanoate and palmitate as substrates. Enzymes from DF7 and F4 retained more than 75% activity after 18 h with butyrate and valerate as substrates, respectively. B9 on the other hand, initially demonstrated high stability after 1 hour of incubation followed by a drop in activity to 70% after 3 h of incubation. These results fare well in comparison to other studies under similar conditions. For example, in a study by Eggert *et al*. (2001) a variant of an esterase (LipB, EC 3.1.1.1) from *B. subtilis* was found to be stable at pH 5.0 and 45°C for 1 hour.

Specificity of enzymes from DF3, DF7, F4 and X towards both the shorter and longer aliphatic acyl chains over the 18 hour incubation period indicates the broad range of substrates these enzymes are able to act upon. The stability of these enzymes is a desirable characteristic and would offer an advantage in potential industrial applications. However, for the purpose of this study the addition of these enzymes to pulp as a pre-treatment step would be optimal up to 2-3 h. Similar results were reported by Massadeh and Sabra (2011) where a lipase isolated from *Bacillus stearothermophilus* remained stable at a pH range of 7.0 to 9.0 after incubation for 1 hour at 30°C, with a residual activity remaining above 50% for pH 7.0, 8.0 and 9.0. However, extremophilic organisms are capable of producing hardier lipases. A thermostable lipase from *Geobacillus thermodenitrificans* IBRL-nra was found to have an optimal temperature of 65°C, at which it retained its initial activity for 3 h. Its highest lipase activity was reported at pH 7.0

and stable for 16 h at 65°C (Balan *et al*., 2012). Borkar *et al*. (2009) reported a lipase from a *P. aeruginosa* strain which was found to be completely stable at 55°C after 2 h at pH 6.9. A lipase from a psychrotolerant *P. fluorescens* strain was active at a temperature range of 15 to 65°C, however, it exhibited maximum activity at 45°C and pH 8.0. This enzyme demonstrated high stability, retaining 100% and 70% of its activity after an incubation period of 45 and 100 min, respectively, at 45°C and pH 8.0. This particular lipase also showed a broad substrate specificity acting on *p*-nitrophenyl esters with C_8-C_{18} acyl groups as substrates (Gökbulut and Arslanoğlu, 2013).

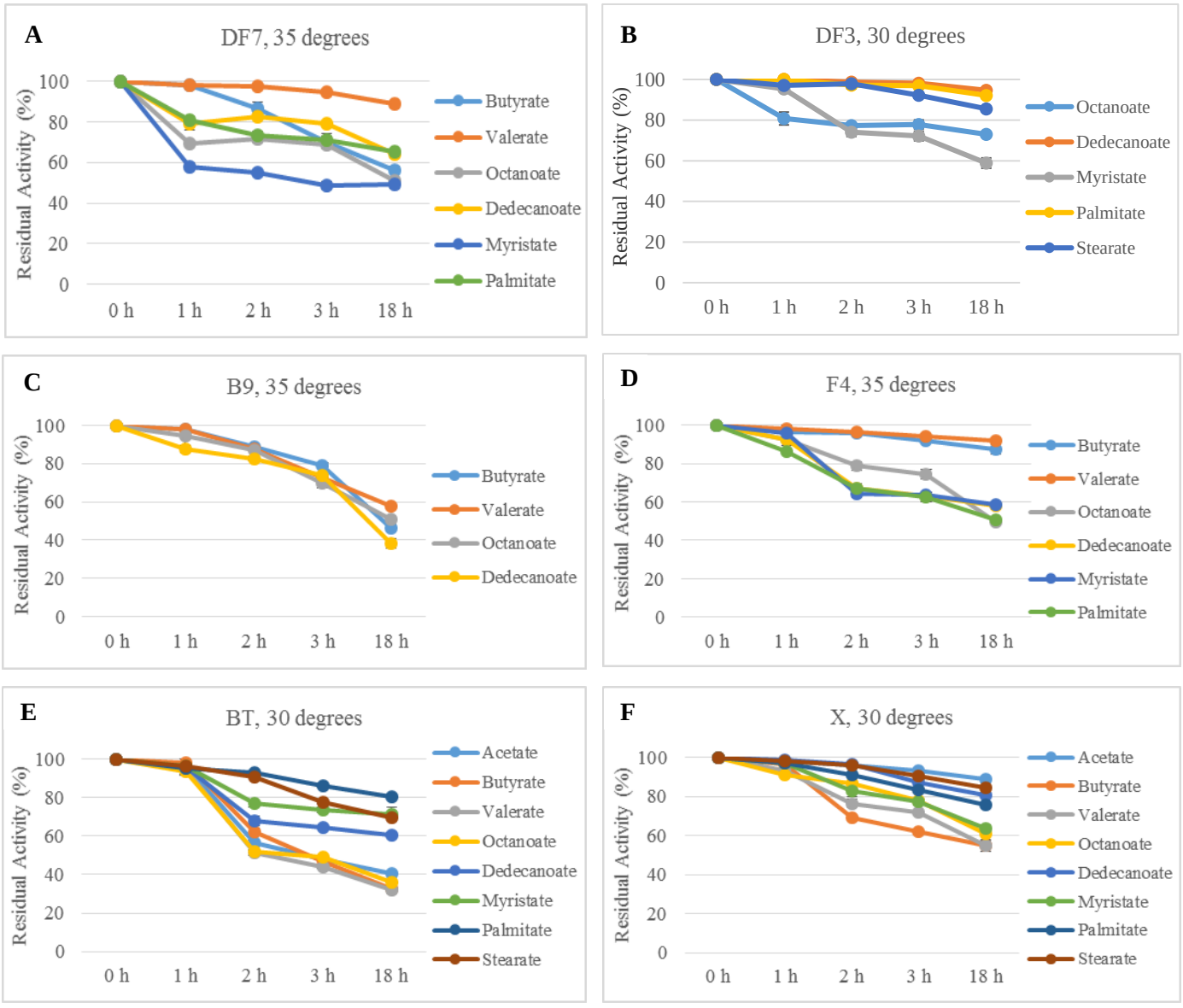

Figure 5.8: Stability of esterases/lipases from DF3 (A); DF7 (B); B9 (C); F4 (D); BT (E) and X (F) at optimum temperature and pH.

Many researchers elect to clone genes, coding for enzymes of interest in order to increase activity and improve production (Abdel-Fattah and Gaballa, 2008; Brod *et al*., 2010; Cedillo *et al*., 2012; Chriș *et al*., 2013; Lehmann *et al*., 2014). However, in industry this may not be a practical approach as screening of clone libraries involves conventional agar plate-based methods, which would require approximately 10 000 petri plates, each containing 10 000 clones. This is time-consuming and would greatly increase expenditure (Mathur *et al*., 2005). The enzyme activities observed in this study are comparable to, if not higher, than those of lipases and esterases which have not been modified or cloned (Table 5.7). The activities recorded in this study (up to 60 U/ml) could be invaluable in the reduction of pitch formation in the pulp and paper industry. In addition, the enzymes described here are indigenous to *Eucalyptus* wood species and have not been modified in any way, thus making them feasible and ideal for industrial applications. This is particularly the case for the acid-bisulphite pulping process used to produce dissolving pulp, as this process involves acidic pH process conditions which would be suitable for the enzymes described in this study.

Table 5.7: Comparison of optimal temperature and pH of some lipases and esterases isolated from different bacteria.

Isolate	Enzyme	pH	Temperature (°C)	Enzyme Activity (U/ml)	Reference
Bacillus thuringiensis	Lipase	5	30	62	This study
Bacillus THL027	Lipase	7	70	8.3	Dharmsthiti and Luchai (1999)
Bacillus sp. strain MC7	Lipase	8.5	60	3	Emanuilova *et al*. (1993)
Bacillus coagulans BTS-3	Lipase	8.5	55	1.16	Kumar *et al*. (2005)
Bacillus thermoleovorans ID-1	Lipase	7.5	65	0.52	Lee *et al*. (1999)
Geobacillus zalihae sp.	Lipase	6.5	65	0.15	Rahman *et al*. (2007)
Pseudomonas aeruginosa LP602	Lipase	8	55	3.5	Dharmsthiti and Kuhasuntisuk (1998)
Pseudomonas aeruginosa KM110	Lipase	7-10	45	0.76	Mobarak-Qamsari *et al*. (2011)
Pseudomonas gessardii	Lipase	3.5	30	156	Ramani *et al*. (2010)
Burkholderia multivorans	Lipase	7	30	58	Gupta *et al*. (2007)
Burkholderia multivorans V2	Lipase	8	37	14	Dandavate *et al*. (2009)
Burkholderia cepacia RGP-10	Lipase	7	50	60	Rathi *et al*. (2001)
Burkholderia sp. ZYB002	Lipase	8	65	22.8	Shu *et al*. (2012)
Enterococcus durans NCIM5427	Lipase	4.6	30	207.6	Vrinda (2013)
Streptomyces exfoliates LP10	Lipase	6	37	6.9	Aly *et al*. (2012)
Salinivibrio sp. strain SA-2	Lipase	7.5	50	5.1	Amoozegar *et al*. (2008)
Pantoea sp.	Esterase	4	35	53	This study
Anoxybacillus gonensis A4	Esterase	5.5	60-80	0.8	Faiz *et al*. (2007)
Bacillus sp. strain DVL2	Esterase	7	37	5.2	Kumar *et al*. (2012a)
Bacillus licheniformis	Esterase	8-8.5	45	12	Alvarez-Macarie *et al*. (1999)
Geobacillus sp. AGP-04	Esterase	8	60	3.62	Ghati and Paul (2014)
Geobacillus sp. DF20	Esterase	7	50	27.9	Özbek *et al*. (2014)
Lactobacillus brevis NJ13	Esterase	8	50	48.12	Kim *et al*. (2013)
Acaligens faecalis	Esterase	8	30	0.27	Poornima and Kasthuri (2016)
Burkholderia fungorum A216	Esterase	6.5	37	0.014	Jiao *et al*. (2014)
Achromobacter denitrificans strain SP1	Esterase	8	50	89.5	Pradeep *et al*. (2015)
Janthinobacterium lividum	Esterase	7	30	0.00568	Park *et al*. (2001)
Pseudomonas sp. KWI-56	Esterase	7.5	22	51.6	Sugihara *et al*. (1994)

5.5 Conclusions

In the present work, a cellulase-free cocktail of lipolytic and other enzymes was obtained from microorganisms indigenous to South African *Eucalyptus* wood chips. Lipases and esterases showed optimal activity at moderate temperatures (30 and 35°C) and acidic pH range (pH 4.0 and 5.0). The enzymes' stability and activity on a broad range of lipophilic substrates could lead to potential biotechnological applications in the removal of lipophilic components that cause pitch problems in the manufacture of high purity chemical pulps such as dissolving wood pulp. The inclusion of laccases have the potential to assist in further degradation of these problematic lipophilic compounds. Considering that the purpose of the study was not to purify the extracts, in so reducing activity and increasing the cost of production, the ability of the crude extract was evaluated as is, since the end application would be to produce and apply the enzyme extracts on-site at commercial pulping mills. The application of these enzymes produced by indigenous microflora will aid in reducing cost and is a greener alternative to chemical treatments. Future work will focus on applying these enzymes directly to the pulped wood chips and evaluating their potential to reduce the agglomeration of lipophilic compounds that cause pitch formation during pulping.

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CHAPTER SIX

HETEROLOGOUS EXPRESSION OF ESTERASES AND LIPASES FROM BACTERIA INDIGENOUS TO *Eucalyptus* spp. WOOD CHIPS USING CULTURE-DEPENDENT AND INDEPENDENT APPROACHES

6.1 Abstract

Culture-independent metagenomic approaches are novel methods that eliminate the use of limiting culture-dependent techniques. This study investigates both methods to clone and express lipolytic enzymes for application in the pulping industry to reduce pitch formation. The recombinant esterases characterized in this study belong to Carbohydrate Esterase Family 1 (CE1), based on sequence analysis. The acetylesterase activity was 147.8 U/ml with *p*-nitrophenyl valerate (C_5) at pH 4.0 and 30°C. Optimal carboxylesterase activity of 127.1 U/ml was seen with *p*-nitrophenyl butyrate (C_4) at pH 4.0 and 35°C. A significant increase in acetylesterase relative activity from 160 to 203% occurred in the presence of agents such as CTAB and K^+ ions. Preliminary pulping trials revealed a notable decrease in lipophilic extractives such as fatty acids and sterols that contribute to pitch formation. An overall lipophilic reduction of 63% and 78% was observed for pulp pre-treatments with acetylesterase and carboxylesterase, respectively. The recombinant enzymes were optimally active in the acidic pH range, remained relatively stable for up to 3 h, and were capable of reducing the lipophilic content of the pulp considerably, making them suitable candidate biopulping catalysts.

Key words: *Cloning, esterases, biopulping, substrate specificity*

6.2 Introduction

The number of microorganisms cultured to date represents only a small fraction (1%) of all microorganisms on earth, therefore a constant increase in the number of novel enzymes is expected (Rastogi and Sani, 2011; Nikolaivits *et al*., 2017). The metagenomic approach has been found to be a resourceful method for the isolation of novel and functional genes directly from environmental DNA libraries (Park *et al*., 2007; Banik and Brady, 2010; Lam *et al*., 2015). Some of these novel enzymes exhibit properties that make them attractive candidates for application in biotechnological and pharmaceutical industries (Alma'abadi *et al*., 2015; Coughlan *et al*., 2015; Thies *et al*., 2016; Krüger *et al*., 2017; Ribeiro *et al*., 2017). Enzymes that are active and stable in extreme conditions of pH, temperature or salinity are a valuable resource in industry (Barroca *et al*., 2017; Madhavan *et al*., 2017; Poli *et al*., 2017). They have various applications in biotechnology for food processing; production of paper, oil, detergents; and the medical and fine chemical industries (Haki and Rakshit, 2003; Lokko *et al*., 2017; Mishra *et al*., 2017). Most metagenomic studies have been correlated to community structure (Nealson and Venter, 2007; Mackelprang *et al*., 2011; Delmont *et al*., 2012), however with high throughput and next generation sequencing technologies, it is now possible to efficiently identify genes encoding enzymes for several bioconversion processes (Lee *et al*., 2008; Mardis, 2008; DeAngelis *et al*., 2010; Kim *et al*., 2013).

The discovery of unique enzymes from environmental samples can be achieved by screening metagenomic libraries using two main strategies: function-based or sequence-based screening (Li *et al*., 2009; Culligan *et al*., 2014; Lam *et al*., 2015; Jameson *et al*., 2017). A major drawback of function-based screening arises when heterologous host systems are used in the production of the enzyme (Padmanabhan *et al*., 2011; Rossum *et al*., 2013; Liebl *et al*., 2014; Rosano and Ceccarelli, 2014). In some instances, no protein is produced due to variation in codon usage or unstable mRNA secondary structure (Angov, 2011; Gaspar *et al*., 2013; Pasotti and Zucca, 2014). This technique may also be limited due to problems in identifying appropriate screening methods for the various enzymes (Banerjee *et al*., 2010), thus the sequencing method is preferred. Sequencing involves searching protein databases for similar sequences to those obtained from the metagenome in order to determine the enzyme which the sequence encodes (Tringe *et al*., 2005; Pearson, 2013; Sharpton, 2014). Due to complex microbial species present in natural environments, there are obstacles to acquiring full length gene sequences, termed contigs, which need to be large enough to contain complete open

reading frames (ORFs) coding for catalytic enzymes (Allgaier *et al*., 2010; DeAngelis *et al*., 2010).

Lipolytic enzymes such as lipases (EC 3.1.1.1, triacylglycerol hydrolases) and esterases (EC 3.1.1.3, carboxyl ester hydrolases) are known to catalyze both the hydrolysis and synthesis of ester compounds (Jaeger and Eggert, 2002; Bornscheuer, 2002; Gupta *et al*., 2004). Lipases and esterases are found in plants, animals and microorganisms (Barros *et al*., 2010; Andualema and Gessesse, 2012). *Pseudomonas* and *Bacillus* spp. are well-known bacterial sources of lipases and esterases (Gupta *et al*., 2004; Ramani *et al*., 2010; Gurung *et al*., 2013; Veerapagu *et al*., 2013; Kumar *et al*., 2016). Bacterial lipases are classified into six families, with Family 1 (true lipases) further divided into six subfamilies (Jaeger *et al*., 1999; Guerrero *et al*., 2013). Carbohydrate esterases are currently classified in 15 families in the Carbohydrate-Active Enzyme (CAZy) database based on their amino acid sequence and structural folds (Lombard *et al*., 2014). Carbohydrate Esterase Family 1 (CE1) include acetylxylan esterases (EC 3.1.1.72), feruloyl esterases (EC 3.1.1.73), carboxylesterases (EC 3.1.1.1), *S*-formylglutathione hydrolases (EC 3.1.2.12), diacylglycerol *O*-acyltransferases (EC 2.3.1.20) and thehalose 6-*O*-mycolyltransferases (EC 2.3.1.122) (Nakamura *et al*., 2017). Lipases and esterases are important in the pulp and paper industry to reduce or eliminate pitch deposit formation caused by the agglomeration of lipophilic compounds released from the wood material during pulping (Sithole, 2000; Jegannathan and Nielsen, 2013). These pitch deposits cause black spots in the pulp, reducing pulp quality and gumming up the machinery, resulting in increased mill shutdowns for cleaning and maintenance (Gutiérrez *et al*., 2010).

Several factors, such as toxicity of proteins to heterologous hosts and a requirement for chaperone proteins to achieve correct folding and functional lipase expression are reported to complicate lipase/esterase gene expression (Rosenau *et al*., 2004; Pauwels *et al*., 2006; Liu *et al*., 2013). Also, the low homology detected between different lipase genes renders PCR and cloning a complex process. One strategy for obtaining new lipase genes is to construct a library from a lipase-producing organism and screen recombinant hosts expressing a functional lipase (Zuo *et al*., 2010; Selvin *et al*., 2012; López-López *et al*., 2014; Yan *et al*., 2017). The recombinant clones producing lipases can be identified by direct detection of lipid hydrolysis (Li *et al*., 2009; Gricajeva *et al*., 2016; Sun *et al*., 2016) or fluorescent indicators such as Rhodamine B (Kouker and Jaeger, 1987; Lanka and Latha, 2015). Novel lipase and esterase

genes have previously been isolated using this method (Ferrer *et al*., 2005; Elend *et al*., 2006; Kim *et al*., 2006; Zheng *et al*., 2013; Peña-García *et al*., 2016).

In this study a two prong approach was implemented in the search for lipases and esterases. Initially a bacterial metagenome, obtained from *Eucalyptus* spp. woodchips was screened for genes encoding lipases and esterases. CLC Genomics Workbench (version 4.0.3; CLC Bio, Cambridge USA) was used for primer design followed by PCR for amplification of putative lipases/esterases using metagenomic DNA as template, ligation into a suitable vector, cloning, and screening for activity of recombinant enzyme. The second approach involved the screening, isolation, cloning and expression of a lipase and two esterases from *Bacillus* sp. (isolated from *Eucalyptus* spp.) for potential future application in the pulp and paper industry.

6.3 Materials and Methods

The culture independent and dependant techniques were implemented in this study (Figure 6.1). The culture independent technique involved analysis of next generation sequencing data and mining of the bacterial metagenome for potential lipolytic enzymes. The culture dependent technique on the other hand involved traditional microbiological and molecular techniques.

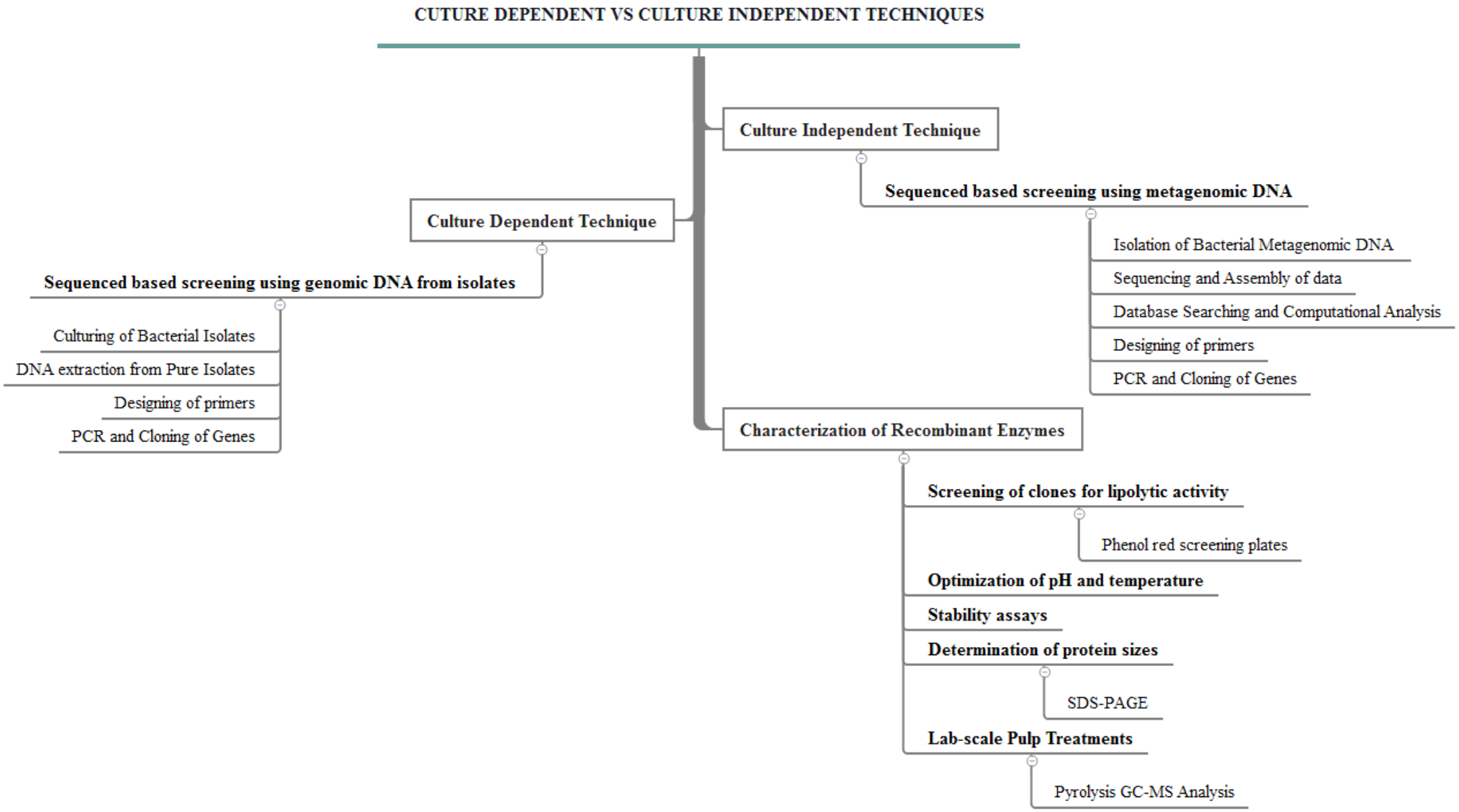


Figure 6.1: Schematic outline of the two approaches used to clone esterase genes and characterisation of the recombinant esterases produced.

6.3.1 Assembly and analysis of metagenomic data

Reads were assembled using the following default parameters on the CLC Genomics Workbench (version 4.0.3; CLC Bio, Cambridge USA): mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.5 and similarity 0.8 (Nnadozie, 2017, unpublished). Assembled nucleotide sequences were submitted using the BLAST search and retrieval system on the National Center for Biotechnology Information (NCBI) database. Those identified as lipases/esterases were further analysed. Open Reading Frames (ORFs) with the conserved catalytic domain GXSXG were located on specific contigs. Primers were designed using the CLC Bio program.

6.3.2 Features of the bacterial metagenome

Of the 4 183 586 sequences (totaling 828 041 196 bps), 96.3% were predicted to be protein features, of which 67.8% of features have been annotated using at least one of the M5NR protein databases, 32.2% have no significant similarities to the protein database (orfans) and 76.2% were assigned to functional categories. A total number of 1 854 727 (368 588 439 bases) raw reads were obtained, averaging 199 bp per read. An average length of 600 bp was obtained for 98 495 contigs. Approximately 510 bacterial species were discovered, and the predominant phyla were Proteobacteria (>80%), Bacteriodetes (>10%), Acidobacteria (>5%) and Actinobacteria (>5%).

6.3.3 Bacterial isolates

6.3.3.1 Culturing and DNA extraction

Five grams of wood chips from a commercial wood chip pile and individual *Eucalyptus* spp. were thoroughly washed by vortexing with 5 ml of phosphate buffer (pH 8.0) for 5 min. The washings were serially diluted and spread onto nutrient agar (Merck, South Africa) incubated at 37°C for 36 h (Merck, South Africa). Colonies were selected and purified from the spread plates based on size, shape, pigmentation, margin, consistency and elevation then purified (Ramnath *et al*., 2014). Genomic DNA was isolated from the pure bacterial isolates using the ZR Fungal/Bacterial DNA Kit (Zymo Research, United Sates) and the manufacturer's instructions.

6.3.3.2 Identification of isolates

Ribosomal RNA genes were amplified using the genomic DNA of pure cultures as template. The universal 16S primer set and amplification conditions are described in Table 6.1. Amplification reactions (50 µl) contained 1.25 mM $MgCl_2$, 0.125 µM forward and reverse primers, 0.2 mM dNTPs, 0.25 U SuperTherm Taq DNA polymerase (Southern Cross Biotech, South Africa), and approximately 200 ng of template DNA (measured with a NanoDrop 1000 Spectrophotometer, Thermo Scientific, USA). PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems, United States). The amplicons were electrophoresed on 1% agarose gels (SeaKem, United States) in 1× Tris-Acetate EDTA running buffer at 90 V for 45 min stained in 0.5 µg/ml ethidium bromide, visualized using a Chemi-Genius 2 BioImaging System (Syngene, United States) and positive amplicons sequenced (Inqaba Biotech, South Africa). The edited sequences were entered into the BLAST algorithm (Altschul *et al*., 1990) for identification of microorganisms.

6.3.3.3 Primer design, PCR and cloning

Several lipase and esterase gene sequences from *Bacillus* spp. and other strains identified from the metagenomic data were downloaded from the NCBI database and aligned using CLC Bio. Primers were then designed based on the consensus sequence and tested *in silico* to determine their effectiveness in amplifying specific regions. Lipolytic genes were amplified with *B. thuringiensis* genomic DNA (lab culture collection) and metagenomic DNA as template and synthesized primers and amplification conditions listed in Table 6.1. Amplification reactions (50 µl) contained 1.25 mM MgCl2, 0.125 µM forward and reverse primers, 0.2 mM dNTPs, 0.25 U SuperTherm Taq DNA polymerase (Southern Cross Biotech, South Africa), and approximately 20-200 ng of DNA template (NanoDrop 1000 Spectrophotometer, Thermo Scientific, USA). PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems, United States) and the amplified product was examined by electrophoresis. The amplicons were excised and purified with GeneJET Gel Extraction kit (Fermentas) and ligated into pJET 1.2/blunt cloning vector (Thermo Scientific). Heat shock transformations were performed and the recombinant plasmid DNA restricted to confirm insert and then sequenced. Nucleotide and protein sequence analysis was performed with the BLAST program, and homology alignments were performed with CLC Bio software. A three-dimensional model of the recombinant proteins was generated with the software used by the automated protein homology-modelling server SWISS-MODEL (Swiss Institute of Bioinformatics) (Kiefer *et al*.,

2009). The acetylesterase from *B. cereus* (PDB code 2qm0.1) and carboxylesterase from *B. cereus* (PDB code 2h1i.1) with sequence identities of 85% and 96%, respectively to the acetylesterase and carboxylesterase in this study, were used as templates during model building.

6.3.3.4 Expression and purification of recombinant esterases

Positive clones were inoculated into LB/amp broth and incubated overnight at 37°C, 200 rpm and plasmid DNA isolated using the Plasmid MiniPrep Kit (Fermentas). Inserts were then ligated to pET 22b and transformed into chemically competent *E. coli* BL21 (DE3) cells for expression. The transformants were grown in LB broth containing 50 μg/ml ampicillin at 37°C and 180 rpm. When the culture density reached 0.5 at OD_{600}, 1 mM IPTG (isopropyl-D-1-thiogalactopyranoside) was added, incubated again and sampled every hour over 6 h. Cells were harvested by centrifugation at 7850 x *g* for 10 min, resuspended in lysis buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) and disrupted by ultrasonic treatment for 10 min in 10 s intervals. The cell lysate was centrifuged at 7850 x *g* for 10 min at 4°C, the supernatant was recovered and used as the crude enzyme extract.

Table 6.1: Primers and PCR conditions.

Primer	Sequence	PCR Conditions
Metagenomic DNA		
BPhyLip forward primer	TTGCTCTTGACCAATGTACCTTTG	Initial denaturation step at 94°C for 5 min, 30 cycles at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min and the final extension at 72°C for 5 min, and preservation at 4°C
BPhyLip reverse primer	CTAGAAGTTGTTCCCCAGCATCCG	
BGluHp forward primer	CTGATGCGCTCGACGCGG	
BGluHp reverse primer	TCATGCTGCCTGCTCCTCATCG	
Bacterial DNA		
B. thuringiensis carboxylesterase forward primer	ATGAAATTAGCATCTCCG	Initial denaturation step at 95°C for 5 min, 30 cycles at 95°C for 1 min, annealing at 50°C for 30 sec, and extension at 72°C for min and the final extension at 72°C for 5 min, and preservation at 4°C
B. thuringiensis carboxylesterase reverse primer	TTACCAATCTAGTTGCTCCA	
B. thuringiensis serovar *kurstaki* carboxylesterase forward primer	ATGATGAAACATGTTTTTCA	Initial denaturation step at 95°C for 5 min, 30 cycles at 95°C for 1 min, annealing at 47°C for 30 sec, and extension at 72°C for 1 min and the final extension at 72°C for 5 min, and preservation at 4°C
B. thuringiensis serovar *kurstaki* carboxylesterase reverse primer	TCACCCATCGTTAATTGA	
B. thuringiensis BMB171 acetylesterase forward primer	ATGAGTCAAACAATAGGGA	
B. thuringiensis BMB171 acetylesterase reverse primer	TCAGTCTGCCAATATTTCC	
B. thuringiensis acetylesterase forward primer	ATGAGAATAAAACAGTTAAAAC	
B. thuringiensis acetylesterase reverse primer	TTAGAAATTTAAGAACTTGG	
B. thuringiensis serovar *thuringiensis* str. IS5056 thioesterase forward primer	ATGCAGAAGACTAAACTTT	
B. thuringiensis serovar *thuringiensis* str. IS5056 thioesterase reverse primer	CTATTTCTTCAATATTTTCATTT	
B. thuringiensis HD-771 thioesterase forward primer	ATGCAGAAGACTAAACTT	Initial denaturation step at 95°C for 5 min, 30 cycles at 95°C for 1 min, annealing at 44°C for 30 sec, and extension at 72°C for 1 min and the final extension at 72°C for 5 min, and preservation at 4°C
B. thuringiensis HD-771 thioesterase forward primer	TTAATTTTCCTATAATACTCT	

6.3.4 Screening for lipolytic activity

All clones were pre-cultivated in LB broth supplemented with ampicillin (100 µg/ml) and induced with IPTG (final concentration of 1 mM). For detection of esterase and lipase activity phenol red screening plates were used. Phenol red olive oil/tributyrin agar plates were prepared as follows (g/L); 0.01% (w/v) phenol red, 0.1% (w/v) $CaCl_2$, 1% (v/v) substrate, 2% (w/v) agar and pH adjusted to 7.3-7.4 with 0.1 N NaOH (Salihu *et al*., 2011). The media was supplemented with 1% tributyrin or olive oil for esterase and lipase, respectively (Rai *et al*., 2014). Untransformed *E. coli* was used as a control. Plates were inoculated with 20 µl of crude enzyme extract followed by incubation at 37°C for 48 h.

6.3.5 Enzyme assays

The lipolytic activity of the recombinant enzymes was quantified spectrophotometrically by measuring the release of *p*-nitrophenol from *p*-nitrophenyl (*p*-NP) esters at 405 nm. Various aliphatic acyl chain lengths were used to determine esterase [*p*-NP acetate (C_2), *p*-NP butyrate (C_4), *p*-NP valerate (C_5)] and lipase [*p*-NP octanoate (C_8), *p*-NP dodecanoate (C_{12}), *p*-NP myristate (C_{14}), *p*-NP palmitate (C_{16}), and *p*-NP stearate (C_{18})] activity. The substrate mixture consisted of 0.5 mM *p*-NP substrate in methanol, 50 mM Tris-HCl buffer (pH 8.0) and 0.1% Triton X-100. The standard assay contained 200 µl of substrate mixture and 20 µl of the enzyme extract, which was incubated at 37°C for 1 h. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 nM of *p*-NP per min under the assay conditions. Lipase/esterase activity was calculated from the formula derived from the Beer-Lambert Law (Desai *et al*., 2011). The appropriate extinction coefficient for each substrate under these assay conditions was used to calculate activity (Hu *et al*., 2010). All assays were conducted in triplicate.

6.3.6 Effects of temperature and pH on lipase/esterase activity and stability

The effect of temperature on enzyme activity in the crude extract and purified enzymes was determined by conducting assays as described previously at different temperatures ranging from 25 to 50ºC (with 5°C increments) and various *p*-NP esters as substrates (Bülow and Mosbach, 1987). Temperature stability of purified enzyme was determined by incubating the enzyme at various temperatures (25 to 50ºC) and estimating residual enzyme activity after incubation for 1, 2, 3 and 18 h. The effect of pH on enzyme activity was determined by assaying enzyme activity over a pH range of 3.0 to 12.0 in the appropriate buffers: citrate–phosphate

buffer (pH 3.0 to 6.0), Tris–HCl buffer (pH 7.0 and 8.0), carbonate–bicarbonate buffer (pH 9.0 and 10.0) and sodium-bicarbonate and sodium-phosphite buffer (pH 11.0 and 12.0) (Bülow and Mosbach, 1987). Stability of the purified enzyme over a range of pH was also determined by measuring the residual activity after incubating the enzyme in the above mentioned buffer systems (pH 3.0 to 12.0) for 18 h at the optimum temperature (Lailaja and Chandrasekaran, 2013). All assays were conducted in triplicate.

6.3.7 Effects of additives on enzyme activity

To determine the effects of additives on esterase activity, the enzymes were treated for 30 min with various additives which included 1 mM metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, and Zn^{2+}), 30% (v/v) organic solvents [acetone, butanol, dimethyl sulfoxide (DMSO), ethanol, glycerol, isopropanol, and methanol], 1% (v/v) detergents [cetyl trimethylammonium bromide (CTAB), sodium dodecyl sulphate (SDS), Tween-20 and Tween 80], and 1 mM enzyme inhibitors [ethylenediaminetetraacetic acid (EDTA) and 2-mercaptoethanol]. Residual activity was determined by measuring the enzyme activity under the standard assay conditions. All assays were conducted in triplicate.

6.3.8 Protein analysis

Protein concentrations were determined using the Bradford method with bovine serum albumin (Sigma) as the standard (Bradford, 1976). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE were performed using the method of Laemmli (1970). PageRuler^{TM} Plus Prestained Protein Ladder was used as the molecular weight standard (10 to 250 kDa) (Thermo Fisher Scientific) and proteins were visualized after staining with Coomassie brilliant blue R-250 and decolorized with Coomassie destaining solution.

6.3.9 Laboratory-scale pulp treatment trials

The acid-bisulphite pulp of two *Eucalyptus* species, *E. dunnii* and *E. grandis*, was used in lab-scale enzyme treatment trials. Approximately 0.5 g of washed pulp was combined with a total volume of 3 ml of liquid (buffer and enzymes) in a test tube and incubated at 37°C for 4 h. Following treatment, pulp samples were freeze dried overnight for Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) analyses.

6.3.10 Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) analysis

The lipophilic compounds in the pulp were characterized using pyrolysis-GC-MS. A multi-shot pyrolyzer, EGA/PY-3030 D, (Frontier Lab, Japan) attached to an ultra-alloy capillary column (30 m × 0.25 mm, 0.25 μm) was used for analysis. The samples were pyrolysed at 550°C for 20 s and the interface temperature set at 350°C. The chromatographic separation of the volatile components released by pyrolysis was performed using an ultra-alloy column. The injection temperature was 280°C and the column flow rate 1.0 mL/min with helium used as a carrier gas. The temperature was programmed as follows: 50°C for 2 min; rate 3°C/min up to 200°C and held for a further 4 min. The ion source and interface temperatures were set to 200°C and 300°C, respectively. The scan range used for mass selective detector was 40-650 m/z. The pyrolysis products were identified by comparing their mass spectra with the mass spectrum NIST library attached to the instrument (modified from Sithole and Watanabe, 2013).

6.3.11 Statistical analysis

Results shown here are the means of three independent determinations. Standard deviations for each of the experimental results were calculated using Microsoft Excel software and represented as error bars.

6.4 Results and Discussion

The features of the bacterial metagenome used in this study are comparable to other similarly small metagenomic studies, where the metagenome is of low complexity, the targeted microbiome is small or insufficient sequence data is available (Lorenz and Schleper, 2002; Tringe *et al*., 2005). The limitations of metagenomic sequencing and assembly are the differences in the abundance of each species in the community and the size of their genomes. This has an influence on sequencing coverage and results in under representation of less abundant species and over representation of abundant species. As a result, coverage of assemblies produced is significant, and can be used to determine quality of the data in terms of species abundance. 16S rRNA-based techniques are also known to be restricted by short read lengths, sequencing errors (Quince *et al*., 2011), problems in evaluating operational taxonomic units (OTUs) (Huse *et al*., 2010), and variances resulting from diverse regions selected (Youssef *et al*., 2009). The members of the bacterial community were similar to those reported in other microbial community studies under different environmental conditions (Foong *et al*., 2010; Romero *et al*., 2014; Pascual *et al*., 2016; Won *et al*., 2017). Numerous

metagenomic studies have reported on the production of lipases and esterases from Proteobacteria (Martínez-Martínez *et al*., 2013), Bacteriodetes (Holmes *et al*., 2011), Acidobacteria (Faoro *et al*., 2012; Kielak *et al*., 2016) and Actinobacteria (Jiménez *et al*., 2014).

Mining the metagenome for lipolytic enzymes

Twenty-one and 62 putative lipase and esterase sequences, respectively, were identified from the metagenome. A phylogenetic tree of the lipases and esterases mined from the metagenome was constructed to determine the relatedness of the identified enzymes (Figure 6.2). InterProScan 5.0 was used to confirm identities of the enzymes, resulting in the use of 14 and 55 lipases and esterases, respectively, in the construction of the trees. The 14 lipases clustered into two clades – one consisting only of the GDSL lipase from *Zymomonas mobilis* and the sister clade comprising the other 13 lipases including six phospholipases- enzymes that assist in the breakdown of phospholipid components of cell membranes (Alberts *et al*., 2002). The lipases of *Rhodococcus* sp. and *Streptomyces himastatinicus* clustered with the phospholipases from several species: *Chryseobacterium gleum*, *Burkholderia* sp., *Acinetobacter ursingii*, *Halobacillus* sp., *Terriglobus roseus* and *Lentisphaera araneosa*. The identified GDSL lipase of *Zymomonas mobilis* appears not to be closely related to all the other lipases, indicating that the lipases could belong to families other than the GDSL family. A similar observation was made for the esterases. Two clades derived from a common ancestor – ferulolyl esterase of *Burkholderia* sp. H160 and *B. gladioli* BSR3 in one clade and all other esterases in the sister clade. A number of branches offered bootstrap values of 100, indicating good support and reliability of the phylogenetic tree (Figure 6.2B). Some of the closely related enzymes with good support include; the esterase and phosphodiesterase of *Chryseobacterium gleum*, phosphodiesterase of *C. gleum* and sialate *O*-acetylesterase of *Sphingobacterium* sp., esterase of *C. gleum* and enterochelin esterase of *Klebsiella* sp.. Phosphodiesterases of other species represented in the tree are widely distributed, indicating that diverse mutation events, caused by habitat/substrate, gave way to enzyme evolution with subsequent in variation and divergence of their DNA sequences (Castro-Fernandez *et al*., 2017).

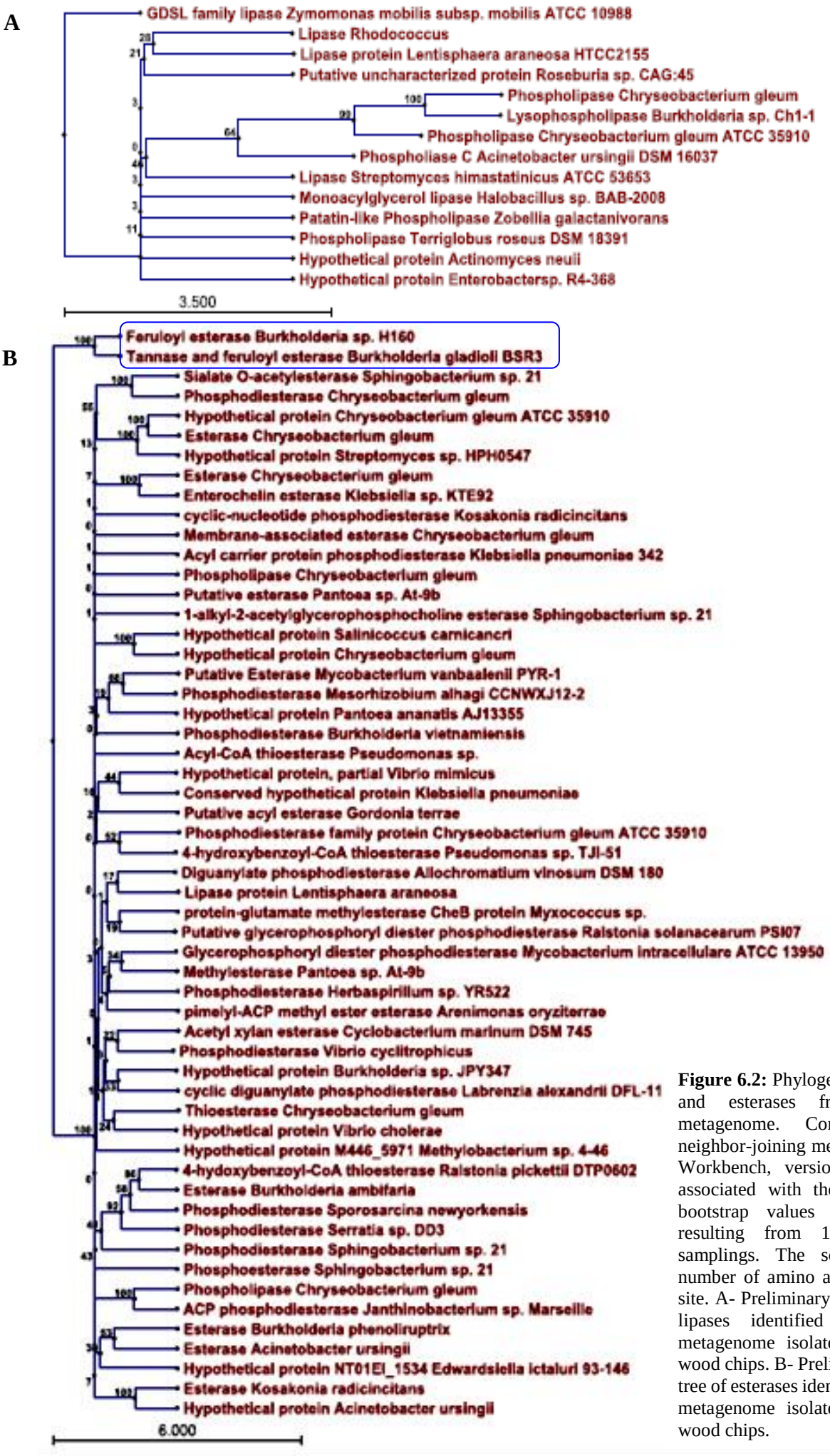


Figure 6.2: Phylogenetic trees of lipases and esterases from the bacterial metagenome. Constructed by the neighbor-joining method using CLC Bio Workbench, version 7. The numbers associated with the branches refer to bootstrap values (confidence limits) resulting from 1,000 replicate re-samplings. The scale represents the number of amino acid substitutions per site. A- Preliminary phylogenetic tree of lipases identified in the bacterial metagenome isolated from *Eucalyptus* wood chips. B- Preliminary phylogenetic tree of esterases identified in the bacterial metagenome isolated from *Eucalyptus* wood chips.

Of the 21 lipases and 62 esterases, two lipases and 11 esterases were confirmed by using BLAST on the NCBI database (Table 6.2). These contigs were then examined for large open reading frames (ORFs) for the genes encoding the lipase/esterase of interest. None of the confirmed lipase and esterase gene sequences identified through the BLAST program have been previously characterized. Thioesterases from *Chryseobacterium gleum* and *Ralstonia picketti* were selected for further study as these organisms are able to grow in a wide pH range and at high temperatures, respectively. Thioesterases exhibit esterase activity (splitting of an ester into acid and alcohol, in the presence of water) specifically at a thiol group, resulting in the degradation of lipolytic compounds present in wood and pulp. A lipase identified from *Burkholderia* sp. was also identified as an enzyme of interest, as it may have a temperature range of 30 to 40°C and a pH range of 4.5-6.5. The feruloyl esterase identified from *Burkholderia gladioli*, belongs to the family of hydrolases, and specifically acts on carboxylic ester bonds.

Table 6.2: Identification of putative esterases and lipases from the bacterial metagenome derived from *Eucalyptus* spp. wood chips.

	No.	Contig	Length	Microorganism	Enzyme	Previously characterized	Properties of microbe
Esterase	1	1043	211	*Acinetobacter ursingii*	Esterase SGNH Hydrolase-type esterase domain	No	30 and 37°C, utilization of: citrate, glutarate, L-aspartate, azelate, D-malate, 4-hydroxybenzoate, ethanol
	2	1444	127	*Chryseobacterium gleum*	Thioesterase	No	Grows luxuriously at pH 9.0 and tolerates up to pH 12.0, 30°C
	3	2065	252	*Acinetobacter ursingii*	Hypothetical protein	No	Refer to 1
	4	2327	403	*Pantoea* sp. At-9b	Putative esterase	No	30-37°C, pH7.0
	5	330	119	*Pantoea* sp. At-9b	Protein-glutamate methylesterase	No	30-37°C, pH7.0
	6	3301	472	*Sphingobacterium* sp. 21	Sialate-O-acetylesterase	No	30°C, pH 6.5-7.0
	7	4536	265	*Pseudomonas* sp. TJI-51	Acyl-CoA thioesterase	No	37°C, pH 6.5
	8	4582	191	*Kosakonia radicincitans*	Esterase YqiA	No	Not available
	9	463	153	*Burkholderia gladioli*	Feruloyl esterase	No	30°C-40°C (depending on sp.)
	10	5104	145	*Ralstonia picketti* DTP0602	Thioesterase	No	15-42°C, saline solution
	11	5611	388	*Klebsiella variicola*	Putative esterase		30°C, pH 7.0
Lipase	1	5472	347	*Burkholderia* sp. Ch1-1	Lipase GDSL 2	No	30°C-40°C (depending on sp.), pH 4.5-6.5
	2	6226	244	*Roseburia* sp. CAG:45	Lipase, class 3	No	37°C

The NCBI database was searched for complete gene sequences for lipases and esterases from various bacterial species mined from the metagenome. Based on sequence alignments of these genes, primers were designed (BPhyLip and BGluHp) and the metagenomic DNA isolated from bacteria indigenous to *Eucalyptus* sp. was used as a template in PCR. Successful amplification with the primer set BPhyLip resulted in an amplicon of approximately 1350 bp, potentially encoding a polypeptide with an inferred molecular mass of 50 kDa (Figure 6.3). The band of interest was excised and purified for further processing.

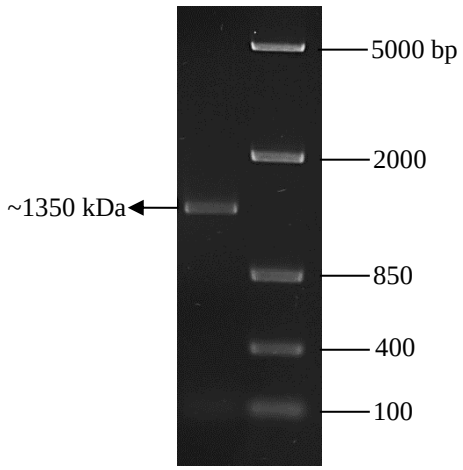


Figure 6.3: Amplification of lipase gene from bacterial metagenomic library using designed BPhyLip primer set.

The amplicon theoretically consists of the complete putative lipase/esterase gene from *Bacillus* sp. and was ligated into the pTZ57R/T vector (InSTA Clone PCR Cloning Kit, Thermo Scientific). The obtained recombinant plasmid was propagated in *E. coli* DH5a, isolated and digested with XBal and BamHI and the DNA fragment ligated into the expression vector pET14b. Unfortunately, cloning was unsuccessful as the identity of the enzyme was not confirmed with sequencing. The failed attempt to clone from a bacterial metagenome isolated from *Eucalyptus* wood may be attributed to the complex microbial species present and chimeric genes resulting in the correct gene size but not coding for the predicted protein. This is not uncommon, as there are reported shortcomings when heterologous host systems are implemented in the production of certain enzymes (Knietsch *et al*., 2003; Lorenz and Eck, 2005; Keasling, 2010). These obstacles may be due to variation in codon usage, lack of a stable mRNA secondary structure or incorrect conformation (Rai and Padh, 2001; Sørensen and Mortensen, 2005; Yin *et al*., 2007). Further attempts to clone from the metagenomic DNA was

abandoned and the study was then focussed on cloning lipases/esterases from pure bacterial isolates obtained from *Eucalyptus* wood chips.

Heterologous expression of esterases and lipases from bacteria indigenous to *Eucalyptus* spp. wood chips

Amplification of *B. thuringiensis* acetylesterase and carboxylesterase genes yielded amplicons approximately sizes 800 and 600 bp, respectively (Figure 6.4). The fragments were excised, purified, ligated into pTZ57R/T and transformed into *E. coli* DH5α. Following verification of the size of putative esterase genes, the genes were sequenced (GenBank accession nos.: acetylesterase MF787225, carboxylesterase MF787226). In addition, recombinant clones were spotted onto screening plates to confirm esterase activity. Sequencing results revealed a 628 bp and 781 bp ORFs encoding the *B. thuringiensis* acetylesterase and carboxylesterase, respectively. These sequences where then compared with esterase sequences from other *Bacillus* spp. downloaded from NCBI and aligned using CLCBio workbench.

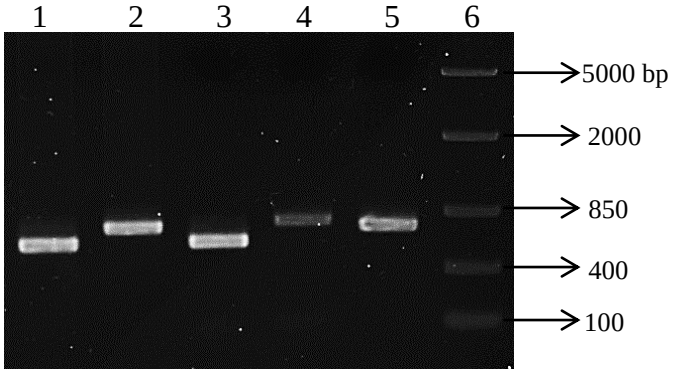


Figure 6.4: Agarose gel of amplicons of *B. thuringiensis* (N3): Lane 1- *B. thuringiensis* serovar *kurstaki* carboxylesterase; lane 2- *B. thuringiensis* BMB171 acetylesterase; *B. thuringiensis* (D7): Lane 3- *B. thuringiensis* serovar *kurstaki* carboxylesterase; lane 4- *B. thuringiensis* carboxylesterase; lane 5- *B. thuringiensis* BMB171 acetylesterase; lane 6- marker.

The recombinant acetylesterase shares 97% similarity with the lipases from *B. thuringiensis* BMB 171 (ADH05901.1) and *B. thuringiensis* serovar *kurstaki* str. YBT-1520 (AHZ50187.1), 96% with *B. thuringiensis* (OFD04831.1), 95% with *B. thuringiensis* (OFD11772.1) and 40% with *B. thuringiensis* serovar *israelensis* (APF32645.1) and *B. thuringiensis* IBL 4222 (EEM98798.1) (Figure 6.5). Alignment of the recombinant carboxylesterase with other *Bacillus* spp. esterases revealed 96% similarity with the esterases of *Bacillus* sp.

(WP_030027185.1), *B. cereus* (WP_000975492.1) and *B. thuringiensis* (WP_060631851.1), 95% with both *Bacillus* spp. (WP_043315358.1 and WP_000975476.1), and 90% with *B. weihenstephanensis* (WP_070144428.1) (Figure 6.6). Both esterases in this study have the conserved motif Gly-X-Ser-X-Gly which is observed in CAZy family CE1. The Ser residue of this motif is the main feature of the active site for fatty acid-esterification (Zuo *et al*., 2010).

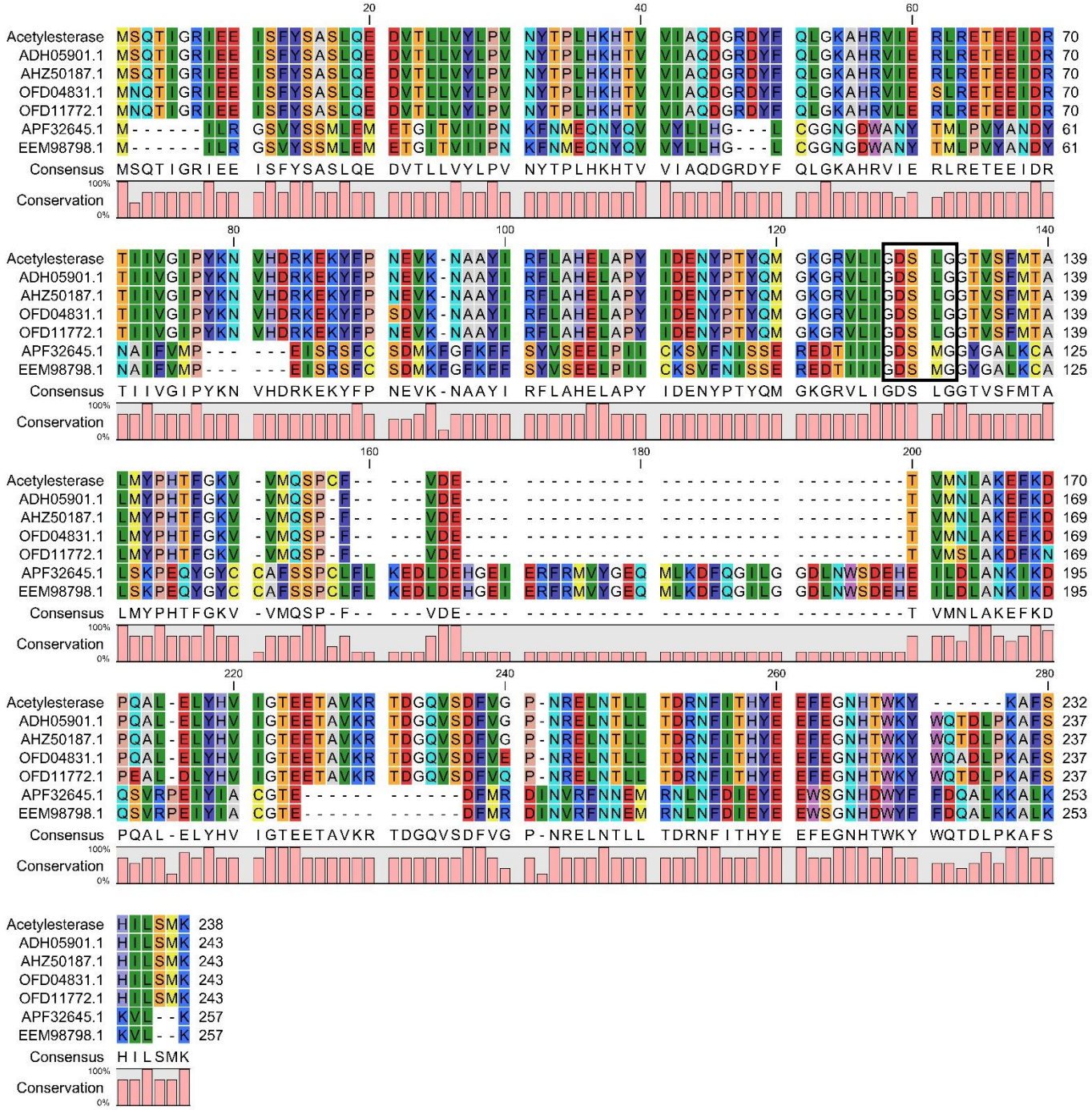


Figure 6.5: Amino acid sequence alignment of acetylesterases from *Bacillus* spp. The amino acid sequence of the recombinant acetylesterase were aligned with esterase sequences of *B. thuringiensis* BMB 171 (ADH05901.1), *B. thuringiensis* serovar *kurstaki* str. YBT-1520 (AHZ50187.1), *B. thuringiensis* (OFD04831.1), *B. thuringiensis* (OFD11772.1), *B. thuringiensis* serovar *israelensis* (APF32645.1), and *B. thuringiensis* IBL 4222 (EEM98798.1) (downloaded from the NCBI database) using the CLC Bio program. The black box highlights the conserved catalytic domain (GXSXG). The numbers at the top and in the right column indicate the position in the sequence.

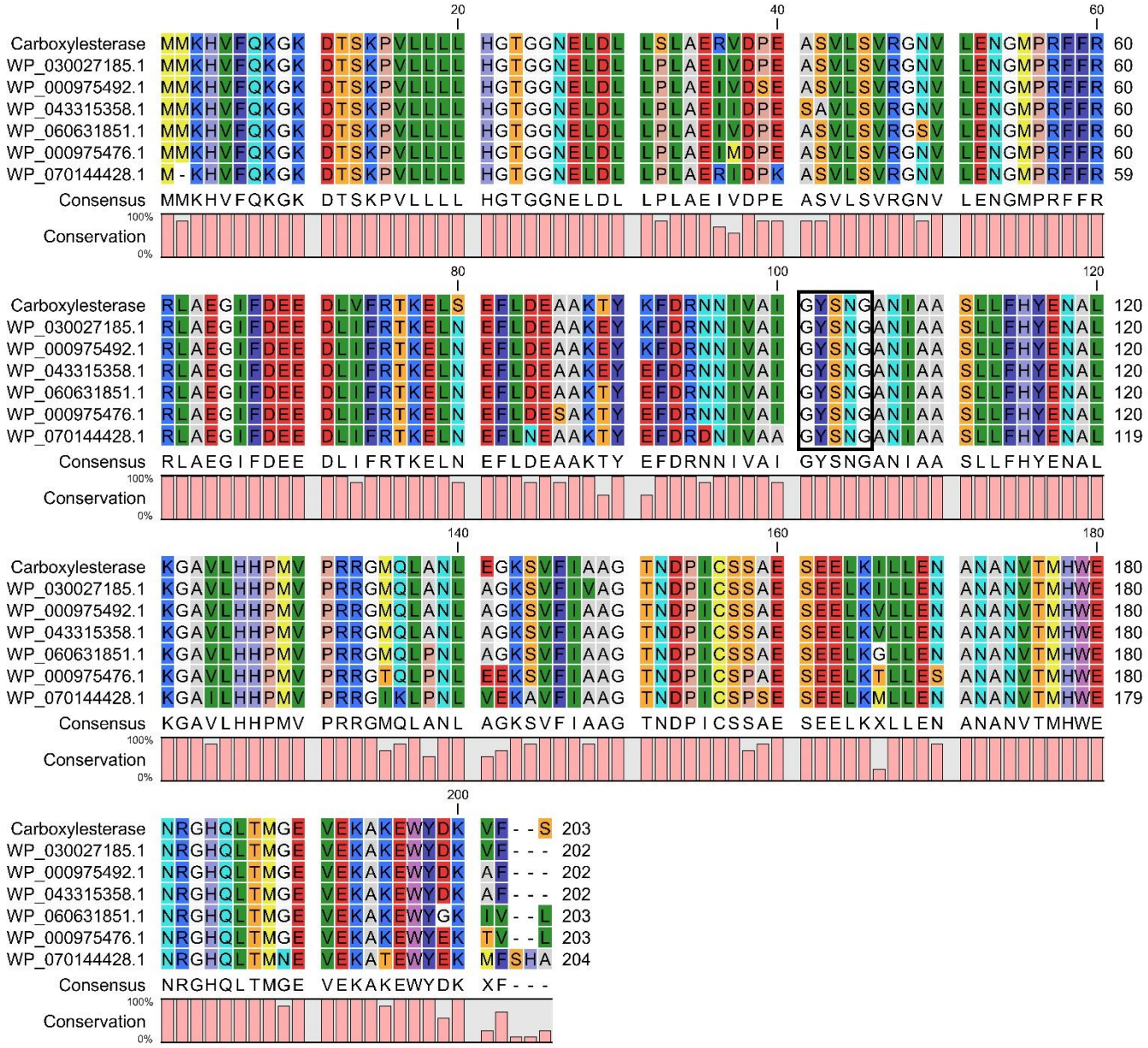


Figure 6.6: Amino acid sequence alignment of carboxylesterases from *Bacillus* spp. The amino acid sequence of the recombinant carboxylesterase were aligned with esterase sequences of *Bacillus* sp. (WP_030027185.1), *B. cereus* (WP_000975492.1), *Bacillus* sp. (WP_043315358.1), *B. thuringiensis* (WP_060631851.1), *Bacillus* sp. (WP_000975476.1) and *B. weihenstephanensis* (WP_070144428.1). The black box highlights the conserved catalytic domain (GXSXG). The numbers at the top and in the right column indicate the position in the sequence.

The three-dimensional (3-D) structure of the recombinant acetylesterase and carboxylesterase was modelled with the acetylesterase from *B. cereus* (PDB code 2qm0.1; 85% identity) and the carboxylesterase from *B. cereus* (PDB code 2h1i.1; 96% identity), respectively as templates. Modelling revealed the typical helical alpha/beta sheets of α/β hydrolases (Figure 6.7) (Kang *et al*., 2011). Comparison of the recombinant 3-D structures and sequences to the templates

revealed that the catalytic triad of the recombinant esterases may consist of Asp222, His255 and Ser226 for the acetylesterase, and Asp153, His184 and Ser157 for the carboxylesterase. These three amino acid residues appear to be located in close proximity to one another, suggesting the region of the active site.

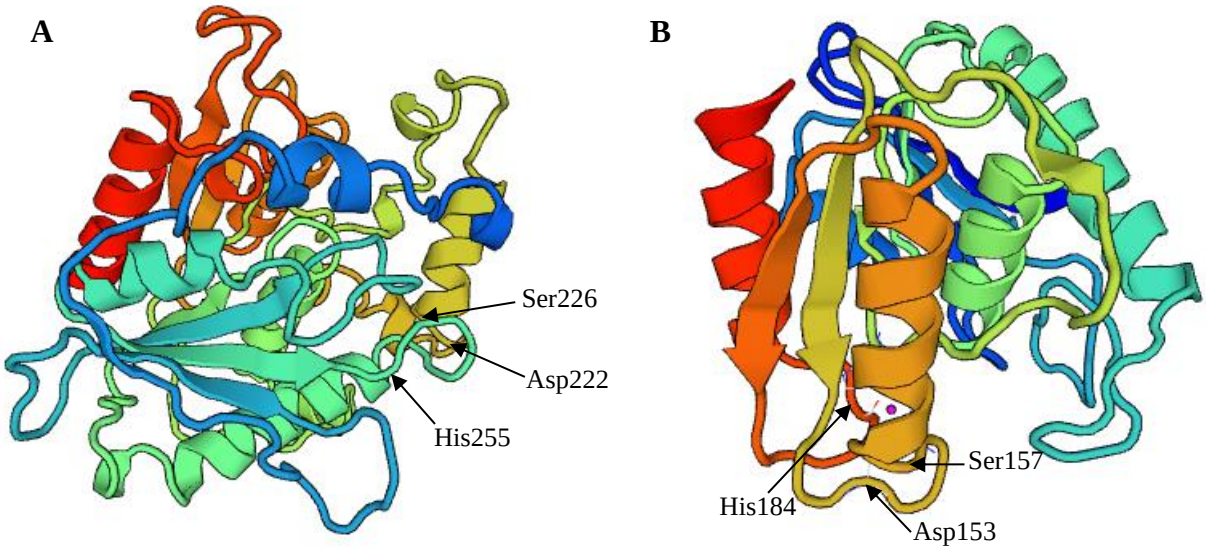


Figure 6.7: Schematic three-dimensional representation of the recombinant (A) acetylesterase and (B) carboxylesterase. The putative catalytic triad consists of Ser226, Asp222 and His255 in acetylesterase and Ser157, Asp153 and His184 in carboxylesterase, as indicated. Models built using SWISS-MODEL and Swiss-PdbViewer (Guex and Peitsch, 1997).

High-level expression of up to 147.8 U/ml for recombinant esterases was attained with the pET22b expression vector, controlled by a T7 promotor. The target protein was directed to the periplasm using the signal sequence leader (pelB) present in the vector. Under standard expression conditions, accumulation of the enzymes in the periplasmic space occurred, confirmed by sonication of intracellular extracts and the appearance of bands of approximately 22.2-29.6 kDa in size on SDS-PAGE. The expression of *Bacillus* lipases/esterases has been indicated in the accumulation of inclusion bodies inside *E.coli* hosts (Jia and Li, 2005; Morabbi Heravi *et al.*, 2009). Hence, the formation of inclusion bodies may be attributed to the high level of expression under the T7 promoter, as observed with Morabbi Heravi *et al*. (2009). The recombinant esterase was optimally expressed after 4 h of induction at 37°C. Numerous attempts to purify the enzymes using a HiTrap Q FF column prepacked with Q Sepharose Fast Flow (anion exchange) were unsuccessful. Binding of the proteins to the column may have

been affected by the pH of the buffers tested. Another reason would be aggregation of the protein, a common occurrence when expressing recombinant proteins (Wang *et al*., 2010). Samples were therefore concentrated and partially purified using spin columns, followed by excision and purification of the bands of interest (Figure 6.8). Following purification, specific activity increased to 104.77 and 148.91 μmol/mg for acetylesterase and carboxylesterase, respectively, with a purification fold of 17.6 for acetylesterase and 1.2 for carboxylesterase.

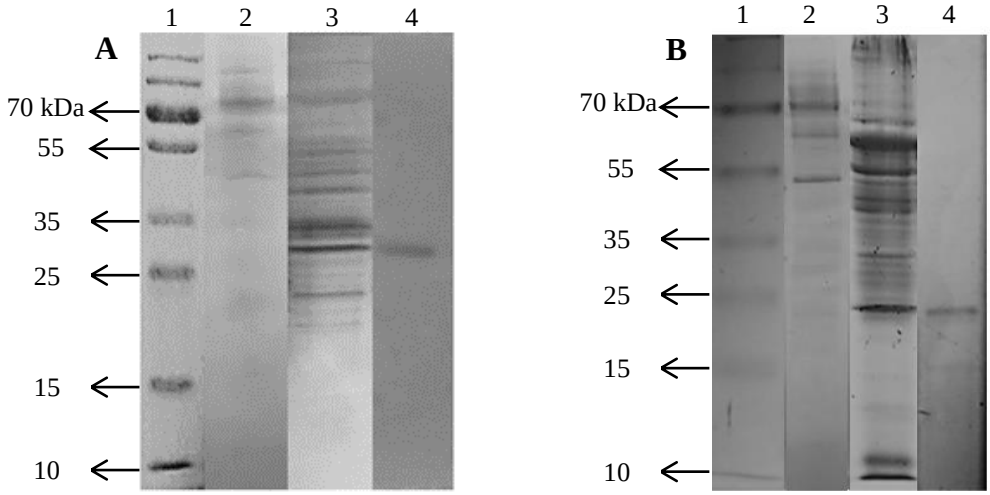


Figure 6.8: Purification of the cloned acetylesterase and carboxylesterase using spin columns, excision and purification of bands. SDS-PAGE gels of (A) acetylesterase: lane 1- marker; lane 2- untransformed *E. coli*; lane 3- induced clone; lane 4- purified clone; and (B) carboxylesterase: lane 1- marker; lane 2- untransformed *E. coli*; lane 3- induced clone; lane 4- purified clone.

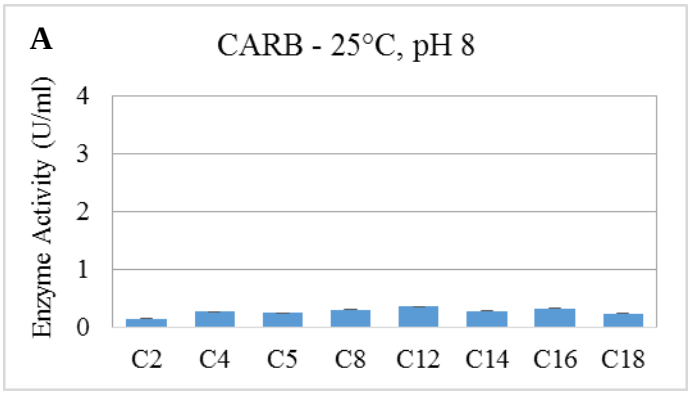
Extracellular and intracellular lipase/esterase activity of the selected clones were investigated at pH 8.0 and 37°C with acetate and butyrate as substrates (commonly used in initial investigations). Greater lipolytic activity was observed for the intracellular extracts compared to the extracellular (Table 6.3), and were consequently used in further characterization assays. Extracts were also tested for the presence of cellulases. Endoglucanase and exoglucanase activity was low (<0.035 U/ml) and therefore would not significantly impact the quality of pulp in this study.

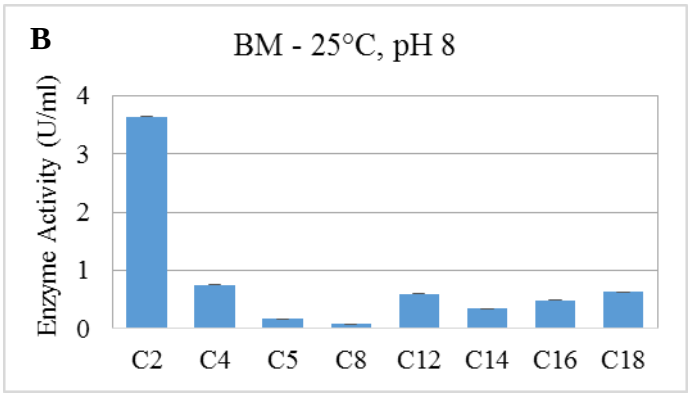
Table 6.3: Enzyme assays for esterase and cellulase activity of crude extract and determination of protein concentration of pure enzymes, intracellularly and extracellularly.

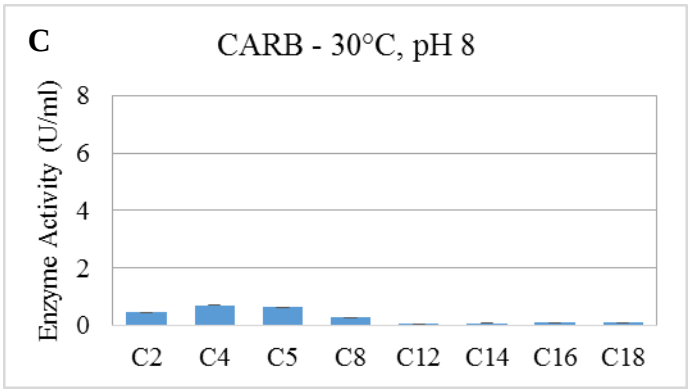
	Acetate (U/ml) ±SD		Butyrate (U/ml) ±SD		Endoglucanase Activity (U/ml) ±SD	Exoglucanase Activity (U/ml) ±SD	Protein Conc. (µg/ml) ±SD	
	Ext.	**Int.**	**Ext.**	**Int.**			**Ext.**	**Int.**
B. thuringiensis acetylesterase	2.55±0.25	8.4±0.04	5.75±0.24	87.8±0.11 [a]	0.035±0.18[a]	0.030±0.32 [a]	2.57±0.48	1410.7±0.82
B. thuringiensis carboxylesterase	5.71±0.14	98.4±0.31	8.98±0.21	127.1±0.17 [a]	0.031±0.36 [a]	0.021±0.18 [a]	3.84±0.33	853.55±0.15

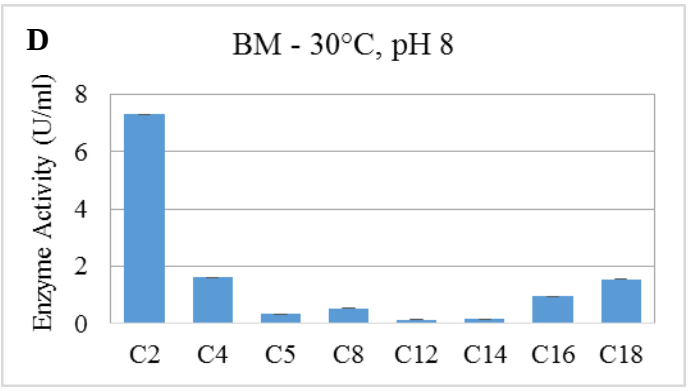
Analyses conducted in duplicate. SD – standard deviation. p-value: a <0.05; b >0.05

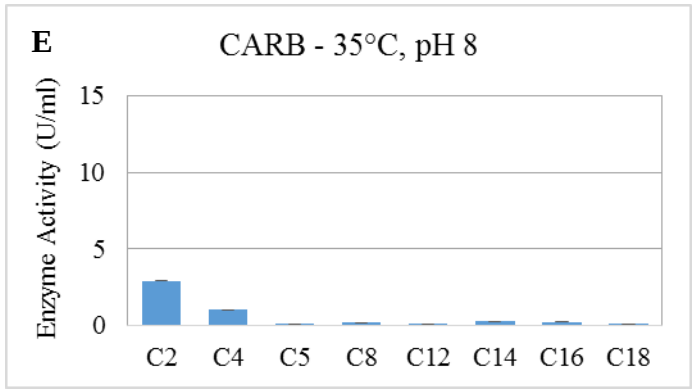
p-Nitrophenyl esters with various aliphatic acyl chain lengths (C_2-C_{18}) were used to determine substrate specificity of the recombinant esterases. These results show that the purified enzymes are esterases due to their preference for short-chain organic acid esters, consistent with other reports for esterolytic enzymes from different sources (Ayna *et al*., 2013; Chuang *et al*., 2011; Fu *et al*., 2011). Initially, at the standard assay pH of 8.0, 50°C and 45°C appeared to be the optimum temperatures for the carboxylesterase (17.5 U/ml) and acetylesterase (22.2 U/ml), respectively (Figure 6.9). However, once the optimum pH was determined at the respective temperature optima, the assays were repeated at the optimum pH and various temperatures. Tenfold and six fold higher activities were obtained with pH and temperature optima shifts as follows: 50°C and 45°C at pH 8.0 for both enzymes to 35°C and 30°C at pH 4.0 for both carboxylesterase (127.1 U/ml) and acetylesterase (147.8 U/ml), respectively. This change in optimum conditions have been observed in other studies (Ramnath *et al*., 2017; Ertuğrul *et al*., 2007; Ghatora *et al*., 2006). Kontkanen (2006) reported a pH optimum of 7.0 with longer chain esters, and between 5.0-5.5 with shorter chain esters for a steryl esterase from *Melanocarpus albomyces*. The author attributed the difference in pH optima to the effect of the pH on the ionisation of amino acids other than catalytic amino acids either within or beyond the active site, that influence the stability of the active conformation of the enzyme and interaction between the enzyme and different substrates. This highlights the necessity for determining optimum pH and temperature using both approaches.

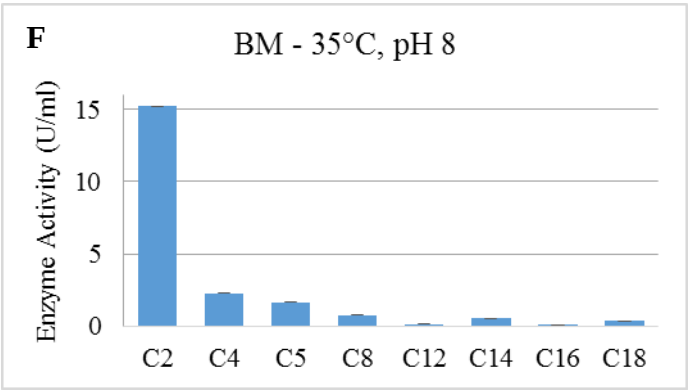

A
CARB - 25°C, pH 8
Enzyme Activity (U/ml)
0
1
2
3
4
C2
C4
C5
C8
C12
C14
C16
C18

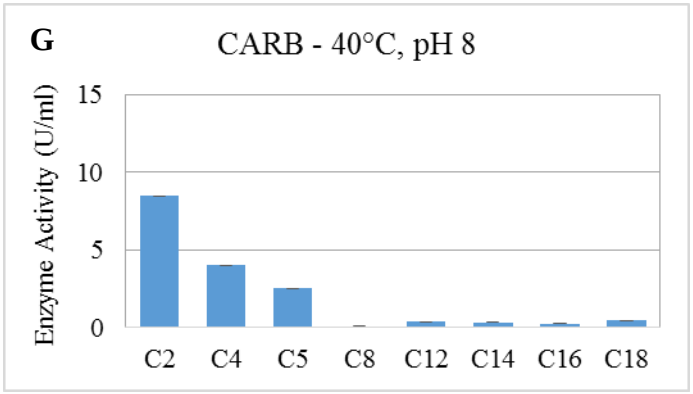

B
BM - 25°C, pH 8
Enzyme Activity (U/ml)
0
1
2
3
4
C2
C4
C5
C8
C12
C14
C16
C18

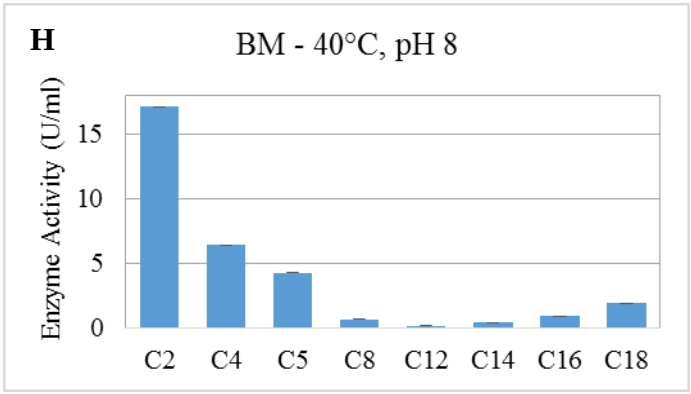

C
CARB - 30°C, pH 8
Enzyme Activity (U/ml)
0
2
4
6
8
C2
C4
C5
C8
C12
C14
C16
C18


D
BM - 30°C, pH 8
Enzyme Activity (U/ml)
0
2
4
6
8
C2
C4
C5
C8
C12
C14
C16
C18


E
CARB - 35°C, pH 8
Enzyme Activity (U/ml)
0
5
10
15
C2
C4
C5
C8
C12
C14
C16
C18


F
BM - 35°C, pH 8
Enzyme Activity (U/ml)
0
5
10
15
C2
C4
C5
C8
C12
C14
C16
C18


G
CARB - 40°C, pH 8
Enzyme Activity (U/ml)
0
5
10
15
C2
C4
C5
C8
C12
C14
C16
C18


H
BM - 40°C, pH 8
Enzyme Activity (U/ml)
0
5
10
15
C2
C4
C5
C8
C12
C14
C16
C18

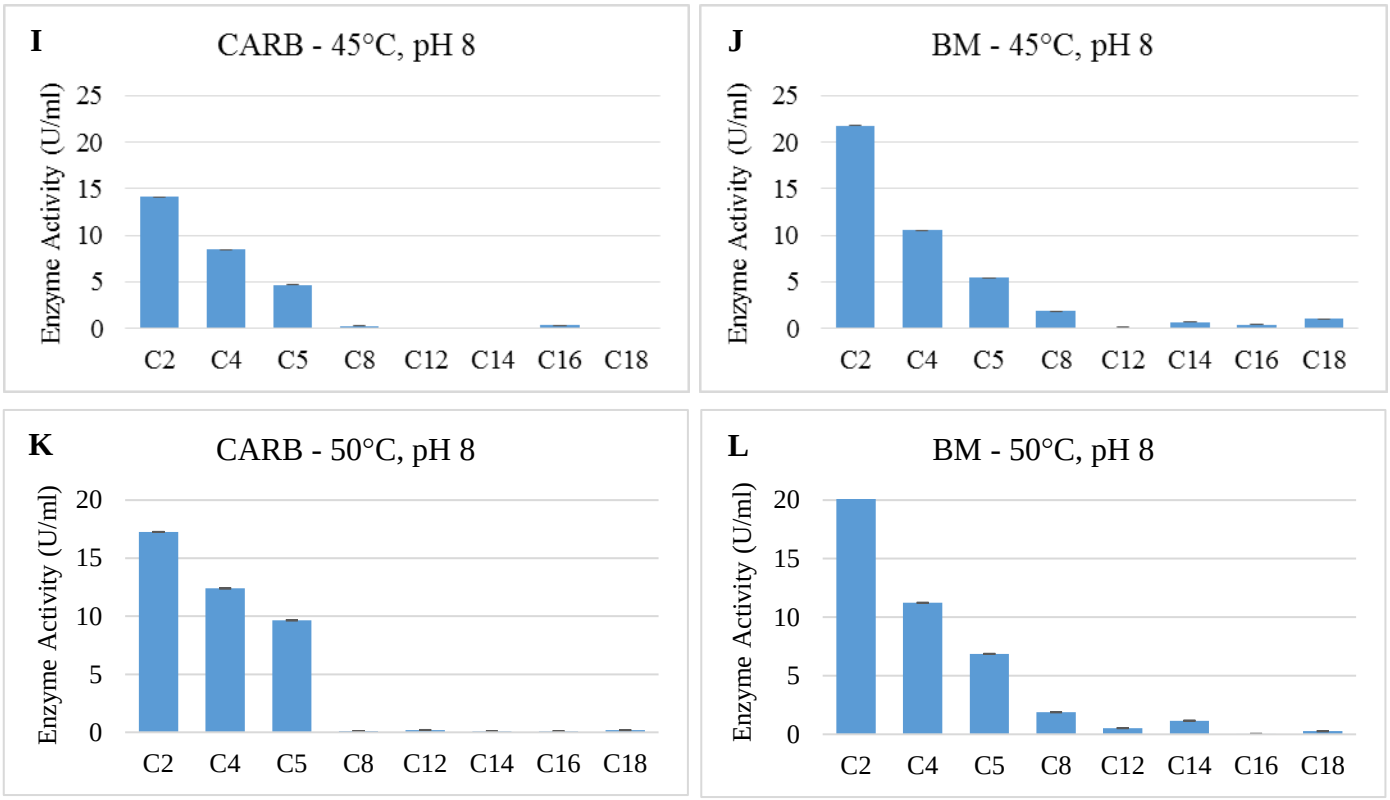


Figure 6.9: Effect of temperature and substrate on carboxylesterase (CARB) and acetylesterase (BM) activity at pH 8.0.

Acetylesterase displayed highest activity (147.8 U/ml) with valerate (C_5) at pH 4.0, followed by butyrate (C_4) with 87.8 U/ml at pH 3.0 (Figure 6.10). Carboxylesterase demonstrated highest specificity towards butyrate (C_4) with 127.1 U/ml at pH 4.0, followed by acetate (C_2) with 98.4 U/ml at pH 5.0. These esterases are more active in the acidic pH range from 3.0 to 5.0 and were able to maintain activity above ~80 U/ml depending on the substrate. A drastic reduction in activity was observed from pH 6.0 to 8.0, and minimal activity from pH 9.0 to 12.0. The performance of these esterases are superior to those from other bacterial species under similar conditions. The esterase of a *Bacillus* sp. strain DVL2 showed optimal activity of 5.2 U/ml at pH 7.0 and 37°C (Kumar *et al*. 2012). Faiz *et al*. (2007) reported on an esterase from *Anoxybacillus* A4 with highest activity of 0.8 U/ml at pH 5.5 and 60°C. A steryl esterase from *Chromobacterium viscosum* exhibited maximum activity of 130 nkat/mg at an acidic pH 4.0 and 40°C (Kontkanen *et al*., 2004). The observed pH optimum of 4.0 and 5.0 presented by the recombinant esterases in study is not uncommon. A thermostable carboxylesterase EstGtA2 from *Geobacillus thermodenitrificans* (AEN92268) has been reported to hydrolyse a broad variety of *p*-nitrophenyl esters of different acyl chain lengths (Charbonneau, 2013), which correlates with the behaviour observed for the carboxylesterase (~30 U/ml) with octanoate as

a substrate at pH 3.0 to 5.0 (Figure 6.10). The acidic nature of the esterases is beneficial for application in the pulping industry, particularly in the acid-bisulphite pulping process, for the production of dissolving pulp (Engström *et al*., 2006).

Sterols, fatty acids (C_{8-26}), including several α and ω-hydroxyfatty acids, and long chain aliphatic alcohols, are the main lipophilic extractives in unbleached pulp (Gutiérrez *et al*., 2001; Freire *et al*., 2005). Medium chain fatty acids typically result from triglycerides degraded during pulping (Schönfeld and Wojtczak, 2016). Freire *et al*. (2005) reported that elemental chlorine free (ECF) bleaching of *Eucalyptus* spp. modified the behaviour of extractives up to 80%, and that a pre-treatment step would therefore be necessary to achieve complete removal of these pitch causing compounds. Approximately 80% of the aliphatic extractives are removed from pulp during bleaching, constituting 90% of the long-chain aliphatic alcohols, 70% of the fatty acids and 70% of the sterols. The degradation of β-sitosterol by chlorine dioxide causes a decrease in sterol, and the high amounts of 24-ethylcholestene-3,5,6-triol identified in the bleached pulp is evidence of this (Freire *et al*., 2006; Silvério *et al*., 2007a,b). However, the lipophilic content (mostly fatty acids and alcohols) may increase during the various stages of pulping due to increasing accessibility in the pulp fibre, thus increasing the risk of pitch deposit formation (Freire *et al*., 2005). Esterases have the ability to hydrolyse both water-soluble substrates generally comprised of short chain fatty acids, and water-insoluble substrates having long chain fatty acids (Kontkanen, 2006). Therefore, the pre-treatment of the brown pulp with esterases could substantially accelerate the degradation of pitch compounds present. Reducing the total extractives content of the pulp and modifying the composition of the pulp could assist in decreasing the risk of pitch formation during bleaching. In addition, this would increase pulp strength, improve machine operations, reduce energy requirements and costs related to pulp processing (Wang *et al*., 2007).


A
Acetate
U/ml
140
120
100
80
60
40
20
0
pH3
pH4
pH5
pH6
pH7
pH8
pH9
pH10
pH11
pH12
BM
CARB
B
Butyrate
U/ml
140
120
100
80
60
40
20
0
pH3
pH4
pH5
pH6
pH7
pH8
pH9
pH10
pH11
pH12
BM
CARB
C
Valerate
U/ml
140
120
100
80
60
40
20
0
pH3
pH4
pH5
pH6
pH7
pH8
pH9
pH10
pH11
pH12
BM
CARB

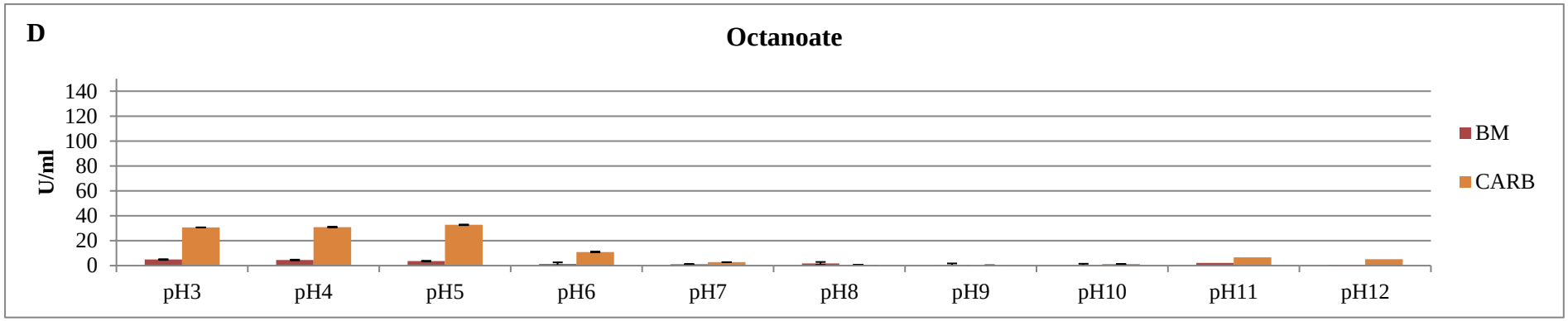


Figure 6.10: Substrate specificity of acetylesterase (BM) and carboxylesterase (CARB) at various pH levels (A-D). Optimum pH of 4.0 for both enzymes, with greatest specificity towards valerate for acetylesterase and butyrate for carboxylesterase. Longer acyl chains yielded minimal activities (data not shown).

The carboxylesterase activity was relatively stable over a period of 18 h at 35°C and pH 4.0, unexpectedly with dodecanoate and stearate as substrates (Figure 6.11). Conversely, the carboxylesterase exhibited the greatest reduction in activity over the period tested with its optimum substrates, acetate and butyrate. Acetylesterase maintained relatively stable activity of >70% at 30°C and pH 4.0 with butyrate as a substrate. The application of these esterases in industry would be feasible as shorter treatment times would be favoured (esters were completely degraded in 3 h). Triglycerides are reduced to medium chain fatty acids during pulping (Gutiérrez *et al*., 1998; Schönfeld and Wojtczak, 2016). Therefore, the treatment of brown pulp with these enzymes as a pre-treatment step prior to bleaching would assist in targeting the short to medium chained fatty acids present.

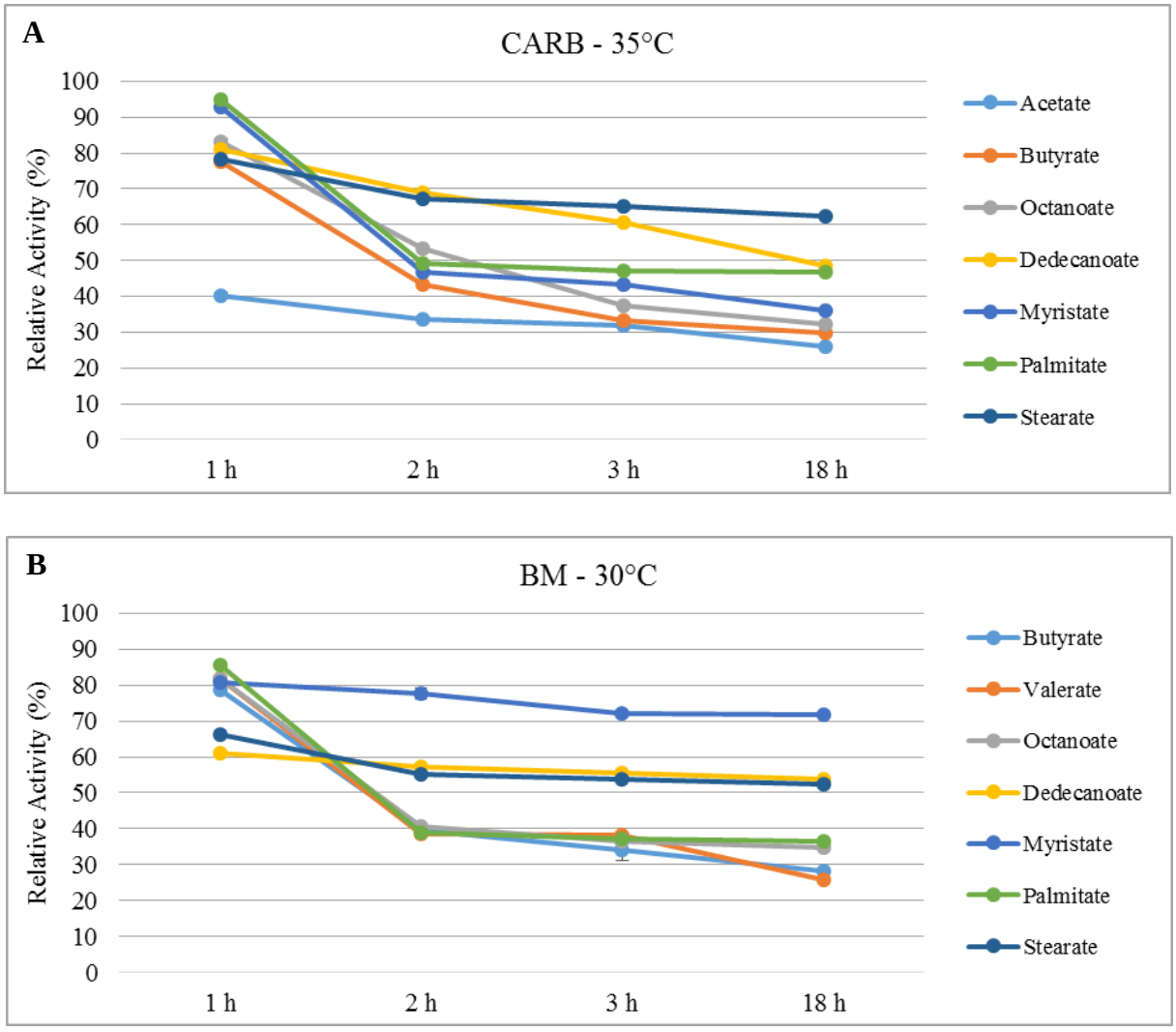


Figure 6.11: Stability profiles of the carboxylesterase (A) and acetylesterase (B) at optimum pH and temperature over a period of 18 h.

The effect of organic solvents, surfactants, metal ions and inhibitors on esterase activity were examined by determining residual activity of the enzyme solution after incubation for 30 min at room temperature (Table 6.4). Acetylesterase activity was enhanced by the presence of ethanol (101.1%), methanol (128.5%), acetone (102.9), Ca^{2+} (128.7%), Mg^{2+} (124.7%), and Na^{+} (115.6%). Similarly a tributyrin esterase isolated from *Lactobacillus plantarum* was found to be moderately stimulated by Ca^{2+} and Mg^{2+} (Gobbetti *et al*., 1997). Stability in the presence of organic solvents such as ethanol, methanol and acetone is an essential and valuable characteristic for biotechnological applications (Soliman *et al*., 2007). The observed effect with calcium ions is not unexpected as it is known to stabilize lipolytic enzymes (Cao, 1993). Significant increase in activity was observed with CTAB (159.9%) and K^{+} (202.7%). The acetylesterase was able to retain 93% and 91.6% of its original activity when incubated with the test inhibitors EDTA and 2-mercaptoethanol, respectively. The minimal influence of EDTA on enzyme activity, suggests that this esterase may be a non-metalloenzyme (Brod *et al*., 2010). Strong inhibition effects were observed in the presence of butanol (38.5%), glycerol (42.5%), SDS (42.3%) and Tween-20 (36.5%). The activity of carboxylesterase in comparison was less affected by the additives. The most significant effects observed were with K^{+} which increased activity to 174.2%. Additives with a significant negative effect on activity were butanol (39.2%), glycerol (43.8%), SDS (30.1%) and Tween-20 (28.6%). These results were similar to other reports (Ayna *et al*., 2013; Brod *et al*., 2010; Castro-Ochoa *et al*., 2005; Kumar *et al*., 2005). BTL-1 and BTL-2 lipases described by Castro-Ochoa *et al*. (2005) in particular, showed increased activity in the presence of methanol, ethanol and acetone. It has been suggested that a thin layer of water molecules remains firmly bound to the enzyme acting as a barrier for the enzyme allowing it to maintain its native conformation in the presence of inhibitors (Nawani *et al*., 1998).

Table 6.4: Effects of organic solvents, surfactants, metal ions, and inhibitors on the activity of the recombinant esterases.

Additives	Relative Activity (%)	
	Acetylesterase ±SD	Carboxylesterase ±SD
Control	100	100
Organic solvents (30% [v/v])		
Acetone	102.9±0.25[a]	100.2±0.18[a]
Butanol	38.5±0.66	39.2±0.58
DMSO	77.8±0.31	75.3±0.26
Ethanol	101.1±0.37[a]	97.6±0.10[a]
Glycerol	42.5±0.29	43.8±0.47
Isopropanol	95.3±0.13[a]	90.8±0.23[a]
Methanol	128.5±0.28[a]	101.4±0.27[a]
Surfactants (1% [v/v])		
CTAB	159.9±0.11[a]	104.8±0.15[a]
SDS	42.3±0.56	30.1±0.43
Tween-20	36.5±0.35	28.6±0.29
Tween-80	49.1±0.25	42.9±0.12
Metal ions (1 mM)		
Ca^{2+}	128.7±0.35[a]	97.6±0.28[a]
Co^{2+}	65.1±0.28	60.8±0.19
Cu^{2+}	89.1±0.22	78.5±0.24
K^{+}	202.7±0.18[a]	174.2±0.14[a]
Mg^{2+}	124.7±0.15[a]	101.5±0.11[a]
Mn^{2+}	63.7±0.21	57.3±0.31
Na^{+}	115.6±0.13[a]	110.9±0.29[a]
Zn^{2+}	57.4±0.20	62.5±0.51
Inhibitor (1 mM)		
EDTA	93.0±0.78[a]	94.6±0.81[a]
2-Mercaptoethanol	91.6±0.57[b]	98.2±0.46[b]

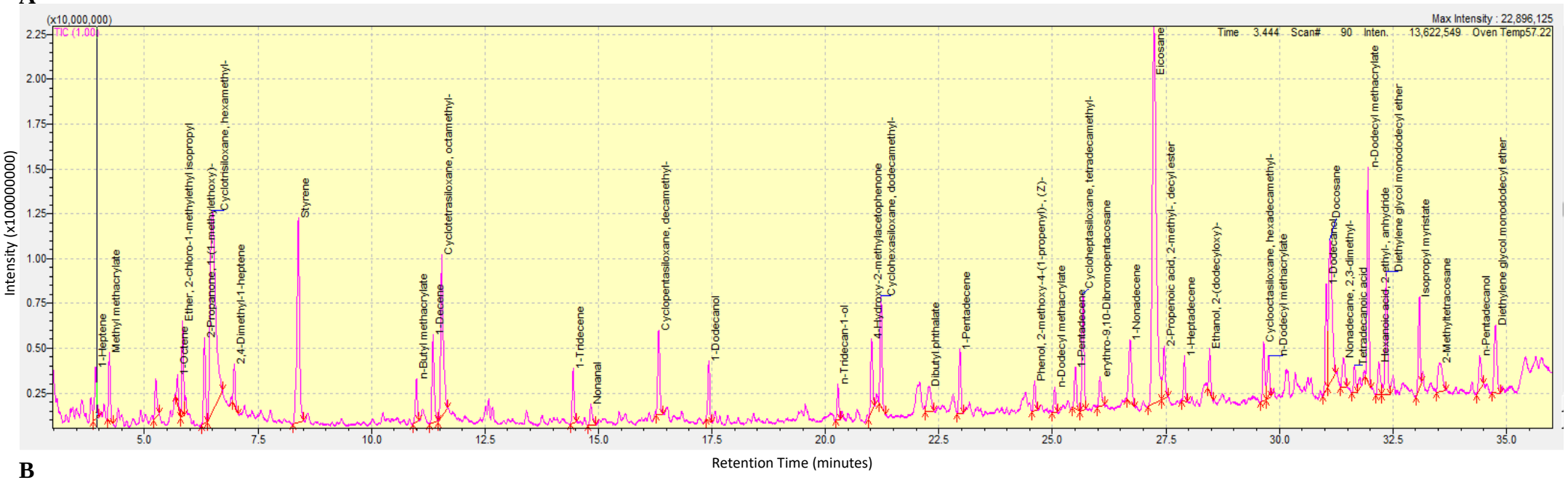
SD – standard deviation. p-value: a <0.05; b >0.05.

Although the lipid content in all eucalypt wood species is relatively similar and naturally low, it is the lipophilic extractive content that causes pitch deposit formation that poses a threat (Sithole, 2000). The Py-GC/MS chromatogram of the treated pulp of *E. dunnii* and *E. grandis* are shown in Figure 6.12 and are the breakdown products of all organic compounds in the pulp samples. The major lipophilic compounds identified by Py-GC-MS in *E. dunnii* and *E. grandis* were sterols, fatty acids, fatty alcohols, glycerides and small amounts of squalene and alkyl ferulates (Figure 6.12). Most of these compounds have been identified as major contributors to pitch formation as reported by other researchers (Gutiérrez *et al*., 1999; Rencort *et al*., 2007; Silvério *et al*., 2007a,b; Prinsen *et al*., 2012). Usually, fatty acids are detected at shorter retention times followed by hydrocarbons, sterols and sterol esters/triglycerides (Rencoret *et al*., 2007; Moodley, 2011). Triglycerides were not detected in either *E. dunnii* or *E. grandis* pulps as they were probably hydrolysed during bisulphite cooking. This is a common occurrence when pulping wood chips (Gutiérrez *et al*., 1999). The profile of wood resin

components generated after treatment of pulp differed substantially compared to the original organic profile of the pulp. Diverse patterns of degradation for the lipophilic extractives were noted upon application of the recombinant esterases. A reduction in the sterol ester content was observed with synchronized increase in free sterols. A similar effect was observed for pine wood treated with *Ophiostoma ainoae* (Martínez-Íñigo *et al*., 1999) and eucalypt wood with *Ceratocystis variabilis* (Gutiérrez *et al*., 1999). The recombinant esterases characterized in this study have demonstrated their ability to degrade lipophilic compounds reported to be responsible for pitch deposit formation. Fatty alcohols represented a small portion of the total extractives analyzed before treatment with the recombinant esterases. Dodecan-1-ol, n-pentadecanol, tetradecanol and hexadecane-1-ol were the major components in this group. Following treatment, a significant amount of the fatty alcohols were removed. The recombinant esterases therefore also had an impact on fatty alcohols. Reduction of fatty acids was also observed. Short chain fatty acid degradation was anticipated as these esterases demonstrated optimal activity with $C_{2\text{-}5}$ esters as substrates. However, it was interesting to note that the hexadecanoic acid (C_{16}) present in the untreated *E. grandis* pulp (0.004 mg) was reduced to 0.0008 mg and 0.0007 mg when treated with acetylesterase and carboxylesterase, respectively. This constitutes a ≥80% reduction in hexadecanoic acid which is a long chain fatty acid. This behaviour highlights the flexibility of these esterases when presented with complex substrates. Treatment of *E. dunnii* pulp resulted in an overall reduction of 63.2% and 77.7% when the recombinant acetylesterase and carboxylesterase, respectively, were added. Greater reduction in lipophilic compounds was observed with the carboxylesterase for *E. dunnii* treated pulp, however, treatment of *E. grandis* pulp with acetylesterase was found to be more effective with a 72.7% reduction compared to the 51.9% observed with the carboxylesterase.

Pulp treated with a combination of these esterases with other suitable lipolytic enzymes such as lipases and laccases and the additives that increase the activity of these enzymes, such as CTAB and K+ ions, could ensure complete lipophilic compound removal. The enzyme-based treatment can be applied post pulping (as tested in this study) or during the bleaching process where the pH level is between 3.0 and 5.0 at a suitable temperature.

A


(x10,000,000)
TIC (1.00)
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Time 3.444 Scan# 90 Inten. 13,622,549 Oven Temp57.22
Intensity (x100000000)
Retention Time (minutes)
1-Heptene
Methyl methacrylate
1-Octene
Ether, 2-chloro-1-methylethyl isopropyl
2-Propanone, 1-(1-methylethoxy)-
Cyclotrisiloxane, hexamethyl-
2,4-Dimethyl-1-heptene
Styrene
n-Butyl methacrylate
1-Decene
Cyclotetrasiloxane, octamethyl-
1-Tridecene
Nonanal
Cyclopentasiloxane, decamethyl-
1-Dodecanol
n-Tridecan-1-ol
4-Hydroxy-2-methylacetophenone
Cyclohexasiloxane, dodecamethyl-
Dibutyl phthalate
1-Pentadecene
Phenol, 2-methoxy-4-(1-propenyl)-, (Z)-
n-Dodecyl methacrylate
1-Pentadecene
Cycloheptasiloxane, tetradecamethyl-
erythro-9,10-Dibromopentacosane
1-Nonadecene
Eicosane
2-Propenoic acid, 2-methyl-, decyl ester
1-Heptadecene
Ethanol, 2-(dodecyloxy)-
Cyclooctasiloxane, hexadecamethyl-
n-Dodecyl methacrylate
1-Dodecanol
Docosane
Nonadecane, 2,3-dimethyl-
Tetradecanoic acid
n-Dodecyl methacrylate
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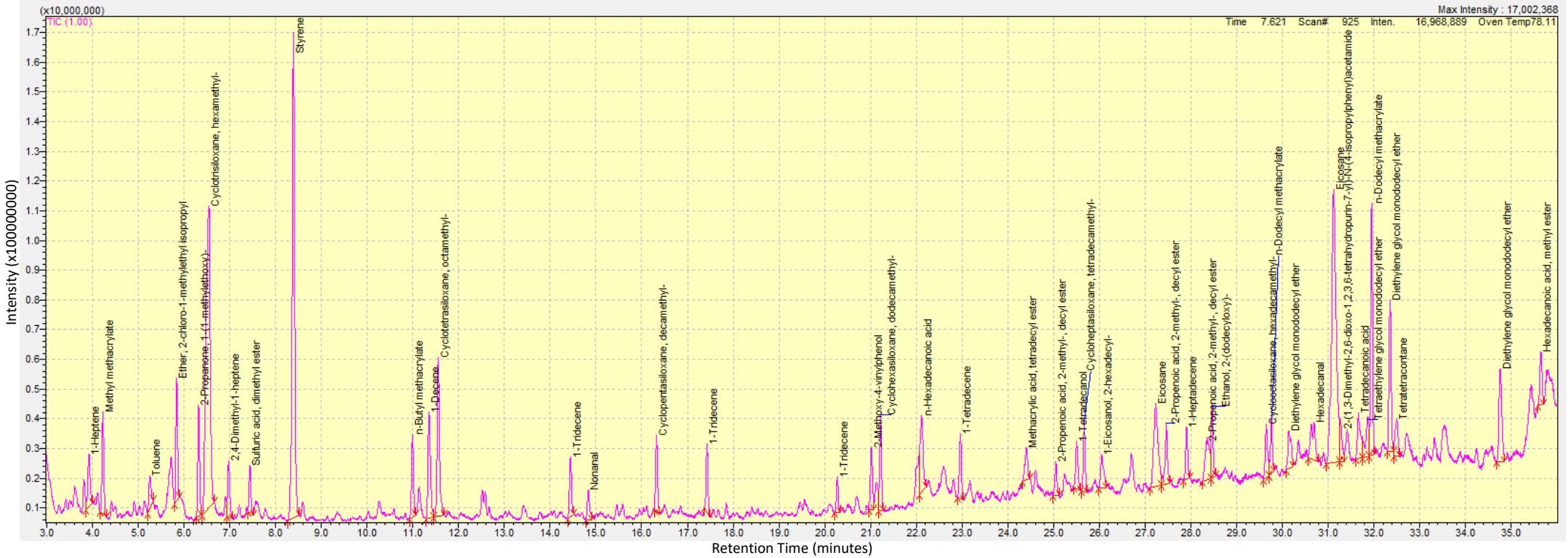
Isopropyl myristate
2-Methyltetracosane
n-Pentadecanol
Diethylene glycol monododecyl ether

B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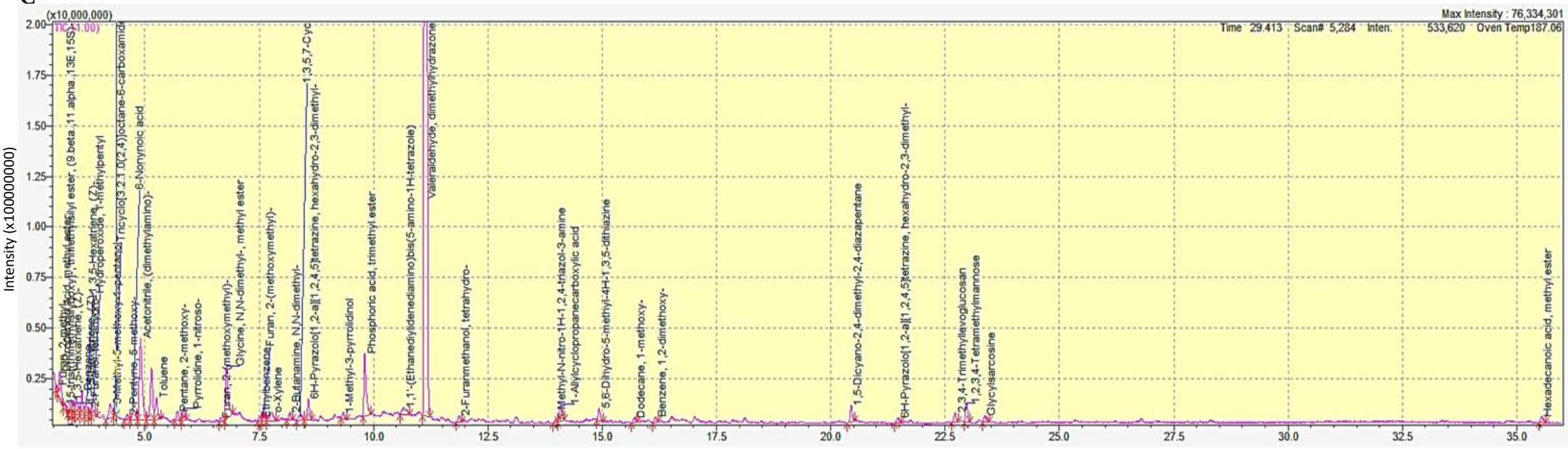
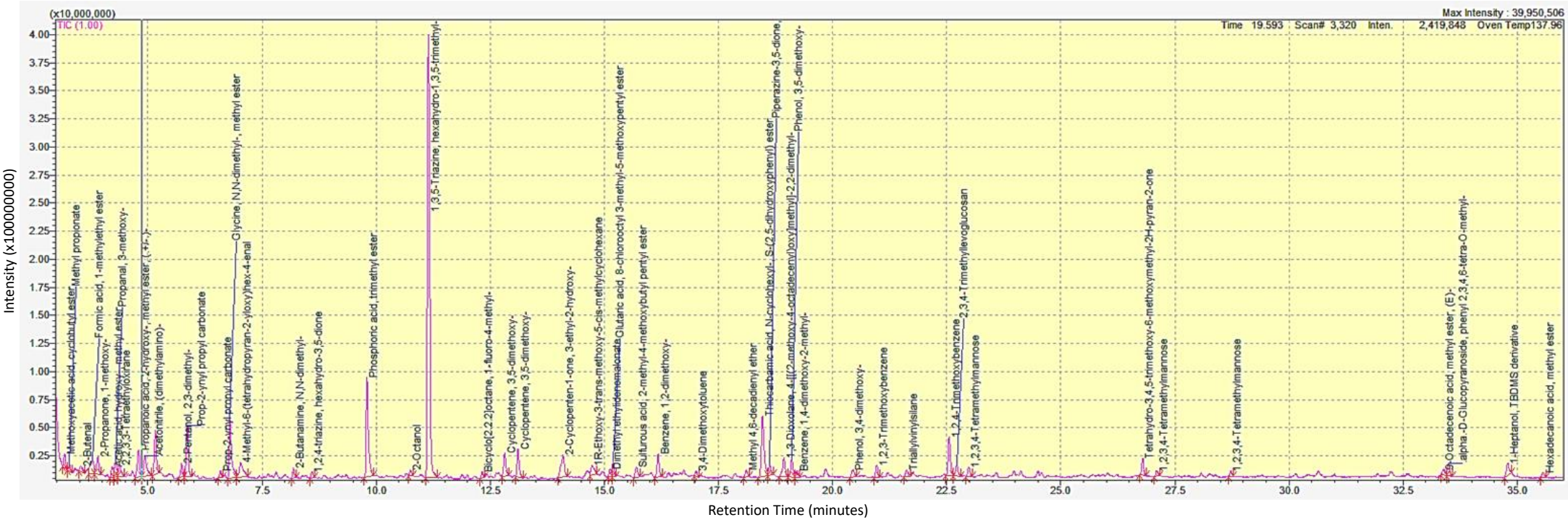
Time 7.621 Scan# 925 Inten. 16,968,889 Oven Temp78.11
Intensity (x100000000)
Retention Time (minutes)
1-Heptene
Methyl methacrylate
Toluene
Ether, 2-chloro-1-methylethyl isopropyl
2-Propanone, 1-(1-methylethoxy)-
Cyclotrisiloxane, hexamethyl-
2,4-Dimethyl-1-heptene
Sulfuric acid, dimethyl ester
Styrene
n-Butyl methacrylate
1-Decene
Cyclotetrasiloxane, octamethyl-
1-Tridecene
Nonanal
Cyclopentasiloxane, decamethyl-
1-Tridecene
1-Tridecene
2-Methoxy-4-vinylphenol
Cyclohexasiloxane, dodecamethyl-
n-Hexadecanoic acid
1-Tetradecene
Methacrylic acid, tetradecyl ester
2-Propenoic acid, 2-methyl-, decyl ester
1-Tetradecanol
Cycloheptasiloxane, tetradecamethyl-
1-Eicosanol, 2-hexadecyl-
Eicosane
2-Propenoic acid, 2-methyl-, decyl ester
1-Heptadecene
2-Propenoic acid, 2-methyl-, decyl ester
Ethanol, 2-(dodecyloxy)-
Cyclooctasiloxane, hexadecamethyl-
n-Dodecyl methacrylate
Diethylene glycol monododecyl ether
Hexadecanal
Eicosane
2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydropurin-7-yl)-N-(4-isopropylphenyl)acetamide
n-Dodecyl methacrylate
Tetradecanoic acid
Tetraethylene glycol monododecyl ether
Diethylene glycol monododecyl ether
Tetratetracontane
Diethylene glycol monododecyl ether
Hexadecanoic acid, methyl ester

C


(x10,000,000)
TIC (1.00)
Max Intensity : 76,334,301
Time 29.413 Scan# 5,284 Inten. 533,620 Oven Temp187.06
Intensity (x100000000)
Retention Time (minutes)

D


(x10,000,000)
TIC (1.00)
Max Intensity : 39,950,506
Time 19.593 Scan# 3,320 Inten. 2,419,848 Oven Temp137.96
Intensity (x100000000)
Retention Time (minutes)

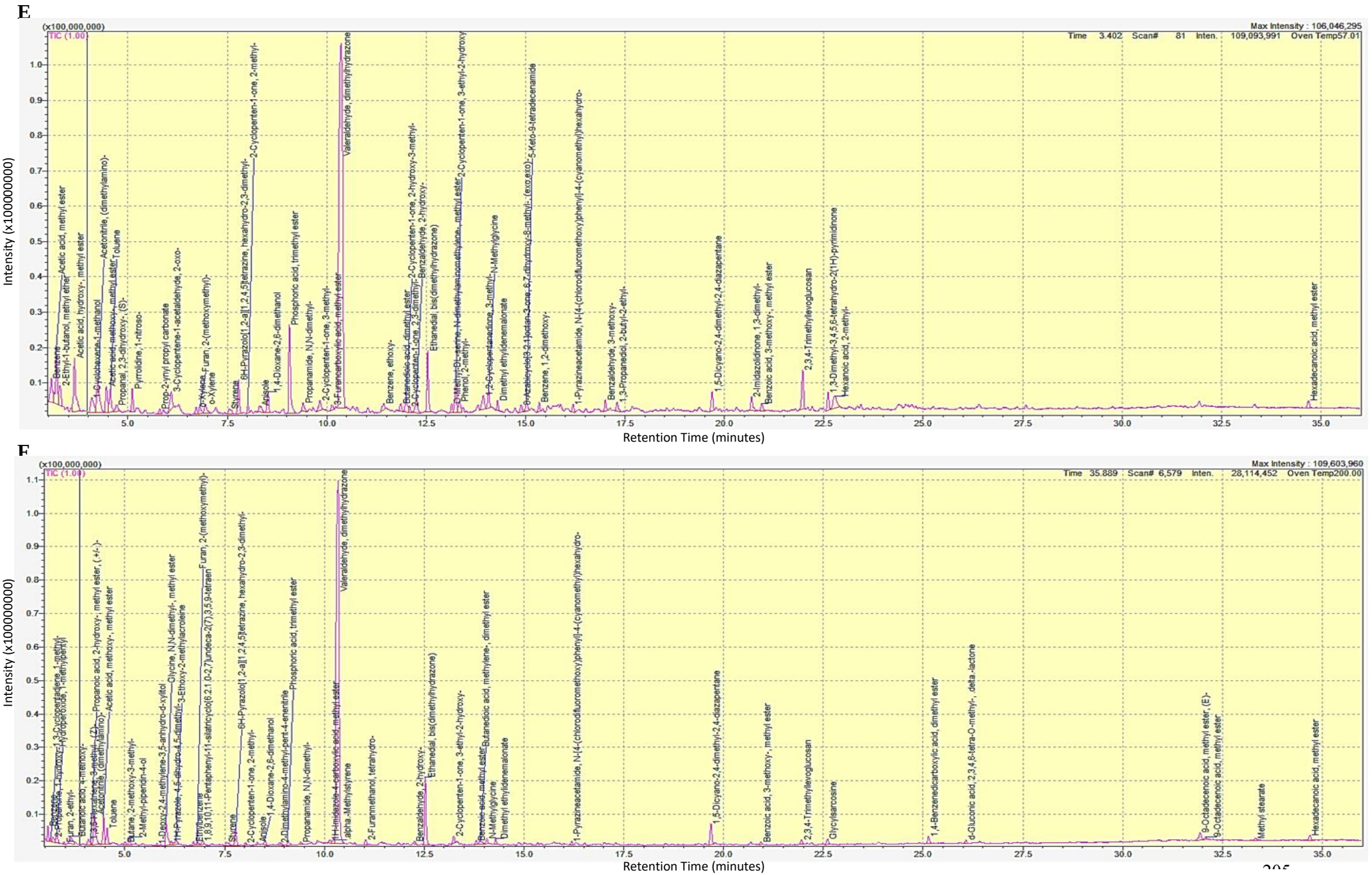


Figure 6.12: Chromatograms of the organic residues after pyrolysis with tetramethylammonium hydroxide (TMAH) as the methylating agent. Pulp before enzyme treatment: A- *E. dunnii*; B- *E. grandis*. *E. dunnii* pulp after enzyme treatment with C- acetylesterase and D- carboxylesterase. *E. grandis* pulp after enzyme treatment with E- acetylesterase and F- carboxylesterase.

6.5 Conclusions

The genes coding for an acetylesterase and carboxylesterase from *B. thuringiensis* were successfully cloned and expressed. The acetylesterase exhibited the highest specific activity at pH 4.0 and 30°C for *p*-NP valerate, followed by *p*-NP butyrate. Activity was considerably enhanced by CTAB and K^+ and to a lesser extent by methanol, Ca^{2+} and Mg^{2+}. Notable inhibition was observed with butanol, glycerol, SDS and Tween 20. Carboxylesterase exhibited highest specificity at pH 4.0 and 35°C for *p*-NP butyrate, followed by *p*-NP acetate. Activity was significantly increased by K^+ and adversely affected by butanol, glycerol, SDS and Tween 20. The treatment of *E. dunnii* pulp was most effective with carboxylesterase whilst treatment of *E. grandis* pulp was most effective with acetylesterase. Preliminary results have shown that the efficiency of lipophilic extractive removal is dependent on the type of compounds present and the specificity of the enzyme applied towards these compounds. Additional structural and biochemical characterization is necessary to improve our understanding of these esterases and their mechanism of action. Additional research is currently underway to establish the combined effects of esterases and lipases on the removal of pitch causing compounds. This would greatly benefit the pulping industry if the complete removal of lipophilic compounds was attainable.

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CHAPTER SEVEN

OPTIMIZATION OF LIPOLYTIC TREATMENT FOR THE CONTROL OF PITCH COMPONENTS IN *Eucalyptus* spp. USING RESPONSE SURFACE METHODOLOGY

7.1 Abstract

Pitch deposition during pulping is problematic and causes reduction in pulp quality and obstruction in the pulp and papermaking equipment. The deposition is caused by coagulation of pitch components, also known as wood resin or lipophilic extractives. Dissolving pulp requires high purity cellulose fibres, therefore the removal of lipophilic extractives, lignin, and hemicelluloses is essential for high-grade dissolving pulp production. In this study the action of lipolytic enzymes such as lipases, esterases and laccases, in combination with ligninases, xylanases and other accessory enzymes from bacterial and fungal isolates (indigenous to *Eucalyptus* spp.) were evaluated for the reduction of lipophilic content in *Eucalyptus* dissolving pulp. Response surface methodology was used to determine the optimal enzyme combinations and reaction conditions for maximal reduction of lipophilic content in *E. dunnii* and *E. grandis* pulp. Three independent variables, pH (3.0-5.0), enzyme dosage (3-9 U/ml), and reaction time (2-6 h) were tested. Lower pH and enzyme dosage gave the greatest response for the reduction of lipophilic content. Reaction time, however, had no effect on the response. Reduction in lipophilic content was optimal in *E. dunnii* pulp treated with 3 U/ml enzyme for 4 h at pH 4.0, whereas for *E. grandis,* 6 U/ml enzyme, pH 5.0, and 4 h was optimal. The enzymatic pre-treatment of pulp resulted in 8.4% reduction in kappa number of the pulp, showing significant delignification with this enzyme treatment. Increases in viscosity were also observed, contributing to the improvement of the pulp properties. This is the first report describing the combined application of lipases, esterases and laccases in the treatment of dissolving pulp for the reduction of pitch-causing compounds. Use of this lipolytic-xylano-lignolytic combination in the pulping industry will assist in making the process eco-friendly and economical.

Key words: *Response surface methodology, lipases, esterases, pitch, biopulping*

7.2 Introduction

Sappi Southern Africa Limited is the world's largest producer of dissolving pulp, which is a high-grade cellulose pulp (95-98% cellulose) used to produce microcrystalline cellulose, viscose rayon, lyocell, ethers, and cellophane, among many others (Figure 7.1) (Christov *et al*., 1998; Sappi, 2017). To generate high-grade cellulose pulp, maximum removal of lignin, hemicelluloses and extractives are required during the pulping process (Christov *et al*., 1998). The extractives released during pulping of wood and bleaching of pulp have a negative impact on the quality and yield of pulp generated (Ogunwusi, 2012) and on the functioning of the machinery, as extractives tend to coalesce to form sticky pitch deposits which gum up the machinery and halt production (Farrell *et al*., 1997). This has major financial implications in commercial industries, increasing production time and profits. Tackling and resolving the issue of pitch deposit formation would greatly benefit the pulping industry and streamline the production of dissolving pulp.

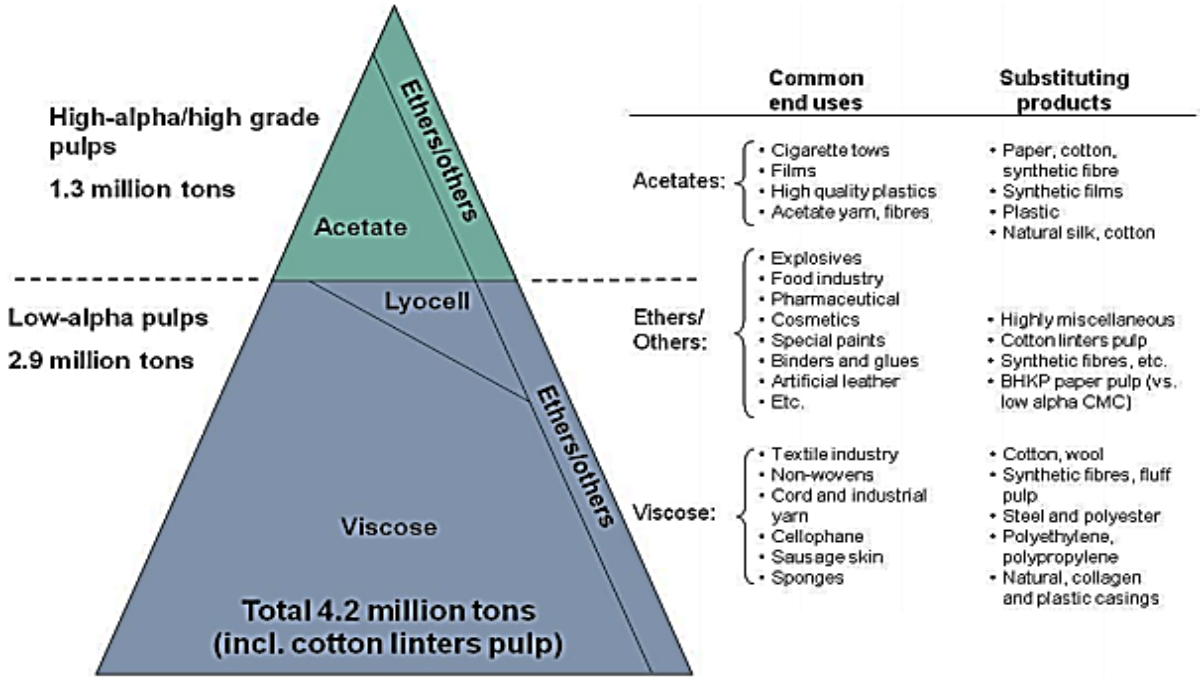

Figure 7.1: Grades of cellulose pulp and their end uses (Suhonen and Oksanen, 2016).

Pitch components comprise less than 10% of the total weight of wood material, yet they are a major challenge in pulping. Pitch components are typically comprised of fatty acids, fatty alcohols, resin acids, hydrocarbons, steroids, triterpenoids and triglycerides (Allen, 1975). The major pitch components in *Eucalyptus* kraft pulp are sitosterol, ketositosterol and steroid ketone (Table 7.1). Different lipophilic extractives may cause pitch problems along the entire

pulping and bleaching process depending on their chemical nature and the process used (Holmbom, 2000). The use of total chlorine-free bleaching, which Sappi has implemented, increases the severity of pitch problems due to the low reactivity of bleaching materials with pulp lipids (Freire *et al*., 2006).

Table 7.1: Different types of lipophilic extractives present in various pulps (*Eucalyptus*, spruce and flax) (Gutiérrez *et al*., 2006a,b; Virk *et al*., 2012).

Fatty and resin acids	Myristic acid, palmitic acid, oleic acid, linoleic acid, arachidic acid, behenic acid, lignoceric acid, cerotic acid, montanic acid, and abietic acid, eicosanic acid, decosanoic acid, tetracosanoic acid, cis-9-octadecanoic acid, pinolenic acid, eicosatrienoic acid
Fatty alcohols	*n*-docosanol, *n*-tetracosanol, *n*-hexacosanol, *n*-octacosanol, *n*-triacontanol, and *n*-dotriacontanol, eicosanol
Sterol, sterol esters, sterol glycosides	Sitosterol, stigmasterol, fucosterol, cholesteryl palmitate, cholesteryl oleate, cholesteryl linoleate, sitosteryl 3-*β*-D glucopyranoside, and 7-oxositosteryl 3-*β*-D glucopyranoside
Alkanes	Octadecane
Triglycerides	Triheptadecanoin and trilinolein
Steroid ketones	Stigmastan-3-one, stigmasta 3,5-dien-7-one, and 7-oxositosterol

There are traditional means of addressing pitch-related problems in mills; they include wood seasoning (storage of logs outdoors) and the addition of additives such as alum, talc, etc. However, biotechnological approaches are preferred due to their non-toxic nature, reduction in chemical consumption and waste, and improved pulp quality (Bajpai, 1999). Many studies have been conducted on the treatment of wood or pulp with enzymes or microorganisms to assist in reducing the compounds that contribute to pitch formation (Bajpai, 1999; Rocheleau *et al*., 1999; Calero-Rueda *et al*., 2002; Jones, 2005; Josefsson *et al*., 2006; Dubé *et al*., 2008; Gutiérrez *et al*., 2011). Enzymes used include lipases, esterases, and in recent times, laccases. Lipases (EC 3.1.1.3) hydrolyse long-chain acyl groups (Litthauer *et al*., 2002; Ellaiah *et al*., 2004), whereas esterases (EC 3.1.1.1) hydrolyse the ester bonds of water-soluble fatty acid esters with short-chain acyl groups (Verger, 1997). The majority of studies have focussed on the addition of one of these groups of lipolytic enzymes to remove pitch components. However, esterases act on a limited number of substrates such as glycerides and sterol esters, and therefore are not effective in the degradation of other components of pitch deposits (Gutiérrez *et al*., 2009). As a result, enzymes acting on a broader range of substrates need to be investigated. Another group of enzymes recently associated with the removal of recalcitrant lipophilic compounds as well as lignin, are laccases (EC 1.10.3.2) commonly supplemented

with mediators (Zhang *et al*., 2005; Gutiérrez *et al*., 2006b; Valls *et al*., 2009; Virk *et al*., 2013). The use of a laccase-mediator system was reported to be proficient in the removal of lipophilic compounds from pulp, regardless of the pulping material used (Molina *et al*., 2008; Valls *et al*., 2009). The pulping method employed also has an influence on the type of compounds that are involved in pitch formation. Sulphite pulping for example, used commercially for the past 27 years (Grant, 1994), generates higher amounts of extractives compared to kraft pulping. This is due to the acidic conditions, whereas alkaline conditions dissolve and degrade wood resin (Sithole *et al*., 2010). In South Africa, the largest producer of dissolving pulp currently uses acid-bisulphite pulping and would therefore need solutions to the problem of excessive extractive accumulation. The traditional methods of pitch control have become outdated due to the space required (seasoning) and toxic nature of chemicals, hence, pulp and paper mills around the world are considering more environmentally friendly alternatives.

A successful pitch control strategy using fungi is the application of an albino strain of *Ophiostoma piliferum* (commercially available as Cartapip 97). However, Cartapip 97 was unable to efficiently remove free sterols from hardwoods and resin acids from softwoods (Martínez-Íñigo *et al*., 1999; Dorado *et al*., 2000). Direct application of an enzyme in the treatment of pitch has also been achieved with lipases such as Resinase® A2X (Novozymes, Bagsvaerd, Denmark). This commercial enzyme has only been successful in controlling pitch in mechanical, thermal and sulphite pulping of softwoods (Gutiérrez *et al*., 2001). Hence, enzyme treatments are dependent on type of wood and pulping process employed. There remains a gap in the research for effective enzyme treatments for the reduction or elimination of pitch deposits in acid-bisulphite pulp of *Eucalyptus* wood species.

Optimization of treatment conditions is an essential step in creating an efficient and cost-effective treatment method. Response Surface Methodology (RSM) is commonly used in the optimization of biochemical and chemical processes (Bas and Boyaci, 2007). Its popularity is due to its capacity to analyse effects of multiple independent variables and their interactive influences simultaneously whilst involving a limited number of experiments (Khuri and Cornell, 1996). In particular, the Box-Behnken design, which was used in this study, requires fewer trials in a 3-factors 3-levels experimental design. Furthermore, being a modified central composite experimental, it is an independent, rotatable quadratic design, in which the treatment combinations are at the midpoints of the edges of the process space and at the centre (Ragonese *et al*., 2002). The acceptability of the proposed model is then examined using the diagnostic

checking tests provided by analysis of variance (ANOVA). The response surface plots, in turn, can be applied to analyze the surfaces and determine optimum conditions. A number of studies have successfully used RSM to optimize process conditions (Liu *et al*., 2012; Virk *et al*., 2013; Chauhan *et al*., 2013; Neiva *et al*., 2014; Hermawan *et al*., 2015).

In this study, we propose to treat the pulps of two popular *Eucalyptus* species with a combination of lipases, esterases and laccases to potentially eliminate all lipophilic compounds that contribute to the formation of pitch deposits. To our knowledge this would be the first such report on pitch control. Optimized treatments of pitch components in *Eucalyptus grandis* and *E. dunnii* were achieved using RSM. The simultaneous influence of pH, dosage and reaction time on lipophilic content of the pulp was evaluated and the quality of the unbleached pulps was compared to the control pulp experiments.

7.3 Materials and Methods

7.3.1 Material

Eucalyptus wood species *E. dunnii* and *E. grandis* were obtained from a plantation in KwaZulu-Natal (South Africa). Logs from approximately 10-year old trees were chipped to average dimensions of 12 mm x 5 mm x 2 mm and dried at room temperature for 2 weeks. Pulping was performed according to the acid-bisulphite process currently implemented at Dissolving Wood Pulp (DWP) mills. The unbleached pulp was collected after the washing step for experimental trials. Bleaching was not necessary as the effectiveness of potential lignin degrading enzymes produced by indigenous bacteria were also considered.

7.3.2 Enzymes

The following enzymes were selected based on our present study (Chapters 5 and 6). Lipases were isolated from bacteria indigenous to *Eucalyptus* species and the esterases were cloned using sequence-based screening and genomic DNA from a bacterial isolate also cultivated from *Eucalyptus* species. Due to the nature of this project, the authors did not wish to purify the sample, but only to remove any potential cellulases that may be present. Cellulases would negatively impact dissolving pulp, however, the presence of xylanases, ligninases and other accessory enzymes would assist in improving the quality of pulp. Enzyme mixtures were therefore maintained as cellulase-free, lipolytic, hemicellulolytic and ligninolytic suspensions, to improve the overall quality of pulp. Laccases were included based on a study extolling their dual benefit of pitch reduction and lignin removal (Virk *et al*., 2012; Rencoret *et al*., 2016).

Based on previous screening and activity data from colleagues, two fungal strains expressing satisfactory laccase activity were selected. Since the optimum temperatures of the lipases and esterases were 30 and 35°C, respectively, laccase activity was tested at these temperatures as the final application was to create an enzyme cocktail for effective removal of lipophilic extractives from pulp. It is accepted that when fungi are grown at pH 5.0, laccases will be produced in excess, however most studies show that a pH range of 3.6 to 5.2 is acceptable for enzyme production (Ghatora *et al*., 2006; Madhavi and Lele, 2009). These fungal strains were also tested for the lipolytic abilities.

Table 7.2: Characteristics of enzymes to be used in cocktail mixtures for pulp treatments.

Isolate	Enzyme	Optimum pH	Optimum Temperature	Substrate Specificity
BT	Lipase	5.0	30°C	Dodecanoate, myristate, octanoate, acetate
DF7	Lipase	4.0	35°C	Dodecanoate, octanoate, valerate, butyrate
B9	Lipase	4.0	35°C	Valerate, dodecanoate, butyrate, octanoate
DF3	Lipase	4.0	30°C	Palmitate, dodecanoate, myristate, octanoate
F4	Laccase/Lipase	4.0	35°C	Dodecanoate, palmitate, octanoate, myristate
X	Laccase/Lipase	5.0	30°C	Dodecanoate, stearate, myristate, octanoate
BM	Esterase	4.0	30°C	Valerate, butyrate, myristate, acetate
CARB	Esterase	5.0	35°C	Butyrate, acetate, myristate, octanoate

Abbreviations: BT – *Bacillus thuringiensis*, DF7 – *B. thuringiensis*, B9 – *Pantoea* sp., DF3 – *Curtobacterium flaccumfaciens*, F4 – *Paecilomyces formosus*, X – *Phialophora alba*, BM – recombinant acetylesterase, CARB – recombinant carboxylesterase.

Based on activity (Table 7.3), the following enzyme cocktails were designed for the pulp treatments. Eight treatments comprising enzyme cocktails and individual enzymes were applied (Table 7.4). Rationally, the combination of lipases and esterases would yield a greater degradation of lipophilic compounds as opposed to lipase or esterase only, since lipases target long acyl chains whereas esterases target short acyl chains. As a result they were not tested independently. Laccases were tested individually and in combination with lipases and esterases to establish their effects.

Table 7.3: Activity and substrate specificity of the enzymes.

	Substrate 1	U/ml	Substrate 2	U/ml	Substrate 3	U/ml	Substrate 4	U/ml
DF3	Palmitate	62.8	Dodecanoate	56.5	Myristate	49.7	Octanoate	39.9
DF7	Dodecanoate	51.1	Octanoate	42.8	Valerate	37.9	Palmitate	35.3
B9	Dodecanoate	42.9	Palmitate	23.5	Butyrate	18.7	Octanoate	18.6
F4	Dodecanoate	46	Octanoate	26.3	Myristate	25.7	Butyrate	25.1
BT	Dodecanoate	62.4	Myristate	51.1	Octanoate	47.7	Acetate	47.3
X	Dodecanoate	44.4	Stearate	35.1	Myristate	32	Octanoate	30.2
BM	Valerate	147.8	Butyrate	87.8	Myristate	15.1	Acetate	8.4
CARB	Butyrate	127.1	Acetate	98.4	Myristate	34.7	Octanoate	32.7
F4	Syringaldazine	2.0		-		-		-
X	Syringaldazine	3.1		-		-		-

Table 7.4: Enzymes, producing organisms, and treatment combinations.

Enzymes	Isolates	Combinations
Lipase cocktail 1:	DF3 and BT and F4	Lipase 1 and Esterase 1
Lipase cocktail 2:	DF7 and X	Lipase 1 and Esterase 2
Lipase cocktail 3:	B9 and F4	Lipase 2 and Esterase 1
Esterase 1:	BM	Lipase 2 and Esterase 2
Esterase 2:	CARB	Lipase 3 and Esterase 1
Laccase 1:	F4	Lipase 3 and Esterase 2
Laccase 2:	X	Laccase 1
		Laccase 2

7.3.3 Experimental design and analyses

Response surface methodology (RSM) with the Box-Behnken design was used to investigate the influence of three variables on the lipophilic content of pulp and to statistically determine the optimum combination of enzyme dosage, pH and reaction time. The main, interaction, and quadratic effects of the variables on the modification of the pulp by enzymatic treatment was also assessed. A 3-factor, 3-level design was applied to investigate the quadratic response surfaces and construct second-order polynomial models. Therefore, each variable was coded and run at three independent levels:-1, 0, and 1 (Table 7.2). The effects of reaction time (2, 4 and 6 h), dosage (3, 6 and 9 U/ml) and pH (3.0, 4.0 and 5.0) on the lipophilic content of the pulp were determined. The interaction of the three parameters was also examined. The nonlinear quadratic model was as follows (Eq. [1]):

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 \sum_{i=1}^{k} \sum_{j=i+1}^{k} b_{ij} X_i X_j + \varepsilon \quad \text{… Eq. [1]}$$

Y is the predicted response related to each factor level combination, b_0 is the interception coefficient, b_i is the linear coefficient, b_{ii} is the quadratic coefficient, b_{ij} is the interaction

coefficient, X_i is the independent variable under study, k is the number of factors studied and ε is the random error. The analysis of variance (ANOVA) technique was utilized to evaluate the competence of the developed model. The significant relationships in the model equations were assessed using p-value and F-value; and the degree of fitness of the models were evaluated by the R-squared values and the lack of fit test. Response surfaces, residual plots, and the optimum conditions were attained through these models. Regression analysis and estimation of coefficients as well as all other analyses were achieved using Design Expert v10 (StatEase, USA) as well as Statistica software (Version 13, Dell Inc., USA) as a comparison point. Optimal treatment conditions from the RSM were validated by treating the pulp at the determined optimum enzyme dosage, pH, and reaction time. Statistical considerations included; coefficient, standard error and T-value. Coefficient is the regression coefficient of the term, which represents the contribution of the term to the variation in the response. Standard Error is the standard deviation of the regression coefficient and the T-value is the normalized regression coefficient, which is equal to Coefficient/Standard Error (Abdi, 2003).

Table 7.5: Experimental design (Box-Behnken) showing actual values of independent variables (dosage, pH and reaction time) tested.

Trial	Dosage (U/ml)	pH	Time (h)
1	3	5	4
2	3	3	4
3	3	4	2
4	6	4	4
5	6	4	4
6	3	4	6
7	9	3	4
8	6	5	6
9	6	3	2
10	9	4	6
11	6	3	6
12	6	5	2
13	9	4	2
14	9	5	4
15	6	4	4

A total of 120 trial experiments were conducted for each of the *Eucalyptus dunnii* and *E. grandis* wood species tested.

7.3.4 Enzyme treatments

A method for the treatment of pulp slurry with enzymes, adopted from Bajpai *et al*. (2003) was used. Two millilitres of buffer (at the appropriate pH) was added to 0.5 g oven-dried unbleached pulp and vortexed briefly to ensure saturation of the pulp sample. Following the Box-Behnken design, different combinations and dosages of enzymes were added to the pulp suspensions. The final volume of the enzyme cocktails was one millilitre, bringing the total volume of the pulp suspensions to three millilitres per experiment to ensure optimal dispersion of the enzymes. Pulp suspensions were incubated at 30°C and 180 rpm for varied periods of time. The enzymatic reaction was terminated by boiling for 5 min to deactivate the enzymes. The control samples contained no enzymes. Samples were freeze dried overnight to prepare for Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) analysis. The lipophilic profiles of the treated samples were examined and compared to the untreated samples.

Based on the results from the RSM design, the ideal combination of enzymes, pH, dosage and incubation period was selected for large scale trials. Five hundred grams of unbleached oven-dried pulp was set up in three-litre flasks. After addition of the cocktail of enzymes and appropriate buffer, the total weight of the pulp suspension reached two kilograms. Flasks were incubated at 30°C for 4 h at 200 rpm. Samples were collected and freeze dried overnight for Py-GC-MS analysis. The remainder of the treated pulp was drained to remove excess liquid and analysed for various parameters as described further on.

7.3.5 Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) analysis

Py-GC/MS permits direct analysis of the original natural material, as the pyrolytic unit is able to generate volatile fragments, which can then be analyzed using GC/MS (Sithole *et al*., 2013). Py-GC-MS was employed for the characterisation of lipophilic compounds in the pulp samples. Samples of approximately 0.2 mg were weighed out and five microliters of tetramethylammonium hydroxide (TMAH) (Sigma Aldrich, USA) was added directly to the sample as a derivatizing agent to increase detection of compounds (Fukushima *et al*., 2009). A multi-shot pyrolyzer, EGA/PY-3030 D, (Frontier Lab, Japan) attached to an ultra-alloy capillary column (30 m x 0.25 mm, 0.25 μm) was used (GCMS-QP2010 SE, Shimadzu, Japan). The samples were pyrolysed at 550°C for 20 s and the interface temperature was fixed at 350°C. The chromatographic separation of the volatile components released by pyrolysis was performed using an ultra-alloy column. The injection temperature was set to 280°C and the column flow rate was set to 1.0 mL/min with helium used as a carrier gas. The temperature

was programmed as follows: 50°C for 2 min; rate 3°C/min up to 200°C and held for a further 4 min. The ion source and interface temperatures were set to 200°C and 300°C, respectively. The scan range used for mass selective detector was 40-650 m/z. The peaks of the pyrolysis-chromatograms attained were interpreted with GC-MS Postrun Analysis software (Shimadzu, Japan) by pairing each peak with the compound listed in spectral libraries provided by the NIST (National Institute of Standards and Technology) library attached to instrument (modified from Sithole and Watanabe, 2013). In the majority of the instances quality matches above 85% were reported, but in certain cases, lower quality matches (minimum 70%) were considered as the degradation fragments seemed more suitable. It is generally accepted, as in other analytical studies, that the chromatographic peak area of a compound can be reasoned as linear with its quantity and correspondingly, the peak area percentage linear to its composition. Changes can therefore be detected in the relative content of products by evaluating the peak area percentage of each identified compound (Lu *et al*., 2011; Carvalho *et al*., 2015). The compounds of interest that corresponded to lipophilic extracts were calculated by dividing the area percentage by 100 and then multiplying by the mass of the sample (~0.25 mg). The sum of lipophilic compounds detected for each sample was then recorded as the response for the RSM design experiments.

7.3.6 Analyses of treated pulps

Quality of treated pulp was determined by assessing viscosity (T203 om-94), final pulp yield, acid-insoluble lignin (T222 om-88), soluble lignin and kappa number (T236 cm-85) (Forestry and Forest Products Research Centre, CSIR, Personal Communication, 2014). The carbohydrate content of treated pulp was characterized by High Performance Liquid Chromatography (HPLC) for quantification of cellulose and hemicelluloses (glucose, mannose, arabinose, xylose, rhamnose, and galactose) using the Dionex ICS-5000 plus with a CarboPac PA1 column (4 x 250 mm) with pulsed amperometric detection (Thermo Fischer Scientific, United States) (T249 cm-85; Wallis *et al*., 1996; Wright and Wallis, 1996).

7.4 Results and Discussion

Response surface methodology was conducted using the Box–Behnken design to determine optimum enzyme treatment conditions for reduction of lipophilic content in the wood pulp of two *Eucalyptus* species. The relationship between three regulated factors (dosage, pH and reaction time) with three levels per factor (-1, 0 and 1) and one variable factor (lipophilic content) was determined (Table 7.6). For the experimental design 15 permutations (trials) of conditions were tested on the eight enzyme combinations for each wood species, resulting in

120 experimental trials per Eucalypt species. Due to the large number of models generated from this study, the tables and graphs of one experimental trial of each Eucalypt spp. will be represented here. Readers may find the other data outputs in the supplementary files (Appendix 7A and 7B).

Table 7.6: Experimental results of independent variables (dosage, pH and reaction time) on the dependent variable (lipophilic content) of selected *E. dunnii* and *E. grandis* pulp.

***E. dunnii* (Lipase 1 vs Esterase 1)**				
Trial	**Dosage (U/ml)**	**pH**	**Time (h)**	**Lipophilic content (mg)**
1	9	5	6	0.0259
2	9	5	2	0.0073
3	3	3	4	0.0081
4	6	4	6	0.0145
5	3	4	4	0.0016
6	9	4	4	0.0055
7	6	5	4	0.0114
8	6	3	2	0.0119
9	3	5	6	0.0200
10	6	3	6	0.0245
11	3	5	2	0.0109
12	6	5	4	0.0132
13	6	4	2	0.0107
14	6	5	4	0.0164
15	9	3	4	0.0208
***E. grandis* (Lipase 3 vs Esterase 1)**				
Trial	**Dosage (U/ml)**	**pH**	**Time (h)**	**Lipophilic content (mg)**
1	9	4	4	0.02520
2	6	3	2	0.02714
3	3	4	4	0.02186
4	6	4	2	0.02952
5	3	3	4	0.01842
6	3	5	6	0.02236
7	6	4	6	0.03044
8	6	3	6	0.02608
9	6	5	4	0.01434
10	6	5	4	0.02260
11	3	5	2	0.02216
12	9	5	6	0.02372
13	6	5	4	0.01990
14	9	3	4	0.02026
15	9	5	2	0.01630

Interaction effects of variables on modification of the lipophilic content of the pulp

Model terms (factors) that are significant are required to obtain a good fit for a particular model. The results obtained from Eq. [1] were analyzed by ANOVA to assess the "goodness of fit". ANOVA and the probability value (p-value) were used to test the significance of the regression model. pH and shorter reaction times significantly reduced lipophilic content as evidenced by their p-values: 0.03 and 0.007 for *E. dunnii* and 0.02 and 0.04 for *E. grandis*, respectively (p-value<0.05) (Table 7.7). Dosage and longer reaction times appeared to have less of an effect on the lipophilic content. The 'F-value' is the ratio of mean regression sum of squares divided by the mean error sum of squares, which tells us if the means between two populations are significantly different (Williams, 2015). For both *Eucalyptus* species, pH and time appeared to be significantly different. Degrees of freedom for the model is the number of regression coefficients for the effects included in the analysis (Williams, 2015). In this case, for both wood species two coefficients may be responsible for the main effect which, considering all other statistical data, would be expected to be pH and time. Higher R^2 values are generally an indication of a better fit, as the model is parallel to the observations. Evaluation of R^2 should be performed with caution as a higher value does not necessarily mean that the model suitably expresses the relationship between the predictors and the responses (Neiva *et al*., 2014). If an effect is added to a model, even if it's noise, the R^2 will increase. Therefore, R^2 is not necessarily a good indicator for the quality of a model, as a model could be built with R^2=100% with only noise (Alexander *et al*., 2015). Although the R^2 values of 0.9073 (*E. dunnii*) and 0.8600 (*E. grandis*) were not closer to 100%, the shortfall may have been due to the lack of noise, consequently making the obtained R^2 values suitable in this study. "Effect" is a measure of how much the response value (Y) changes when the value of the corresponding term (factor) in the model changes from the low level to the high level (Alexopoulos, 2010). For *E. dunnii* the effect value is negative for pH and time indicating that the high value of the variable is better (Figueiredo Filho, 2013). The results from the statistical analyses reveal that efficiency of the removal of pitch forming compounds may be addressed by implementing the statistical significant parameters mentioned above.

Table 7.7: ANOVA and regression coefficients of the response surface quadratic model for the response variables of *E. dunnii* and *E. grandis*.

***E. dunnii* Lipase 1 and Esterase 1**							
Effect Estimate	**Effect**	**Std. Err.**	**t(8)**	**p-value**	**Coefficient**	**Std. Err. Coefficient**	**Regression Coefficient**
Mean/Interc.	0.013475	0.001162	11.59400	0.000003	0.013475	0.001162	0.123704
(1) Dosage (L)	0.004725	0.002847	1.65970	0.135556	0.002363	0.001423	0.003482
Dosage (Q)	0.002021	0.002095	0.96448	0.363050	0.001010	0.001048	-0.000225
(2) pH (L)	-0.00147	0.002533	-0.58395	0.575346	-0.000740	0.001267	-0.060823
pH (Q)	-0.00751	0.002378	-3.15785	0.013438	-0.003755	0.001189	0.007510
(3) Time (L)	0.011025	0.002847	3.87264	0.004724	0.005513	0.001423	-0.006002
Time (Q)	-0.00437	0.002095	-2.09004	0.070014	-0.002190	0.001048	0.001095
ANOVA	**Sum of Squares**	**Degree of Freedom**	**Mean Square**	**F-value**	**p-value**		
(1) Dosage L+Q	0.000060	2	0.000030	1.842422	0.219719		
(2) pH L+Q	0.000162	2	0.000081	4.996204	0.039084		
(3) Time L+Q	0.000314	2	0.000157	9.682822	0.007304		
Error	0.000130	8	0.000016				
Critical Values	**Observed minimum**	**Critical Values**	**Observed Maximum**				
Dosage	3.000000	7.753608	9.000000				
pH	3.000000	4.049237	5.000000				
Time	2.000000	2.741199	6.000000				
***E. grandis* Lipase 3 and Esterase 1**							
Effect Estimate	**Effect**	**Std. Err.**	**t(8)**	**p-value**	**Coefficient**	**Std. Err. Coefficient**	**Regression Coefficient**
Mean/Interc.	0.023622	0.000852	27.73667	0.000000	0.023622	0.000852	-0.045711
(1) Dosage (L)	0.000170	0.002086	0.08149	0.937052	0.000085	0.001043	0.003143
Dosage (Q)	0.002336	0.001535	1.52140	0.166653	0.001168	0.000768	-0.000260
(2) pH (L)	-0.00293	0.001856	-1.58082	0.152575	-0.001467	0.000928	0.040510
pH (Q)	0.005247	0.001743	3.01082	0.016793	0.002624	0.000871	-0.005247
(3) Time (L)	0.001870	0.002086	0.89642	0.396203	0.000935	0.001043	-0.008581
Time (Q)	-0.00452	0.001535	-2.94673	0.018518	-0.002262	0.000768	0.001131
ANOVA	**Sum of Squares**	**Degree of Freedom**	**Mean Square**	**F-value**	**p-value**		
(1) Dosage L+Q	0.000020	2	0.000010	1.160652	0.360929		
(2) pH L+Q	0.000115	2	0.000057	6.578181	0.020445		
(3) Time L+Q	0.000083	2	0.000041	4.743397	0.043805		
Error	0.000070	8	0.000009				
Critical Values	**Observed minimum**	**Critical Values**	**Observed Maximum**				
Dosage	3.000000	6.054584	9.000000				
pH	3.000000	3.860200	5.000000				
Time	2.000000	3.793332	6.000000				

For both model equations (Eq. 2 and 3), coefficients with negative signs were observed. For the *E. dunnii* model B, AB and A^2 have negative signs whereas A, C, AC, BC, B^2 and C^2 have positive signs. The *E. grandis* model has negative signs for B, AB, A^2 and B^2, while A, C, AC, BC, C^2 have positive signs. A positive sign for the coefficients in the model indicates a positive interaction while the negative sign suggests the opposing effect of each factor (Anupam *et al*., 2015). Generally the positive effect correlates with an increase in response when the variable increases while the negative effect correlates with a decrease in response when the variable increases (Brown and Brown, 2012). In this study, a decrease in response when the variable deceases would be ideal, as lower dosages and reaction times would be better suited to industry conditions.

Model equations:

E. dunnii

$$\text{Lipophilic content} = 0{,}00689583 + 0{,}00305\,A - 0{,}000739583\,B + 0{,}00532727\,C - 0{,}00275\,AB + 0{,}002375\,AC + 0{,}000740909\,BC - 0{,}00202083\,A^2 + 0{,}00751042\,B^2 + 0{,}00437917\,C^2 \quad \text{… Eq. [2]}$$

E. grandis

$$\text{Lipophilic content} = 0{,}0256608 + 0{,}000380909\,A - 0{,}00146708\,B + 0{,}000625455\,C - 0{,}00118364\,AB + 0{,}001805\,AC + 0{,}00123818\,BC - 0{,}00233583\,A^2 - 0{,}00524708\,B^2 + 0{,}00452417\,C^2 \quad \text{… Eq. [3]}$$

Key: A-dosage; B-pH; C-time

The effects plots allow visual assessment of the effects of factors and factorial interactions on response. The three-dimensional (3-D) surface graph in this study presents the combined effect of dosage and pH on response which represents the lipophilic content of the pulp, as shown in Figure 7.2. In this study, enzymes were used to degrade the lipophilic compounds in pulp, therefore, a reduction in the lipophilic content would be regarded a positive response. Generally with RSM designs, the red coloration of the graph is an indication of optimized conditions with greatest response, however, in this study the opposite is true. Areas with green coloration are interpreted as optimal conditions for the treatment of pulp as lowest lipophilic content would be observed here. Response surfaces corresponding to the experimental results showed that the most effective of the independent variables in reducing lipophilic content was pH. For *E. dunnii* pulp, improved reduction in lipophilic content was observed with more acidic conditions (pH 3.0) and lower enzyme dosages, whereas *E. grandis* showed greater reduction in lipophilic content at pH 5.0 and higher enzyme dosages. Variable optimum reaction periods were

observed. The lipophilic content of *E. dunnii* pulp was dependent on pH and time, indicated by the response surface with a steep slope (Figure 7.2A), and displayed lower lipophilic reduction potential at higher dosages and longer reaction times. These results show that at lower dosages for both wood species the lipophilic reducing potential of the enzyme combination reached the highest level with the median reaction time tested.

Depending on the effectiveness of the treatment, the lipophilic content of the pulps ranged from <0.01 mg to 0.07 mg in *E. dunnii* pulp and from <0.01 mg to 0.05 mg in *E. grandis* pulp. There were some differences in the types of lipophilic degradation products observed for both pulps which may explain why the same enzyme combinations and conditions yielded different responses. Similar observations were made in another study where a distinct difference was shown between the dissolving pulp of *E. dunnii* and a *E. grandis* x *urophylla* clone (GU-W) (Kilulya, 2012). This may explain why lower dosages of lipases, esterases and laccases were sufficient to reduce the lipophilic content of the *E. dunnii* pulp. *E. grandis* in contrast, required the maximum enzyme dosage for laccase 1 and 2, lipase 1 and 2 and esterase 1 and 2, however, the lowest lipase 3 enzyme dose was sufficient to reduce lipophilic content. Overall, across the pH range (pH 3.0-5.0), lower dosages of enzymes and shorter reaction times produced the greatest reduction in the lipophilic content of the pulp (Figure 7.2). These response surface graphs are supported by the t and p values obtained (Table 7.7). The higher the value of t and the lower the value of p, the more significant is the corresponding coefficient term (Vanderghem *et al*., 2012). Lipophilic content of the pulp was strongly dependent on pH and to a minimal degree on time and dosage.

The general trend of the 3-D contour plot for *E. dunnii* revealed that longer reaction times, higher pH levels and lower dosages were most efficient in reducing lipophilic content of the pulp. The increase in pH led to a substantial reduction of lipophilic compounds in the pulp, up to 97.7% at pH 4.0, after 4 h and 3 U/ml. The maximum reduction in lipophilic content in *E. grandis* pulp was 70.7% under the following treatment conditions: pH 5.0, 4 h and a dosage of 6 U/ml.

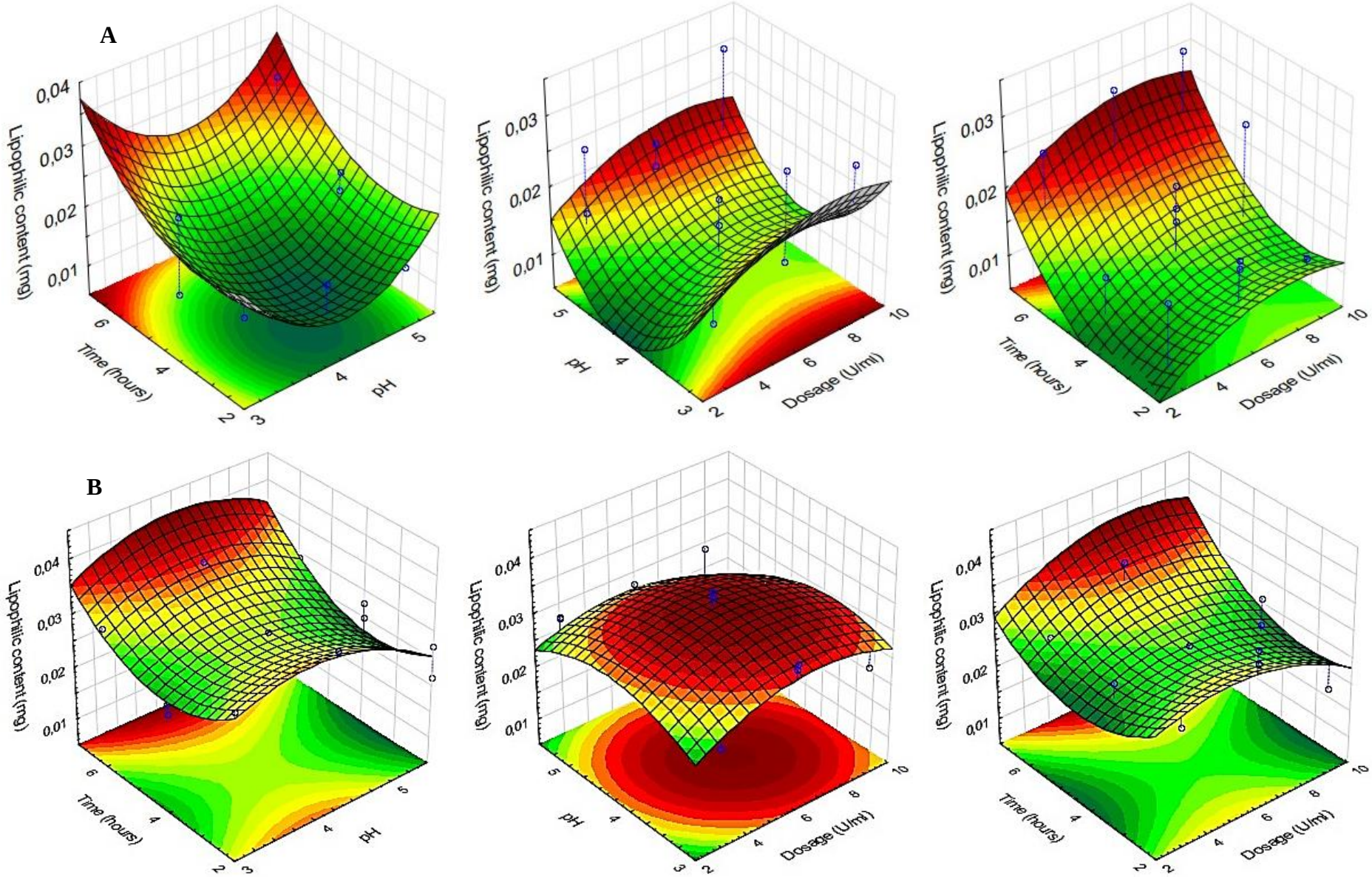

Figure 7.2: Response surface plots showing the effects of enzyme dosage (U/ml), pH and reaction time (hours) on response (lipophilic content of *Eucalyptus* sp. pulp (mg)). **A:** effect of lipase 1 and esterase 1 on *E. dunnii* pulp. **B:** effect of lipase 3 and esterase 1 on *E. grandis* pulp.

The normal plots of residuals, the residuals versus the predicted response, the residuals versus experimental trial, and the actual versus predicted responses for *E. dunnii* and *E. grandis* models are shown in Figure 7.3 and 7.4, respectively. The actual results plotted against the predictive results, with the points aligned around the 45° line, implies that the model defines the true behaviour of the system and that the errors are evenly distributed, thus supporting the aptness of the fit (Yuliwati *et al*., 2012) and its use for interpolation (Myers and Montgomery, 1995). In the normal probability plot the model again fits the data well as all points occur on the line, following a normal distribution. Some scatter is to be anticipated, however, if there is a general lack of fit and the data forms a perceptible pattern around the line, then the variable may be transformed should it be used for further analysis. This step was not necessary in the current analysis. A close fit of the points to the line was observed for both *Eucalyptus* species, indicating that the model fits the data appropriately. In Figures 7.3A, 7.3B, 7.4A and 7.4B, the residuals versus the predicted response and the residuals versus the experimental trials are randomly scattered above and below the x-axis with no observable pattern, however, all the points lie between ±3.00 and since the sum of the residuals are always zero, this suggests that the proposed models are suitable and reliable (Yi *et al*., 2011). The actual and predicted responses in Figure 7.3D and 7.4D demonstrate an almost linear relationship with slight deviations. These evaluations validate the association of the model with the experimental data. Therefore, the models can be considered satisfactory for prediction as well as optimization of the pre-treatment of pulp. In addition, results from the Statistica and Design Expert software yielded the same results, however, Design expert had a more user friendly interface compared to Statistica.

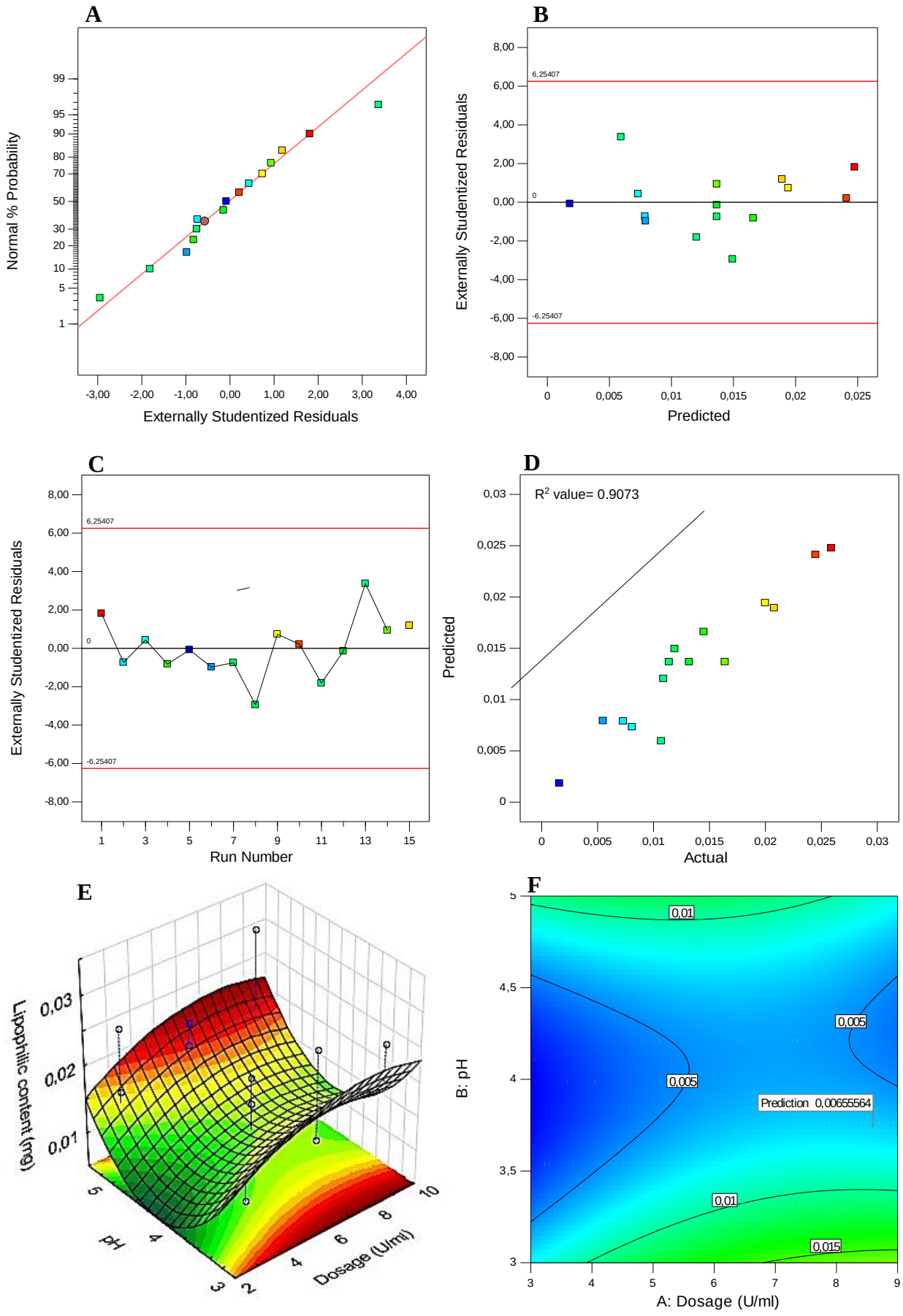

Figure 7.3: Diagnostics and model graphs for lipophilic content reduction of *E. dunnii* pulp. A: normal probability plot of residuals, B: residuals versus predicted, C: residuals versus trial number, D: predicted versus actual values of the lipophilic content of treated pulp, E: response surface, and F: contour plot.

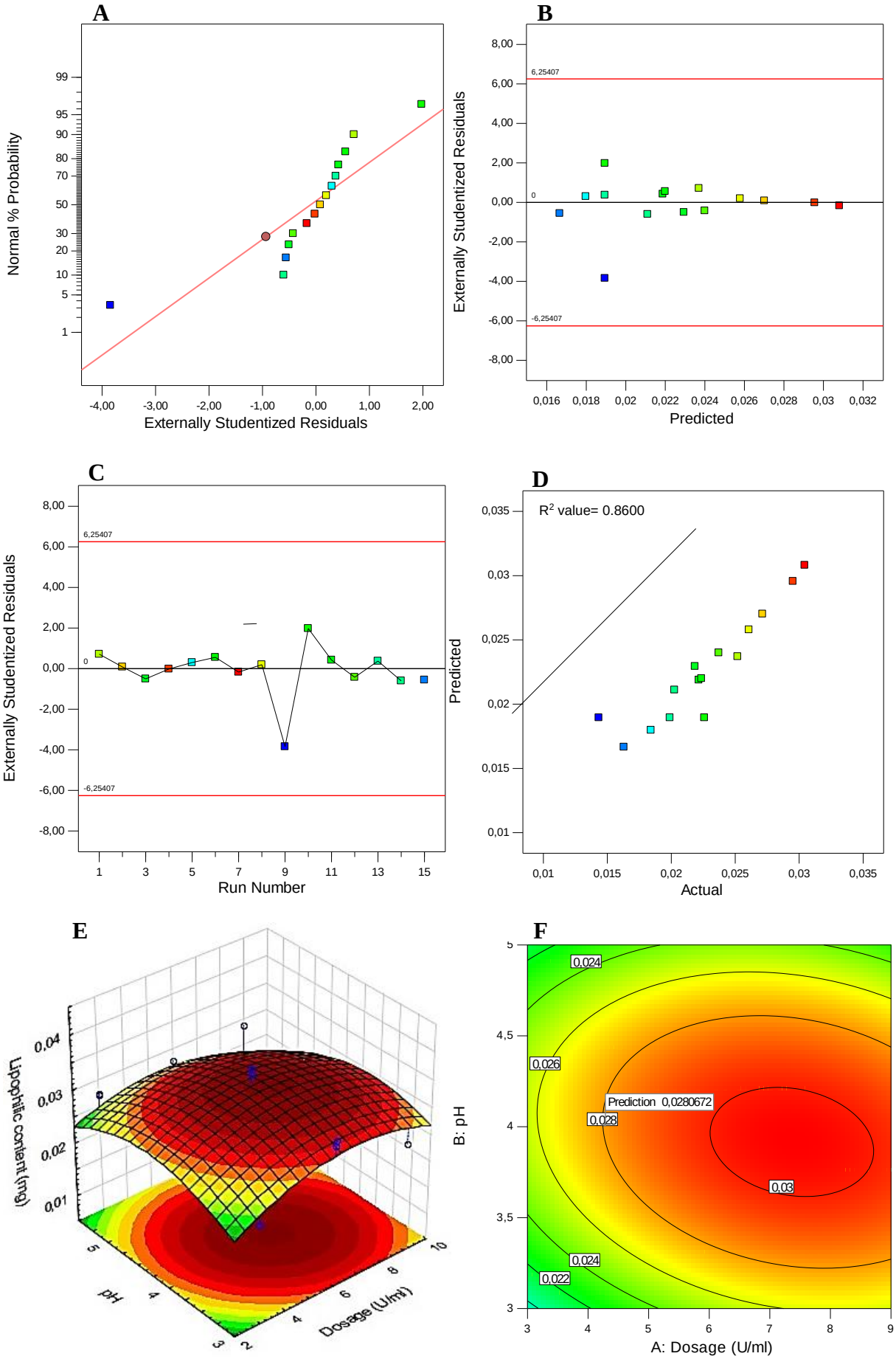

Figure 7.4: Diagnostics and model graphs for lipophilic content reduction of *E. grandis* pulp. A: normal probability plot of residuals, B: residuals versus predicted, C: residuals versus trial number, D: predicted versus actual values of the lipophilic content of treated pulp, E: response surface, and F: contour plot.

Optimizing a number of settings for each response may lead to incompatible settings for the factors. However, it is possible for a number of responses to be optimized simultaneously. A balanced setting has to be found that gives the most suitable values for all the responses, and this may be achieved with desirability functions. The relationship between predicted responses on a dependent variable and the desirability of responses is called the desirability function (Derringer and Suich, 1980; Raissi, 2009). Contour plots show the levels of overall response desirability produced in different regions of the plane defined by pairs of independent variables, where each region of the plane represents a different combination of the levels of the two variables and their effect on the response. This enables one to visualize the influence of changes in the levels of each predictor variable on responses of each dependent variable, as well as the overall desirability of the responses. This is beneficial when determining how quickly overall response desirability changes as the predictor variable changes for each independent variable, thus enabling a differentiation between "inert" and "active" factors. A higher desirability of reduced lipophilic content in *E. dunnii* was observed at pH 4.0 and 2-4 U/ml enzyme dosage over 2-4 h (Figure 7.5). Desirability was reduced with the combined effects of higher dosages, longer incubation times and higher or lower pH levels. For *E. grandis* highest desirability was observed at pH 3.0 and 5.0 either 2 or 10 U/ml enzyme over 4 h. Decline in desirability was observed with treatment combinations of pH 4.0, 2 or 6 h of incubation, as well as, 2 or 6 U/ml of enzyme.

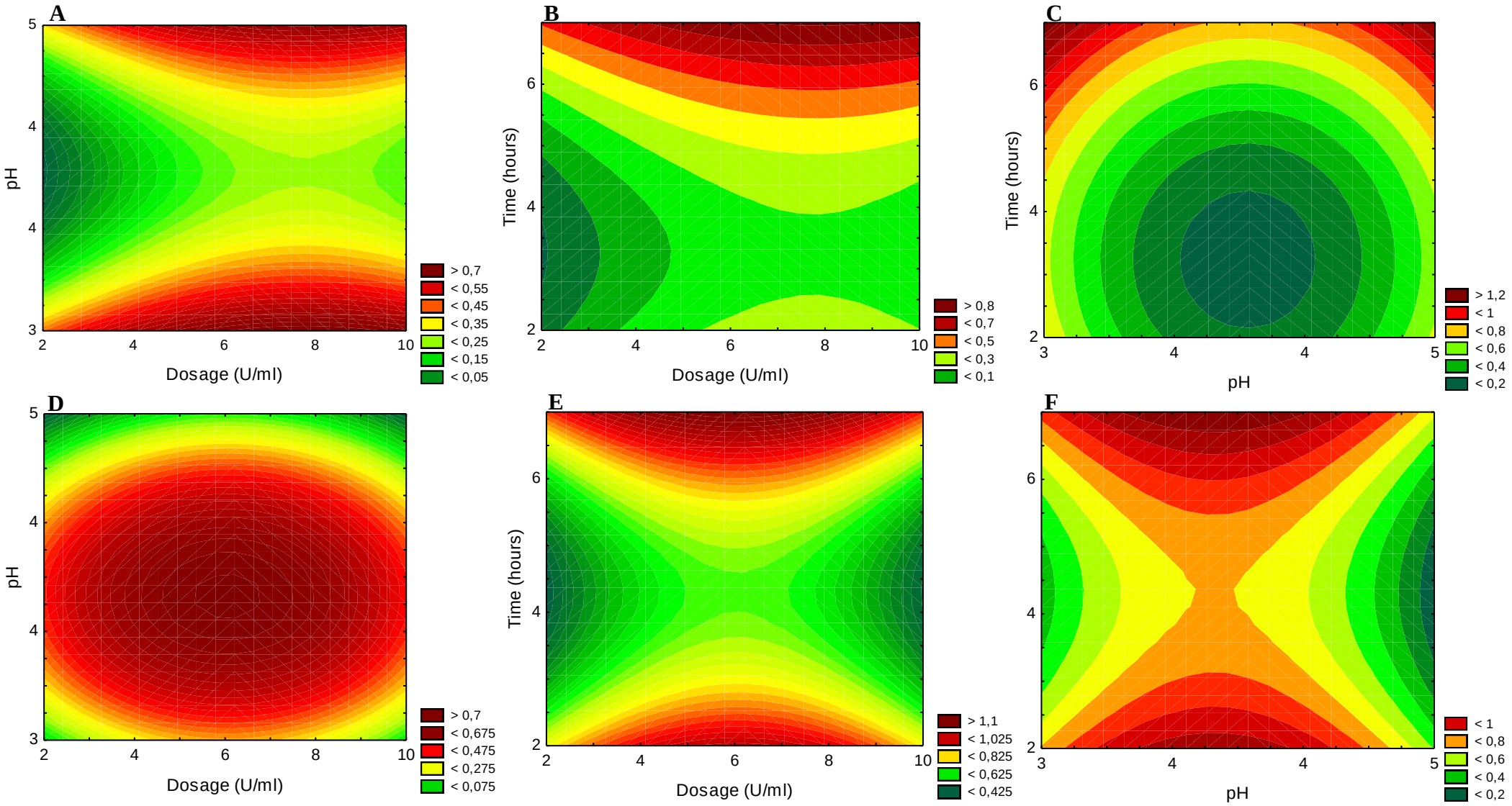

Figure 7.5: Desirability surface/contours of *E. dunnii* (A, B, C) and *E. grandis* (D, E, F). Independent variables (pH, time and dosage) and dependent variable (lipophilic content) were used in a quadratic fit method.

Optimization of enzymatic treatment to improve degradation of lipophilic compounds present in pulps

Based on the analyses of the RSM data the optimized combination of enzymes was ascertained to be Lipase 1/Esterase 1 and Lipase 3/Esterase 1 for *E. dunnii* and *E. grandis*, respectively. It was not surprising that Lipase 1 was most effective in reducing lipophilic compounds, since this cocktail of enzymes was composed of enzymes that displayed the highest enzyme activity. Lipase 1 cocktail comprised of DF3 (lipase), BT (lipase) and F4 (laccase) which demonstrated activities of 62.8 U/ml, 62.4 U/ml and 2 U/ml with *p*-NP palmitate, *p*-NP dodecanoate and syringaldazine, respectively. Esterase 1 is an acetylesterase which demonstrated 147.8 U/ml activity with *p*-NP valerate. It appears the optimal combination of enzymes for the most effective degradation of lipophilic extractives in pulp would be the combined action of lipases, esterases and laccases.

The optimal enzyme combinations of Lipase 1/Esterase1 and Lipase 3/Esterase 1 for *E. grandis* and *E. dunnii*, respectively, degraded a substantial amount of the pulp lipophilic extractives belonging to the sterol, fatty acid and ester fractions. This was evident from the complete elimination of a number of peaks in the pyrograms of both *E. grandis* and *E. dunnii* pulp (Figure 7.6). Pyrograms of all additional trials may be found in Appendix 7C.

Free sitosterol, sitosterol esters and fatty acids, the major lipophilic compounds in the *Eucalyptus* pulps, were entirely eliminated by treatment with the enzyme cocktails. Treatment of *E. dunni* pulp with the enzyme combination Lipase 1 and Esterase 1 removed most of the pitch-inducing lipophilic compounds (98%) present. This was due to the substrate specificity of the lipases for *p*-NP palmitate and *p*-NP dodecanoate and esterase for *p*-NP valerate, which represent the long and short chain esters commonly found in wood resin and pitch formations. For *E. grandis* only 71% of the extractives was removed. Nevertheless, such reductions would still be very beneficial in the pulp and paper industry. This study, therefore, demonstrates the effectiveness of a combination of lipase, esterase and laccase enzymes to remove lipophilic compounds from acid-bisulphite pulp. Similar observations of pulp treatment with lipases (Blanco *et al*., 2009), esterases (Calero-Rueda *et al*., 2004) and laccases (Gutiérrez *et al*., 2006b) have been reported in other studies. However, this study is unique as the actions of all three enzymes in the removal of pitch components were investigated. In addition, reduction in the lignin content of the pulps was also observed. This would be beneficial to the pulping

process as lower amount of chemicals would be required during bleaching, thus reducing cost and the amount of waste generated (Virk *et al*., 2013).

The removal of lipids by the enzyme cocktails resulted in the release of various oxidized derivatives that were either absent or present in low quantities in the untreated pulps. Regardless, the total lipid content in the pulps was significantly decreased, and the most problematic pitch causing compounds (1-dodecanol, n-tridecan-1-ol, 2-propenoic acid, tetradecanoic acid, hexanoic acid, n-pentadecanol, 1-tetradecanol, heptadecane and heneicosane) were completely eliminated. Similar observations were made by Gutiérrez *et al*. (2009) who used microbial and enzymatic treatments for the removal of pitch.


A
(x10,000,000)
TIC (1.00)
Max Intensity : 22,896,125
Time 3.444 Scan# 90 Inten. 13,622,549 Oven Temp57.22
Intensity (x100000000)
Retention Time (minutes)
1-Heptene
Methyl methacrylate
1-Octene
Ether, 2-chloro-1-methylethyl isopropyl
2-Propanone, 1-(1-methylethoxy)-
Cyclotrisiloxane, hexamethyl-
2,4-Dimethyl-1-heptene
Styrene
n-Butyl methacrylate
1-Decene
Cyclotetrasiloxane, octamethyl-
1-Tridecene
Nonanal
Cyclopentasiloxane, decamethyl-
1-Dodecanol
n-Tridecan-1-ol
4-Hydroxy-2-methylacetophenone
Cyclohexasiloxane, dodecamethyl-
Dibutyl phthalate
1-Pentadecene
Phenol, 2-methoxy-4-(1-propenyl)-, (Z)-
n-Dodecyl methacrylate
1-Pentadecene
Cycloheptasiloxane, tetradecamethyl-
erythro-9,10-Dibromopentacosane
1-Nonadecene
Eicosane
2-Propenoic acid, 2-methyl-, decyl ester
1-Heptadecene
Ethanol, 2-(dodecyloxy)-
Cyclooctasiloxane, hexadecamethyl-
n-Dodecyl methacrylate
1-Dodecanol
Docosane
Nonadecane, 2,3-dimethyl-
Tetradecanoic acid
n-Dodecyl methacrylate
Hexanoic acid, 2-ethyl-, anhydride
Diethylene glycol monododecyl ether
Isopropyl myristate
2-Methyltetracosane
n-Pentadecanol
Diethylene glycol monododecyl ether
B
(x10,000,000)
TIC (1.00)
Max Intensity : 16,037,158
Time 25.244 Scan# 4,450 Inten. 14,014,078 Oven Temp166.22
Intensity (x100000000)
Retention Time (minutes)
Docosyl heptafluorobutyrate
9-Desoxo-9-xi-hydroxy-3,7,8,9,12-pentaacetate ingol
Acetic acid, methoxy-, methyl ester
Heptadecafluorononanoic acid, butyl ester
Prop-2-ynyl propyl carbonate
Ethanamine, 2-hydrazino-N,N-dimethyl-2-oxo-
Tomatidine
1,3,5-Triazine, hexahydro-1,3,5-trimethyl-
Batrachotoxinin A, 7,8-dihydro-O3-methyl-, (8.beta.,20.xi.)-
[2-(N,N-Dimethyl)]-1,2-propanediamine
2-Cyclopenten-1-one, 3-ethyl-2-hydroxy-
Propanoic acid, pentafluoro-, 4-[2-[(2,2,3,3-
Ergostane-3,5,6,12,25-pentol, 25-acetate, (3.beta.,5.alpha.,6.beta.,12.beta.)-, 3TMS derivative
Benzene, 1,2-dimethoxy-
Z-26-Pentatriacontene-2-one
Pentatriacontane, 1-bromo-
3,4-Dimethoxytoluene
2-(4,5-Dimethyl-benzothiazol-2-ylamino)-3,3,3-trifluoro-2-pentanoylamino-propionic acid ethyl ester
Tetracontane, 3,5,24-trimethyl-
2-Hexenoic acid, 3-methyl-, methyl ester
Pentatriacontane, 1-bromo-
2-Hydroxyhexadecyl butanoate
2,6-Dimethoxy-4-aminopyrimidine
Benzenesulfonamide, 2-iodo-N-(1-adamantyl)-
Pentatriacontane, 1-bromo-
1,2,3-Trimethoxybenzene
Pentatriacontane, 1-bromo-
2,3,4-Trimethylevoglucosan
Succinic acid, dodecyl 2,4,5-trifluorobenzyl ester
Benzene, 1,2,3-trimethoxy-5-methyl-
9-Desoxo-9-xi-hydroxy-3,7,8,9,12-pentaacetate ing
1,2,3,4-Tetramethoxybenzene
dodecanamide, N-[2-methoxy-4-[[(4-nitrophenyl)sulfonyl]amino]phenyl]-
Tetrahydro-3,4,5-trimethoxy-6-methoxymethyl-2H-pyran-2-one
Phen-1,3-diol, 4,5,6-trimethoxy-2-propionyl-
N-[4-Bromo-2-(2-chlorobenzoyl)phenyl]-3,4,5-trimethoxibenzamide
Pentatriacontane
D-(+)-Raffinose, permethyl
1,2,3,4-Tetramethylmannose
2-Amino-6,7-bis[3,4-dichlorobenzyl]-4-hydroxypteridine

Figure 7.6: Pyrograms illustrating chemical composition of treated and untreated pulps of *E. dunnii* and *E. grandis*. A: untreated *E. dunnii* pulp, B: treated *E. dunnii* pulp, C: untreated *E. grandis* pulp, D: treated *E. grandis* pulp.

Effect of optimized enzymatic treatments on unbleached pulp properties

Fatty acids and sterols were the dominant lipophilic extractives in the treated pulp samples of *E. dunnii* and *E. grandis*. Due to unavailability of chlorine dioxide, the authors were unable to conduct bleaching of the treated pulp. However, based on the objectives of this study, this was not critical.

The optimal enzyme treatment for *E. dunnii* with Lipase 1/Esterase 1 at pH 4.0 and lower enzyme dosage (3 U/ml) for 4 h resulted in an 8% decrease in kappa number, thus increasing the brightness for the pulp before bleaching (Table 7.8). For *E. grandis* on the other hand, the optimal enzyme treatment of Lipase 3/Esterase 1 at pH 5.0 for 4 h and intermediate enzyme dosage (6 U/ml) resulted in a 2% decrease in kappa number. Kappa number is a measure of the amount of residual lignin in the pulp. The higher the kappa number value, the greater the amount of bleaching chemicals required to brighten the pulp. The kappa numbers of pulps leaving the digester are typically about 30 for softwoods and 20 for hardwoods in kraft mills that employ conventional cooking methods (Axegård *et al*., 2003). The kappa numbers of treated pulp in this study were 12.64 and 14.0 for *E. dunnii* and *E. grandis*, respectively. These kappa numbers were lower than the levels for the control experiments for both wood species (*E. dunnii*- 13.8; *E. grandis*- 14.29). As a result, enzymatic pre-treatment of unbleached pulp reduced the kappa number, consequently reducing the amount of chemicals that would be required during bleaching. These results are similar to those obtained by extended cooking of wood, which traditionally enables kappa numbers of the pulp to be further reduced (softwoods- 20 and hardwoods- 14) (Suurnäkki *et al*., 1995) in the digester, inevitably resulting in reduced strength and yield losses. Prior to bleaching, the limiting factor for kappa number reduction is the decline of pulp strength (Stratton *et al*., 2004). The kappa number of dissolving pulp is usually <0.5, which is easily achieved during the bleaching process (Vehmaa, 2013) with pulp yields between 52.3-54.4%. Similar yields were observed by Seca and Domingues (2006). However, subsequent to bleaching, final pulp yields could decrease to 35-49%. Factors that influence these values are wood quality (species, moisture, etc.) and dissolving pulp quality for end product application (microcrystalline cellulose, rayon, acetate, etc.) (Vehmaa, 2013).

Table 7.8: Chemical composition of treated and untreated (control) *E. dunnii* and *E. grandis* pulp.

	E. dunnii **Control ±SD**	***E. dunnii*** **Treated ±SD**	***E. grandis*** **Control ±SD**	***E. grandis*** **Treated ±SD**
AIL (%)	5.0±0.56	6.0±0.43[a]	5.8±0.32	5.4±0.22 [a]
ASL (%)	3.6±0.48	5.5±0.37 [a]	5.5±0.25	5.5±0.35 [a]
Kappa No.	13.8±0.42	12.6±0.41 [a]	14.3±0.48	14.0±0.41 [a]
Pulp Yield (%)	52.5±0.53	52.3±0.54 [a]	54.4±0.12	54.0±0.58 [a]
Viscosity (cP)	612.80±0.31	637.02±0.21 [a]	630.42±0.19	638.59±0.30 [a]

Abbreviations: AIL = Acid-Insoluble Lignin, cP = centipoise, SD - standard deviation. Values represented here are mean values for duplicate pulping experiments. p-value: a <0.05; b >0.05.

Improvements were also observed for other physical properties of the enzyme-treated pulps. Viscosity increased from 612.80 cP to 637.02 cP for *E. dunnii* and from 630.42 cP to 638.02 cP for *E. grandis* (Table 7.8). This gives an indication that intact cellulose chains were better preserved in enzyme-treated pulps. Acid-insoluble lignin and acid-soluble lignin increased in *E. dunnii* from 5 to 5.9% and 3.6 to 5.5%, respectively. However in *E. grandis* slight decreases were observed for both acid-soluble and insoluble lignin implying more efficient removal of lignin fibres from the *E. dunnii* compared to *E. grandis*. This may be attributed to action of ligninases and hemicellulases that hydrolyse the linkages between lignin and hemicellulose side groups.

Overall, the enzyme cocktail used had a positive effect on the *Eucalyptus* pulps by enhancing viscosity, and lowering the kappa number by removing lignin. The amounts of acid-insoluble (klason) lignin, soluble lignin and monosugars measured in the treated and untreated pulp are shown in Table 7.8 and 7.9. The pulp produced after the treatment had a lower kappa number than the control samples, as well as higher pulp viscosity. Hemicelluloses are known to protect cellulose chains (Palme *et al*., 2016), hence, their removal would improve extraction of cellulose fibres, resulting in higher pulp viscosities. The presence of hemicellulases, such as xylanases, present in the enzyme cocktail could be assisting in the degradation of hemicelluloses. The glucose content was high amongst all *Eucalyptus* species, indicating minimal cellulose degradation during pre-treatment of the brown pulp (Table 7.9). The properties of the treated pulp conforms to the standards of high-grade dissolving pulp (Table 7.10), making this pre-treatment step suitable for the commercial pulping industry.

Table 7.9: Carbohydrate composition (as percentage of total sugars (%) of treated and untreated (control) *E. dunnii* and *E. grandis* pulp.

	E. dunnii **Control ±SD**	***E. dunnii*** **Treated ±SD**	***E. grandis*** **Control ±SD**	***E. grandis*** **Treated ±SD**
Arabinose	0.15±0.24	0	0.04±0.25	0
Galactose	0.33±0.31	0	0.4±0.28	0
Glucose	89.14±0.15	88.75±0.15[a]	88.46±0.13	88.03±0.11 [a]
Mannose	0.52±0.21	0	0.35±0.11	0
Rhamnose	4.27±0.23	2.99±0.12 [a]	1.57±0.21	1.46±0.26 [a]
Xylose	0.15±0.15	0	0.04±0.13	0

Std Dev – standard deviation. p-value: a <0.05; b >0.05.

Table 7.10: Properties of dissolving wood pulp (Vehmaa, 2013).

	Eucalyptus **wood**		**South American Dissolving Pulp Producer** ***Eucalyptus*** **Specifications**		
	Lab scale	Mill scale	Standard	Special	High grade
Viscosity	400-550	520	350-599	250-599	500-599
Extractives	<0.2	-	<0.2	<0.15	<0.1
*Alpha Cellulose	94.5-97.4	94.7	94.5-96	95-96	95.5-96.5
Pentosans	3-4	3	3-4	2.5-3.5	1.5-2.5

*Alpha cellulose: undegraded, higher-molecular-weight cellulose content in pulp

7.5 Conclusions

Quadratic models containing three independent variables were found to appropriately define the lipophilic content of the pulp material with correlations between the actual and predicted values of the response variables having a fairly high R^2 value. Statistical modelling using the Box-Behnken design and RSM recognised pH and dosage of enzyme cocktail as the most influential factors for reducing the lipophilic content of *Eucalyptus* pulp. The experimental results obtained were validated by the predictions of the mathematical models. The optimum enzyme cocktail and reaction conditions predicted for maximum reduction of lipophilic compounds in *E. dunnii* pulp comprised of lipases, acetylesterase and laccases at a dosage of 3 U/ml for 4 h at pH 4.0. *E. grandis* pulp on the other hand should be treated at pH 5.0, for 4 h with an enzyme dosage of 6 U/ml (lipase, acetylesterase and laccase). The results from this study suggest that combining various lipolytic enzymes can enhance reduction of lipophilic content of *Eucalyptus* pulps, thus reducing pitch deposit formation. In addition, by utilizing partially crude enzyme cocktails, the presence of ligninolytic and hemicellulolytic enzymes assisted in the delignification of the pulp.

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CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

8.1 General Discussion

In our current environmental predicament, industries worldwide are focused on reducing their carbon footprint and directing their processes towards greener alternatives. The pulp and paper industry is no exception. Their processes have been modified by the incorporation of biocontrol agents that serve the dual purpose of reducing chemical consumption and the amount of waste generated, so too, reducing production costs. One of the key challenges encountered in the pulping of wood material is the formation of pitch deposits. These deposits are caused by accumulation of lipophilic compounds (triglycerides, fatty acids, fatty alcohols and esters) naturally found in wood material and leads to the formation of black pitch deposits, impacting the quality of pulp and functioning of machinery (del Río *et al*., 2000; Freire *et al*., 2005; Valto *et al*., 2012) as well as affecting the quality of downstream products made from pulps (Back and Allen, 2000). Therefore, the creation of an enzyme cocktail that is able to function as both a biopulping and deresinating agent would greatly benefit the pulping industry. Various studies have investigated bacteria and fungi as potential sources of lipolytic enzymes for the reduction or elimination of wood resin components and pitch deposits. However, none have considered the indigenous microflora of wood material (particularly *Eucalyptus* wood species) and their ability to produce enzymes targeting the problem of wood resin and pitch deposits. To our knowledge pulping mills make use of commercially available enzymes, instead of producing them in-house. The exploration of alternative sources for these enzymes, potentially being produced on-site, could reduce costs associated with purchasing and transporting chemicals and enzymes. In addition, the unique combination of lipolytic enzymes investigated in this study marks this study as novel.

This study has revealed considerable variation in lipophilic content amongst the different species of *Eucalyptus*. *E. nitens* had a higher lipophilic content than *E. dunnii* which is contrary to another study by Kilulya *et al*. (2014) in which the inverse was reported. In addition, variation in chemical characteristics amongst *Eucalyptus* of different site qualities was observed with *E. dunnii* site quality 3 (SQ3) exhibiting a higher amount of hot water and

solvent extractives compared to *E. dunnii* site quality 4 (SQ4). Kilulya *et al*. (2014) reported similar findings on site quality. The differences in the chemical composition of the trees may be attributed to the environmental conditions as well as the soil composition at the plantation (Foelkel and de Assis, 1995; Klash *et al*., 2010). Lipophilic extractives of wood appear to be substantially influenced by clay soil and organic matter, therefore sandy sites are recommended for the production of *Eucalyptus* trees with lower amounts of lipophilic extractives (Kilulya, 2012). Consequently, care needs to be taken when selecting trees for experimental studies, as standardized sampling techniques should be maintained within wood species. Another important factor to consider is duration of storage of wood material. After 6 months of storage at -20°C wood chips exhibited notable degradation in the chemical structure of wood, particularly with regard to lipophilic, lignin, xylan and cellulose content. Storage for this period of time clearly had negative effect on the integrity of the wood material and would inadvertently affect the results of any study. The changes observed in the wood may be attributed to the activity of the microorganisms present in the wood. Analysis of the microbial communities in the wood revealed subtle changes in some samples and major changes in other samples, before and after storage.

Knowledge of the lipophilic profile of *Eucalyptus* species is vital, as this insight enables the development of highly specific enzyme cocktails capable of targeting lipophilic compounds present in pulp. In this study, lipases, esterases and laccases were obtained from native strains as well as recombinant hosts either as pure enzyme preparations or crude, concentrated extracts with the cellulase activities removed. The enzymes were characterized by a somewhat tedious process that evaluated substrate specificity in all permeations of the experimental design. Changes in pH and temperature were important as they influenced substrate specificity. This finding is significant as most studies do not investigate substrate specificity under various experimental conditions, and instead optimize pH and temperature conditions by determining temperature optima under standard assay pH and then testing pH optima under optimal temperature for enzyme activity and thereafter determining substrate specificity under these optimized conditions (Gökbulut and Arslanoğlu, 2013; Prasad, 2014). pH is particularly important when treating pulp with enzymes, as the acidic nature of bisulphite pulp can influence the functioning and specificity of the enzyme applied. Sulphite pulps also contain higher amounts of extractives compared to alkaline pulps (kraft), due to the acidic nature of the pulping process the resins in the wood are not degraded, unlike with alkaline pulps in which the resin is broken down by saponification and hydrolysis (Sithole *et al*., 2010). Highest

lipolytic activity of 45-61 U/ml was observed for enzymes from selected bacteria isolated from *Eucalyptus* species. The results reported here fall within the range of other lipolytic enzymes described from environmental microorganisms such as a lipase from *Pseudomonas gessardii* which produced 156 U/ml of activity at a pH of 3.5 and 30°C (Ramani *et al*., 2010). Faiz *et al*. (2007) reported the esterase activity of an *Anoxybacillus gonensis* strain with 0.8 U/ml activity at pH 5.5 and 60°C. In comparison, the recombinant esterases produced in this study displayed much higher activity than the lipases. This is not unexpected as the recombinant *E. coli* strains were induced to over produce these esterases. Optimal activity of 147.8 U/ml was observed for the recombinant acetyl esterase at pH 4.0 and 30°C, whereas the recombinant carboxylesterase was 127.1 U/ml at pH 4.0 and 35°C. Agents such as CTAB and K^+ ions further stimulated activity of acetyl esterase. Fungal laccases were also included in this study as a 60-100% reduction in lipophilic compounds in wood and pulp has previously been reported (Gutiérrez *et al*., 2006; Molina *et al*., 2008).

In the application phase of this study various combinations of enzymes were tested on unbleached pulp and their ability to reduce the lipophilic content and lignin of the pulp evaluated. As the crude enzyme cocktails were rendered to be cellulase-free by the removal of cellulases, the presence of other hemicellulolytic and lignolytic enzymes are expected in the enzyme cocktails, as they are commonly produced by microorganisms colonizing lignocellulosic material such as *Eucalyptus* wood species. The combined effects of recombinant esterases, lipases and laccases significantly reduced the lipophilic content in the pulp. A 97.7% reduction was achieved in *E. dunnii* pulp treated at pH 4.0, dosage of 3 U/ml and a 4 h reaction time. At pH 5.0, dosage of 6 U/ml and 4 h, a 70.7% reduction in lipophilic content was observed for *E. grandis*. The enzymes described here maintained up to 95% of their activity over 2-3 h thus evidencing this combination of enzymes as suitable for the removal of wood resin from acid-bisulphite pulp. Impressive reductions in sterols and fatty acids were observed; these materials constitute the most abundant components in wood extractives responsible for pitch formation (Valto *et al*., 2012). In addition, unsaturated lipophilic and phenolic compounds would elevate consumption of bleaching reagents. Phenolic compounds were present in high concentrations in *E. dunnii* and *E. grandis* pulp and are reported to be produced by the plants in response to biotic and abiotic stresses, notably water stress (Ramakrishna and Ravishankar, 2011), which is typical in Brazil and especially in South Africa, which is currently in the midst of a severe drought season. Other pulp properties were

also improved, such as; increased viscosity, improved removal of lignin and decrease in kappa number, with minimal impact on pulp yield.

8.2 Recommendations

The most valuable recommendation emanating from this study would be evaluating substrate specificity and enzyme activity under different experimental conditions. This will ensure that true optimized conditions of the enzyme have been established, ultimately utilizing the enzyme to its full potential. The lipolytic enzymes examined displayed variable substrate specificity and activity levels depending on the pH and temperature conditions of the treatments. Therefore knowledge of the composition of wood resins is vital when selecting enzymes for their degradation.

The low amounts of lipophilic extractives present in *E. grandis* as well as its good kraft and dissolving pulp properties supports the widespread use of this species in pulping. *E. grandis* is the most widely planted eucalypt species to date (Sappi, 2012). The pulping properties of other species such as *E. smithii* may not be as desirable as *E. grandis*, but the lower risk of pitch formation makes it favourable for pulping. Consideration of pulping properties and lipophilic content of each *Eucalyptus* species when pulping could potentially reduce the risk of pitch formation without the need for pretreatment steps. This may work well in small scale mills, however, in a large commercial setting this may not always be feasible, thus additional strategies such as enzyme pretreatments are valued.

In addition, changes in bacterial and fungal communities were observed after storage, which should be taken into consideration when conducting laboratory scale trials. It is recommended that if storage is necessary under laboratory conditions, wood material should be retained for a maximum of 3 months at -20°C as per other studies in this area of research.

Prior knowledge of the lipophilic profiles of *Eucalyptus* wood species assisted in improving the selection of lipases and esterases to be used in the enzyme cocktails. Furthermore, the crude enzyme cocktails or individual recombinant esterases described here could have potential applications in other commercial industries such as the detergent, fuels, and leather industries. This combination of enzymes could have substantial impact on various avenues of bioindustry, particularly with their ability to function at acidic pH levels and affinity for a broad range of

substrates. The implementation of these enzymes is also an environmentally friendly alternative to chemicals currently being utilized.

8.3 Future possibilities

The class of enzymes described in this study exhibit catalytic pliability and robustness which makes them highly attractive as potential industrial biocatalysts in various commercial processes, particularly the pulp and paper industry. The benefits of using indigenous microorganisms has been demonstrated and further research into detailed characterization of these microorganisms and their enzymes would provide a wealth of potentially useful biocatalysts. Studies on improving the properties of these individual enzymes by using mutation methods, recombinant DNA technology and protein engineering, to develop a superior enzyme cocktail would be innovative research. One step further would be to incorporate effective lignin degrading enzymes to complement the optimized enzyme cocktail. This has the potential to ensure complete removal of residual lignin in the pulp, thus completely eliminating the need for the addition of chemicals in the pulping and bleaching process. This would go a long way towards achieving ‘greener’ technologies.

8.4 References

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