Repolymerisation of Lignins extracted from wood pulps through Enzymatic Modification to Establish Viable Valorisation Routes

by

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Thesis presented in partial fulfilment
of the requirements for the Degree

of

MASTER OF ENGINEERING

(CHEMICAL ENGINEERING)

In the Faculty of Engineering
at Stellenbosch University

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March 2021

DECLARATION

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ABSTRACT

Lignin is an amorphous polymer providing structure and strength to the cell walls in plants. In the process of making pulp the lignin is removed or solubilised from woodchips and burnt for steam production. Interest in alternative higher value applications of these lignins has increased considerably. Furthermore, the isolation and characterisation techniques of lignin have improved, thus leading to a better understanding of their structure. In order for lignin to be utilised, there is a requirement for its modification to improve its reactivity due to the fact that the pulping process results in a significant alteration of its structure – specifically by the repolymerisation of the lignin. Ligninolytic enzymes have shown significant potential in repolymerising these degraded lignins. The potential high value application of repolymerised lignins include their use as additives as reinforcements in polymeric packaging, composites and active packaging. In this project, six different industrial lignins from the South African pulping industry, including lignosulphonates, Kraft, and soda lignins, were isolated, characterised and reacted with commercial laccase (51003) and Lignin peroxidase, i.e (LiP) enzymes to cause repolymerisation, in order to achieve a higher molecular weight. The lignin was treated with the enzyme for a period of 24 hours with various enzyme dosages and lignin concentrations. As the lignosulphonates are water-soluble, they were able to be tested at higher lignin concentrations than the non-soluble alkaline lignins which had a solubility limit. Repolymerisation occurred for almost all the lignins treated with the laccase or LiP enzymes, resulting in a subsequent increase in molecular weight. Molecular weight was increased up to 5.68-fold as was observed for soda lignin treated with LiP. The highest molecular weight increase with the laccase treatment was 3.79-fold for Kraft (hardwood origin) lignin. For the laccase treatments an increase in molecular weight was accompanied by a decrease in the lignins' phenolic content. However, this was not observed for the LiP, indicating that this enzyme was able to repolymerise both the phenolic and non-phenolic lignin component. Depolymerisation was observed at low enzyme dosages in the LiP experiments (0.067 U/g lignin). It was noticed that lower molecular weight lignins are more reactive and allow more efficient interaction with the enzymes in comparison to higher molecular weight lignins.

The economic analysis of a proposed enzymatic modification process from the spent liquor was determined, however, owing to the high operating costs of the proposed processes for all six investigated lignins, together with the low projected income from the sales of the treated lignin, the enzymatic valorisation process is deemed not feasible at this stage. The lowest minimum required selling price of the sodium lignosulphonate (hardwood-softwood mix origin) was R250 421 per ton lignin. It is recommended that investigations into decreasing the reaction time, increasing the lignin concentration, and methods to reuse the enzymes could be considered.

OPSOMMING

Lignien is ’n amorfiese polimeer wat struktuur en krag aan die selwande in plante bied. In die proses om pulp of papier te maak, word die lignien verwyder of oplosbaar gemaak uit houtspaanders en gebrand vir stoomproduksie. Belang in alternatiewe hoër waarde toepassings van hierdie lignien het aansienlik verhoog. Verder, die isolasie- en karakteriseringtegnieke van lignien het verbeter, wat dus tot ’n beter verstaan van die struktuur lei. Vir lignien om gebruik te word, is daar ’n behoefte vir sy modifikasie om sy reaktiwiteit te verbeter as gevolg van die feit dat die pulpproses ’n beduidende verandering van sy struktuur tot gevolg het – spesifiek by die herpolimerisasie van die lignien. Ligninolitiese ensieme het beduidende potensiaal gewys in herpolimerisasie van hierdie gedegradeerde lignien. Die potensiële hoë waarde toepassing van herpolimeriseerde lignien sluit hul gebruik as bymiddels as versterkings in polimeriese verpakking, samestellings en aktiewe verpakking in. In hierdie projek is ses verskillende industriële lignien van die Suid-Afrikaanse verpulpingsindustrie, insluitend lignosulfonate, kraft, en sodalignien, geïsoleer, gekarakteriseer en gereageer met kommersiële lakkase (51003) en Lignienperoksidase, i.e. (LiP) ensieme wat herpolimerisasie veroorsaak, om hoër molekulêre gewig te bereik. Die lignien is behandel met die ensiem vir ’n periode van 24 uur met verskeie ensiemdosisse en lignienkonsentrasies. Omdat die lignosulfonate wateroplosbaar is, kon hulle getoets word by hoër lignienkonsentrasies as die nie-oplosbare alkaliese lignien wat ’n oplosbaarheidsbeperking het. Herpolimerisasie het voorgekom vir amper al die lignien behandel met die lakkase of LiP-ensieme, wat ’n verhoging in molekulêre gewig tot gevolg gehad het. Molekulêre gewig is verhoog tot 5.68-voud soos waargeneem vir sodalignien behandel met LiP. Die hoogste molekulêre gewig verhoging met die lakkasebehandeling was 3.79-voud vir kraft (hardehout afkoms) lignien. Vir die lakkasebehandelinge is die verhoging in molekulêre gewig vergesel deur ’n afname in die lignien se fenoliese inhoud. Dit was egter nie waargeneem vir die LiP nie, wat aandui dat hierdie ensiem beide die fenoliese en nie-fenoliese lignienkomponent kon herpolimeriseer. Depolimerisering is waargeneem by lae ensiemdosisse in die LiP-eksperimente (0.067 U/g lignien). Dit is opgelet dat laer molekulêre gewig lignien meer reaktief is en meer effektiewe interaksie met die ensieme toegelaat het in vergelyking met hoër molekulêre gewig lignien.

Die ekonomiese analise van ’n voorgestelde ensiematiese modifikasieproses van die gebruikte pulploog is bepaal, maar weens die hoë bedryfskostes van die voorgestelde prosesse vir al ses ondersoekte lignien, saam met die lae geprojekteerde inkomste van die verkope van die behandelde lignien, is die ensiematiese valorisasieproses beskou as nie uitvoerbaar op hierdie stadium nie. Die laagste minimum vereiste verkoopsprys van die sodiumlignosulfonaat (hardehout-sagtehout-mengsel afkoms) was R250 421 per ton lignien. Dit word aanbeveel dat ondersoeke in die reaksietyd, verhoging van die lignienkonsentrasie, en metodes om die ensieme weer te gebruik, oorweeg word.

TABLE OF CONTENTS

List of Tables

List of Figures

NOMENCLATURE

ABTS	2,2' - azino-bis (3-ethylbenzthiazoline-6 sulfonate)
AQ	Anthraquinone
ATR	Attenuated Total Reflectance
BHT	Butylated Hydroxytolvene
CAPEX	Capital Cost Estimate
CEPCI	Chemical Engineering Plant Cost Index
C-NMR	Carbon Nuclear Magnetic Resonance Spectroscopy
FCI	Fixed Capital Investment
FC-reagent	Folin Ciocalteu's phenol reagent
FT-IR	Fourier Transform Infrared Spectroscopy
GPC	Gel Permeation Chromatography
H	Coumaryl alcohol
H2O2	Hydrogen Peroxide
HAA	3-hydroxyanthranilic
HBT	1-hydroxybentzotriazole
IRR	Internal Rate of Return
KH-S-N	Kraft Lignin, Hardwood origin, Sappi
KS-S-N	Kraft Lignin, Softwood origin, Sappi
LiP	Lignin Peroxidase
LSD	Least Significant Difference
MgO-S-S	Magnesium Lignosulphonate, Hardwood, Sappi
MN	Number average molecular weight
MRSP	Minimum Required Selling Price
MW	Weighted average molecular weight
Mw/MN	Polydispersity
NaOH	Sodium hydroxide
Nas-M-PR	Sodium Lignosulphonate, Mixed, Mpact
NaS-S-T	Sodium Lignosulphonate, Hardwood, Sappi
NMR	Nuclear Magnetic Resonance
NPP	New Production Cost
NPV	Net Present Value
NSSC	Neutral Sulphite Semi-Chemical
PAMSA	Paper Manufacturers Association of South Africa
PI	Polydispersity Index
Q	Coniferyl alcohol
S	Sinapyl alcohol
S-S-S	Soda Lignin, Bagasse origin, Sappi
TCI	Total Capital Investment
TDC	Total Direct Costs
TGA	Thermogravimetric analysis
UV	Ultraviolet Spectroscopy

CHAPTER 1: INTRODUCTION

1.1 Contextual Background

Lignin is a heterogeneous, amorphous bio-polymer that is the second-most abundant substance in the plant world (Fengel and Wegener, 2003). Lignocellulose plant biomass consists of three structural biopolymers in its cell walls, namely cellulose, hemicelluloses and lignin (Axelsson *et al.*, 2012). Lignin provides structure and strength to the cells walls. It makes the cell wall resistant to insect attack and decay, and it aids in controlling the fluid flow within the plant (Laurichesse and Avérous, 2014).

In the pulp and paper industry, the lignin is removed from the lignocellulose biomass in a by-product, referred to as black liquor or spent liquor. The liquor consists of lignin, spent pulping chemicals and other dissolved wood constituents (Svensson, 2008). The liquor is burnt as a fuel for the production of steam, electricity, and to recover the pulping chemicals used in the pulping process (Kouisni *et al.*, 2016). Worldwide it is estimated that approximately 50 million tons of these 'technical' lignins are produced in the paper and pulping industry, where only 2 percent is commercialized into low- value products such as additives, surfactants, antioxidants and fillers (Gouveia, 2014; Laurichesse and Avérous, 2014).

The exact chemical structures of technical lignins are not known, as they are dependent on various factors such as the biomass origin, the conditions during its growth, the pulping process used to remove the lignin, and the method by which lignin is isolated from pulping liquors. All of these impact on lignins' chemical structures, especially pulping and isolation, which cause extensive degradation to the lignin and contaminating it with its inorganic pulping chemicals (Laurichesse and Avérous, 2014; Espinoza-Acosta *et al.*, 2016; Gordobil *et al.*, 2016a). This limits the high-value application capabilities of these technical lignins as the low molecular weight lignins obtained from pulping liquors are not well-suited to applications such as polymer blends, carbon fibres and active packaging, therefore there is a need to develop processes to repolymerise these low-molecular weight lignins (Bruijnincx *et al.*, 2016).

Most research into lignin valorisation investigates the utilizing of these technical lignins by further breaking them down into their monomeric units. However, little research investigates the repolymerisation of these lignin resulting in a higher molecular weight lignin as well as the industrial application of these processes. In order to increase the molecular weight of these lignin, they need to be altered physically or chemically (Areskogh, 2011; Wells, Kosa and Ragauskas, 2013) .

In nature, the lignin is formed by the polymerisation of three basic phenylpropanoid units, namely guaiacyl, syringyl and p-coumaryl, where these units form ether and carbon-carbon bonds (Kong *et al.*, 2018). This process is initiated by oxidoreductase plant-produced enzymes such as peroxidases and laccases, which form the complex structure (Gouveia, 2014). Peroxidase is a class consisting of enzymes such as lignin peroxidase, manganese peroxidase and versatile peroxidase, which utilises hydrogen peroxide as an oxygen-donor, and laccases which are fungal enzymes requires oxygen as an oxidant to cause the polymerisation (Liu, Luo and Zheng, 2018).

Enzymatic modification of technical lignin is an environmental-friendly approach to upgrading these lignin. Enzymes are specific, selective and allow for direct lignin modification without the formation of undesired products, with minimal energy demand and minimal generation of hazardous wastes (Chan, Paice and Zhang, 2020).

The South African paper and pulping industry contributes significantly to the economy, society and the environment in South Africa. The sector contributed R24.13 billion to the South African GDP, which is 0.53% of the country's total GDP, while employing 150 000 people across the country in 2019 (PAMSA, 2019). The sector has initiatives and organisations such as Paper Manufacturers Association of South Africa (PAMSA) that investigate improvements in the sector by better resource and waste utilization, combatting climate change and moving towards a greener economy.

The use of black liquor, which is considered carbon neutral and renewable to produce steam and/or electricity in the paper and pulping industry, will reduce the mills' reliance on the national power grid and reduces the sector's carbon footprint (PAMSA, 2019). Studies have shown that in some pulping mills, 60% more technical lignin is produced than is required to meet the internal energy requirements of the pulping process (Gordobil *et al.*, 2016b). This surplus of black liquor, results in mills experiencing bottlenecks at their recovery boilers. This influences the mills pulping capacity, thus resulting in the excess black liquor being stored in tanks (Nagy *et al.*, 2010; Namane, 2016). Storing the black liquor will decrease the lignin quality over time (Govender, 2020). As lignin is such a carbon-rich source, by only burning it for energy production is an underutilization of its potential. In realising the potential application capabilities of lignin, the industry has started investigating alternative higher value application opportunities for technical lignin (Sena-Martins, Almeida-Vara and Duarte, 2008). Pulping mills which do not have excess black liquor, would require the lignin application to add more value than the current economic worth of lignins as an energy source.

The aim of this project was to repolymerise technical lignin from the South African paper and pulping industry through enzymatic modification. The lignins were characterised to predict how they would respond to the enzymes. The extent of the repolymerisation which was be achieved by the two enzymes laccase and lignin peroxidase and the six lignins, were compared by their ability to repolymerise. In order to determine if enzymatic modification of lignins was economically feasible, a techno-economic analysis was performed.

1.2 Thesis layout

This thesis is structured as follows:

Chapter 1 provides an introduction and gives the contextual background to the study.

Chapter 2 reviews the literature on the study, starting with the structure of lignin, followed by the various pulping processes as a possible source of lignin. Then the availability of various valorisation methods are examined for these technical lignin, before moving onto enzymatic modification of lignin and various polymerisation studies using enzymes. The chapter ends with explaining the aims and objectives of the study.

Chapter 3 details the methods used to characterise the various lignin samples, and it presents the results from the characterisation of the various lignin samples, such as the compositional, structural and thermal analysis.

Chapter 4 details the methods used to enzymatically modify the various lignin samples, and it presents the results found from the characterisation of the various treated lignin samples, such as compositional, structural and thermal analysis.

Chapter 5 presents the techno-economic evaluation of enzymatically modifying these technical lignin.

Chapter 6 contains the general conclusion of the study and any recommendation for future work.

CHAPTER 2: LITERATURE

2.1 Lignin

2.1.1 Background

A French chemist Anseleme Payen (1795-1871) recovered two different products when he treated wood with caustic soda and nitric acid. The first product he found was called “cellulose” and the second he described as an “encrusting material,” now known as lignin, which was embedded within the cellulose (Laurichesse and Avérous, 2014). Lignocellulosic biomass is mainly plant cell wall material that consists of three biopolymers: cellulose, hemicellulose and lignin, where in woody plant dry matter lignin contributes between 15 – 40 wt% (Axelsson *et al.*, 2012; Laurichesse and Avérous, 2014).

Figure 1 depicts the ultrastructure of a wood cell wall. The figure shows that the cellulose molecules are bonded in parallel to each other with the lignin and hemicellulose in-between. The cell wall consists of several layers where the lignin concentration decreases from the outer to the inner layer (Azadi *et al.*, 2013). The lignin in the outer layer binds the adjacent cells together. However, the lignin in the inner layer of the cell wall acts as a natural bonding agent with the hemicellulose and the cellulose microfibrils to provide rigidity (Azadi *et al.*, 2013).

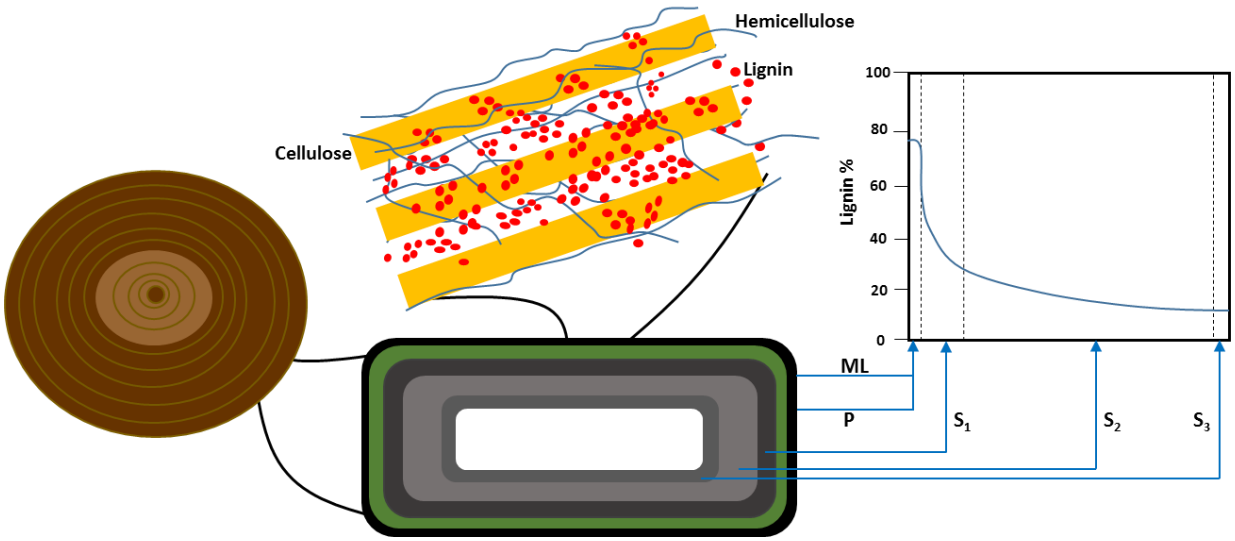

Figure 1: The ultrastructure of wood cell wall and a schematic diagram of the lignin distribution within the middle lamella (ML), primary wall (P) and secondary wall layers (S) redrawn from Azadi *et al.*(2013)

Cellulose, a linear biopolymer, is a polysaccharide (carbohydrate) and is the most abundant organic molecule found on Earth. It is used in areas such as construction, animal feed, but predominately in the papermaking process for the production of electricity, and recently for the production of bioethanol (Bugg *et al.*, 2011). Hemicelluloses are heterogeneous polymers that are both linear and branched, and which interact with cellulose by strengthening the cell walls (Schoemaker and Piontek, 1996). Lignin provides strength and structural support to the cell, as well as resistance to decay and insect attack, and

it controls the fluid flow in the plant by aiding in the hydrophobic nature of the cell walls (Laurichesse and Avérous, 2014).

Lignin is predominately obtained in the paper and pulping industry, where the lignin is removed from the lignocellulosic material using physical or chemical measures. The resulting pulping liquor contains these technical lignins (Areskogh, 2011).

2.1.2 Lignin Structure

Lignin is a complex heteropolymer with phenylpropanoid monomers that are bound together by various carbon to carbon bonds and by irregular ethers (Conacher, 2018). The basic structure of lignin monomers consists of a phenolic ring with a hydroxyl group (-OH) and a propane side chain, $C_9H_{10}O_2(OCH_3)_n$ (n is the ratio of methoxyl groups) (Gouveia, 2014). There are three main phenylpropane monomers, namely p-coumaryl alcohol , sinapyl alcohol (additions of a methoxyl group ($-COH_3$)) and coniferyl alcohol (addition of two methoxyl groups), which are illustrated in Figure 2 (Doherty, Mousavioun and Fellows, 2011). These monomers are also referred to by their aromatic moieties, i.e. p-hydroxyphenyl (H) for p-coumaryl alcohol, syringyl (S) for sinapyl alcohol, and guaiacyl (G) for coniferyl alcohol (Ek, Gellerstedt and Henriksson, 2009).

Lignin is synthesized by random polymerisation of these three phenylpropane monomers, known as monolignols. These monolignols undergo an enzyme-mediated dehydrogenation which will result in the formation of radical monolignol units. The oxidation of these monolignols causes polymerisation by radical-radical coupling to form the polymer lignin (Chakar and Ragauskas, 2004; Conacher, 2018). The enzymes causing the radical formations are the plant-based enzymes peroxidase and laccase (Duval and Lawoko, 2014). The monolignols are produced within in the wood cells cytoplasm and move to the cell wall where the polymerisation with various noticeable linkages forming between them will take place (Areskogh, 2011). These linkages include the β-O-4 aryl ether linkage which also occurs the most; aryl ether (α-O-4), diphenyl (4-O-5), as well as carbon-carbon linkages such as biphenyl (5-5), pinoresional (β-β), phenylcoumaran (β-5) and diphenyl methane (Thβ-1). These linkages have been indicated on the schematic representations of lignin in Figure 3 (Laurichesse and Avérous, 2014). The abundance of chemical sites on the lignin molecule provides opportunity for the chemical modification of the polymer, to play a central role in new chemical formations (Laurichesse and Avérous, 2014). However, because the lignin molecule is complex, it has been challenging to be able to identify and quantify the various structures and linkages in lignin, especially when determining the degree of esterification (Zakzeski *et al.*, 2010).

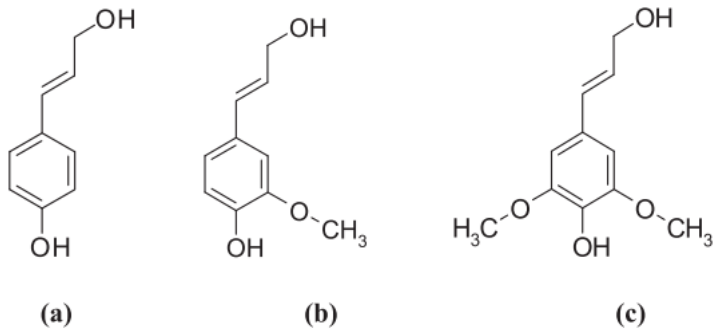

Figure 2: Monolignol monomer species. (a) *p*-Coumaryl alcohol (b) coniferyl alcohol (c) sinapyl alcohol redrawn from (Doherty, Mousavioun and Fellows, 2011)

The complexity of the lignin polymer is caused by various factors including, the variation in linkage structures and functional group types between different biomass origins, environmental conditions, various locations within a specific plant and the process used to isolate the lignin. Various lignin models have been produced to explain the various properties of different native lignins (García *et al.*, 2009). There are three main types of lignin that are classified according to their monolignol content and these include softwoods, also called guaiacyl lignin, as they are mainly formed from coniferyl alcohols (G) and hardwoods, also called syringyl-guaiacyl lignin, as they consist of similar amounts of coniferyl (G) and sinapyl alcohol (S) phenylpropane and grasses which are called HGS-lignin. The different structures of these are shown in Figure 3. Softwoods are gymnosperms (where the ovules are not protected) and hardwoods are angiosperms (where the ovules are protected). The different monolignol ratios and linkages present in the different types of lignin are summarized in Table 1 and Table 2, respectively. The percentage dry mass, composition and molecular weight of lignin, differ from plant to plant. Softwoods contain the highest concentration of lignin, then hardwoods, and lastly grasses (Zakzeski *et al.*, 2010). The weight percentages of lignin in softwood, hardwood and grasses are typically 27–33 %, 18–25 % and 17–24 %, respectively (Azadi *et al.*, 2013).

Table 1: Percentage of H,S and G-monomers present in hardwood, softwood and grass lignin adapted from Ek, Gellerstedt and Henriksson (2009)

	Percentage H-monomer (p-coumaryl)	**Percentage S-monomer (sinapyl)**	**Percentage G-monomer (coniferyl)**	**Reference**
Hardwood	0-8	46-75	25-50	(Ek, Gellerstedt and Henriksson, 2009)
Softwoods	<5	Trace	>95	
Grasses	5-33	20-54	33-80	

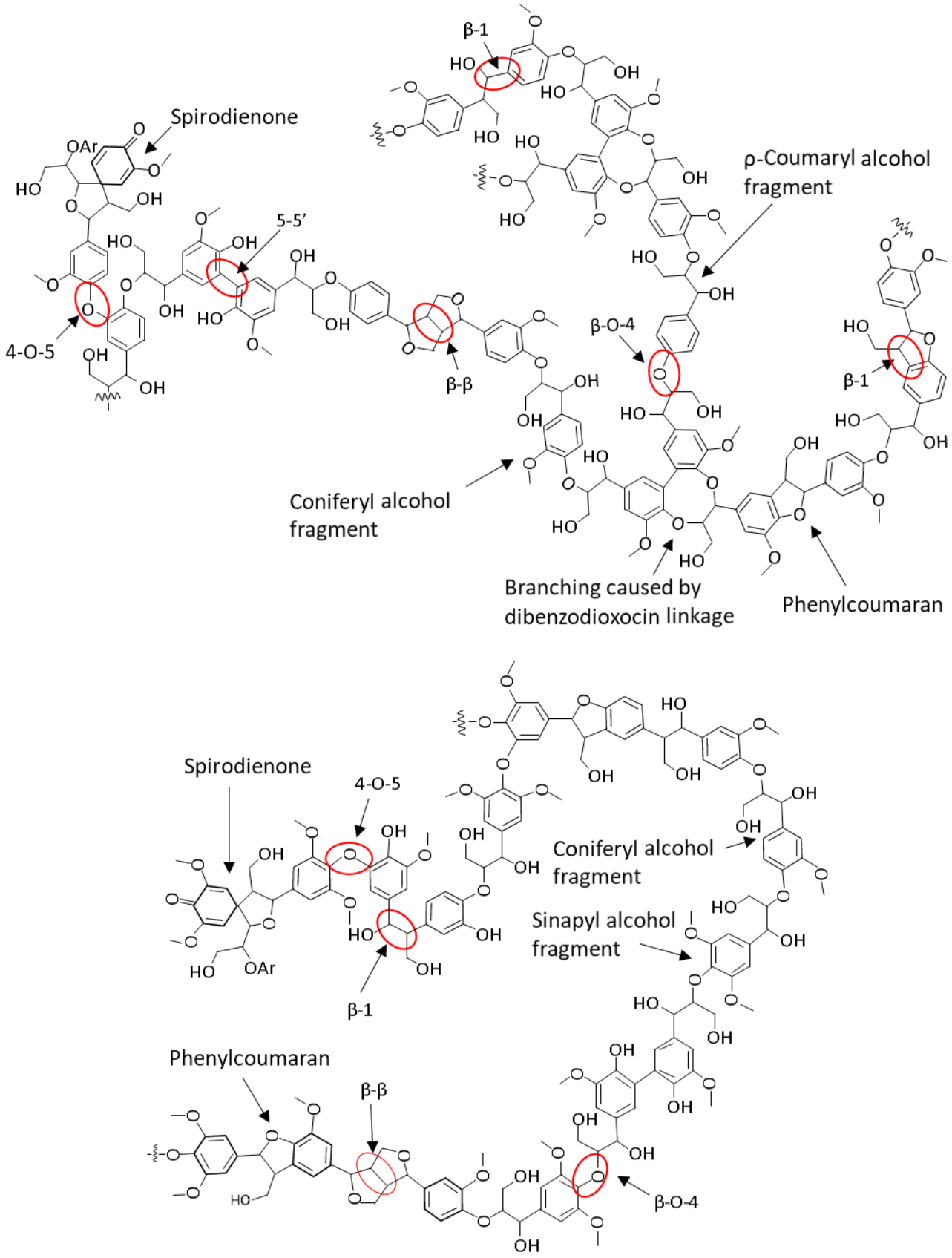

Figure 3: The Schematic representation of softwoods (A) and hardwoods (B) redrawn from Zakzeski et al. (2010))

Table 2: The estimated percentage of each type of linkage found in softwood and hardwood lignin (Chakar and Ragauskas, 2004; Gouveia, 2014)

Type of Linkage	Approximate percentage linkage		**Reference**
	Softwoods (%)	Hardwoods (%)	
β-O-4	45-50	60	(Chakar and Ragauskas, 2004; Gouveia, 2014)
α-O-4	6-8	6-8	
4-O-5	4-8	6	
5-5	18-25	5	
β-1	7-10	7	
β-β	3	3	
β-5	9-12	6	

As no definitive structure of lignin exists, only models have been determined which indicate the complexity of lignin as a polymer and the lack of methods available to extract the lignin in its native form (Areskogh, 2011). The development of various analytical techniques to help characterise and clarify the structure of the lignin molecule is evolving, especially owing to the abundance of the molecule and the potential products it can provide, and it will be investigated in this study (Conacher, 2018).

2.1.3 Lignin Characterisation Methods

Lignin is seen to be one of the most difficult biopolymers to characterise (Chakar and Ragauskas, 2004). Characterising the lignin by determining the lignin polymer size, quantifying the chemical linkages and functional groups present are essential to design and explain the performance of the enzymatic-catalytic reactions to aid in determining the possible applications for these lignins. In order to characterise this complex polymer, various techniques are used and have been summarised in Table 3 (Rinaldi *et al.*, 2016).

The ash content of the lignin, shows the percentage of inorganics present in the lignin, which indicates the purity of the lignin (Mansouri and Salvadó, 2006). When determining the chemical composition of the lignin, techniques such as Gas Chromatography with Mass Spectrometry (GC-MS) have been used to determine the volatile compounds of lignin released from lignins. However, many issues such as the calibration standards that are not commercially available and that should be synthesised, have resulted in these methods poorly representing the volatile compounds present, and hence they should be used in conjunction with other methods. Methods such as Thermogravimetric Analysis (TGA), which is performed under an inert gas, has shown to be effective in quantifying mass losses, due to the release of volatile compounds (Rinaldi *et al.*, 2016). During thermal heating, the lignins are degraded to form volatile compounds, which gives insight into the thermal stability of the lignin. Methods such as Gel Permeation Chromatography (GPC) have been successful in indicating the lignin molecular weight and the size distribution (Owhe, 2020). Care must be taken when choosing the solvent for the polymer to be fully dissolved. To improve the solubility and lower the polarity of the lignin in different solvents, methods of chemical derivatisation may be applied to modify its hydroxyl groups; these methods include acetylation, flurobenzylation and flurobenzoylation (Esakkimuthu, 2020). Elemental analysis gives insight into the atomic C/H and C/O indices and the heating value which can also be measured directly (Rinaldi *et al.*, 2016).

There are various methods that are used to determine the functional groups present in lignin. Fourier Transform Infrared Spectroscopy (FTIR) is the most utilized method, as it has a high sensitivity, is non-destructive, has short analysis time and samples can be used directly in solid form, and it is easy to perform. However, the only drawback is that the results are not quantitative. The absorption bands of the FTIR spectrum have been extensively researched and have been assigned to represent various functional groups (Esakkimuthu, 2020). Nuclear Magnetic Resonance (NMR) techniques are commonly used to determine the structural composition of the lignin molecules, where commonly used techniques include H-, C-, P- and F-NMR. Ultraviolet Spectroscopy (UV) is another quick method to determine the phenolic content present in lignins and results have been comparable to H-NMR results. However, there are limitations such as the resulting absorbance that is directly proportional to the lignin purity, as well as the method is dependent on the lignin to be fully dissolved in the medium, which does not always occur owing to lignin solubility limits (Esakkimuthu, 2020).

The methods used to characterise lignin are always improving. The determination of the free phenolic content, as well as the molecular weight determination of lignin is an important part of lignin valorisation (Esakkimuthu, 2020). This study utilizes characterisation techniques which will aid in determining the effect of the enzymatic modification before and after the experiments, and where the determination of the molecular weight change of the lignin (GPC) will give the best indication whether repolymerisation has occurred. Other characterisation techniques like FTIR, elemental analysis, phenolic content and TGA, will be done to give insight into the structure and properties of the lignin and hopefully the influence it has on the catalytic action of the various enzymes.

Table 3: Various characterization methods of lignin

Characterisation Method	Method Determines	References
Ultraviolet Spectroscopy (UV)	Determine the amount of phenolic end groups present in the lignin (Folin-Ciocalteu Reagent)	(Areskogh *et al.*, 2010a)
Fourier Transform Infrared Spectroscopy (FTIR)	The functional groups in the lignin	(Mansouri and Salvadó, 2006)
Elemental Analysis	Carbon, Hydrogen, Nitrogen, Oxygen and Sulphur contents in the lignin	(Ai, Wang and Huang, 2015)
Gel Permeation Chromatography (GPC)	The average molecular weight (M_w)), number average (M_n) and the polydispersity (M_w/M_n) of the lignin samples	(Naron *et al.*, 2017)
Gas Chromatography	Identifies the volatile components	(Esakkimuthu, 2020)
Ash Content	The percentage of inorganics in the lignin compound	(Sameni, Krigstin and Sain, 2016)
Nuclear Magnetic Resonance Spectroscopy (NMR)	Structural compositions: functional groups, linkages and it identifies the carbon-hydrogen framework of an organic compound.	(Bergeron, 1984)

2.2 Pulp and Paper Industry as a source of Lignin

The pulp and paper industry is the main commercial source of lignin (Doherty, Mousavioun and Fellows, 2011). Other areas where lignin is produced as a by-product is in cellulosic bioethanol production, an industry that is globally in its infancy. The second-generation cellulosic ethanol is seen as one of the promising alternatives for liquid fossil fuels, especially with the goal to replace 30% of fossil fuels with alternatives such as biofuels by the year 2030 (Cotana *et al.*, 2014). Thus, a large amount of lignin may be produced from this industry in future. It is estimated that 300 billion tons of lignin exist in the biosphere where the majority of lignin is combusted as a fuel, while only a portion (less than 2%) is utilized in value-added application (Naron, 2019).

In each pulping process, the lignin will undergo various structural changes during the isolation from the biomass, hence the properties, structure and composition of the isolated lignin is very different from the native lignin present in wood (before the isolation) (Azadi *et al.*, 2013). These isolated lignin are referred to as technical lignin or spent lignin, and their names are derived from their type of isolation (Laurichesse and Avérous, 2014).

During the pulping process, pulping chemicals are used to selectively solubilize the lignin from the lignocellulose material so that the remaining material can be used to make paper. The lignin is solubilized by degrading and/or derivatising the lignin bonds, resulting in lignin fragments of a lower molecular weight (Gouveia, 2014). The change in the molecular weight is dependent on the pulping process used. In the chemical pulping processes the lignin, hemicellulose, and a small amount of cellulose will dissolve in the liquor. These compounds and the inorganics of the liquor are referred to as black liquor or spent liquor. Delignification without degrading the cellulose fibres is seen as an important part of the pulping process. The black liquors' organic components are recovered to be utilized in the production of steam for the plant, while the inorganics are recovered to be used again in the pulping process (except in the soda pulping process).

The delignification process is done, chemically or with some delignification occurring during semi-mechanical pulping, where there is a combination of mechanical and chemical processes. (Fengel and Wegener, 2003). The chemical pulping processes namely, Kraft pulping, sulphite pulping, soda, ionic liquids and organosolv pulping, are processes that utilize chemicals to degrade and dissolve the lignin to remove it from the lignocellulose material (Zakzeski *et al.*, 2010). The semi-mechanical pulping processes, such as the Neutral Sulphite Semi-chemical (NSSC) process, utilizes an initial chemical process followed by mechanical refining (Fengel and Wegener, 2003). Technical lignin can be divided into two main categories: Sulphur containing lignin (lignosulphonates and Kraft lignin) and sulphur-free lignin (soda and organosolv lignin) (Espinoza-Acosta *et al.*, 2016). The structures of sulphur-free lignins have a moderate molecular mass in comparison to sulphur containing lignin and they resemble native lignin more than sulphur lignin do (Domenek *et al.*, 2014), where the Kraft lignins have been depolymerised more extensively than the lignosulphonates (Ragauskas *et al.*, 2014). The exact structure of technical lignin are unknown, because the various isolation methods of lignin from the lignocellulose biomass alter its structure. However, many studies focusing on the characterisation of lignin have provided insights into

the biosynthesis of lignin (Zakzeski *et al.*, 2010). This study focusses on the industrial lignin from the Kraft, soda, and sulphite-pulping processes and are discussed below.

2.2.1 Kraft Pulping Process

Kraft lignin is the by-product from the Kraft pulping process. The Kraft process makes up 90% of the total pulp production capacity in the world, making it the most dominant pulping process (Azadi *et al.*, 2013). The Kraft process uses an aqueous solution containing sodium hydroxide and sodium sulphide (white liquor) to solubilize lignin and hemicellulose from the wood chips (containing lignocellulosic biomass) into what is referred to as black liquor (Duval and Lawoko, 2014) .

The lignin, together with other components of the black liquor, is removed from the digesters during washing in the pulping process. During the cooking time in the digester the lignin is depolymerised by cleavage of the aryl ether bonds, resulting in the lignin being dissolved into the white liquor (Duval and Lawoko, 2014). The delignification process occurs in three stages. The first stage is controlled by diffusion and it occurs at a temperature around 150°C. The second stage occurs at a temperature between 150–170°C (pH 14), where the bulk of the delignification occurs and the final stage occurs at a higher temperature (Doherty, Mousavioun and Fellows, 2011). Approximately 90–95% of the lignin content in the wood chips is dissolved into the aqueous solution (black liquor) during the cooking (Vishtal and Kraslawski, 2011). The dissolved lignin and the spent Kraft-pulping chemicals form a liquid stream, called weak black liquor, which is then removed from the pulp by washing (Tran and Vakkilainnen, 2008). Other compounds such as hydroxyl carboxylic acids and polysaccharides are also found in the black liquor, due to the Kraft- pulping chemicals causing fragmentation of cellulose's inter-units and converting the hemicelluloses (Naron, 2019). The weak black liquor is sent to the Kraft recovery process as depicted in Figure 4. In the Kraft recovery process the weak black liquor is first concentrated in evaporators in order to be burnt in the recovery boilers to produce steam, and then electricity for the pulping plant. The remaining spent chemicals are recovered, causticized and reused in the digester (Tran and Vakkilainnen, 2008).

Over 1.3 billion tons of weak black liquor is processed globally per year, which corresponds to 200 million tons of dry black liquor solids per year. This will produce 700 million tons of high pressure steam. Thus, black liquor is considered to be the fifth most important fuel in the world, next to coal, natural gas, oil and gasoline (Tran and Vakkilainnen, 2008). The high-pressure steam is utilized in the high energy integrated Kraft pulping process, thus making it difficult to utilize the lignin from the pulping process for alternative applications. However, if a fraction of the lignin is taken away from the fuel and used in the production of speciality or fine chemicals, it may become economically viable if the price of the produced chemicals exceeds that of the cost of an alternative fuel to replace the lignin in boilers, once all the downstream processing costs have been accounted for (Rinaldi *et al.*, 2016).

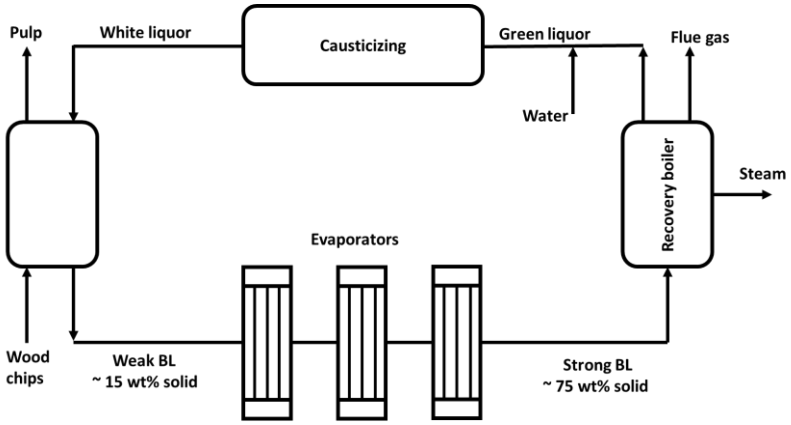

Figure 4: Simplified flow diagram of the Kraft recovery process redrawn from Azadi *et al.* (2013)

Kraft lignin can be isolated from the black liquor by a pH-controlled precipitation. Since lignin water solubility decreases as the black liquor's pH decreases, and the other black liquor components such as sugars and inorganic compounds remain water-soluble at various pH values, acidification will precipitate the lignin from the liquor. The precipitation is very efficient and the resulting lignin has a very low ash content, however, the precipitation itself will also change the lignin structure (Gouveia, 2014). The structure of the Kraft lignin is highly modified during the Kraft pulping process where approximately 70 – 75% of the hydroxyl groups will become sulfonated, and a higher number of condensed structures within the lignin will occur. These structural changes are caused by the extensive bond cleavage of the α-aryl and β-aryl ether linkages (Lange, Decina and Crestini, 2013; Laurichesse and Avérous, 2014). In order for the lignin to dissolve during the process, the sulphide ions react with an important linkage in lignin β-O-4, and this causes the lignin to degrade. The increase in the cleavage of these ether bonds results in the increase in free phenolic groups (Brodin, 2009). Kraft lignin will generally contain little sulphur (1.5 – 3 wt.%) within its structure in comparison to the lignin obtained from the sulphite pulping process (4.8 wt.%) (Azadi *et al.*, 2013).

Kraft lignin is soluble in basic, alkali and highly polar organic solvents (Lange, Decina and Crestini, 2013), especially at a pH above 10, where most of the commercially produced Kraft lignin are sulfonated, so that it becomes water-soluble (Azadi *et al.*, 2013). This causes issues in terms of further modification of the Kraft lignin as the lignin will not be soluble at the lower pH (between 3 and 6) required by, for example, the enzyme modification processes (Gouveia *et al.*, 2013). Previous studies have shown the value in the modification of Kraft lignin into low or high molecular weight compounds. The degradation of Kraft lignin yields low molecular weight compounds such as phenols, dimethyl sulfoxide and aromatics such as vanillin, while a high molecular weight Kraft lignin has been used to produce adhesives, carbon fibres and thermally , stable copolyester (Wells, 2015).

2.2.2 Soda Pulping Process

Soda lignin is produced through the soda pulping process. Soda pulping was the first chemical pulping process and was patented in 1845 (Doherty, Mousavioun and Fellows, 2011). The soda pulping process utilizes high alkaline solutions (sodium hydroxide) to treat lignocellulosic materials such as sisal, bagasse,

wheat straw, hemp or kenaf to obtain soda lignin as a by-product under similar conditions to the Kraft pulping process (Espinoza-Acosta *et al.*, 2016). The lignin extraction in the soda pulping process is based on hydrolytic cleavage of the native lignin (Laurichesse and Avérous, 2014). The soda pulping process is becoming the most dominant pulping process when treating non-woody materials, mainly because of the development of effective experiment technology and lower chemical recovery costs (Doherty, Mousavioun and Fellows, 2011).

The soda pulping process is similar to the Kraft pulping process, although sodium hydroxide is the only active chemical used. The reactions involved to remove the lignin, include the scission of the α and β linkages (Naron, 2019). The addition of the additive anthraquinone to the process, has an effect on the stabilization of the carbohydrates and the dissolution of lignin, called the anthraquinone (AQ) alkaline sulphite process. However, the removal rate of lignin in the Kraft process is still faster than in the soda process (Azadi *et al.*, 2013).

Soda lignin is sulphur-free, making it attractive for the production of sulphur-free products and polymer applications (Duval and Lawoko, 2014). Soda lignin properties include a lower molecular weight (1 000 – 15 000 g/mol) and a higher phenolic content in comparison to Kraft lignin and lignosulphonates (Thakur *et al.*, 2014). The soda lignin is recovered from the soda pulping process by methods such as acid precipitation, maturation and filtration, for it to be used for alternative applications (Strassberger, Tanase and Rothenberg, 2014).

2.2.3 Sulphite Pulping Process

The sulphite pulping process makes up 10% of the global pulping processes. The lignin produced by the process is referred to as lignosulphonate (Lange, Decina and Crestini, 2013). In the sulphite pulping process the wood chips are treated with sulphur dioxide dissolved in water (H_2SO_3) and a metal bisulphite (HSO_3^-) at a temperature between 125–145 °C and a pH between 1 and 2 (Tyhoda, 2008). The different metal bisulphites are calcium, sodium, magnesium or ammonium (Fengel and Wegener, 2003). Therefore, sulphur dioxide, hydrogen sulphite ions and sulphite ions are involved in the process with the amounts of these dependent on the pH of the reactions in the digesters (Naron, 2019). The delignification occurs in three stages, i.e. sulphonation, hydrolysis and condensation. During sulphonation the lignin is softened by making it more hydrophilic. The second stage which is hydrolysis, causes the lignin to be broken down by the random degradation of the ether bonds in the lignin. These lignin fragments are solubilized into the liquor by the addition of sulphonic acid groups at various positions on the lignin (Areskogh, 2011). The insoluble cellulose is then separated by filtration from the solubilized lignin.

Lignosulphonates are water-soluble anionic polyelectrolytes, which contain a large amount of charged groups (sulphonic, hydroxyl, phenolic and carboxylic acid groups) making them different from other technical lignins (Areskogh *et al.*, 2010b; Vishtal and Kraslawski, 2011). Sulphonate groups covalently link to the lignosulphonates, resulting in hydrophilic properties, despite lignosulphonates having a hydrophobic backbone. There are various proposed lignosulphonate structural models, such as spherical micelle, randomly branched polyelectrolytes and ellipsoidal flat particles, which are used to predict and explain the behaviours of lignosulphonates (Areskogh, 2011). This combination of hydrophobic and hydrophilic properties within lignosulphonates, has resulted in them being used in many applications as

plasticizers and technical surfactants (Areskogh *et al.*, 2010a). The molecular weight of lignosulphonates is relatively high (1 000 – 250 000 g/mol) in comparison to Kraft and soda lignins, with a broad dispersion of the weight (Vishtal and Kraslawski, 2011). Low molecular weight lignosulphonates act as dispersants, whereas high molecular weight lignosulphonates at low concentrations, have shown to act as flocculants (Chan, Baker and Beeckmans, 1976). The structure characteristics of lignosulphonates is shown in Figure 5.

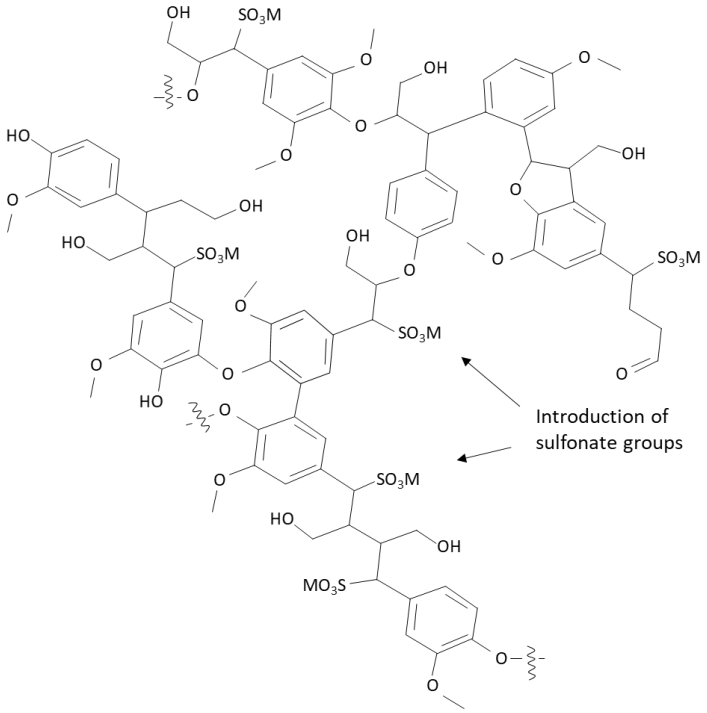

Figure 5: Model depicting the structural characteristics of lignosulphonate lignin redrawn from Zakzeski *et al.* (2010)

Over the years various modifications to the sulphite pulping process caused the improvement of the pulp yields and reaction kinetics, namely acid sulphite, bisulphite and neutral sulphite semi-chemical (NSSC) pulping (Naron, 2019). Table 4 summarizes the various sulphite pulping modifications applied to the industry.

Table 4: Modifications made to sulphite pulping process adapted from Naron (2019)

Process	pH	Base	Active reagent	Pulp type
Acid sulphite	1-2	Na^+,Mg^{2+}, Ca^{2+}, H_4N^+	HSO_3^-, H^+	Dissolving pulp Chemical pulp
Bisulphite	2-6	Na^+,Mg_2^+, H_4N^+	HSO_3^-, H^+	Chemical pulp High-yield pulp
Neutral sulphite semi-chemical	6-9	Na^+, H_4N^+	HSO_3^-, SO_3^{2-}	High-yield pulp

2.3 Lignin Valorisation

The movement towards more sustainable and renewable sources of fuel, energy and chemical products, has increased interest in finding alternatives to current petroleum-based products. Lignin has shown great potential as it is a carbon-neutral and an abundant renewable resource (Welker *et al.*, 2015). Lignin properties provides a great opportunity for application in the manufacture of higher-value products. These properties include its polyphenolic structure, thermal stability, antioxidant, thermoplasticity, and its antimicrobial properties (Upton and Kasko, 2015).

Currently high volumes of lignin are being produced and very few commercial applications of these technical lignin exist to completely realize the potential of lignin. Furthermore, the current lignin-based commercial products that are produced, have not always been superior in quality to the existing products (Vishtal and Kraslawski, 2011). There is a definite underutilization and misapplication of technical lignin (Gouveia, 2014).

The various applications require lignin with specific properties such as certain molecular weights, weight distributions, purity, as well as certain functional groups to be present within the molecule (Gouveia, 2014). Table 5 summarises the various properties of various lignin from the pulping industry.

Table 5: Properties of various technical lignin. Extracted from Vishtal and Kraslawski (2011)

Parameter	Kraft Lignin	Soda Lignin	Lignosulphonates
Ash Content (%)	0	0.7-2.3	4-8
Moisture Content (%)	3-6	2.5-5	5.8
Carbohydrates (%)	1-2.3	1.5-3	-
Acid Soluble lignin (%)	1-4.9	1-11	-
Nitrogen (%)	0.05	0.2-1	0.02
Sulphur (%)	1-3	0	3.5-8
Molecular weight (M_w)	15 00-25 000	1 000-15 000	1 000-15 0000
Polydispersity (PI)	2.5-3.5	2.5-3.5	4.2-7
Production status	Industrial	Industrial	Industrial

Since technical lignins are non-homogenous, it is difficult to use lignins in various applications. Methods to isolate high-purity lignin from the liquors, are being developed. Thus far, methods such as ultrafiltration, selective precipitation, including lignoboost™ and solvent extraction have shown potential (García *et al.*, 2009). The various methods and recovery of technical lignin are summarised in Table 6.

Table 6: Summary of various separation methods of Technical lignin. Adapted from Vishtal and Kraslawski (2011).

Type of Technical Lignin	Separation Methods	Status	Reference
Kraft Lignin	Selective Precipitation (pH change)	Industrial	(Loutfi, Blackwell and Uloth, 1991)
	Ultrafiltration	Industrial	(Jönsson, Nordin and Wallberg, 2008)
Soda Lignin	Selective Precipitation (pH change)	Laboratory/ Pilot	(Mousavioun and Doherty, 2010)
	Ultrafiltration	Laboratory	(Toledano *et al.*, 2010)
Lignosulphonates	Ultrafiltration	Industrial	(Restolho *et al.*, 2009)

Methods have been developed but not implemented commercially to produce syngas through the gasification of the black liquor. The syngas can be converted to other products such as methanol with the use of a catalyst (Abdelaziz *et al.*, 2016). Owing to lignin antioxidant properties, studies into pharmacological applications of lignin and its derivatives have increased. Studies have found that lignin and its derivatives have potential applications in improving the current research into obesity, thrombosis, diabetes, viral infections and cancer (Vinardell and Mitjans, 2017). Lignin can also be applied to fertilizers, acting as a controlled or slow-release additive (Welker *et al.*, 2015). One of the highest value applications of lignin is in the production of carbon fibres. Current research has focussed on Kraft lignin replacing the main feedstock of carbon fibres, polyacrylonitrile, which is expensive to produce. Carbon fibres produced by Kraft lignin have been found not to meet the mechanical characteristics (lower strength) of carbon fibres produced from polyacrylonitrile (Norberg, 2012). However, a study of higher molecular weight Kraft lignin, gave improved mechanical properties in the carbon fibre (Brodin, 2009). Lignin has shown potential as a phenol substitute in the production of phenol-formaldehyde resins, owing to lignin high phenolic content structure. As phenols are petroleum-based, toxic, corrosive and flammable, alternative substituents have been tested in an attempt to reduce economic and environmental effects, without compromising the final product quality. Studies have shown that a 20–50% substitution of lignin is possible while maintaining the resin's important properties (Khan and Ashraf, 2007).

Lignin has also shown potential in the field of bioplastics. Currently bioplastics contain synthetic fibres, which could potentially be replaced with lignin, owing to its availability and good mechanical properties (Yang, Ching and Chuah, 2019). Lignin can improve the biodegradability of the bioplastic, act as a stabilizer (as the phenolic hydroxyl groups can scavenge free radicals), and act as a plasticiser in bioplastics. However, modification to the lignin structure is required in order to improve the lignin compatibility and dispersion in the polymer blends (Yang, Ching and Chuah, 2019). Lignin can be applied to other synthetic polymers such as in polystyrene and polyethylene to improve their thermal stability (Govender, 2020).

Lignin polyphenolic structure provides interesting properties such as antioxidant activity, which can be applied to active packaging for the purpose of protection of light and sensitive goods (Domenek *et al.*, 2014). Another area where lignin are used, is in polymer blends where a challenge has been to improve

the dispersion of the lignin. However, calcium lignosulphonates have been polymerised with laccase and have shown an increase in the dispersion property of the lignin (Prasetyo *et al.*, 2010).

The depolymerisation of lignin is a widely studied topic owing to lignin being a renewable resource. Current depolymerisation methods include processes such as pyrolysis, hydrogenolysis as well as some oxidations. These methods focus on the degradation of technical lignin into its monomeric units (Upton and Kasko, 2015). The oxidation process causes the cleavage of lignin C-O and C-C bonds and/or altering its structure. This depends on the type of oxidant, catalyst and temperature the lignin is exposed to. Various oxidants have been investigated, namely H_2O_2, O_2, nitrobenzene and enzymes such as laccase. Low molecular weight products are produced, e.g. carboxylic acids, aldehydes and alcohols. The production of vanillin is a well-known and an investigated lignin oxidation process (Margellou and Triantafyllidis, 2019). Table 7 summarises the current and potential products of lignin. A risk-reward diagram of the main possible lignin application is shown in Figure 6.

Table 7: Indication of current and potential product applications of lignin adapted from Gouveia (2014)

MULTI-POLARITY PRODUCTS		**MATERIALS**	**AGRICULTURE**	**HIGH-PURITY VALUE APP.**
Dispersion	**Others**			
ceramics	complexing agents	phenolic resins	soil rehabilitation	antibacterial effects
oil well drilling	flocculating	polyurethanes	slow-release fertilizers	HIV inhibition
clay bricks & tiles	heavy metal binders	epoxies	artificial humus	digestion regulation
cements	ion exchanging	particle boards	humus	antioxidants
concrete	water softening	resin boards	encapsulation	plant immunology
gypsum board	protein coagulants	rubber reinforcing	composting aid	growth stimulators
dyestuffs	destabilization of oil emulsions	bloc copolymers	manure experiment	oxygen scavengers
electrolytes	corrosion protection	polyesters	humus improvement	hydrogels
paper sizing	anti-scaling	composites	soil stabilization	**MISCELLANEOUS**
emulsion	metal cleaners	polyolefin	insecticides	energy production
wax	grinding aids	biodegradables	granulation	diesel fuel
asphalt		carbon sieves	pelletizing	foam stabilizers
bitumen		activated carbons	chelates	binders
micronutrients		carbon fibres		tanning agent
		heat resistance		hydrophobization
		antioxidants		absorbents
		anti-inflammation		
		paper bounding		
		paper bounding		

Polymerisation of these reclaimed technical lignin offers the opportunity for increasing the technical lignin molecular weight to improve or apply to new applications. Various higher-molecular weight applications of lignin, include adhesives, copolyester and carbon fibres (Wells, 2015). There are various

polymerisation methods that can be applied to alter the properties of lignin, using enzymatic or non-enzymatic methods. Enzymatic modification of lignin is an energy-efficient and eco-friendly approach, which promotes long-term environmental stability and is effective as the enzymes are highly selective towards their substrates to upgrade lignin (Gouveia, 2014). However, for this method of lignin valorisation to be viable, large quantities of certain enzymes are required at a low cost (Conacher, 2018). Enzymatic modification has shown a high specificity of the type of radical generated, mild reaction conditions, and few undesired by-products production (Areskogh *et al.*, 2010a). Enzymes such as laccase and lignin peroxidase have shown great potential in polymerizing lignin, while they are also able to depolymerise it under certain conditions (Conacher, 2018).

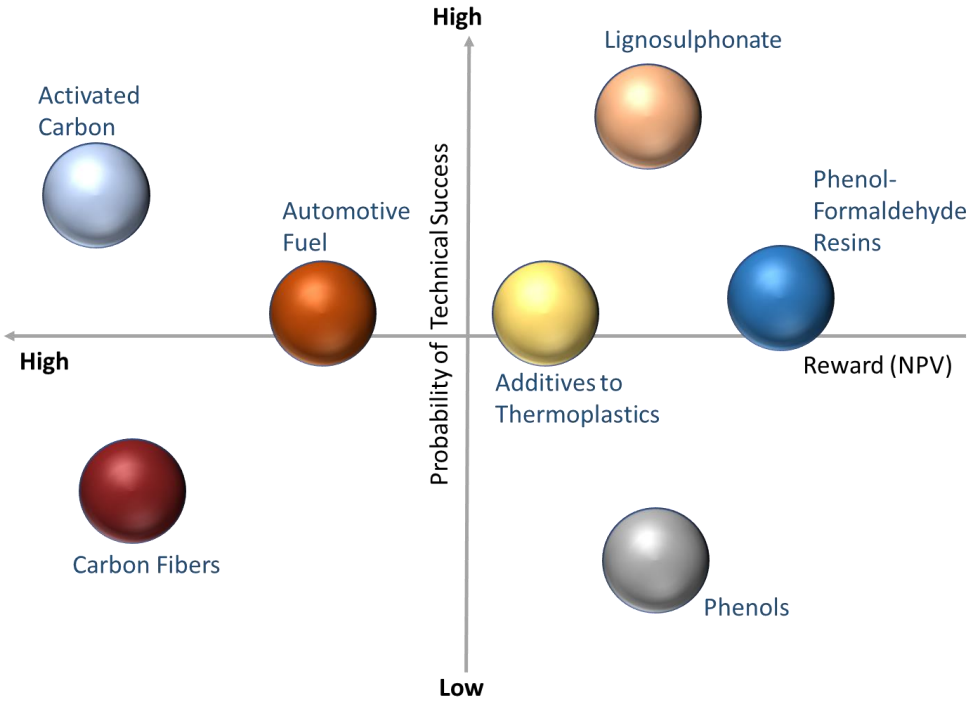

Figure 6: Risk-reward diagram of possible lignin applications redrawn from Gouveia (2014)

This project will investigate the repolymerisation of lignins present in pulping liquors via enzymatic modification. The free radicals that are formed by the activity of peroxidases or laccases are important from an industrial view – the formation of these radicals will increase the reactivity of these lignin molecules. These radicals will catalyse repolymerisation in a non-enzymatic manner, forming a newer higher-molecular weight lignin with new linkages (Sena-Martins, Almeida-Vara and Duarte, 2008).

2.4 Lignin modification enzymes

2.4.1 Lignin-degrading organisms

There are a limited number of organisms capable of degrading lignin with fungi that are responsible for most of the wood decay in nature, aided by some bacteria (Bugg *et al.*, 2011). Various extracellular enzymes which differ in composition and their role in the decomposition pathway, are produced by these

fungi. These fungi are classified as either brown-rot, soft-rot, or white-rot fungi, based on the characteristics of the rotten wood (Gouveia, 2014).

A brown-rot *Basidiomycetes* fungus produces a cocktail of enzymes that are able to degrade cellulose, lignin and hemicellulose. The degradation of the cellulose and hemicellulose occurs quickly, however, the enzymes produced to degrade lignin are very restricted to mainly modifying the lignin. Brown-rot fungi present more frequently in softwoods than in hardwoods. The degraded wood has a brownish colour which is caused by the increase in modified lignin and decrease in carbohydrates left behind in the wood (Dashtban *et al.*, 2010; Gouveia, 2014). Soft-rot fungi are deurteromyces or ascomycetes. They grow in more severe environments and are produce enzymes which are able to degrade all the components of wood. However, this occurs at a much slower rate.

White-rot fungi enzymes are able to metabolize lignin in great quantities and could degrade all the cell wall components (Martínez *et al.*, 2005). Most white-rot fungi are basidiomycetes, while some have been identified as ascomycetes. The delignification of white-rot fungi can be selective, where only lignin is degraded, or non-selective where lignin, cellulose and hemicelluloses are also degraded. Different enzymes produced by white-rot fungi, degrade the components at different rates, where variations in degradation can even be seen amongst different strains from the same species (Gouveia, 2014). Various white-rot fungi and bacteria produce and secrete enzymes that are able to degrade or modify the lignin (Chan, Paice and Zhang, 2020).

2.4.2 Lignolytic modification enzymes

Enzymes are macromolecules which are made up of monomeric amino acids linked together by peptide bonds. During chemical reactions the enzyme works as a catalyst. The enzyme will not be consumed in the reaction, just like a catalyst. Catalysis is achieved by lowering the activation energy of the reaction, thus increasing the rate at which the reaction will occur. The catalytic function of the enzyme is determined by its chemical structure which is dependent on the arrangement and variation of the amino acids creating the three-dimensional structure. The active site in the protein is where the catalysis occurs, where the three-dimensional structure around the active site is of particular importance. Thus enzymes do have a higher specificity towards certain substrates.

White-rot fungi produce four major groups of enzymes in order to degrade lignin, namely lignin peroxidase, manganese peroxidase, versatile peroxidase and laccase (Wong, 2009). In nature lignin peroxidase and laccase produced by the plant during biosynthesis, are enzymes involved in the formation of lignin in the cell walls as well as the degradation of lignin. Although the laccase and lignin peroxidases secreted by microbes are not identical to the plant-produced enzymes, they may hold similar catalytic activities. Studies on utilizing these enzymes produced by microbes on lignin, have shown that lignin is depolymerised as well as repolymerised during the experiments, producing a more degraded or higher molecular weight lignin (Harvey, 1997). Therefore these two enzymes were selected to be investigated in this study.

2.4.2.1 Laccase

Laccases are enzymes found in plants, fungi, bacteria or insects, where they have different functions such as detoxification, synthesis of pigments and fruit-body morphogenesis (Martínez *et al.*, 2005). Laccase belongs to the enzyme class oxidoreductases. These enzymes transfer electrons from the donor to the acceptor (Areskogh, 2011). Laccases use oxygen as an oxidant to oxidize phenolic rings in lignin to phenoxy radicals, while producing water as a by-product (Gasser *et al.*, 2012). The reaction mechanism is shown in Figure 7. These radicals can undergo covalent coupling to initiate lignin polymerisation (Dong *et al.*, 2017). The advantage of using laccase is because of the type of oxidant it requires. Other enzymes such as peroxidases require hydrogen peroxide, whereas laccase requires oxygen. The overall reaction showing the catalyses of laccase is summarized in Equation 1 (Pollegioni, Tonin and Rosini, 2015).

Equation 1:

$$4\ benzenediol + O_2 \leftrightarrow 4\ benzosemiquinone + 2H_2O$$

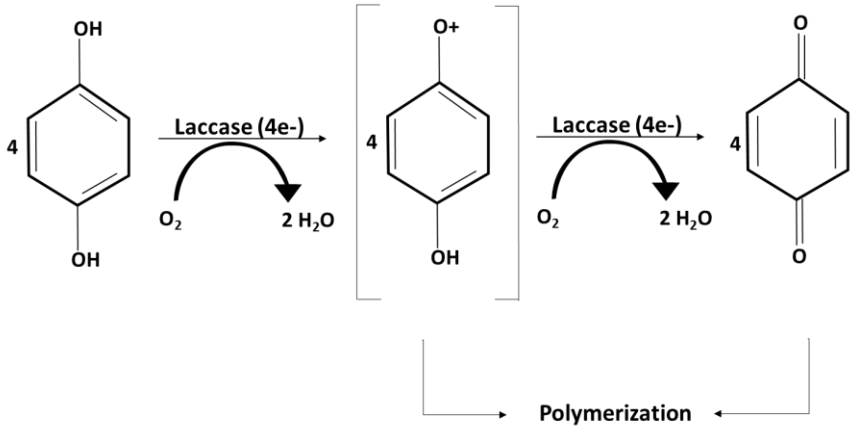

Figure 7: General mechanism of phenolic compound oxidation by laccase action from Gonçalves, Silva and Cavaco-Paulo (2015)

The laccase enzyme has an active site containing four copper ions: type 1 (T1 with 1 Cu atom), type 2 (T2 with 1 Cu atom) and type 3 (T3 with 2 Cu atoms) as shown in Figure 8. The enzymes resting from these ions are in a 2+ oxidation state (Pollegioni, Tonin and Rosini, 2015). The reaction mechanism that has been proposed for laccase is the so-called two-site, ping-pong, bi-bi reaction mechanism (Areskogh, 2011). The reaction starts with the oxidation of the substrate by the copper at the T1 site, which accepts an electron from the substrate and transfers it to the T2/T3. Dioxygen will bind to the T2/T3 site by accepting the electron and is then reduced to form water. The reduction of dioxygen to water requires four electrons, hence T1 must oxidise four substrates to transfer the required electrons (Wong, 2009; Areskogh, 2011). An advantage of lignin is its low substrate specificity and it can oxidise a range of chemical compounds such as diphenols, aminophenols and aryl diamines (Wong, 2009).

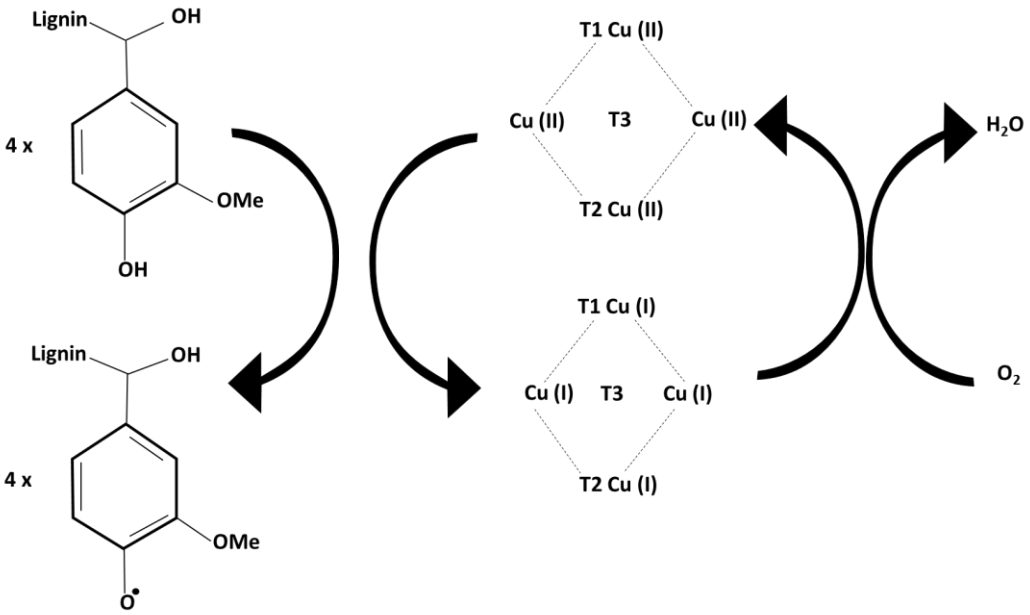

Figure 8: The reaction mechanism and structure of laccase redrawn from Areskogh (2011)

The activity and stability of laccase is dependent on pH and temperature. The optimum pH for laccase is highly dependent on the substrate. The pH affects the charges of the amino acids in laccase, and pH changes from the optimum can alter the enzymes' structure and also affect the enzymes' activity. Slight changes in pH are reversible, however, at high acid or a high basic pH, irreversible activity losses will occur. Operating at the optimum temperature is beneficial for the stability and activity of laccase. When operating at a very high temperature, the enzyme will become denatured, resulting in a sudden loss of activity (Gouveia, 2014).

There are various substances that can inhibit, slow down or stop the enzyme's activity. This can be caused by other substances similar to the substrate occupying the enzyme's active site, by a substance changing the enzyme's formation if added to a different area other than the active site on the enzyme, and in some cases when the concentration of the substrate is too high, it causes it to block the access to the active site. The presence of other substances has resulted in the loss of enzymatic activity, and these substances include various ions (azide, halides, cyanide, fluoride, thiocyanide and metal ions), fatty acids or chelating agents (Kunamneni *et al.*, 2007).

There are two problems to consider in oxidising lignin. Firstly, the lignin's structure as a 3-dimensional polymer, results in possible difficulties in fitting into the active sites of enzymes, and secondly its redox potential. Laccases' redox potential is relatively low (V ≤0.8), meaning the laccase is restricted to the oxidization of phenols, anilines and benxenethiols as components in lignin (Pollegioni, Tonin and Rosini, 2015). The degradation of the β-1 linkage causes the formation of phenoxy radicals, which leads to the cleavage of alkyl-aryl and aromatic rings (Wong, 2009). Native lignin is suggested to contain as much as 70% non-phenolic groups – these require a redox potential of at least 1.5V in order to be oxidised. As laccase has a low redox potential, the oxidation of the non-phenolic substrates is only possible in the presence of a mediator. Most studies have looked at mediators such as ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate)), HBT (1-hydroxybenzotriazole) and HAA (3-hyroxyanthranilicacid). The

mediator interacts with laccase in a cyclic reaction, where the oxidation of the laccase forms a higher potential mediator, which will interact with the substrate (not able to be oxidised by laccase) in a non-enzymatic manner and returns to its reduced state. This interaction between laccase and the mediator is different to general mediator-substrate chemistry. One important difference is that the mediators do not increase the redox potential of laccase (Wong, 2009; Areskogh, 2011). Currently the ideal mediator for laccase has not been found as studies show that the mediators have low stability, are slow to regenerate, or do not regenerate at all. Studies that focused on the depolymerisation of lignin by laccase, have shown that repolymerisation of lignin occurs if there are no mediators present. However, if mediators are present, then depolymerisation of the lignin can occur. This makes polymerising lignin challenging by utilizing laccase with the aid of a mediator to oxidise not only the phenolic substrates but the non-phenolic substrates (Gasser *et al.*, 2012).

This project will focus on the laccase enzyme produced from *Myceliophthora thermophile* (Novozymes NS51003), i.e. a commercially produced laccase from this organism.

2.4.2.2 Lignin peroxidase (LiP)

Lignin peroxidase (abbreviated to LiP) forms part of the extracellular fungal class peroxidases (Pollegioni, Tonin and Rosini, 2015). LiP was first discovered in *Phanerochaete chrysosporium* in 1983, which is a white-rot fungus. The crystal structure of LiP contains many amino acid residues and water molecules, a heme group, four carbohydrates molecules and two calcium ions (Chan, Paice and Zhang, 2020). The enzyme LiP is referred to as a glycoprotein, which catalyses oxidative depolymerisation of lignin that is dependent on H_2O_2.

LiP has been found to oxidise phenolic, non-phenolic lignin compounds and various organic compounds (Wong, 2009). LiP is assumed to oxidize aromatic rings via long-range electron transfer of the lignin. Unstable cation radicals, which are produced by the unspecified oxidation of the aromatic rings, will react further and undergo different non-enzymatic reactions such as scission of the Cα-Cβ and C4 ether linkages. This will cause the release of aromatic aldehydes and demethoxylations with methanol release (Gasser *et al.*, 2012). The non-phenolic lignin compounds have shown to be oxidised to aryl cation radicals. The overall reaction equation is summarized in Equation 2 (Pollegioni, Tonin and Rosini, 2015).

Equation 2:

$$1{,}2\ -\ bis(3{,}4-dimethoxyphenyl)propane\ -\ 1{,}3-diol+\ H_2O_2\ \leftrightarrow\ \ 3{,}4-dimethoxybenzaldehyde+1-(3{,}4-dimethoxyphenyl)ethane-1{,}2{,}-diol+\ H_2O$$

The catalytic cycle of LiP is a typical peroxidase cycle and consists of two main steps. First the presence of hydrogen peroxide (H_2O_2) initiates the oxidation of two electrons of the native LiP enzyme, which produces a ferryl cation radical intermediate, referred to as Compound I (LiP-I). The second stage involves two consecutive reductions of Compound I by the substrate donating the electrons, resulting in Compound II (LiP-II) and then back to LiP native form (Wong, 2009). The catalytic cycle of LiP is summarised in Figure 9. The concentration of the hydrogen peroxide is important: Excess addition of hydrogen peroxide and the absence of a reducing substrate can cause heme bleaching, which results in the inactivation of the enzyme referred to as Compound III (LiP-III). However, by spontaneous

autoxidation or oxidation of the substrate, the radical Compound III can return to its native form (Chan, Paice and Zhang, 2020).

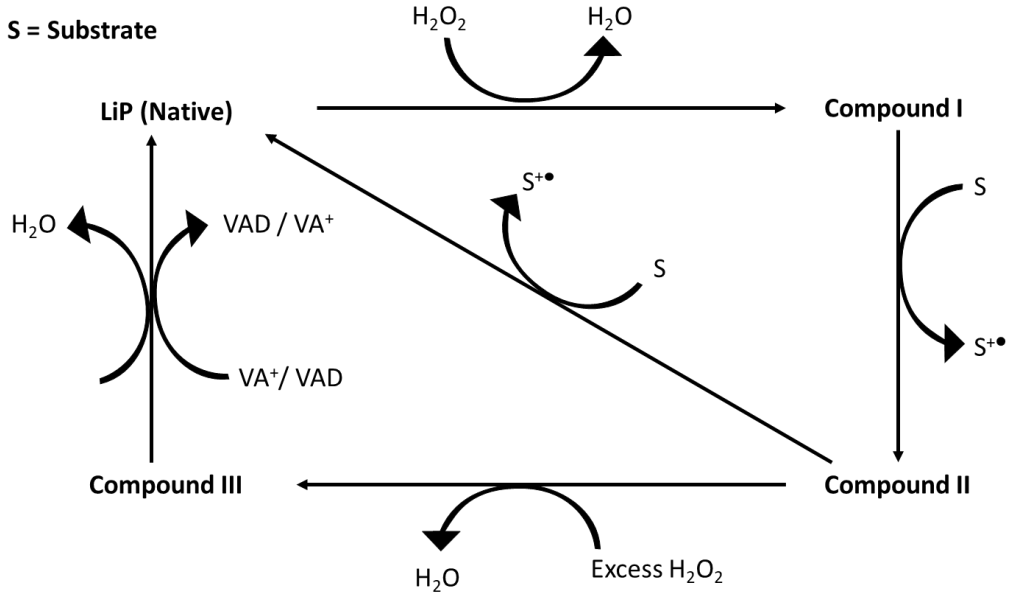

Figure 9: The catalytic cycle of LiP redrawn from Wong (2009)

LiP has an unusually low pH optimum of about 3 (Wong, 2009). The pH and hydrogen peroxide concentration plays an important role in the stability of the enzyme; any major deviations cause the inactivation of the enzyme. The redox potential of LiP is higher than for laccase, which allows the LiP to catalyse the oxidation of the non-phenolic lignin substrates without a mediator (Dashtban *et al.*, 2010). Although mediators are not needed, the addition of smaller molecule mediators such as veratryl alcohol has shown to increase the activity of LiP, and hence the rate at which lignin is degraded. Theoretically LiP should be able to degrade lignin to smaller compounds such as vanillin, veratraldehyde and syringaldehyde methyl ether, but in practice this has not happened. Supposedly the combination of lignin as a macromolecule as well as LiP results in the incompletion of the depolymerisation. The activity of the enzyme has shown to be dependent on the size of the substrate (Chan, Paice and Zhang, 2020). LiP has been found to polymerise lignin, yielding a higher molecular weight, because the LiP provides no mechanism to remove lignin fragments. Hence, the formed radical and fragments are susceptible to repolymerisation (Hammel *et al.*, 1993).

The research on the LiP enzyme is limited and further analysis into its interaction with lignin is needed, especially on the production of the enzyme. Various methods have been explored such as genetically modifying organisms. These include methods such as baculovirus expression vector systems and the addition of mineral nutrients in the growth medium. However, they are either not suitable for commercialisation or they are expensive. Research to improve the commercial production of this enzyme is required if it is to be used as a method to upgrade technical lignin (Chan, Paice and Zhang, 2020).

2.5 Lignin polymerisation studies

Lignin is an inexpensive and abundant renewable raw material. It has a potential high value and viewed as being non-toxic. When treated chemically or enzymatically it can be converted into higher value products (Mattinen *et al.*, 2008).

The potential for lignin production in the pulp and paper industry is 50 million tons per year. From the technical knowledge and characteristics of lignin it could replace fossil resources. However, this has not occurred, because of the low purity standard of the technical lignin, and as a result, lignin is used in the energy production for the pulping process (Gosselink *et al.*, 2004). The properties of lignin, its size, composition, linkages and functional groups, make it interesting compound, however, its various properties also restrict it from being applied to specific applications such as adhesives, polymer blends and carbon fibres. To overcome this structural modification of lignin it is needed to produce the ideal lignin for the type of application (Gouveia, 2014). An increased molecular weight lignin has shown in improved mechanical properties in the carbon fibres produced, while in polymer blends higher molecular weight lignin aids in acting as a thermos-oxidation stabilizer in bio-based polymers (Brodin, 2009; Ludmila *et al.*, 2015).

Much of the reported research on the valorisation of lignin has been focused on the depolymerising of lignin, while some of these enzymatic depolymerisation methods have also shown occurrences of repolymerisation. Table 8 summarises the various studies that investigated the repolymerisation of technical lignin.

A study was done where various lignosulphonates were treated with a commercially produced laccase (NS51003 and NS51002). Various lignin concentrations (1, 10 and 100 g/L) and enzyme dosages ranging between 1 and 10 U/L were investigated. The calcium lignosulphonates showed the highest molecular weight increase of 20-fold after 24 hours, where the sodium lignosulphonates only achieved a 2.6-fold increase. It was found that the higher the lignin concentration, the higher the molecular weight increase achieved over the 24-hour period. A low enzyme dosage (1U/L) did not yield any molecular weight increase at any concentration. The study also revealed that as the molecular weight of the lignin increased, the phenolic content of the lignin decreased, leading to a conclusion that there is a strong relationship between the molecular weight increase and the consumption of phenols. It was also noted that various factors are important to achieve a maximal molecular weight increase, namely the reaction time, the phenolic content, sulphonate content and the initial molecular weight of the lignosulphonates (Areskogh *et al.*, 2010a).

Another study using the commercially produced Laccase (NS51003), reacted with Kraft lignin with a biomass origin – *Eucalyptus globulus*. The study investigated what effect various mediators would have on the reaction, however, it was found that higher molecular weight increases were achieved when no mediator was present. This study also found that there is a relationship between the molecular weight increase and the consumption of phenols (Gouveia *et al.*, 2012). The polymerisation reaction is the random coupling of phenoxy radicals, which have been produced by laccase oxidation of the phenolic end groups (Areskogh, 2011).

Table 8: Various chemical and enzymatic polymerization studies in literature

	Initial Lignin material		Oxidation agent	Reference
E N Z Y M A T I C	Technical lignin	*Eucalyptus globulus* Kraft lignin	*Mycellophtora thermophila* - laccase; mediators	(Gouveia *et al.*, 2012)
		Commercial Softwood Kraft lignin Commercial Hardwood Kraft lignin Birch organosolv lignin	*Streptomyces ipomoea* - laccase *Melanocarpus albomyces* - laccase	(Moya *et al.*, 2011)
		Commercial lignosulphonates	*Myceliophtora thermophila* - laccase *Trametes villosa* - laccase	(Areskogh *et al.*, 2010a, 2010b)
		Commercial lignosulphonates	*Trametes villosa*; HBT mediator *Bacillus subtilis* bacteria; HBT mediator *Trametes hirsuta* - laccase; HBT mediator *Myceliophtora thermophila* - laccase; HBT mediator	(Prasetyo *et al.*, 2010)
		Eucalyptus globulus Kraft lignin	Polyoxometalate; laccase	(Dos Santos, Rudnitskaya and Evtuguin, 2012)
		Flax Soda lignin Spruce Enzymatic mild acidolysis lignin	*Trametes hirsuta* - laccase	(Mattinen *et al.*, 2008)
		Lignosulphonate (Calcium, Sodium) Kraft Lignin	*Trametes villosa* - laccase	(Mai *et al.*, 2002)
		Soda-anthraquinone Steam Explosion Lignin	*Phanerochaete chrysosporium* - LiP	(Majeke *et al.*, 2020)
		Coniferyl alcohol	*Phanerochaete chrysosporium* - LiP	(Sarkanen *et al.*, 1991)
		Kraft Lignin Steam Explosion Lignin Sodium Lignosulphonate	*Trametes villosa* - laccase	(West *et al.*, 2014)

The relationship between molecular weight and phenolic groups was also discovered in a study of Kraft lignin treated with laccase by Ai, Wang and Huang (2015). Where the lignin after modification showed a molecular increase, an increase in the condensed structures and the phenolic hydroxyl and methoxy groups decreased (Ai, Wang and Huang, 2015). A study of jute fibres treated with laccase showed an increase in phenolic hydroxyl, aliphatic hydroxyl and methoxy groups and a decrease in molecular weight (Zhang *et al.*, 2014).

West et al. (2014) found in his study using Kraft, steam-exploded lignin and sodium lignosulphonates that the enzymes are not able to act on, precipitated lignin, hence a compromise must be found between the

lignin solubility and the optimum operating pH of the enzyme. He also found that the lower molecular weight lignin showing a higher free phenolic content are more reactive (West *et al.*, 2014). Mattinen et al. found when Flax soda lignin reacted with laccase, the oxidation was also dependant on its solubility as well as the biomass origin of the lignin (Mattinen *et al.*, 2008). When determining the operating conditions of the study, it is important to ensure that the enzymes are not inhibited in some way. A study looking into the inhibition of the laccase enzyme by lignin fragments, found that at high dosages the inhibition of the enzyme is evident (Pamidipati and Ahmed, 2020).

There are very few studies using lignin peroxidase to alter the structure of lignin. In a study by Majeke et al. lignin peroxidase was used to treat Soda-anthraquinone and Steam explosion lignin. A tartrate buffer with an enzyme dosage of 2 U/mg lignin was prepared and after a 24-hour incubation period the soda-anthraquinone achieved a 1.43-fold molecular weight increase. As the study aimed to achieve depolymerisation of the lignin, it was noted that although LiP possesses the ability to both depolymerise and repolymerise, the repolymerisation is more dominant in vitro conditions. It was also found that the maximum thermal degradation temperature of the lignin increased after the enzymatic experiment, suggesting the lignin converted to a higher molecular compound with an improved thermal stability (Majeke *et al.*, 2020). Sarkanen et al. (1991) treated coniferyl alcohols with lignin peroxidase, where it was found that LiP was able to cause polymerisation in the presence of hydrogen peroxide (Sarkanen *et al.*, 1991). The use of LiP to repolymerise lignin requires a more in-depth analysis in the context of South African technical lignins is an area to be explored.

2.6 Aims and Objectives

2.6.1 Aims

The aim of this project was to repolymerise technical lignin from the South African paper and pulping industry through enzymatic modification. To repolymerise these industrial lignin means increasing their molecular weight lignin to improve or create possible higher value products.

In order to distinguish the changes incurred by the enzyme, the lignin will be characterised extensively. The process of the project will follow the following steps:

- The Isolation of technical lignin from the black liquor (where necessary).
- The purification of technical lignin to reduce the ash contents.
- Initial characterisation of the technical lignin includes ash content, elemental analysis, functional group content, molecular weight analysis and thermogravimetric analysis.
- Repolymerisation reactions utilising enzymes, laccase and lignin peroxidase to determine the extent of repolymerisation. The molecular weight and phenolic content will be determined during the experiments.
- Final characterisation of the treated technical lignin includes ash content, elemental analysis, functional group content, molecular weight analysis and thermogravimetric analysis, to determine the effect of the enzymatic modification.
- A techno-economic analysis to determine the industrial application of the process.

2.6.2 Objectives

The specific objectives of the study are:

1. To characterise the physio-chemical characteristics of lignin that would give insight into its interaction with the enzymes.

Various lignins were selected from six pulping mills in South Africa. The lignin's selected varied in biomass origin and in the pulping process. In order to determine which lignin could be repolymerised, various methods of characterization were investigated to determine the structural and chemical composition of the lignin – from literature it could be determined how each lignin would react to the enzymes (Chapter 3).

2. To investigate the extent of the repolymerisation by enzymatic modification.

Two enzymes were investigated: a commercial laccase and a lignin peroxidase (LiP) which was produced at Stellenbosch University. The use of LiP as an enzyme for the repolymerisation of technical lignins is a novel approach, as LiP has mainly been associated with the depolymerisation of lignins. Each lignin was reacted with each enzyme within a range of dosages to determine the optimum operating conditions, which would result in the highest repolymerisation.

3. The comparison of various lignins' ability to repolymerise.

The lignins were characterized after the enzymatic treatment to determine the changes incurred by the enzymatic modification (Chapter 4).

4. To investigate the economic feasibility of utilising enzyme modification to repolymerise technical lignins.

The optimum process conditions (maximum repolymerisation) were used to develop a techno-economic model. This was used to investigate the feasibility of the industrial application of enzymatically modifying lignin. The model utilizes one of the enzymes laccase, and the minimum selling points were determined, as selling prices for higher molecular weight lignins are not available in literature.

CHAPTER 3: CHARACTERISATION OF LIGNIN AND LIGNOSULPHONATES

3.1 Introduction

As lignin has great potential in various applications, it is critical to determine its structural and chemical properties prior to its utilisation, especially as various lignins from industrial sources have variable properties (Naron, 2019). Various applications require lignin with certain characteristics, such as lignin with high phenolic content, and with high thermal degradation temperatures, which is a requirement for polymer applications (Govender, 2020). In order to determine the various properties of lignin, various established methods exist as was explained in Section 2.1.3. The methods require lignin with low impurities so that accurate measurements can be given that would not cause damage to the equipment. Hence, the lignin samples are purified before being characterised (Fatehi and Chen, 2016). This chapter discusses these methods and how they were applied to determine the properties in the lignins, which will aid in the repolymerisation by enzymatic modification.

3.2 Material and Methods

3.2.1 Experimental approach

The experimental approach was to first acquire lignin samples for the study. Some samples from various pulping mills in South Africa were acquired for previous studies. The samples were acquired in 2018 and they had gone through initial sample preparation (isolated from the liquor) and were being stored in powder form. Two additional lignosulphonates were acquired from different pulping mills in South Africa. A total of six samples were investigated and the samples were split into three groups according to their pulping process: Kraft lignin, soda lignin and lignosulphonates. The Kraft and soda lignin required sample preparation including lignin isolation and purification, while the lignosulphonates could not be purified and were prepared by drying the liquor. The samples were characterised using established and cost-effective methods to give insight into the various lignin samples' structure and chemistry.

3.2.2 Lignin Sources

The lignin used in this project was obtained from various pulping processes from mills in South Africa, namely Sappi and Mpact pulping mills. The samples were allocated a sample ID and they were summarised in Table 9. All the samples, except NaS-S-T, were received in black liquor form and were required to be extracted from their liquor and purified further if possible.

There is also a slight difference in the Kraft pulping process used, where KH-S-N has an extra processing step, pre-hydrolysis, where the biomass is treated with steam or hot water to initially remove some of the hemicellulose and some of the lignin before the cooking stage (Govender, 2020). The lignin was extracted from the black liquor and then purified.

S-S-S which is the only non-woody biomass origin lignin investigated, was obtained from Sappi's Stanger Mill from their Soda pulping process. It was received as a black liquor and then isolated and purified.

NaS-S-T was received in powdered form from Sappi's Tugela Mill. The spent liquor was concentrated and then spray-dried. The other lignosulphonates, MgO-S-S and NaS-M-PR, were received in liquor form and required drying.

Table 9: Biomass origin, pulping process and mill source of lignin to be used in this project

Sample ID	Biomass Origin	Pulping Process	Mill Source	Type of Lignin
KS-S-N	*Pinus patula* (softwood)	Kraft	Sappi, Ngodwana	Kraft Lignin
KH-S-N	*Eucalyptus grandis* (hardwood)	Kraft (pre-hydrolysis)	Sappi, Ngodwana	
S-S-S	Sugarcane Bagasse	Soda	Sappi, Stanger	Soda Lignin
NaS-S-T	*Eucalyptus grandis*	Sodium Sulphite	Sappi, Tugela	Lignosulphonates
NaS-M-PR	Hardwood-Softwood-sugarcane Bagasse mixture	Sodium Sulphite (NSSC)	Mpact, Piet Retief	
	P. greggi/E. grandis			
MgO-S-S	*Eucalyptus grandis*	Magnesium Oxide	Sappi, Saiccor	

3.2.3 Lignin Sample Preparation

The pulping liquor contains various number of organic and inorganic substances; the organic components include lignin, low molecular weight substances, and various derivatives from the biomass, while the inorganic components include the chemical pulping chemicals, from the wood and their transformation products. These inorganics can negatively affect the application capabilities of these lignin, hence it is important to isolate and purify them (Tyhoda, 2008).

The lignin samples KS-S-N, KH-S-N and S-S-S were all isolated and purified using the methods described below.

As lignosulphonates are water-soluble, acid precipitation and lignin purification techniques are not suitable methods to isolate or purify the lignin solids. NaS-S-T was received in powder form and required no further preparation, while the MgO-S-S and NaS-S-T which were received in liquor form, required further preparation. Thus, these samples received in liquor form were poured into tinfoil trays and dried at 40°C in an oven for a period of at least 3 weeks. The dried liquor was milled into a powder and sealed in an airtight bag as lignosulphonates are very hydrophilic.

3.2.3.1 Acid Precipitation

The method used to precipitate the lignin from the black liquor was obtained from Naron et al. (2017). According to this method, the black liquor samples are first diluted using distilled water. Sulphuric acid (1N) was added to the diluted black liquor under stirred conditions at room temperature until the pH was 2. The liquor and acid was then left for a period of 24 hours. Thereafter the mixture was centrifuged for

a period of 10 minutes at approximately 7 000 rpm, where the solids are kept and the supernatant was discarded. The solids are washed with acidified water (pH 2), centrifuged and then air dried in foil trays until weight constant (Naron, 2019). The lignin was placed in a tinfoil tray and left to dry. Once dried, the lignin samples were milled with an Ultra Centrifugal Mill to a 0.5 mm particle size and stored. The lignosulphonates did not undergo this process as they will dissolve when added to acid.

The lignin which required isolating from the black liquor (KS-S-N, KH-S-N and S-S-S) was isolated by P. Govender and B. Majeke at Stellenbosch University.

3.2.3.2 Lignin Purification

To ensure that minimal impurities remained, the isolated lignin was further purified according to an acid-purification method described by Naron et al. (2017). The acid-precipitated dried lignin was mixed for 24 hours in a 1 M sulphuric acid solution in a solid liquid ratio of 1.5 (w/v). The mixture was then centrifuged to extract the solids and the supernatant discard. The lignin was then washed with distilled water and centrifuged twice. The ash content of the solids was determined and the process was repeated until the ash content was less than 5%. The lignin was then air-dried in tin foil containers, ground and stored.

3.2.4 Lignin Characterisation methods

The lignin characterisations were done to give insight into the structure and chemistry of the lignin samples. The following characterisation methods were used: Ash content, Elemental analysis, Fourier transform infrared spectroscopy (FTIR), Phenolic content determination, Gel permeation chromatography (GPC) and Thermogravimetric analysis (TGA).

3.2.4.1 Ash content

The ash content of the isolated and purified lignin and the dried lignosulphonates were gravimetrically determined according to the method described by TAPPI (1993). The determination was carried out in crucibles which were cleaned and oven-dried at 900°C for a period of 60 minutes and stored in desiccators to cool. The initial weight of the crucible was recorded, after which approximately 1 g of each sample was added to the dried crucibles and the weight noted. The crucibles were placed in a muffle furnace and heated gradually to 900°C and then kept at that temperature for a period of 24 hours, after which the muffle furnace temperature was decreased and the crucibles removed and left to cool in desiccators. After the heating cycle, the samples had a whitish-grey colour. The crucibles containing the ash samples were weighed to the nearest 0.1 mg, and the ash content was determined according to Equation 3.

Equation 3:

$$Ash\ content\ (\%) = \frac{(Mass\ of\ the\ ash)}{(Initial\ mass\ of\ the\ test\ samples)} \times 100$$

3.2.4.2 Elemental analysis

Elemental analysis was used to determine the carbon, oxygen, hydrogen and sulphur content in the lignin. The analysis was completed by the Central Analytical Facility (CAF) at Stellenbosch University. Samples of dried lignin, between 1–3 mg were analysed by being combusted in a Vario EL Cube Elemental Analyser. The composition of carbon (C), hydrogen (H), nitrogen (N) and sulphur (S) was expressed as a weight

percentage (wt. %), with the weight percent of oxygen that was determined by the difference method. The above-mentioned method was obtained from Ai, Wang and Huang, (2015) and Naron *et al.* (2017).

3.2.4.3 Fourier Transform Infrared spectroscopy (FT-IR)

FT-IR spectra of the lignin were determined in order to determine the functional groups of the lignin. The spectra were obtained by using a Thermo Nicolet Nexus™ model 470/670/870 FT-IR spectrometer, which is equipped with an ATR (attenuated total reflectance) system sampling module hosting a diamond crystal. The lignin samples were recorded at a 4 cm^{-1} resolution and a number of 32 scans per sample within the absorption bands of 4 000-600 cm^{-1} were taken. The background measurement of the clean diamond is used as the initial spectrum and subtracted from the spectra sample. Assignment of the major absorption bands was based on findings from literature (Naron *et al.*, 2017).

3.2.4.4 Phenolic Content Determination

As the enzymes utilize phenols to form radicals, the determination of the phenolic content is important to determine if repolymerisation has occurred. The phenolic content was determined by a method used by Areskogh *et al.*, (2010a) which utilizes the Folin-Ciocalteu reagent to determine the phenolic hydroxyl group content of the lignin samples. A lignin solution was prepared by dissolving a lignin sample in a reaction phosphate buffer (pH 6) at a lignin concentration of 10 g/l. From the lignin solution 1 ml was added to 30 ml of distilled water and 3 ml of Folin-Ciocalteu reagent (FC reagent) and mixed for approximately 8 minutes. After thorough mixing, 10 mL of a 20% sodium carbonate solution was added under stirred conditions and distilled water was added to adjust the final volume to 50 ml. The mixture was stirred for 2 hours after which the absorbance was measured by a spectrophotometer at a wavelength of 760 nm of the blue-coloured samples. A phenol-free sample (no lignin present in the lignin solution) was taken throughout the entire procedure to use as a reference. In order to determine the phenolic content, a calibration curve was set up where the absorbance was plotted against concentration. A standard solution of 5 M vanillin was used as the top level in a series of dilutions. The number of phenolic hydroxyl groups was found by measuring the absorbance at 760 nm and fitting it to the calibration curve as shown in Figure 10. The reference was also a phenol-free sample in the calibration curve.

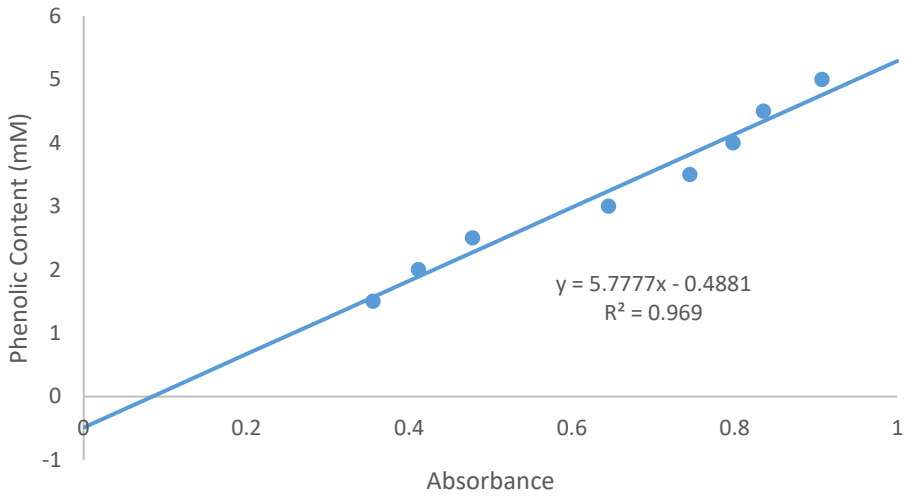

Figure 10: Phenolic content calibration curve (Vanillin)

3.2.4.5 Molecular weight determination

The molecular weight was determined to see if repolymerisation had occurred. Gel permeation chromatography (GPC) determines the average molecular weight (M_w), number average (M_n) and the polydispersity (M_w/M_n) of the lignin samples. The method used is described by Ringena, et al. (2005). The solvent was first prepared: Lithium bromide (0.05M) was dissolved in dimethylsulfoxide and water (90:10, v/v). Approximately 2mg of a dried lignin sample was dissolved in 2 ml of solvent and left for 24 hours at room temperature, after which the samples were then filtered (0.45 µm) and placed in HPLC vials before being analysed. All lignin samples dissolved in the chosen buffer and could be analysed. The samples were analysed in an Agilent 1260 Infinity Quaternary LC, which consisted of an Agilent 1260 Infinity, Quaternary pump, degasser, higher performance auto sampler and a variable wavelength detector. The columns were set up with an Agilent polargel-M Guard (50 x 7.5mm) and two Aqueous Agilent polargel-M (300 x 7.5 mm) columns. The flowrate was set to 0.5 ml/min with a column temperature of 60°C and the wavelength detector to 254 nm at a temperature of 30°C. 40 µL of each sample was injected and analysed for 45 minutes. The instrument was calibrated using polystyrene polymer standards ranging from 180 to 708 000 g/mol.

3.2.4.6 Thermal properties

The thermal degradation of the lignin samples was determined to ascertain the thermal stability of the lignin by using a TGA according to a method described by Naron, (2019) and Majeke *et al.*, (2020). Approximately 10 mg of dried lignin samples was heated on an alumina pan under a nitrogen atmosphere from 50 – 110°C at a heating rate of 50°C/min and then 110–900°C at a heating rate of 100°C/min. The weight loss of the samples was determined to compare it to the temperature, resulting in a degradation curve which would indicate the maximum degradation temperature of the lignin.

3.3 Results and Discussion

3.3.1 Compositional Analysis

The compositional analysis of the purified lignin samples and dried lignosulphonate samples consists of the ash content determination, the elemental analysis and the estimation of the C_9 empirical formulas of the lignin samples. The results from the analyses are summarised in Table 10. The ash content indicates the inorganic compounds attached to the lignin. These inorganics originate from the pulping chemicals added during the pulping process (Gouveia, 2014; Adu-Poku, 2015). It was expected that the ash content that the lignosulphonates (NaS-S-T, NaS-M-PR and MgO-S-S) would be higher than the Kraft and soda lignin (KS-S-N, KH-S-N and S-S-S), as the Kraft and soda lignin have undergone precipitation and purification processes. The ash content of both Kraft lignin agrees with literature, as it was expected that it should be below 1% (Chen, 2014). At 1.4%, the ash content of the soda lignin is within the expected range of 0.7–2.3% (Vishtal and Kraslawski, 2011). The lignosulphonates from the sulphite pulping process showed ash contents between 17–32%, which agrees with the results reported by Govender, (2020) on lignosulphonates from the same pulping mills. As lignosulphonates are unable to be precipitated and purified from the liquor, owing to their solubility in acid and water solutions, this results in a high ash content.

Table 10: Compositional results of various technical lignin

Sample ID	Ash Content	Elemental Analysis					Estimated Empirical Formula from Elemental Analysis
	%	C [%]	H [%]	N [%]	S [%]	O [%]	
KS-S-N	0.3	59.85	5.99	0.04	4.12	30.00	$C_9H_{10.81}S_{0.23}O_{3.38}$
KH-S-N	0.4	59.2	5.6	0.11	4.1	31.0	$C_9H_{10.23}S_{0.23}O_{3.54}$
S-S-S	1.4	52.9	6.4	0.09	1.0	39.6	$C_9H_{13}S_{0.07}O_{5.06}$
NaS-S-T	22.1	31.8	4.9	0.11	6.6	56.6	$C_9H_{16.50}S_{0.70}O_{12.03}$
NaS-M-PR	32.5	24.0	3.7	0.1	10.1	62.1	$C_9H_{16.44}S_{1.41}O_{17.45}$
MgO-S-S	17.3	36.1	6.0	0.1	6.0	51.9	$C_9H_{17.82}S_{0.56}O_{9.70}$

The elemental analysis showed that the carbon content of the Kraft and soda lignin was higher than the lignosulphonates (59.2–59.85%, 52.9% and 24–36.1 %, respectively). This is owing to the proportionally higher ash content present in the lignosulphonate samples. The carbon content of the Kraft and soda lignin is slightly lower than the reported ranges of between 59–64 wt% (Schorr, Diouf and Stevanovic, 2014; Naron *et al.*, 2017). The reported hydrogen content ranged between 5.6–6.4 wt% for the purified lignin, which is slightly higher than in the reported literature of between 5–6 wt% (Naron, 2019). The slight variations in the carbon content could be because of the varying plant origin and purification methods. This should be considered when looking for potential applications for these lignins (Schorr, Diouf and Stevanovic, 2014). While all the nitrogen content present in all the lignin samples was below 0.11%, the amount has originated from the biomass (Govender, 2020). The sulphur content in the lignosulphonates (NaS-S-T, NaS-M-PR and MgO-S-S) and the Kraft lignin (KS-S-N and KH-S-N) was as

expected and was in the range of 4.1–10.1 %, while the soda pulping lignin (S-S-S) which would not be contaminated by sulphur from the pulping process, contained 1%, which probably originated from the biomass source, the precipitation, or the purification process that was used. The range of sulphur present in Kraft lignin is between 4.6–6.3 wt% which is slightly higher than the results (4.1 wt%) found in this study (Naron *et al.*, 2017). This indicates a higher purity lignin which is confirmed by the low ash contents obtained. The results from the elemental analysis were used to estimate the C_9 formulas of each lignin sample. The resulting elemental analysis and estimated formulae are within range and consistent with analysis from literature (Mansouri and Salvadó, 2006; Wörmeyer *et al.*, 2011; Naron, 2019; Govender, 2020).

3.3.2 Structural Analysis

Insight into the structural analysis of the lignin was achieved by determining the functional groups present. Infrared spectra of the lignin samples by Fourier Transform Infrared Spectroscopy (FTIR) were taken to identify the functional groups, and are presented in Figure 11. The spectra shows characteristic lignin structures, where some differences in the intensities of the peaks between the samples are observed. The important functional groups' peak assignments have been summarized in Table 11.

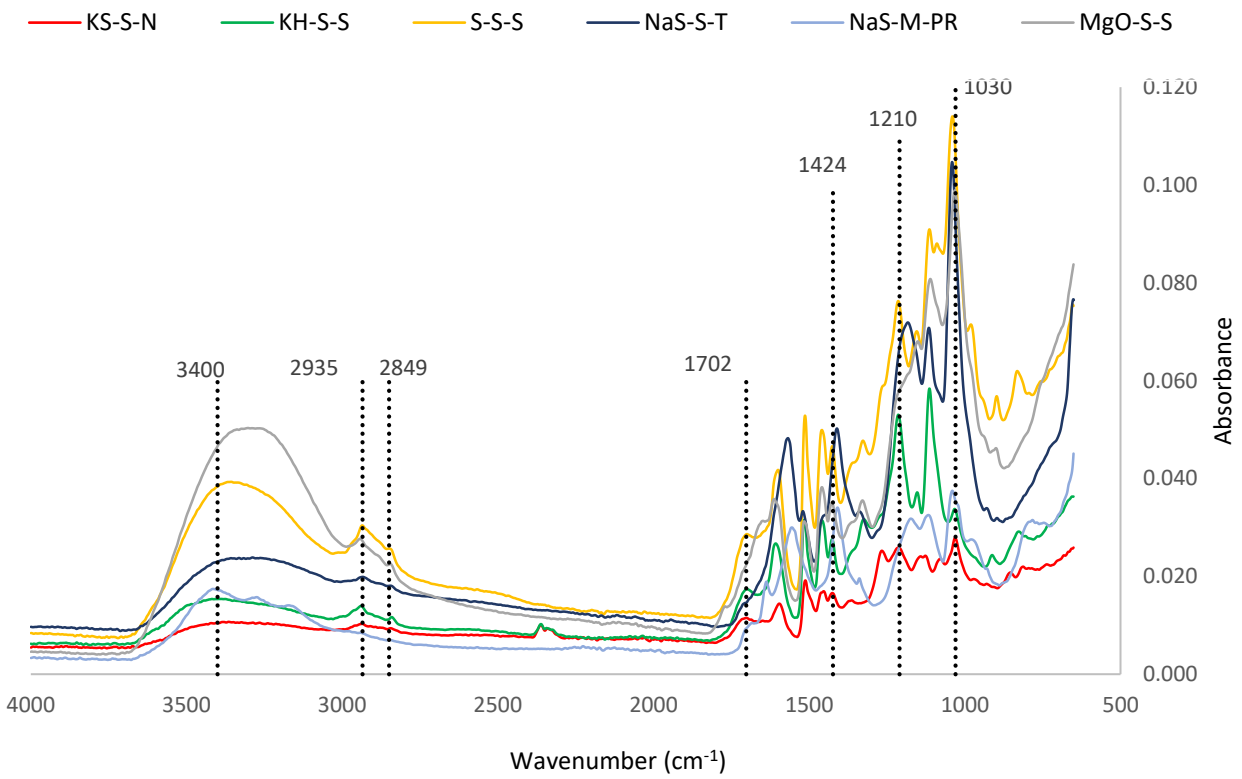

Figure 11: FT-IR spectra of technical lignin samples

The region between 3440–3430 cm^{-1} results from the presence of phenolic or aliphatic hydroxyl groups. The magnesium lignosulphonate, MgO-S-S, and bagasse origin soda lignin, S-S-S, have the highest intensity in this region, and hence contain more hydroxyl groups, thus they are expected to be more

reactive as the hydroxyl groups' influence the reactivity of the lignin (Laurichesse and Avérous, 2014). This would indicate that these lignins have a good potential for repolymerisation as the enzymes utilise the phenolic groups. The peaks at 2935 cm^{-1} show the C-H stretching of methyl and methylene groups. At 2849 cm^{-1} they indicate that the C-H vibrations of $–OCH_3$ groups were visible in all samples without major intensity differences, with the exception of the sodium lignosulphonate, NaS-M-PR, showing no distinctive peak, and the S-S-S lignin showing the highest intensity compared to the other samples.

The major spectral differences between the samples are found in the fingerprint region (1800–800 cm^{-1}), which has been enlarged and can be seen in Figure 12. The presence of unconjugated ketones, carboxyl and ester groups as found in the region 1712–1702 cm^{-1}, showed the highest intensity for S-S-S lignin, indicating that little modification was done to the carbonyl groups. This observation agrees with a study done by Govender (2020), but this disagrees with what was observed by Naron (2019), where both these studies investigated lignin's in the South African pulping industry. The C-H bending of methoxyl groups is seen at a peak of 1457 cm^{-1}, where distinctive peaks were observed for most of the technical lignin, with the exception of two samples where no peak was observed for the NaS-M-PR and a small peak was observed for the Kraft lignin, KS-S-N. Meanwhile in the peak region of 1424-1426 cm^{-1}, indicating the aromatic ring stretching, the KH-S-N showed lignin the lowest intensity and an absence of peaks was observed for the sodium lignosulphonates samples (NaS-S-T and NaS-M-PR). This absence or low peak could indicate either a lower methoxyl group content, or because of the lignosulphonates' high impurity (indicated by the high ash content) it could result in a possible band shift, as distinctive peaks can be seen around 1400 cm^{-1}, however, this peak has no band assignment available in current literature (Govender, 2020). The peak region 1220–1210 cm^{-1} indicates the stretching of phenolic hydroxyl groups and peak region 1044–1030 cm^{-1} indicates the stretching of primary aliphatic hydroxyl groups, including C-O stretching of primary alcohols. KH-S-S and S-S-S showed the highest intensity peaks at 1220–1210 cm^{-1}. The lignosulphonates showed no peaks at 1220–1210 cm^{-1}, but a strong peak intensity at 1044–1030 cm^{-1}, indicating that the lignosulphonates contain more aliphatic hydroxyls with trace amounts of phenolic hydroxyls, or that the peak allocation for the phenolic hydroxyl groups may also have shifted, owing to the lignosulphonates' high impurity (Ignat *et al.*, 2011). As expected all the lignosulphonates have strong intensity peaks at 1190 cm^{-1}, indicating the presence of sulphonate groups. However, the magnesium lignosulphonate, MgO-S-S indicated a lower intensity peak in comparison to the sodium sulphite lignosulphonates, NaS-S-T and NaS-M-PR, which can be explained from the elemental analysis and the ash contents found in Section 3.3.1.

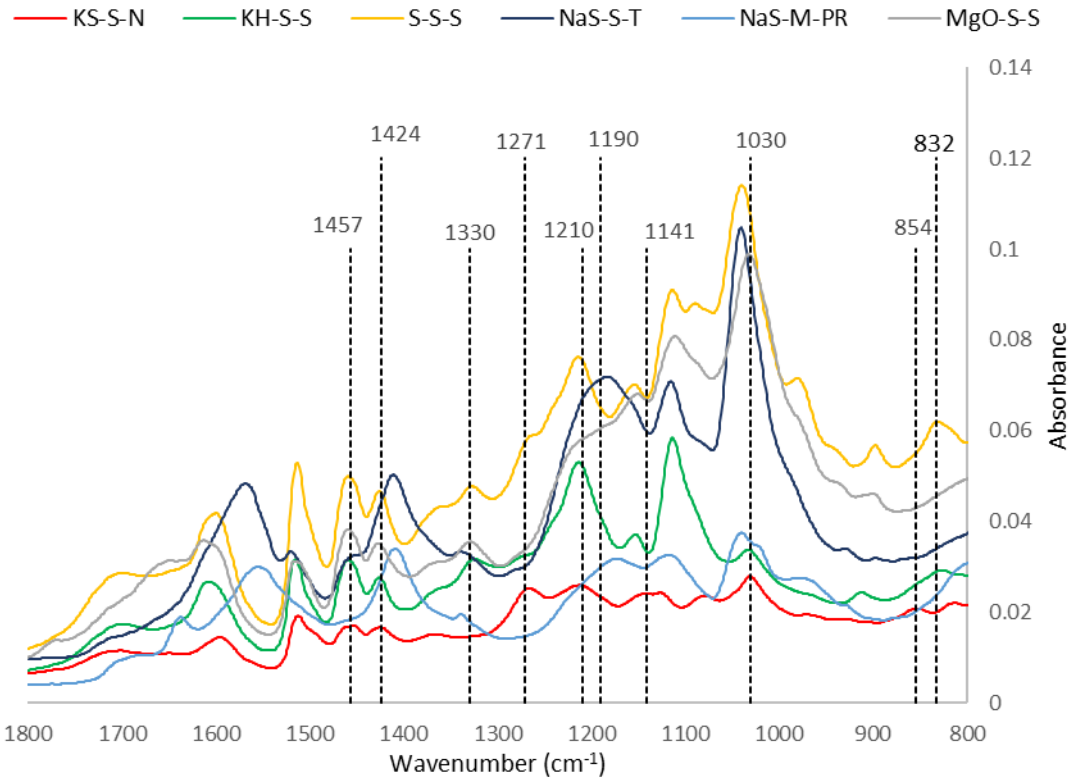

Figure 12: Enlarged FT-IR spectra between the wavenumber of 1800–800 cm^{-1}

The region between 914 and 813 cm^{-1} indicates the C-H bending of the G & S units (lignin monomers), while the peak at 832 cm^{-1} indicates the C-H stretching of the H units. The S-S-S lignin was the only lignin with a peak intensity at 832 cm^{-1} as the lignin origin, bagasse, are grass which contain H, G and S monomers (as discussed in Section 2.1.2). The peak 854 cm^{-1} indicates the C-H bending of the guaiacyl ring, as softwoods contain more G monomers than hardwoods or grasses, it is expected that the softwood origin lignin will have a higher intensity (Gouveia, 2014). Other peaks associated with G units are 1264 and 1141 cm^{-1}. The only softwood origin lignin from the Kraft pulping process, KS-S-N, shows distinct peaks in comparison to the other lignin at these mentioned wavenumbers (854, 1264 and 1141cm^{-1}). The peak 1330 cm^{-1} has shown to indicate the C-O in the syringyl (S monomer). All the lignin investigated except the softwood Kraft lignin, showed peaks at this wavenumber, which is expected as both grasses and hardwoods contain S monomers.

Table 11: FT-IR Absorption peak functional group assignments extracted from Govender, (2020)

Peaks (cm^{-1})	Band Assignment
3 430-3 440, 3 400	Phenolic and aliphatic O-H stretching
2 935	C-H stretching of methyl and methylene groups
2 849	C-H vibration of $–OCH_3$ngroups
1 702-1 712	Unconjugated ketone and aldehyde groups
1 510-1 606, 1 500	Aromatic skeletal vibrations
1 457	C-H bending of methoxyl groups
1 424-1 426	Ring stretching coupled with C-H in plane formation
1 264	C=O stretching of G units
1 220	Stretching of phenolic hydroxyl groups
1 210	G & S rings breathing and C-O stretching (phenolic C-OH and phenolic C-O(Ar) stretching)
1 190	Sulphonic acids/sulphonate groups in lignosulphonates
1 141	C-H bending G units
1 030	Stretching of primary aliphatic OH
1 030-1 044	C-O stretching of primary alcohols
914-813	C-H bending of G & S units
854	C-H out-of-plane deformation typical of guaiacyl aromatic ring structure
832	C-H stretching of p-hydroxyphenylpropane

3.3.3 Molecular weight and Phenolic Content Analysis

The results of the average molecular weight (M_w), number average (M_n), polydispersity and phenolic content for the lignin samples, are shown in Table 12. The molecular weights ranged from 5 657 to 12 156 g/mol, the number averages from 1 744 to 3 811 g/mol, and the polydispersity ranged from 1.73 to 4.3.

Table 12: Molecular weight and Phenolic content characterisation of various technical lignin

Sample ID	Molecular Weight			Phenolic Content (mmol/g)
	Mw (g/mol)	Mn (g/mol)	Polydispersity	
KS-S-N	12 156	2 513	4.37	1.65
KH-S-N	5 657	2 073	2.83	2.23
S-S-S	5 685	1 744	3.26	1.28
NaS-S-T	6 185	3 251	1.732	0.96
NaS-M-PR	6 453	3 189	2.0	0.95
MgO-S-S	6 589	3 811	2.319	1.16

The softwood origin Kraft lignin (KS-S-N) has a higher average molecular weight and polydispersity than the hardwood Kraft lignin (KH-S-N), which is consistent with reports in literature (Fengel and Wegener, 2003; Naron, 2019). As softwood origin lignin contains predominantly guaiacyl units, resulting in the lignin structure being more stable, predominately from the C-C linkages formed at the C5 position of the aromatic ring of the guaiacyl monomer which are difficult to cleave and the β-aryl ether bonds are easily repolymerised (Fengel and Wegener, 2003). In comparison to the β-aryl ether bonds present in the sinapyl, monomers of hardwoods are cleaved easily and have a lower tendency to repolymerise, hence the average molecular weights of hardwoods are lower than softwoods (Naron, 2019). Even though the sodium lignosulphonates (NaS-S-T and NaS-M-PR) were obtained from different pulp mills, similar biomass origin and similar isolation processes, the average molecular weights, number average and polydispersity were similar, indicating the lignin was depolymerised into similar chain lengths and hence have similar average molecular weights.

The phenolic content determined by the spectrophotometric method showed the lignins' phenolic content and the conclusions regarding the intensity of the phenolic hydroxyl functional group bands present in the lignins' results, were similar and are comparable to results in literature (Areskogh, 2011; Gouveia, 2014). The Kraft lignin, KS-S-N and KH-S-N, showed the highest phenolic content of 1.65 and 2.23 mmol/g lignin, respectively, and the sodium lignosulphonates, NaS-S-T and NaS-M-PR, had the lowest of 0.96 and 0.95 mmol/g lignin, respectively. During the delignification in the pulping process the cleavage of the phenyl propane linkages generates these phenolic hydroxyl groups, which indicates the reactivity of the lignin (Gouveia, 2014). The cause of the lignosulphonates lower phenolic content may be owing to the high impurity of the lignin.

3.3.4 Thermal Analysis

A thermogravametric analyser (TGA) commonly determines the thermal decomposition of the organic components of polymers and reveals the loss of weight of the samples in relation to the temperature of thermal degradations. The first derivative of this curve shows the corresponding weight loss rate, and the peak of the curve indicates a single degradation temperature, which can be used to compare the thermal stability of the different lignin samples as summarised in Figure 14.

In Figure 13, the decomposition of the different lignin samples are shown to start at temperatures ranging between 120°C – 250°C with S-S-S and MgO-S-S degrading slightly earlier than the other lignin samples, which agrees with literature (Laurichesse and Avérous, 2014; Govender, 2020). It is believed the initial degradation is as a result of the dehydration of the hydroxyl groups from the benzyl functional groups of the lignin and the cleavage of the α - aryl and β – aryl –aryl links (Laurichesse and Avérous, 2014). The main degradation of the lignin samples occurs between 250°C and 500°C. This agrees with studies found in literature. During this range the KS-S-N, KH-S-N and S-S-S experience a significant mass percentage loss between 39% and 46% while the lignosulphonates, NaS-S-T, NaS-M-PR and MgO-S-S, achieved 26%, 23% and 37% respectively. This is explained by the fact that after a temperature of 300°C the degradation of the aliphatic side chains occur, and after 370°C the cleavage of the carbon-carbon bonds between the lignin structures occurs (Laurichesse and Avérous, 2014). Slight degradation of the purified lignins are observed at temperatures above 500°C where the degradation of some aromatic rings occur (Tejado *et al.*, 2007; Martin-Sampedro *et al.*, 2011), while the lignosulphonates have a higher inorganic content and hence, degradation is still noticed after 600°C. Interestingly, the magnesium sulphite lignosulphonate, MgO-S-S, degrades before the sodium sulphite lignosulphonates, NaS-S-T and NaS-M-PR, which could be attributed to MgO-S-S having a lower ash content (Govender, 2020). The final weight residues recorded at 899°C were found to be between 29.8–32.5% – this range is within the reported ranges in literature (Tejado *et al.*, 2007; Laurichesse and Avérous, 2014; Govender, 2020; Majeke *et al.*, 2020).

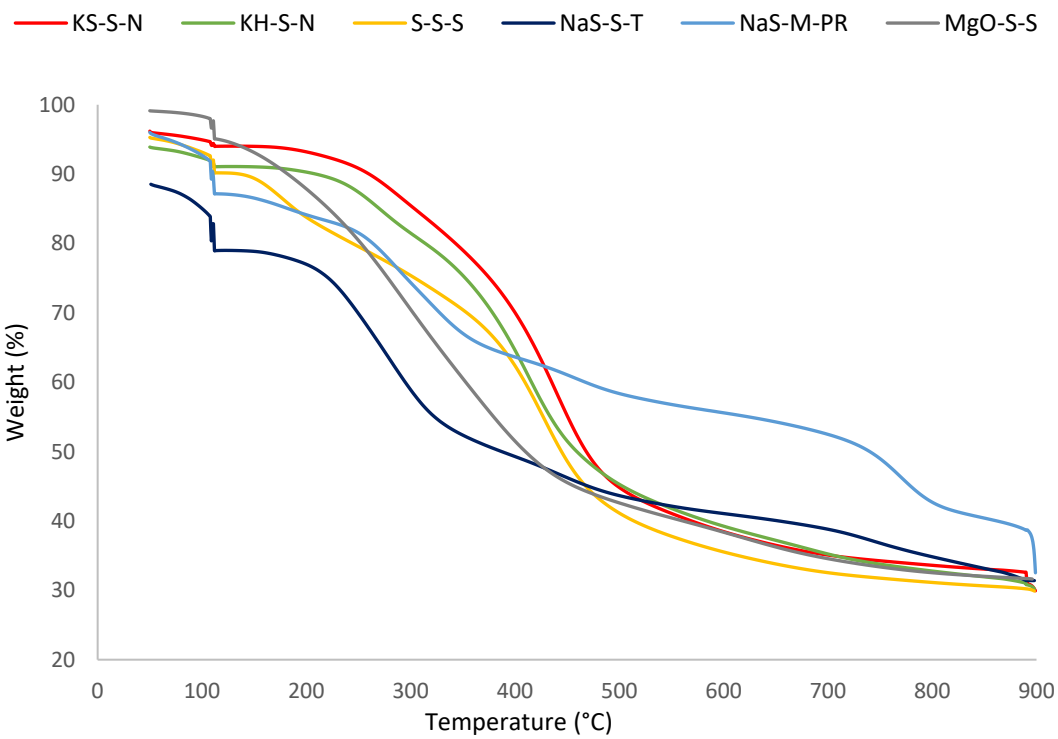

Figure 13: Thermal degradation curves of lignin samples

The rate of degradation of each lignin sample is depicted in Figure 14 where the peaks indicate the maximum degradation rate at a specific temperature. The maximum degradation temperature of the purified lignin was between 407°C – 434°C. which agrees with reports made by Tejado *et al.,* (2007) and

Gouveia, (2014) in literature. The softwood biomass origin lignin KS-S-N, recorded the highest degradation temperature at 434°C which is expected, as softwood lignin contains more G monomers and have more condensed carbon-carbon bonds than hardwood or non-woody lignin, thus making them more thermal stable (Tejado *et al.*, 2007; Naron, 2019). While the lignosulphonates ranged between 276 °C – 307°C which is slightly higher, but within the range of what was reported by Govender, (2020). The cause of the lignosulphonates to degrade at lower temperature is because of a catalytic effect from the higher inorganic content of the lignosulphonates (Naron, 2019; Govender, 2020).

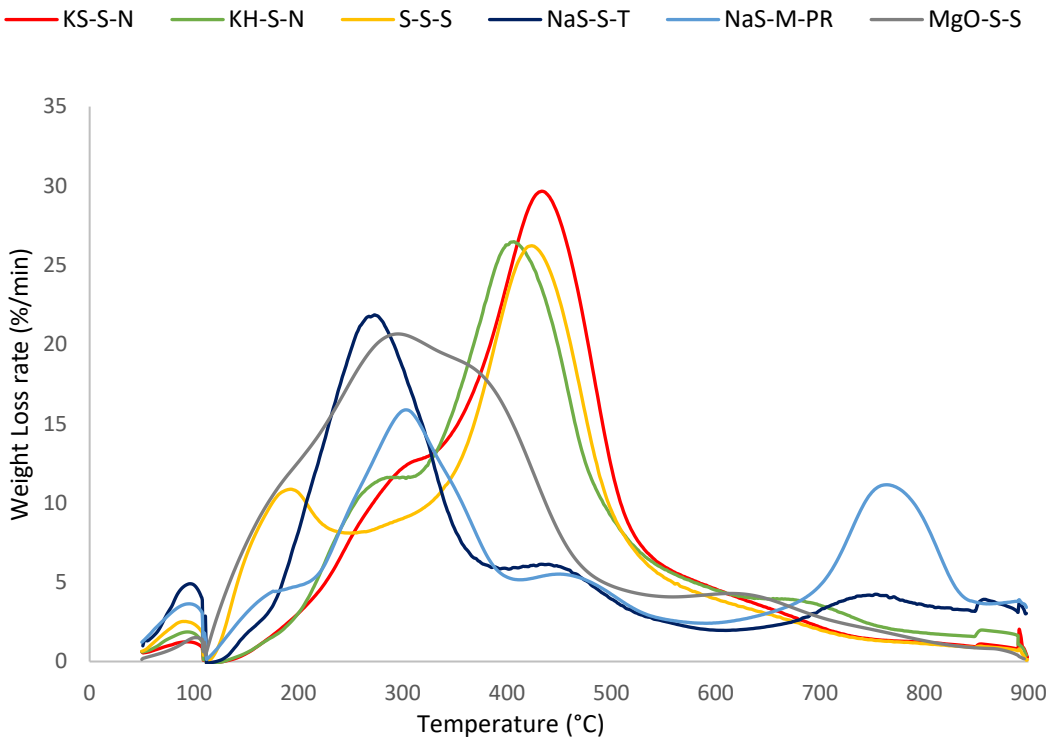

Figure 14: Rate of degradation of lignin samples

CHAPTER 4: ENZYMATIC POLYMERISATION OF TECHNICAL LIGNIN

4.1 Introduction

The use of lignin in higher value applications requires the lignin structure and chemistry to be altered either by depolymerisation or by repolymerisation methods. Studies have shown that lignin can be modified to be used as a value-added low or high molecular weight material. Low molecular weight lignin can be used as raw material for such chemicals as dimethyl sulfoxide, aromatics or phenols. High molecular weight lignin applications include the production of adhesives, carbon fibres, binders, additives (e.g. In cement) and thermally-stable copolyester (Areskogh, 2011; Gouveia, 2014; Wells, 2015). While various methods have been proposed to achieve either low or high molecular weight lignin, the use of enzymes to upgrade lignin has indicated to be an energy-efficient and eco-friendly method (Gouveia, 2014).

4.2 Material and Methods

4.2.1 Experimental Approach

In this study, two enzymes were investigated i.e. laccase and Lignin peroxidase (LiP). The enzymes' optimal operating conditions were sourced from literature and established methods were used to determine the activity of the enzymes. Individual experiments were designed for the enzymatic treatment of each of the purified lignins, i.e. soda and Kraft lignin, as well as for the lignosulphonates with the two enzymes. The original as well as the treated lignins were characterised by established methods to determine the chemistry and structural changes incurred by the enzyme experiments, in order to determine if repolymerisation had occurred.

4.2.2 Materials

The six lignins investigated in Section 3 were treated with two different enzymes which have shown the ability to repolymerise lignin under various conditions. The two enzymes to be investigated are Laccase (NS51003) from *Myceliophthora thermophile,* which was kindly supplied by Novozyme, and Lignin peroxidase (LiP) from *P. chrysosporium,* which was developed in the *Pichia pastoris* expression system as described in Majeke (2020).

4.2.3 Enzyme Activity

4.2.3.1 Laccase

The laccase activity was determined according to a method developed by Hong, Meinander and Jönsson (2002). According to this method, 0.4 mM 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonate), also known as ABTS, is dissolved as a substrate in a 100 mM sodium acetate buffer (pH 5 and at 25°C). The enzymes are diluted appropriately and mixed with the substrate solution (1 mM). The increase in the absorbance is monitored in a spectrophotometer at 420 nm for 5 minutes (using an extinction coefficient, $\varepsilon = 36\ mM^{-1}\ cm^{-1}$ to calculate the activity). The activity is then expressed in units per litre of sample.

4.2.3.2 Lignin Peroxidase

The LiP activity is determined in a spectrophotometer according to a method described by Majeke *et al.* (2020). According to this method, the reactive mixture contains 0.5 ml of enzyme, 1 ml of 125 mM Sodium tartrate buffer (pH 3) and 0.5 ml of 10 mM veratryl alcohol (3,4-Dimethoxylbenzyl alcohol). An amount of 0.5 ml of Hydrogen peroxide (0.4 mM) is added to initiate the reaction. The absorbance is measured in a spectrophotometer for 5 minutes at a wavelength of 310 nm. The absorbance is used to determine the enzyme activity [extinction coefficient, Ɛ = 9,3 mM^{-1} cm^{-1} (Majeke *et al.*, 2020)].

4.2.4 Lignin repolymerisation

Owing to the insolubility of the purified lignin, two different experiments were designed. The purified lignin, Kraft and soda lignin that had limited solubility, could only be investigated at a maximum fixed concentration of 15g/l at various enzyme dosages. The lignin was added to an alkaline NaOH (0.5 mM) solution to produce a homogenous suspension and diluted with the respective reaction buffer to the specified reaction volume as described by Ai, Wang and Huang (2015). The enzyme dosages investigated for the purified lignin ranged from 1 – 30 U/l as summarised in Table 13. As the lignosulphonates are water-soluble, a fractional factorial was designed to investigate lignin concentrations from 15 to 50 g/l and an enzyme dosage from 1 – 20 U/l. However, after the statistical analysis, additional points were added in order to fit the data to the full surface response model. All the investigated points are also summarised in Table 13. Both experiments followed the same experimental procedure as is explained below. The reactions were carried out in two different buffers at different pH's and different reaction temperatures, owing to the enzyme's pH and temperature sensitivity, which was determined from literature. This has been summarised in Table 14.

Table 13: Lignin- Enzymatic investigation of lignin concentrations and enzyme dosages

Purified lignin reaction with laccase and LiP (KS-S-N, KH-S-N and S-S-S)		
Lignin Concentration (g/L)	Enzyme dosages (U/L)	Enzymatic units per gram lignin (U/g)
15	1	0.067
15	5	0.333
15	10	0.667
15	20	1.333
15	30	2
Lignosulphonates reaction with laccase and LiP (NaS-S-T, NaS-M-PR and MgO-S-S) Central composite design with additional runs		
15	1	0.07
15	20	1.33
50	1	0.02
50	20	0.40
32.5 (Centre points: Triplicate)	10.5	0.32
5.75 (Additional)	32.5	0.178
10.5 (Additional)	41.25	0.255
15.25 (Additional)	32.5	0.469
10.5 (Additional)	23.75	0.442

A phosphate buffer (0.1M) at a pH of 6 was prepared for the laccase experiments and an acetic acid buffer (0.1M) at a pH of 3 was prepared for the lignin peroxidase experiments. The phosphate buffer was prepared by adding approximately 800 ml of distilled water into a volumetric flask, 3.669 g of sodium phosphate dibasic, 11.911 g of sodium phosphate monobasic. Thereafter distilled water was added until a final volume of 1 L. The pH of the buffer is measured and adjusted by the addition of hydrochloric acid or sodium hydroxide to a pH of 6. The acetic acid buffer was prepared by adding approximately 800 ml of distilled water into a volumetric flask, 396 mg of sodium acetate, and 5.715 g of acetic acid. Thereafter, distilled water was added until a final volume of 1 L. The pH of the buffer is measured and adjusted by the addition of hydrochloric acid or sodium hydroxide to a pH of 3. As the activation of the Lignin peroxidase is caused by hydrogen peroxide, it was added to the acetic acid buffer at a concentration of 0.4 mM as reported by Majeke *et al.* (2020) for the LiP enzyme.

4.2.5 All the reactions were carried out in closed Erlenmeyer flasks in shake incubators at the desired reaction temperature for a period of 24 hours, as summarised in Table 14Enzyme Activity

4.2.5.1 Laccase

The laccase activity was determined according to a method developed by Hong, Meinander and Jönsson (2002). According to this method, 0.4 mM 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonate), also known as ABTS, is dissolved as a substrate in a 100 mM sodium acetate buffer (pH 5 and at 25°C). The enzymes are diluted appropriately and mixed with the substrate solution (1 mM). The increase in the absorbance is monitored in a spectrophotometer at 420 nm for 5 minutes (using an extinction coefficient, $\varepsilon = 36\ mM^{-1}\ cm^{-1}$ to calculate the activity). The activity is then expressed in units per litre of sample.

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4.2.6 Lignin repolymerisation

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added in order to fit the data to the full surface response model. All the investigated points are also summarised in Table 13. Both experiments followed the same experimental procedure as is explained below. The reactions were carried out in two different buffers at different pH's and different reaction temperatures, owing to the enzyme's pH and temperature sensitivity, which was determined from literature. This has been summarised in Table 14.

Table 13. The flasks contained 50 ml of the reaction buffer for the respective enzymes, the lignin was added according to the desired concentration and was solubilised into the buffer. The respective enzymes were added at the desired dosage to the reaction mixture and placed in the shake incubators at 150 rpm for a period of 24 hours with sampling occurring at various stages – 0, 4, 10 and 24 hours. As samples were taken, their pH was lowered to 2 which caused the enzyme to become inactive. The samples were oven-dried at 50°C and milled for further analysis. A liquid sample was also taken directly from the reaction mixture to immediately determine the phenolic content based on the method described in Areskogh *et al.*(2010a); Gouveia (2014); Majeke *et al.*(2020).

Table 14: Optimal operating conditions of the various enzymes obtained from literature.

		Laccase (NS51003)	**LiP (Stellenbosch University)**
Optimum Conditions from literature	Temperature (°C)	40 – 60 *	30 **
	pH	3 *	2.5-4.5 **
Enzymatic Experiment Conditions	Temperature (°C)	60	30
	pH	6	3
	Reaction Buffer	Phosphate (0.1M)	Acetic Acid (0.1M)
	Experiment time (hrs)	24	24

* According to Manufacturer (Novzymes, 2003)

** According to literature (dos Santos *et al.*, 2016; Majeke *et al.*, 2020)

4.2.7 Treated Technical Lignin characterisation

The lignin characterisations were done before, during and after enzymatic treatment to determine the changes which might have occurred, owing to enzymatic modification. The following characterisation methods were used: Elemental analysis, Fourier transform infrared spectroscopy (FT-IR), Phenolic content determination, Gel permeation chromatography and Thermogravimetric analysis (TGA). The Elemental analysis, FT-IR and TGA were determined before the enzyme experiment (see Chapter 3), and for some selected conditions after 24 hours of laccase or LiP enzymatic treatment. The phenolic content and molecular weights were determined at all the sample time. The sampled points were 0, 4, 10 and 24 hours for the laccase and LiP enzymatic experiments. However, because of running out of time the molecular weights could not be determined for the 4 hours LiP-treated lignin samples.

4.3 Results and Discussion

Extra results from the enzymatic modification as well as the statistical analysis of the results are presented in Appendix A.

4.3.1 Lignin molecular weight changes during enzymatic modification

The molecular weight of the various lignins was determined before, during and after the enzymatic experiments. Table 15Molecular weight increases were observed for almost all the enzymatic experiments tested, with the exception of three lignin-enzyme combinations. It was observed that when KH-S-N was treated with LiP at low doses (0.067 and 0.85 U/g lignin), no increase in molecular weight was observed. Instead, a molecular weight decrease was observed, indicating a slight depolymerisation after the 24 hours (8 115 to 6 227 g/mol and 8 230 to 6 971 g/mol, respectively). The same was observed for the lignosulphonate MgO-S-S (Table 17) which was treated with laccase at a low dose of 0.02 U/g lignin, where no increase in molecular weight was observed (16 447 to 13 614 g/mol). This suggests that there is a minimum required enzyme dosage to induce repolymerisation in the lignin KH-S-N, as it was found that the molecular weight increases as the enzyme dosage increases (0.77-, 0.85-, 1.18-, 1.47- and 1.93-fold). While in the case of the lignosulphonate, the high lignin to enzyme ratio, combination of a 50 U/L Laccase dosage and a 1 g/L lignin dose (0.02 U/g lignin), has caused an inhibition of the enzyme (Kunamneni *et al.*, 2007). This has also been observed in a study by Pamidipati and Ahmed (2020) using lignin fragments.

Table 15, 16 and 17 provides summaries of the molecular weight changes that occurred during the 24-hour enzymatic experiment.

The highest increase in molecular weight of the purified lignins (KS-S-N, KH-S-N and S-S-S) after enzymatic modification of 24 hours, was from 1 639 to 9 315 g/mol (5.68-fold or 82% increase) for the lignin S-S-S at a dosage of 2 U/g lignin of LiP (Table 17). The highest increase in molecular weight by the enzyme laccase was a 3.79-fold increase of KH-S-N at a low 0.067 U/g lignin dosage. In the lignosulphonate enzymatic modification, the highest molecular weight increase achieved was a 4.34-fold of NaS-S-T (3 226 to 14 014 g/mol) by LiP at 0.4 U/g lignin dosage. The highest laccase molecular weight increase was 2.42-fold of NaS-S-T at 1.33 U/g lignin dosage.

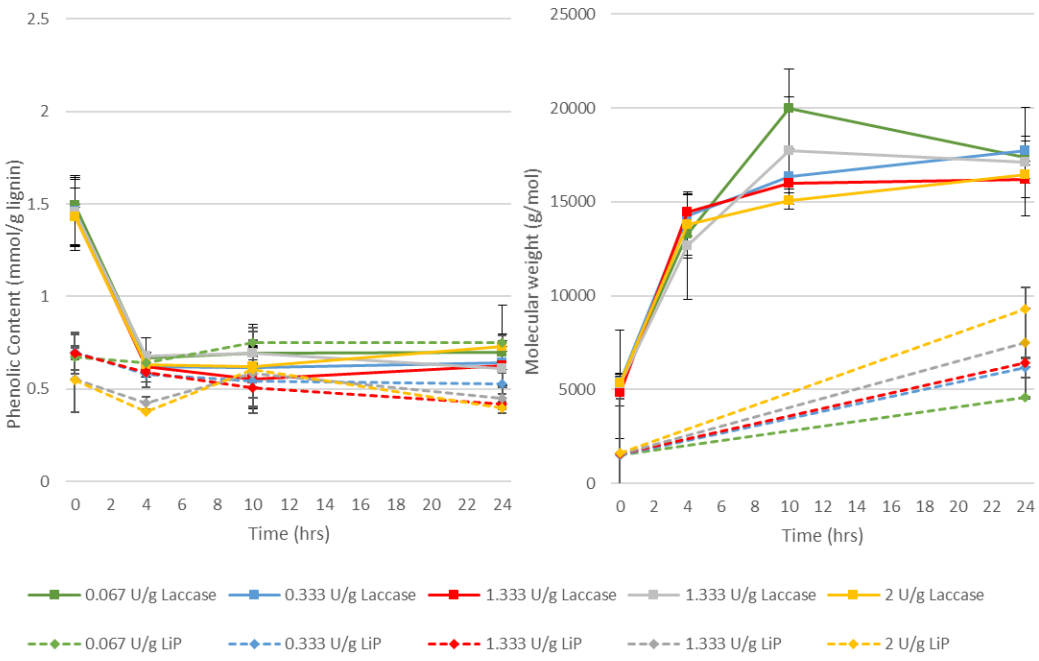

Figure 15: Molecular weight and Phenolic content analysis of S-S-S during enzymatic experiment

Few investigations into the use of enzymatic modification to repolymerise technical lignin have been done. In literature a study done on a *Eucalyptus globulus* biomass origin (hardwood) Kraft lignin and a 75% softwood-25% hardwood mixture Kraft lignin by Gouveia (2014), resulted in a 20-fold and 2-fold molecular weight increase by laccase, respectively. The results in this study where Kraft lignin was from a softwood biomass, the 1.61-fold increase of KS-S-N by laccase is comparable. However, the hardwood Kraft lignin molecular weight increase reported in Gouveia (2014) is much higher than the 3.79-fold increase of KH-S-N by laccase. Sodium lignosulphonates treated with laccase have been investigated by both Mai *et al.* (2002) and Areskogh (2011), where a 2-fold and 2.6-fold molecular weight increase was observed, respectively. In this study the sodium lignosulphonates obtained a 2.2- and 2.42-fold increase in molecular weight, which agrees with previously published literature. According to the knowledge of the author, no study has been done on technical lignin to achieve repolymerisation by LiP. However, a study by Majeke (2020) which aimed to depolymerise Soda lignin, resulted in a 1.43-fold repolymerisation of the lignin. In this study the same origin Soda lignin was investigated. However, at the conditions chosen, a 5.68-fold increase in molecular weight was observed. This is significantly higher than the results obtained in Majeke's (2020) study.

Molecular weight increases were observed for almost all the enzymatic experiments tested, with the exception of three lignin-enzyme combinations. It was observed that when KH-S-N was treated with LiP at low doses (0.067 and 0.85 U/g lignin), no increase in molecular weight was observed. Instead, a molecular weight decrease was observed, indicating a slight depolymerisation after the 24 hours (8 115 to 6 227 g/mol and 8 230 to 6 971 g/mol, respectively). The same was observed for the lignosulphonate MgO-S-S (Table 17) which was treated with laccase at a low dose of 0.02 U/g lignin, where no increase in molecular weight was observed (16 447 to 13 614 g/mol). This suggests that there is a minimum required

enzyme dosage to induce repolymerisation in the lignin KH-S-N, as it was found that the molecular weight increases as the enzyme dosage increases (0.77-, 0.85-, 1.18-, 1.47- and 1.93-fold). While in the case of the lignosulphonate, the high lignin to enzyme ratio, combination of a 50 U/L Laccase dosage and a 1 g/L lignin dose (0.02 U/g lignin), has caused an inhibition of the enzyme (Kunamneni *et al.*, 2007). This has also been observed in a study by Pamidipati and Ahmed (2020) using lignin fragments.

Table 15: Summarizing the Molecular weight fold increase after a 24 hour enzyme experiment of the various Purified lignin's

Sample ID	Molecular weight fold increase after 24hr Enzyme experiment of the Purified lignins				
	0.067 U/g	0.333 U/g	0.667 U/g	1.333 U/g	2 U/g
KS-S-N Laccase	1.58	**1.61**	1.6	1.5	1.56
KS-S-N LiP	1.21	1.11	1.24	1.29	**1.31**
KH-S-N Laccase	**3.79**	3.4	3.58	3.24	3.28
KH-S-N LiP	0.77	0.85	1.18	1.47	**1.93**
S-S-S Laccase	3.24	3.29	**3.34**	3.24	3.06
S-S-S LiP	2.99	4.04	4.14	4.73	**5.68**

When comparing the purified lignin, it was found that lignin with a low initial molecular weight performed better than a higher molecular weight lignin. KH-S-N and S-S-S are of hardwood and grass biomass origin technical lignin, respectively. They have an initial molecular weight of around 5 600 g/mol. When these lignins were treated enzymatically with laccase or LiP, their molecular weight increased after 24 hours. This was higher than KS-S-N, a lignin with a higher initial molecular weight (12 156 g/mol at 0 hrs). This can be seen in Table 15Molecular weight increases were observed for almost all the enzymatic experiments tested, with the exception of three lignin-enzyme combinations. It was observed that when KH-S-N was treated with LiP at low doses (0.067 and 0.85 U/g lignin), no increase in molecular weight was observed. Instead, a molecular weight decrease was observed, indicating a slight depolymerisation after the 24 hours (8 115 to 6 227 g/mol and 8 230 to 6 971 g/mol, respectively). The same was observed for the lignosulphonate MgO-S-S (Table 17) which was treated with laccase at a low dose of 0.02 U/g lignin, where no increase in molecular weight was observed (16 447 to 13 614 g/mol). This suggests that there is a minimum required enzyme dosage to induce repolymerisation in the lignin KH-S-N, as it was found that the molecular weight increases as the enzyme dosage increases (0.77-, 0.85-, 1.18-, 1.47- and 1.93-fold). While in the case of the lignosulphonate, the high lignin to enzyme ratio, combination of a 50 U/L Laccase dosage and a 1 g/L lignin dose (0.02 U/g lignin), has caused an inhibition of the enzyme (Kunamneni *et al.*, 2007). This has also been observed in a study by Pamidipati and Ahmed (2020) using lignin fragments.

Table 15 which indicates the molecular weight fold increases after the 24-hour enzymatic modification. The lower molecular weight lignin allows for a more efficient interaction with the enzyme, as the lignin fragments are smaller and the enzymes' active sites have better access to the substrate, also because the fragments will dissolve better (Areskogh, 2011). This is also seen in the FTIR analysis. In Chapter 3 it was

found that when comparing the purified lignin, S-S-S had the highest peak at the wavenumber assigned to the OH group (3400cm^{-1}), whereas KS-S-S showed the lowest peak.

In Figure 16 and

Figure 17 the peaks at 3 400cm^{-1} are lower for the KH-S-N and S-S-S lignin after the enzymatic modification by both enzymes, whereas in the lignin KS-S-N, a slight change is seen in the phenolic and aliphatic group after the enzymatic experiment (Figure 18). The lower molecular weight lignins respond better to the enzymatic modification as there are more available phenolic end groups for enzymes in comparison to the higher molecular weight lignins, which have a more compact structure (Areskogh, 2011; Gouveia, 2014).

Table 16: Summarizing the Molecular weight increases after a 24-hour enzyme experiment of the various purified lignin

Sample ID	Average molecular weight increases after 24-hour enzyme modification (g/mol)														
	0.067 U/g			0.333 U/g			0.667 U/g			1.333 U/g			2 U/g		
	Initial	Final	Change (%)	Initial	Final	Change (%)	Initial	Final	Change (%)	Initial	Final	Change (%)	Initial	Final	Change (%)
KS-S-N Laccase	14 324	22 561	37	13 418	21 425	37	12 691	20 336	38	12 707	19 149	34	12 673	19 833	36
KS-S-N LiP	11 439	13 852	17	11 556	12 822	10	11 743	14 527	19	11 859	15 165	22	11 685	15 344	24
KH-S-N Laccase	5 607	21 253	74	5 465	18 601	71	5 486	19 664	72	5 492	17 769	69	5 265	17 251	69
KH-S-N LiP	8 115	6 227	-30	8 230	6 971	-18	8 231	9 712	15	8 283	12 189	32	8 385	16 149	48
S-S-S Laccase	5 357	17 369	69	5 385	17 701	70	4 845	16 186	70	5 280	17 132	69	5 372	16 450	67
SSS LiP	1 539	5 990	74	1 529	6 180	75	1 558	6 453	76	1 581	7 481	79	1 639	9 315	82

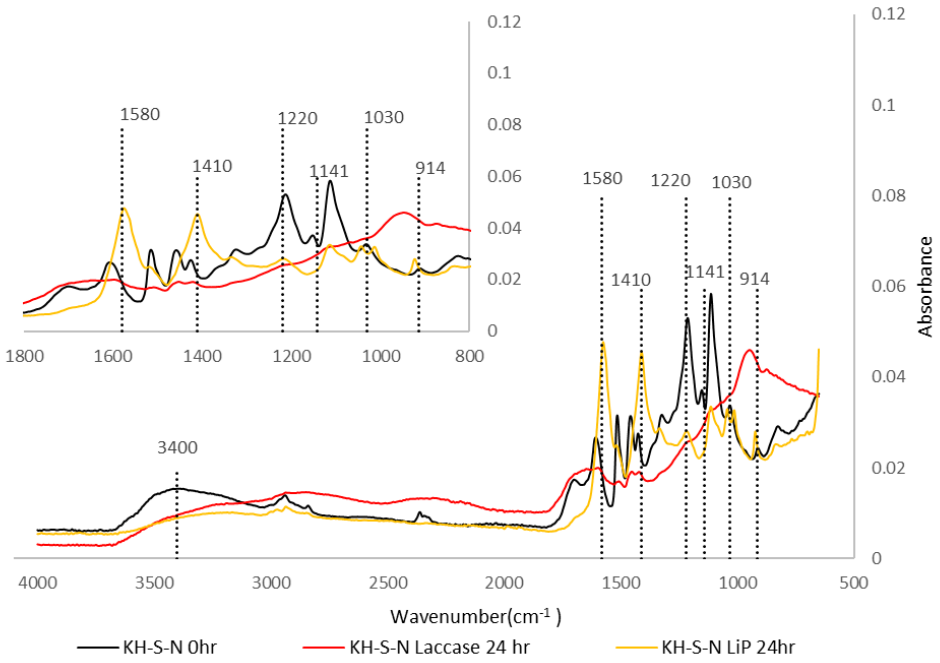

Figure 16: FT-IR Spectra of KH-S-N before and after 24-hour enzymatic experiment, insert enlarges the 'fingerprint' region of the lignin

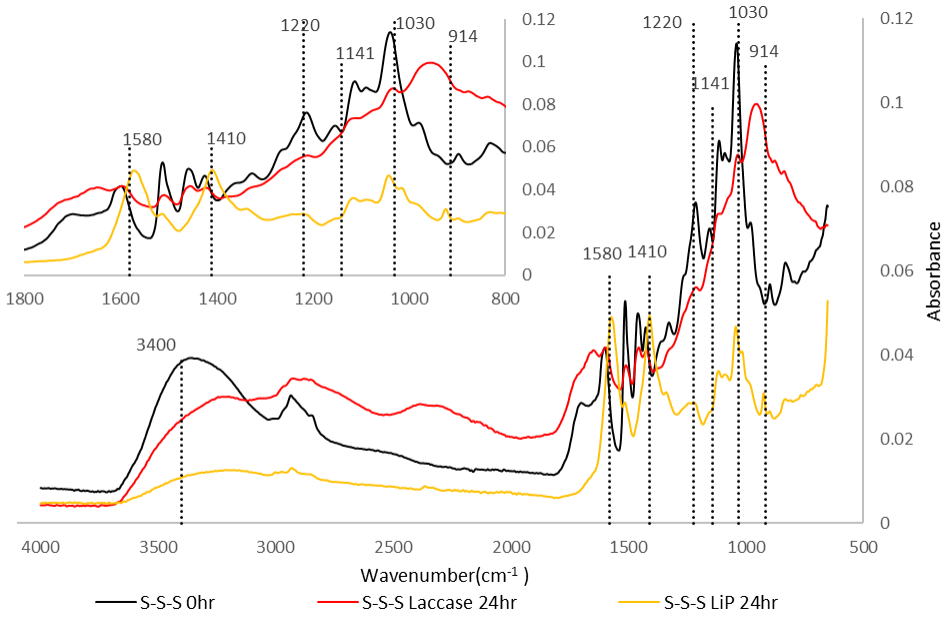

Figure 17: FT-IR Spectra of S-S-S before and after 24-hour enzymatic experiment, insert enlarges the 'fingerprint' region of the lignin

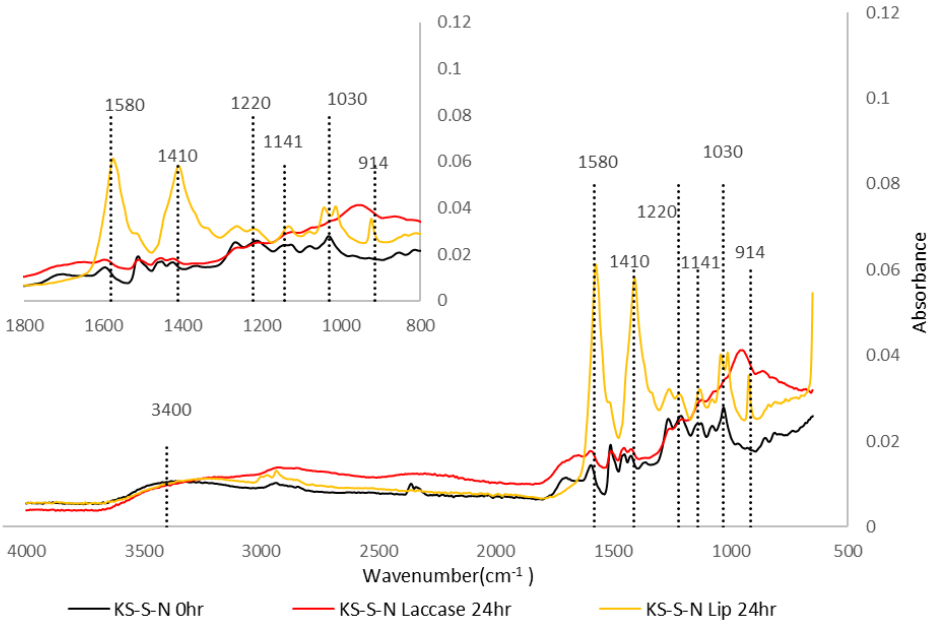

Figure 18: FT-IR Spectra of KS-S-N before and after 24-hour enzymatic experiment – insert enlarges the 'fingerprint' region of the lignin

The hardwood origin lignin, KH-S-N and grass origin S-S-S achieved the highest laccase molecular fold increase, 3.79-fold and 3.34-fold, respectively, while the softwood origin lignin, KS-S-N achieved a 1.61-fold increase. This observation agrees with data from a study where it revealed that the molecular weight of a hardwood lignin, polymerised by laccase was higher than the investigated softwood lignin (van de Pas *et al.*, 2011). In hardwoods the syringyl structure's two methoxyl substituents which are in ortho-positions to the phenolic hydroxyl group, are oxidised by laccase. These electron-donating groups stabilize the phenoxyl radicals and prevent the formation of 5-5' linkages, thus prolonging the lifespan of the radicals, causing a high concentration of radicals which are stable for longer (Cañas and Camarero, 2010; van de Pas *et al.*, 2011). As KH-S-N only has trace amounts of syringyl units (Table 1 in Chapter 2), they result in a lower molecular weight increase in comparison to the grass and hardwood origin lignin, which contain syringyl units.

When comparing the performance of two enzymes with respect to molecular weight increase capability, it was observed that laccase performed better on KS-S-N and KH-S-N in comparison to LiP. In Table 16, the initial molecular weights (0 hrs) of the same lignins differ for the two enzymes. For example, for the enzymatic modification of KS-S-N at a dosage of 0.067 U/g lignin, it was found that the initial molecular weight of the laccase and the LiP is 14 324 and 11 439 g/mol, respectively. The LiP enzymatic modifications were performed at a low pH of 3 in comparison to laccase at a pH of 6. It was observed that in the LiP experiments some of the lignin did not dissolve. There are many factors influencing the solubility of lignin, and molecular weight is one of them. At the lower pH treatments the lower molecular weight lignins' fragments were only able to solubilise, hence lower molecular weights were analysed. It has also been suggested that an enrichment of phenolic hydroxyl groups improves the solubility of the lignin (Evstigneyev and Shevchenko, 2019).

Table 17: Summarizing the molecular weight fold increase after a 24-hour enzyme experiment of the various lignosulphonates

Sample ID	Molecular weight fold increase after 24-hr experiment															
	0.02 U/g	Change (%)	0.07 U/g	Change (%)	0.178 U/g	Change (%)	0.255 U/g	Change (%)	0.32 U/g	Change (%)	0.40 U/g	Change (%)	0.442 U/g	Change (%)	0.469 U/g	Change (%)	1.33 U/g	Change (%)
NaS-S-T Laccase	1.84	46	1.54	35	1.87	47	2.21	55	1.55	35	2.02	51	1.87	47	2.15	53	**2.42**	59
NaS-S-T LiP	2.27	56	2.63	66	3.46	71	3.85	74	3.50	71	**4.34**	77	3.48	71	3.83	72	3.44	71
NaS-M-PR Laccase	1.69	41	1.28	47	1.98	50	1.72	42	1.52	34	1.66	40	**2.12**	53	1.94	48	1.70	41
NaS-M-PR LiP	**2.05**	51	1.89	47	1.89	47	1.86	45	1.88	55	2.03	51	1.89	47	1.88	47	1.89	47
MgO-S-S Laccase	*0.83*	-21	1.88	22	**2.18**	54	2.17	54	1.92	48	1.97	49	2.06	51	2.12	53	1.21	18
MgO-S-S LiP	1.54	35	1.62	38	2.09	52	2.23	55	2.22	47	2.38	58	2.41	59	2.56	61	**2.79**	64

When comparing the lignosulphonates, Table 17 summarises the lignosulphonate enzymatic modifications, where the highest molecular weight increase observed was 4.34-fold (0.4 U/g lignin) and 2.42-fold (1.33 U/g lignin) of NaS-S-T by LiP and laccase, respectively. The lowest molecular weight increase observed was 0.83-fold by MgO-S-S by laccase. Overall NaS-M-PR achieved the lowest molecular weight increases. This could partly be explained by the lignosulphonates' high impurity with an ash content of 32.5% in comparison to NaS-S-T and MgO-S-S (ash content of 22.1 and 7.3%, respectively). The molecular weight increases achieved by laccase or LiP are very similar, with the exception of NaS-S-T when treated with LiP. NaS-S-T LiP achieved the highest molecular fold increases at every dosage in comparison to the other LiP and laccase experiments. The LiP enzyme shows a definite substrate specificity to the structure of NaS-S-T.

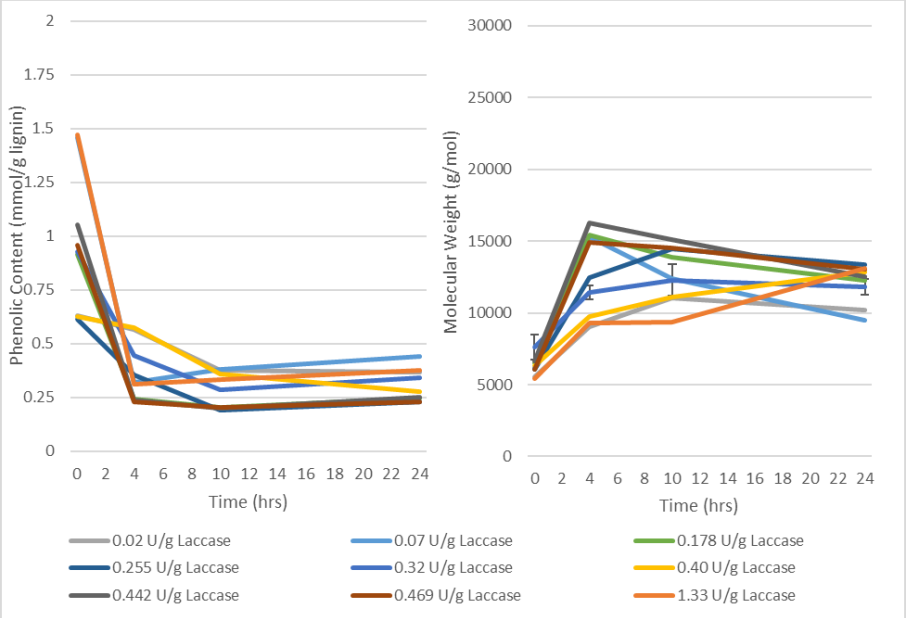

Figure 19: Molecular weight and phenolic content analysis of NaS-S-T during the laccase enzymatic experiment

As part of the statistical analysis of the results, when comparing the change in the molecular weight during the enzymatic treatment, the least significant difference tests were performed. A mean separation was performed using Fischer's least significant difference (LSD 0.05) at a 95% confidence level. The graphs which are presented in Appendix A have been summarised in Table 18. As the LiP molecular weight was not taken throughout the whole experiment, unfortunately LSD tests could not be done for the molecular weight analysis. The graphs show that the molecular weight of the lignin after the enzymatic experiment at 24 hours are significantly different to the molecular weight at the start of the enzymatic experiments for all the investigated lignin.

When comparing the laccase enzymatic modification of the lignosulphonates, there is a significant drop in the enzymatic reaction rate after 4 or 10 hours for some of the enzymatic experiments. After this time there was no significant change in the molecular weight of lignin observed. The LSD tests indicate that after 4 hours there is no significant difference between the molecular weights recorded at 4, 10 and 24

hours (an example is shown in Figure 20). This can partly be explained by the macromolecular structure of the lignosulphonates. Many models of the macromolecular structure have been proposed such as the micelle-like structure, which suggests that the lignosulphonates in solution form a hydrophilic surface and a hydrophobic core (Rezanowich and Goring, 1960). Owing to steric constraints, the phenolic end groups located on the surface of the micelle can only be accessed by the enzyme. Thus, it is possible that when the available phenolic end groups are oxidised, the molecular weight increase levels out, as the remaining groups are inside the micelle and they are not accessible (Areskogh, 2011).

The lignin's of KH-S-N, NaS-S-T and MgO-S-S all reached their maximum molecular weight increase after 4 hours of being treated with laccase. These lignin's have a biomass origin, *Eucalyptus grandis* (hardwood). The softwood origin lignin KS-S-N reached a maximum at 24 hours and had not reached completion. As the structure of softwood lignin is more condensed, this influences the ability of the enzyme to access possible active sites to cause radical formation. Also, if radicals are formed they may not be able to couple together, because of the condensed structure (Kunamneni *et al.*, 2007).

Table 18: Summary from LSD tests

Sample ID	Time taken to reach maximum molecular weight (hr)	Observed change in molecular weight after maximum is reached
KS-S-N Laccase	24	-
KH-S-N Laccase	4	Slightly Increased
S-S-S Laccase	10	Constant
NaS-S-T Laccase	4	Constant
NaS-M-PR Laccase	10	Decreased
MgO-S-S Laccase	4	Decreased

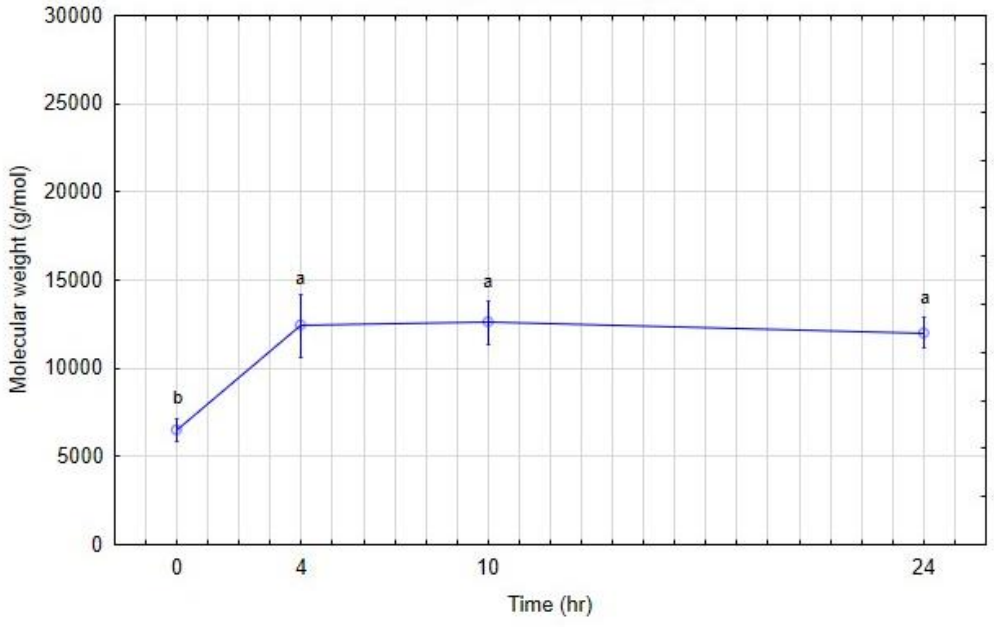

Figure 20: The LSD test results of NaS-S-T treated with Laccase

4.3.2 Phenolic content changes during enzymatic modification

The free phenolic content decreases which were determined during the enzymatic modification, are summarized in Table 19 and Table 20.

All the lignin's treated with laccase achieved a significant decrease in their free phenolic content. As mentioned in the literature study, there is a relationship between the decrease in the free phenolic content and the increase in the molecular weight of the lignin during the enzymatic treatment. Over time the phenolic content of the lignin decreases as the laccase enzyme oxidises the phenols to produce radicals, which are able to repolymerise through a radical coupling process, creating longer chains and thereby increasing the average molecular weight of the lignin (Areskogh, 2011). The results of this showed that the molecular weights of the lignins did increase while the free phenolic content decreased when treated with laccase. However, it is not a linear relationship. For example, when MgO-S-S was treated with laccase, it achieved the largest decrease in the free phenolic content (80.4% at 1.33 U/g lignin) in comparison to the other lignins. However, this did not necessarily correspond with the highest molecular weight increase (1.21-fold at 1.33 U/g lignin). This means that the relationship will not apply to the lignins that reacted with LiP, as this enzyme is able to oxidise the phenolic and non-phenolic groups of lignin.

The lignins enzymatically modified by LiP showed decreases in their phenolic content. However, some lignin's treated at lower enzyme dosages showed increases in their phenolic content after 24 hours. These include the lignosulphonates, MgO-S-S (0.02 U/g lignin) and NaS-M-PR (0.178 U/g lignin), as well as all the purified lignin's, which showed an increase in the phenolic content at a LiP dosage of 0.067 U/g (S-S-S, KH-S-N and KS-S-N). This indicates that at low enzyme to lignin concentrations, repolymerisation of the phenolic end groups does not occur, but rather depolymerisation. The breakdown of the radicals formed occurs as there is fewer radical-radical interactions at the lower dosages. However, MgO-S-S, NaS-M-PR, S-S-S and KS-S-N molecular weights all increased at the above-mentioned dosages (1.54-, 1.89-, 2.99- and 1.21-fold, respectively). This indicates that repolymerisation must have occurred through the oxidation of the non-phenolic groups of the lignin by LiP to cause an increase in the lignins' molecular weight. LiP has shown the ability to cause repolymerisation and depolymerisation. It has been suggested that the repolymerisation is more dominant, as the radicals which are formed by LiP are not removed. Therefore, the formed radicals are susceptible to repolymerise (Álvarez *et al.*, 2011).

Table 19: Summarizing the phenolic content decreases after a 24-hour enzyme experiment of the various Purified lignin

Sample ID	Phenolic content decrease after 24hr Enzyme experiment				
	0.067 U/g	0.333 U/g	0.667 U/g	1.333 U/g	2 U/g
KS-SN Laccase	36.4%	**44.3%**	41.2%	42%	42.5%
KS-S-N LiP	*-9.64%*	9.59%	7.14%	**12.65%**	*-27.9%*
KH-S-N Laccase	21.9%	24.35%	**25.14%**	24.84%	23.43%
KH-S-N LiP	*-14.24%*	13.29%	14.08%	**18.35%**	14.56%
S-S-S Laccase	52.99%	56.06%	56.79%	**58.06%**	48.98%
S-S-S LiP	*-11.9%*	24.28%	**39.23%**	18.50%	27.58%

Table 20: Summarizing the Molecular weight fold increase and the phenolic content decreases after a 24-hour enzyme experiment of the various lignosulphonates

Sample ID	Phenolic content decrease after 24hr experiment								
	0.02 U/g	0.07 U/g	0.178 U/g	0.255 U/g	0.32 U/g	0.40 U/g	0.442 U/g	0.469 U/g	1.33 U/g
NaS-S-T Laccase	41.7%	69.8%	73.2%	62.4%	63.1%	55.5%	**76.1%**	75.9%	74.5%
NaS-S-T LiP	14.4%	42.6%	52.0%	54.2%	50.8%	47.9%	53.9%	51.7%	50.4%
NaS-M-PR Laccase	66.7%	77.6%	77.1%	69.3%	77.4%	72.5%	74.8%	**77.8%**	77.7%
NaS-M-PR LiP	8.3%	6.1%	*-28.8%*	**14.9%**	**14.9%**	8.3%	12.5%	14.7%	1.1%
MgO-S-S Laccase	3.3%	63.2%	58.3%	48.8%	45.6%	27.7%	72.8%	76.3%	**80.4%**
MgO-S-S LiP	*-1.2%*	5.9%	13.9%	*-1.5%*	8.8%	18.9%	24%	18.0%	**44.5%**

The highest phenolic content decrease was 80.4 % of MgO-S-S, caused by laccase treatment at the highest dosage of 1.33 U/g lignin. The highest phenolic content decrease by LiP was 44.5% of MgO-S-S at the highest dosage of 1.33 U/g lignin. This corresponds with the results from Chapter 3, where it was observed that MgO-S-S had the highest peak in the phenolic hydroxyl groups in the FTIR analysis.

As the enzyme LiP is able to repolymerise, the non-phenolic groups and phenolic end groups explain why the highest molecular weight fold increases were obtained by LiP, but low changes in phenolic content were observed in comparison to the laccase-treated lignins.

4.3.3 Structural and thermal property changes

The FTIR analysis of the purified lignins and the lignosulphonates before and after the enzymatic modifications revealed only minor differences, such as the reduction in the phenolic and aliphatic groups (3 400cm^{-1}), which suggest that no structural changes occurred, except the increase in molecular weight increases.

The changes in the thermal stability of the lignin were observed before and after the enzymatic treatments. Slight degradation temperature changes were observed for the laccase-treated lignins, whereas the LiP-treated lignin showed larger differences. This has been summarised in Table 21. It was speculated that an increase in the molecular weight of a lignin will cause an increase in the thermal degradation temperature (Majeke *et al.*, 2020). Also studies where a more polymerised structure was observed, showed a more thermally stable structure with a defined peak (Abdelaziz and Hulteberg, 2017). This was only seen in the LiP results where the thermal degradation temperatures increased as well as the molecular weights of the lignins. The same conclusion was reached by Álvarez *et al.* (2011), where fibre lignin was treated with laccase.

The thermal degradation results of laccase-treated lignins did not correspond to the results shown in literature. Low thermal degradation temperatures were observed around 170°C. Interestingly, the final degradation temperature of NaS-S-T laccase was slightly lower than the initial molecular weight.

Table 21: The thermal degradation temperature changes over a 24-hour enzymatic treatment.

Sample ID	**Thermal degradation temperature changes over the 24 hour enzymatic treatment.**	
	Initial (°C)	Final (°C)
KS-SN Laccase	170	180
KS-S-N LiP	320	400
KH-S-N Laccase	175	175,532
KH-S-N LiP	310	450
S-S-S Laccase	160	175,400
S-S-S LiP	300,465	300,465
NaS-S-T Laccase	450	430
NaS-S-T LiP	200	300
NaS-M-PR Laccase	197	268
NaS-M-PR LiP	438	445
MgO-S-S Laccase	150	175
MgO-S-S LiP	171	250

4.3.4 Optimal conditions

The results from the standardised effects and the overall optimum conditions are summarised in Table 22, where additional and more detailed data such as the surface response curves can be seen in Appendix A. It can be concluded that the enzyme dosage used in the enzymatic experiment has a significant effect on both the molecular weight and the phenolic content outcome. Areskogh (2011) found that the change in the phenolic content was unaffected by the lignin concentration. However, in this study the lignin concentration was only found to be significant when determining the phenolic content in the NaS-M-PR and LiP enzymatic experiment. The overall optimum values were determined where the highest increase in the molecular weight was observed and then the phenolic content overall optimums were obtained where the maximum minimization of the phenolic content was observed. The lignosulphonates overall optimum values obtained for the two are almost identical in terms of enzyme dosage with slight differences in lignin loading.

Table 22: The standardized effects and the overall optimum values to maximize the molecular weight or minimize the phenolic content (PC) for the lignosulphonates

		MgO-S-S				NaS-S-T				NaS-M-PR			
		Laccase		LiP		Laccase		LiP		Laccase		LiP	
Standardized Effect: Significant variable	M_w	Enzyme		Enzyme		Enzyme dosage		Enzyme Enzyme X Lignin		None		Lignin X Enzyme	
	PC	Enzyme		Lignin X Enzyme		None		Lignin X Enzyme		Enzyme		Lignin	
Dosage		(g/L)	(U/L)	(g/L)	(U/L)	(g/L)	(U/L)	(g/L)	(U/L)	(g/L)	(U/L)	(g/l)	(U/L)
Overall Optimum	M_w	32.5	20	15	20	32.5	10.5	32.5	15.5	32.5	10.5	32.5	20
		0.62 U/g		1.33 U/g		0.32 U/g		0.48 U/g		0.32 U/g		0.62 U/g	
	PC	32.5	20	50	20	32.5	10.5	50	15.5	50	15.25	50	20
		0.62 U/g		0.4 U/g		0.32 U/g		0.31 U/g		0.31 U/g		0.4 U/g	

Table 23: The optimum operating conditions for the purified lignins

Sample ID	Optimum operating conditions		
	Enzyme dosage (u/L)	Lignin concentration (g/L)	Units per gram lignin (U/g)
KS-S-N Laccase	5	15	0.33
KS-S-N LiP	30	15	2
KH-S-N Laccase	1	15	0.067
KH-S-N LiP	30	15	2
S-S-S Laccase	10	15	0.667
S-S-S LiP	30	15	2

CHAPTER 5: TECHNO-ECONOMIC EVALUATION OF ENZYMATIC LIGNIN EXPERIMENT TO INCREASE MOLECULAR WEIGHT

5.1 Introduction

In order to determine if enzymatic modification of technical lignin is a potential process route, a techno-economic study is required to determine financial feasibility of the processes. In Chapter 4 it was found that utilizing the enzymes, laccase and LiP are technically feasible, in order to increase the molecular weight of technical lignin. As LiP is not yet available for commercial application, the techno-economic evaluation will utilise the laccase enzyme for the enzymatic modification. Although the LiP produced the highest molecular weight-fold increases, the laccase enzyme did produce more consistent results. It is suggested that these upgraded lignins can be applied to high-value applications such as carbon fibres, polymer blends, adsorbents and hydrogels (Ludmila *et al.*, 2015). In this chapter a techno-economic model was developed to determine the feasibility of producing a higher molecular weight lignin sourced from six South African mills by laccase enzymatic modification.

5.2 Literature

The upgrading of technical lignin is not a new concept, however, upgrading these lignins to increase their molecular weight is. This study and previous studies have focused on experimental, laboratory scale testing of these technical lignins (Areskogh *et al.*, 2010a; Gouveia *et al.*, 2012; Kalliola *et al.*, 2014), where the novelty of the study setup is the addition of the enzyme LiP to repolymerise these lignins, and the varying of the enzyme dosage and a techno-economic analysis for the modification of technical lignin in the South African environment. Furthermore, there is currently no industrial application of utilizing enzymes to increase the molecular weights of technical lignin. Hence, based on the performances demonstrated under the laboratory scale conditions and assuming that these can be replicated during the scale-up to an industrial practice, one needs to assess whether the isolation and enzymatic modification of technical lignin to a higher molecular weight macromolecule, is economically attractive as a potential technology for industrial application.

Two different proposed plants will be designed according to the steps of the laboratory-scale experiments. The first proposed plant will be for the Kraft and soda lignin, as they need to be isolated from the liquor, and the second plant will be for the lignosulphonates which will not be isolated. Isolating the lignin removes significant inorganics attached to the lignin, resulting in a higher purity lignin. Higher purity lignins are required for higher value applications (Smolarski, 2012). The proposed process of enzymatic modification will occur in bioreactors to upgrade the lignin, where steps will be included to solubilise the Kraft and soda lignin for the enzyme to access the substrate (West *et al.*, 2014). The treated lignin will be dried and stored (Gouveia, 2014).

5.3 Methods

5.3.1 Techno-economic analysis steps

The following process steps are followed when conducting a techno-economic analysis and they have been summarised in

Figure 21 in order to construct a process flow diagram. The study's experimental procedure and the literature associated with the proposed process need to be reviewed. The process will follow the steps of the laboratory scale experiments: lignin isolation, purification, enzymatic experiment and product drying. Once the process diagram is constructed, a mass balance will be determined such that the process equipment and utility consumption can be designed and calculated. The capital and operating cost of the proposed process, referred to as CAPEX and OPEX, will then be determined. Financial indicators and profitability analysis such as the total capital investment (TCI), net present value (NPV), and the minimum selling prices (MSPs) of the proposed processes will be determined.

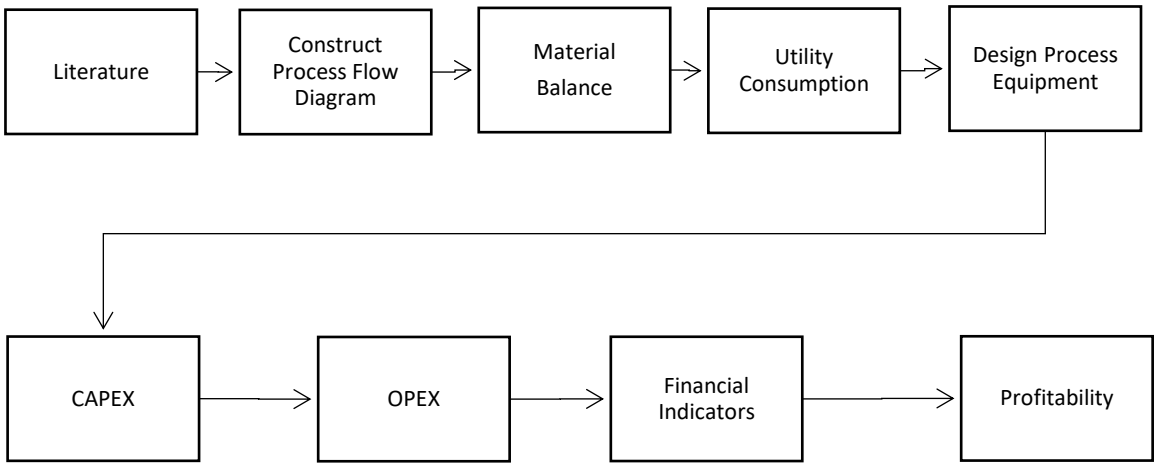

Figure 21: Techno-economic Analysis Process Steps

5.3.2 Process Development

5.3.2.1 Process Scenarios

There are no existing plants in the South African industry to enzymatically upgrade technical lignin from pulping mill liquor sources. Two processes are proposed, the first for the experiment of black liquor from the Kraft and soda pulping processes and the second for the experiment of red liquor from the sulphite pulping process.

The following scenarios for Kraft/Soda process will be investigated:

- Scenario 1: Kraft Black liquor, *Pinus patula* origin (KS-S-N) from Sappi, Ngodwana
- Scenario 2: Kraft Black liquor, *Eucalyptus grandis* origin (KH-S-N) from Sappi, Ngodwana
- Scenario 3: Soda Liquor, Sugarcane Bagasse origin (S-S-S) from Sappi, Stanger

The following scenarios for Sulphite process will be investigated:

- Scenario 4: Sodium sulphite liquor, *Eucalyptus grandis* origin (NaS-S-T) from Sappi, Tugela
- Scenario 5: Sodium sulphite liquor, Mixed origin (NaS-M-Pr) from Mpact, Piet Retief
- Scenario 6: Magnesium liquor, *Eucalyptus grandis* origin (MgO-S-S) from Sappi, Saiccor

5.3.2.2 Process Description and Flow Diagrams

The simulated processes include the preparation of liquor from the mill by drying or the isolation of the lignin, the enzymatic treatment of the lignin, and the post processing. Currently there are no industrial enzymatic modification processes in production to increase the molecular weight of these industrial lignins. The process flow diagrams were developed by modifying the current lignin extraction process and by scaling up the enzymatic reactions done in the study (Chapter 4). Two different processes were developed, one for the Kraft and soda lignin, and another for the lignosulphonates. The process flow diagrams are shown in Figure 22 and Figure 23. Both are batch processes.

In the Kraft and soda lignin a proposed plant was designed from scaling up the experimental work in Chapter 4: The black liquor stream from the paper mills storage tanks will be pumped (P-100A/B) into a mixer (M-101) where water and sulphuric acid will be added (P-101A/B and P102A/B, respectively) and the blend will be mixed for approximately 24 hours. This forms part of the precipitation and isolation step of the lignin and is based on the experimental work done in Chapter 3.2.3. The mixture is pumped (P-103A/B) to an industrial centrifuge (C-100) where the supernatant will be removed, the solids washed and centrifuged again and transported by a conveyor (CV-100A/B) to the reaction vessel (RX-100). The buffer preparation for the enzymatic reaction (Chapter 4.2.4) starts with sodium phosphate dibasic and sodium phosphate monobasic that are transported by conveyor (CV-101A/B and CV-102A/B, respectively), water is pumped (P-104A/B) to be mixed, and then pumped (P-105A.B) to the reaction vessel. As for this study, the solubility of lignin was overcome by initially dissolving the lignin in sodium hydroxide (Chapter 4.2.4), the same is proposed for the plant. Sodium hydroxide will be pumped (P-106A/B) to the reaction vessel to initially dissolve the lignin and then the reaction buffer will be added. The addition of the commercial enzyme laccase will start the enzymatic modification experiment, where reaction will occur for a period of 24 hours under stirred conditions (Chapter 4.2.4). Once the reaction is completed, the pH of the mixture will be adjusted to denature the enzyme. The lignin mixture is pumped (P-108A/B) from the reaction vessel to a dryer (D-100) where the lignin will be dried at 50°C. The lignin is dried in a pan dryer and can be milled into a powder to be sold. Using the pan dryer, corresponds to the drying method of ovens used in Chapter 4.2.4.

The plant proposed for the lignosulphonates as shown in Figure 23, was designed from scaling up the experimental work in Chapter 4: The liquor from the pulping mills storage tanks is pumped (P-100A/B) to

the reaction vessel (RX-100). The buffer preparation will be identical to the Kraft and soda proposed plant, in that sodium phosphate dibasic, sodium phosphate monobasic and water is mixed in M-100 and then pumped (P-102A/B) to the reaction vessel (Chapter 4.2.4). The commercial enzyme laccase is added, and the reaction will occur for a period of 24 hours under stirred conditions. Once the reaction is completed, the pH of the mixture will be adjusted to denature the enzyme. The lignin mixture is pumped (P-104A/B) to be dried in a pan dryer (D-101) at 50°C. Thereafter the lignin can be milled into a powder to be sold. The optimized process conditions from the results in Chapter 4.3.4 are summarized in Table 24.

Table 24: The Optimized Process Conditions for each scenario to be tested

Scenario	Lignin Concentration (g/L)	Enzyme Dosage (U/L)	Temperature (°C)	Reaction pH	M_w Change (g/mol)
Scenario 1 (KS-S-N)	15	5	60	6	13418-21425
Scenario 2 (KH-S-N)	15	1	60	6	5607-21253
Scenario 3 (S-S-S)	15	10	60	6	4845-16186
Scenario 4 (NaS-S-T)	32.5	10.5	60	6	7625-11806
Scenario 5 (NaS-M-PR)	32.5	10.5	60	6	6583-10033
Scenario 6 (MgO-S-S)	32.5	20	60	6	6473-16557

5.3.2.3 Process Assumptions

As information regarding the production volumes of excess liquor, or liquor that is discarded by being pumped into the sea from the various plants are not reported, a confidential and alternative approach was used to design the plant.

The plants were designed with the installation of the largest available jacketed bioreactor with a volume of 30m^3 (Turton *et al.*, 2009), hence, a production capacity of 30m^3 in the bioreactor per day. This production capacity is small in relation to the volumes of black liquor being produced, however, as an initial investigation to compare the various lignins. This will be a batch process in the study where the reaction duration is 24 hours (Chapter 4.2.4). Including the shutdown, cleanup and start-up, the total duration of the enzymatic modification is 26 hours.

Various key assumptions were made and have been summarized below:

- Steady state operations;
- The loss owing to transport, handling or raw materials is negligible;
- The drier efficiency is at 80% (Turton *et al.*, 2016);
- All the lignin dissolves in the reaction vessel;
- The recovery rate of lignin from the isolation is 60% (Govender, 2020);
- The lab conditions can be replicated during scale-up to the industrial process.

The percentage solids and density of the liquor received from the various mills were assumed from literature and have been summarized in Table 24.

Table 25: Pulping mill sources solids percentage and density of liquor obtained from literature

Sample ID	Percentage solids (%)	Density (kg/m^3)	Reference
KS-S-N	42	1300	(Cardoso, de Oliveira and Passos, 2009; Nikolskaya *et al.*, 2019)
KH-S-N	39	1251	(Andreuccetti, Leite and D'Angelo, 2011)
S-S-S	30	1100	(Mamaye *et al.*, no date; Azadi *et al.*, 2013)
NaS-S-T	50	1380	(Nicholas and Cheremisinoff, 2010; Polchem, 2020)
NaS-M-PR	32	1035	(Joachimiak, Wojech and Wójciak, 2019)
MgO-S-S	56	1480	(Marques *et al.*, 2009)

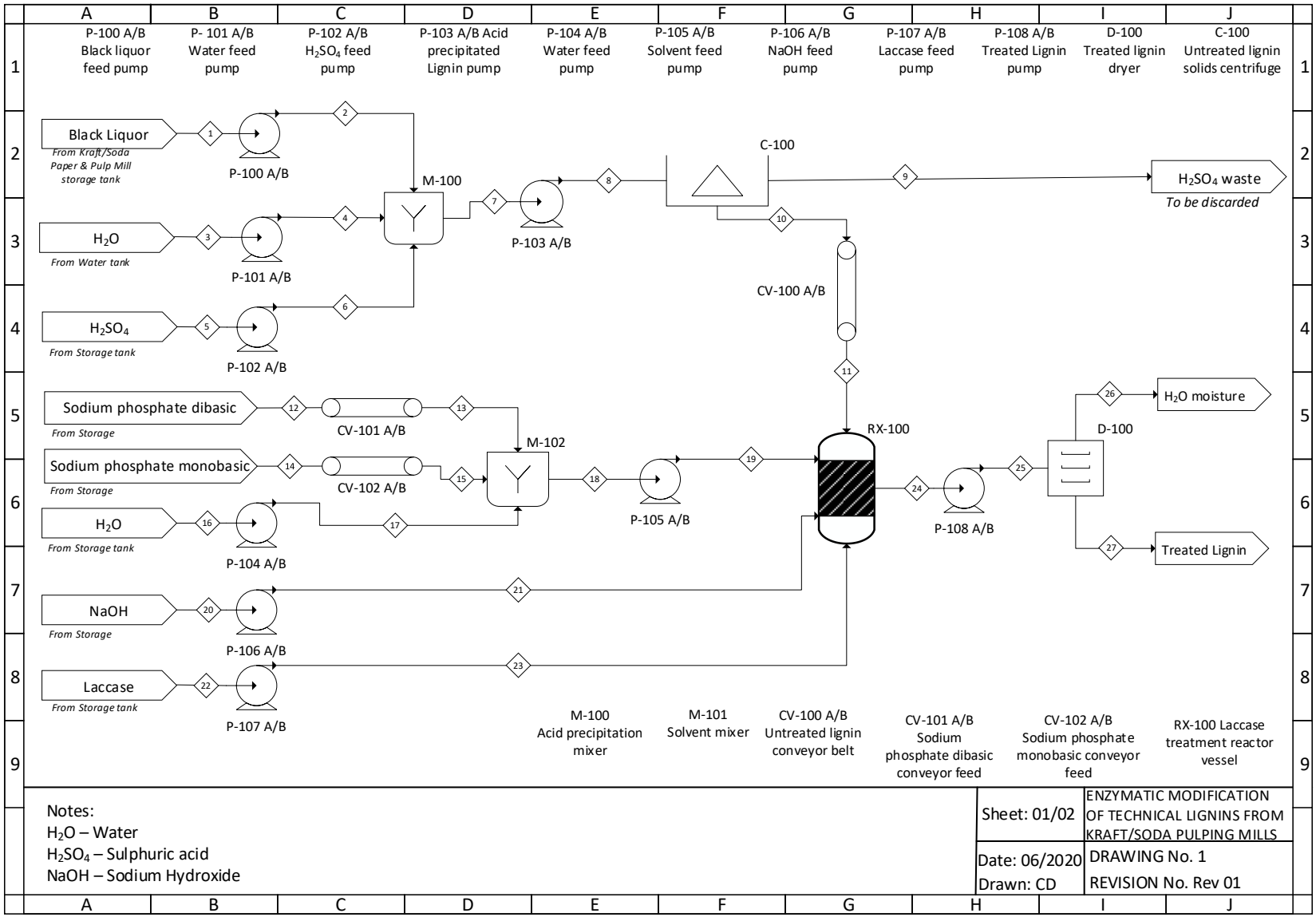

Figure 22: Process flow diagram for the enzymatic modification of Kraft or Soda Lignin from the black liquor

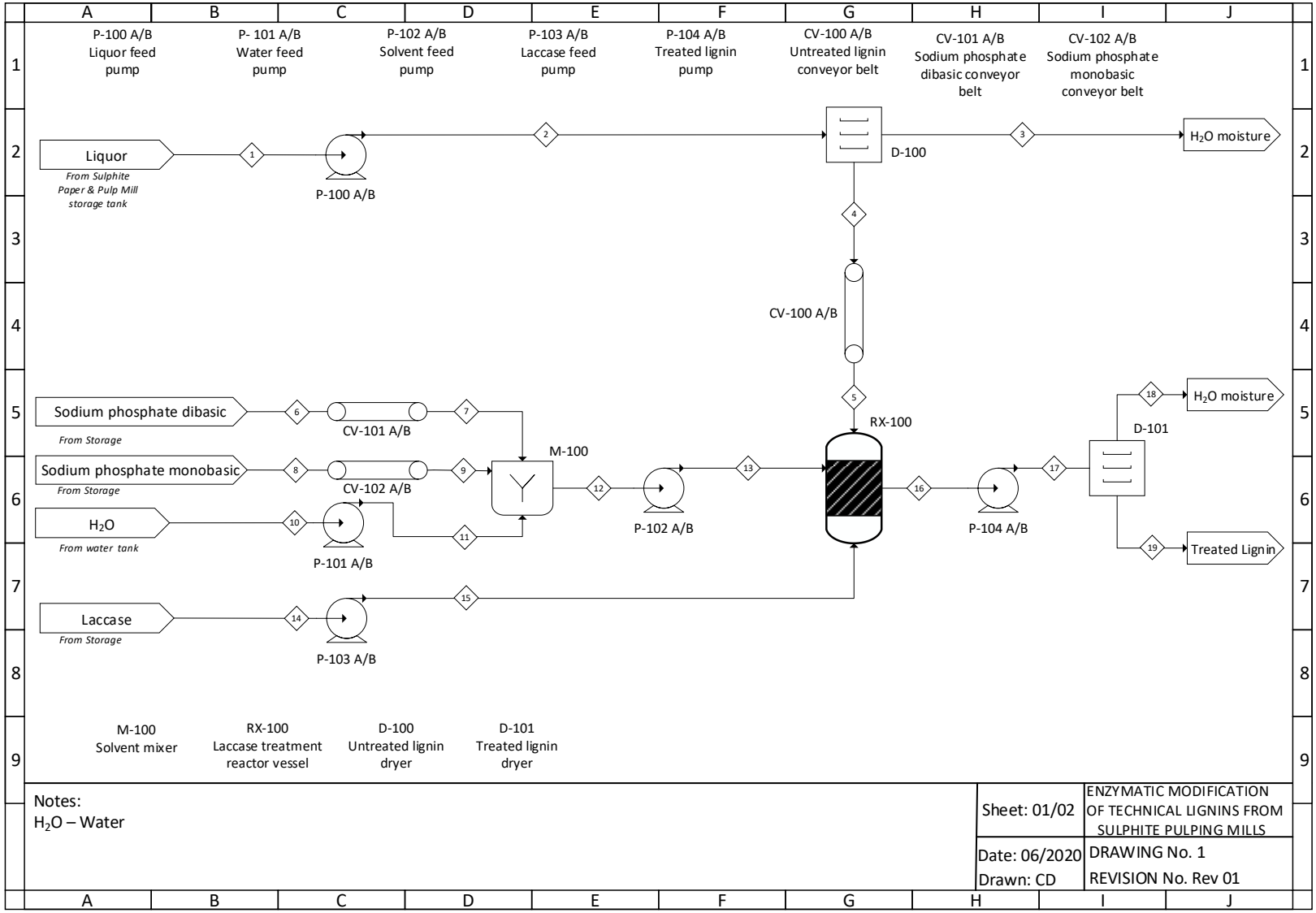

Figure 23: Process flow diagram for the enzymatic modification of lignosulphonates from the spent liquor

5.3.3 Economic Evaluation

5.3.3.1 Capital Cost Estimation

The Capital Cost estimation (CAPEX) was gauged from methods described in Sinnott (2005) and Turton *et al.* (2016). It is assumed that the capital investment, which is required to build the facility and the utility supply, has been made (Turton *et al.*, 2016). The total of all the installed purchased costs is referred to as the new product production cost (NPP).

The total direct costs (TDC) consist of the NPP, the warehouse cost (4% NPP), site development (9% NPP) and additional piping costs (4.5% NPP), which are calculated relative to the NPP. The total indirect costs consist of the field expenses (10% TDC), office construction (20% TDC), contingency (10% TDC) and other costs (10% TDC). The total capital investment (TCI) is determined by adding the fixed capital investment (FCI), which is the combination of the total direct and indirect costs with the working capital (5% FCI).

To determine the NPP, the process equipment purchased costs and the installation costs were calculated. The process equipment purchased costs were determined by sizing the equipment from literature heuristics, and the costs determined from the size parameters according to Equation 4 (Sinnott, 2005).

Equation 4: $$C_e = CS^n$$

Where

C_e is the calculated purchased equipment cost, $ (Price for 2004)
S is the characteristic capacity or size parameter [Units displayed in Table 6.2 in Sinnot (2005)]
C is the cost constant [Table 6.2 in Sinnot (2005) on page 263]
n is the index number for the type of equipment

The heuristics of the pumps were determined and the cost estimation made according to Equation 5 from Turton *et al.* (2016).

Equation 5: $$\log_{10} C_p^o = K_1 + K_2 \log_{10}(A) + K_3[\log_{10}(A)]^2$$

Where

C_p^o is the purchased cost of equipment (2001)
A is the capacity of size parameter of the equipment
K_1, K_2, K_3 values are determined from Table A.1 in Turton et al. (2006) on page 953

As the equipment was determined based on historical cost data, factors such as inflation need to be considered. This was calculated according to Sinnot (2005) as shown in Equation 6.

Equation 6: $$Current\ year\ cost = Previous\ year\ cost \times \frac{CEPCI_{current\ year}}{CEPCI_{previous\ year}}$$

The installation costs were determined according to Humbird *et al.* (2011), where the equipment purchased cost was multiplied by an installation factor.

5.3.3.2 Operation Cost Estimation

The operating cost estimation (OPEX) includes the manufacturing costs, operating costs and general expenses. The plant was envisaged to operate for 8 000 hours in a year as was described by Chimphango (2020) for a valorization of water paper sludge.

5.3.3.2.1 Raw Materials

The determination of the cost of the raw materials was based on the required quantity for the plant's production for a year and the cost of the raw materials. The costs of the various raw materials were determined from literature and is summarized in Table 26. Equation 7 summarizes the formula used to determine the cost of raw materials.

Equation 7:

$$Raw\ material\ cost = Flowrate\ \left(\frac{kg}{hr}\right) \times operation\ time\ (hr) \times material\ cost\left(\frac{R}{kg}\right)$$

Table 26: Proposed plants raw materials unit costs

Raw Material	Cost	Reference
Sodium phosphate dibasic	R236/kg	(Noah Technologies, 2020a)
Sodium phosphate monobasic	R145/kg	(Noah Technologies, 2020b)
Sodium hydroxide	R46/kg	(KCatz, 2020)
Sulphuric Acid Industrial Grade	R18/l	(Reflecta, 2020)
Laccase	R1000/l	(Merck, 2020)

5.3.3.2.2 Utility Costs

The utility costs are summarized in Table 27.

Table 27: Utility Costs Summary

Utility	Cost	Reference
Electricity	R0.84/kWh	(Chimphango, 2020)
Water	R16.96/ton	(Chimphango, 2020)

5.3.3.2.3 Labour Costs

In order to determine the labour costs, the staff requirements for the proposed plant needed to be determined. The quantities of staff are based on other production processes (Chimphango, 2020) and through Turton *et al.* (2016).

The shift operators were determined according to Equation 8 (Turton *et al.*, 2016).

Equation 8: $$N_{OL} = \left(6.25 + 31.7(P^2) + 0.23N_{np}\right)^{0.5}$$

Where

N_{OL} *is the number of operators per shift*
P is the processing steps involved in handling of particle solids
N_{np} *is the number of non-particulate steps*

The salary estimations were based on the average South Africa salary value sourced from Pay Scale South Africa.

Position	Required Quantity	Total Salary/year
Plant Manager	1	R620 415
Plant Engineer	1	R717 491
Maintenance Supervisor	1	R273 035
Maintenance Technician	2	R351 540
Laboratory Manager	1	R392 933
Laboratory Analyst	1	R125 765
Shift Supervisors	2	R758 542
Shift Operators	12	R2 186 304
Administrator	1	R540 489
Accountant	1	R277 859
Secretary	1	R129 248
Total Salaries	24	R6 373 621

The number of operators are six per shift for both designed plants. The operators will be split into two groups where six will operate in a 12-hour shift ever alternative day.

5.3.3.2.4 Other Expenses

The other expenses include the maintenance costs (3% NPP) and property tax (0.7% FCI) – these relations were obtained from Chimphango (2020).

5.3.4 Market Research

There are many lignin applications available that are run at a high volume and a resulting low cost, however, to find a higher value application for lignin, modifications are required (Bangalore Ashok *et al.*, 2018). In the pulping process, there are excess lignosulphonates produced, some of which are discarded or stored and mainly burnt in the recovery boilers a select few produce and sell their lignosulphonates to

various markets. If a high-value application can be determined for a modified lignin with a process plant which is economically feasible, it would be beneficial to the pulping industry (Vishtal and Kraslawski, 2011).Especially since applying the lignin for alternative uses should cover the cost for an alternative fuel source to replace the lignin that are presently going to the recovery boilers. This alternative fuel source could be forest residues or excess biomass such as sawdust from nearby sawmills.

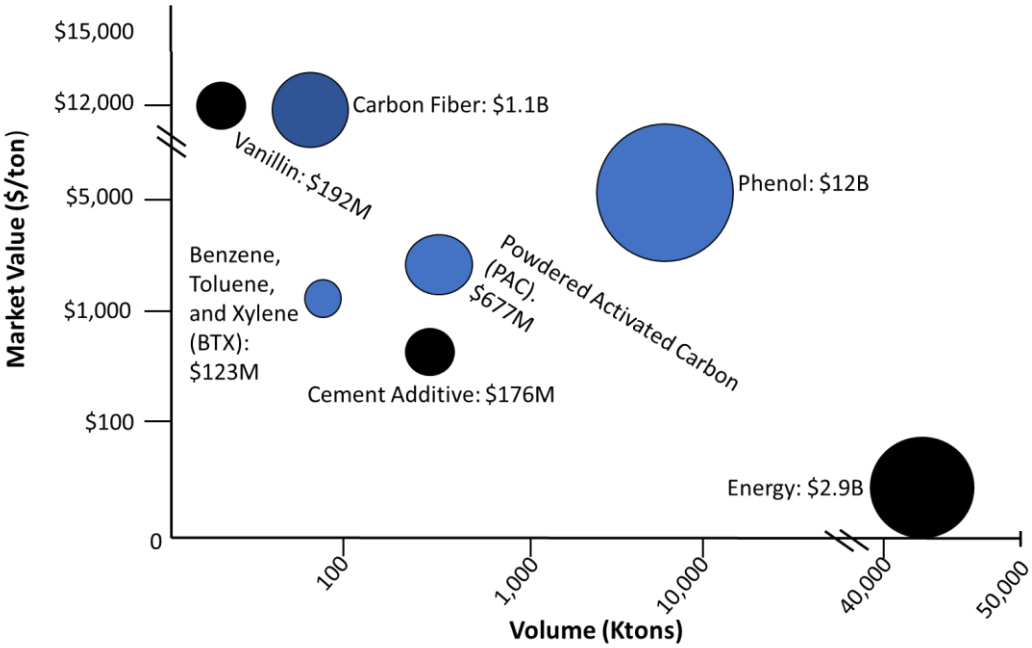

Figure 24: Depiction of the lignin products based on cost and volume redrawn from Cline and Smith (2017)

As lignin is a renewable resource and a rich source of phenols, there are many applications where lignin can replace existing non-renewable sources (Gouveia, 2014). Figure 24 indicates the various applications in terms of their market value. An interesting application where a higher molecular weight lignin has shown promise, is where lignin is used for the production of carbon fibre (Wells, 2015). As some of the current possible applications require further processing or development, the minimum required selling price will be determined for the six scenarios.

5.3.5 Cash Flow sheet Assumptions

In the determination of the NPV and other indicators of economic profitability, the following assumptions were made and are summarised in Table 28. The assumptions are determined from an economic analysis done by Chimphango (2020). The plant will operate for 8 000 hours a year to include time allocated for planned and unexpected maintenance and plant downtown. The plant is assumed to take one year to construct and thereafter a phased production will occur over three years until the plant is running at full capacity in the 4th year. This will allow time for the logistics, markets and training adjustments. The plant is expected to operate for a period of 20 years.

A straight line depreciation of the plant is to be done over a period of 5 years with an annual depreciation of 20%. The sales and operating costs are adjusted for an inflation rate of 4.13%.

Table 28: Cash Flow Assumptions

Assumption	Value
Annual Operating hours	8000h
Scrap value	0
Equity	100%
Working Capital % of FCI	5%
Depreciation Method	Straight Line
Annual Depreciation	20%
Depreciation Period in years	5
Construction Period	1
Salvage value	0
Ramp-up to full capacity:	
1st Year	70%
2nd Year	80%
3rd Year	90%
4th Year	100%
Exchange Rate	R16.50/$
Inflation Rate	4.13%
Income Tax Rate	28%
Internal Rate of Return (IRR)	15%
Minimum Set Hurdle rate	25%
Cost Year for Analysis	2020
Selling price of produced lignin ***Determined from Industry professionals**	R1 622/ton

5.3.6 Profitability Indicators

The NPV and MRSP will be ascertained to determine the profitability of the proposed process. The NPV indicated the cumulative discounted cash flow analysis of the plant at the end of its lifespan. The MRSP indicates the minimum selling price of the lignin to provide an acceptable return to the investor (Turton *et al.*, 2016).

5.4 Results and Discussion

5.4.1 Plant Capacity

The annual production capacity of the proposed plants are summarised in Table 29, which was determined from mass balances. It was decided that each plant will run one bioreactor (30m^3) with a residence time of 26 hours, thus the spent liquor feed amount would be determined from the liquors' properties (solids content) and the efficiencies and recovery rates of the processes. The solids contents of the various liquors were determined from literature sources.

Table 29: Plant Production Capacity

	Unit	Scenario 1 KS-S-N	Scenario 2 KH-S-N	Scenario 3 S-S-S	Scenario 4 NaS-S-T	Scenario 5 NaS-M-Pr	Scenario 6 MgO-S-S
Feedstock							
Feed liquor	ton/day	1.97	2.12	2.76	2.69	4.20	2.40
Solids Content	%	42[a]	39[b]	30[c]	50[d]	32[e]	50[f]
Sodium phosphate dibasic	kg/day	110	110	110	110	110	110
Sodium phosphate monobasic	kg/day	357	357	357	357	357	357
Sodium hydroxide	kg/day	1.20	1.20	1.20	-	-	-
Sulphuric Acid	l/day	4 140	4 140	4 140	-	-	-
Water	l/day	170 550	170 550	170 550	24 690	24 690	24 690
Laccase	l/day	15	3	30	32	32	60
Product							
Treated lignin	Dry ton/day	0.40	0.40	0.40	0.86	0.86	0.86

a- (Cardoso, de Oliveira and Passos, 2009; Nikolskaya *et al.*, 2019)

b- (Andreuccetti, Leite and D'Angelo, 2011)

c-(Mamaye *et al.*, no date)

d-(Nicholas and Cheremisinoff, 2010; Polchem, 2020)

e-(Joachimiak, Wojech and Wójciak, 2019)

f-(Marques *et al.*, 2009)

In the lab-scale experiments, the concentration of the purified lignins was limited, owing to their insolubility at the lower pH values. As the lignosulphonates are soluble at higher lignin concentrations this could be investigated resulting in the optimum concentration conditions higher than the purified lignins, therefore the dry tonnes produced per day is higher for the lignosulphonates than for the Kraft and soda lignin.

5.4.2 CAPEX and OPEX

5.4.2.1 CAPEX

The total capital investment is summarised in Table 30. Of the various proposed process scenarios the KS-S-N required the highest TCI of R 59.01 million, where the MgO-S-S proposed process required the least, R 43 million. There is a big difference in FCI between the Kraft/soda proposed process and the lignosulphonate proposed process, this is because of the isolation process required for the Kraft and soda plant, which adds more equipment requirements. The breakdown of the total installation cost is summarised in Figure 25. The cost of the bioreactors contributed most to the total installation cost for all the scenarios.

Table 30: Total capital investment for the various proposed scenarios

Total Capital Investment (TCI) (R million/year)	Scenario 1 KS-S-N	Scenario 2 KH-S-N	Scenario 3 S-S-S	Scenario 4 NaS-S-T	Scenario 5 NaS-M-Pr	Scenario 6 MgO-S-S
Total Direct Costs (TDC)	35.13	36.51	34.82	26.37	26.36	25.60
Total Installation Cost	29.88	29.76	29.62	22.44	22.42	21.77
Warehouse (4% NPP)	1.20	1.19	1.18	0.90	0.90	0.87
Site Development (9% NPP)	2.69	2.68	2.67	2.02	2.02	1.96
Additional Piping (4.5% NPP)	1.36	1.35	1.35	1.02	1.02	0.99
Total Indirect Costs (60% TDC)	21.08	20.99	20.89	15.82	15.82	15.36
Fixed Capital Investment (FCI)	56.20	55.97	55.71	42.20	42.18	40.95
Working Capital (5% FCI)	2.81	2.8	2.79	2.11	2.11	2.05
Total Capital Investment (TCI)	59.01	58.50	58.50	44.31	44.28	43.00

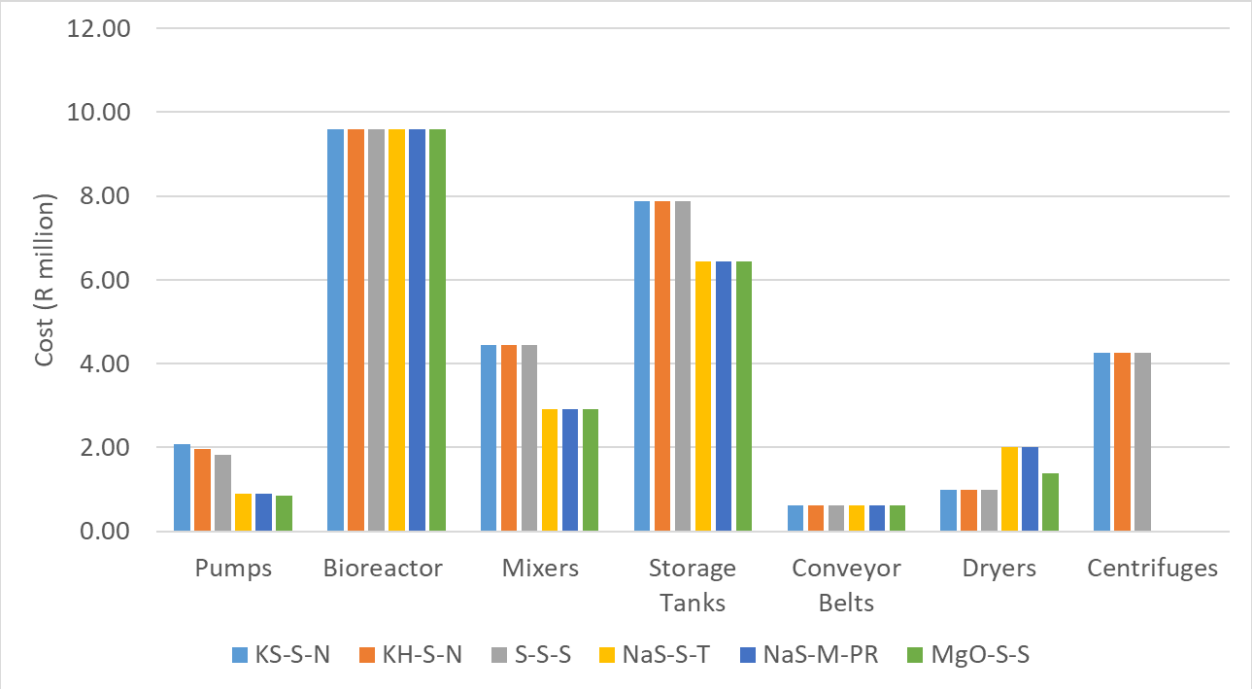

Figure 25: Distribution of installation cost for the TCI

5.4.2.2 OPEX

The total operating costs are summarised in Table 31 for the various scenarios. The scenario with the highest operating costs is scenario, S-S-S (R79.2 million) and the lowest operating costs is Scenario 4 and 5 – NaS-S-T and NaS-M-Pr, respectively (R54.86 million). The difference is mainly as a result of the cost. The cost of the isolation of the lignin in the soda and Kraft designed process (Scenarios 1-3) is also expensive, owing to the high volumes of sulphuric acid and the corresponding costs required. The required buffer chemicals are the same for all the processes, as the same reaction volume was processed every day and the pH of the buffer solution is the same for all six scenarios. The contribution of each of the materials to the total operating costs is summarised in Figure 26. The treatment of the toxic waste from the proposed plants were not included in the OPEX and should be considered in further studies.

Table 31: Total operating costs for the various proposed scenarios

Total Operating Costs (TOC) (R million/year)	Scenario 1 KS-S-N	Scenario 2 KH-S-N	Scenario 3 S-S-S	Scenario 4 NaS-S-T	Scenario 5 NaS-M-Pr	Scenario 6 MgO-S-S
Raw Materials						
Sodium phosphate dibasic	8.0	8.0	8.0	8.0	8.0	8.0
Sodium phosphate monobasic	15.9	15.9	15.9	15.9	15.9	15.9
Sodium hydroxide	0.017	0.017	0.017	-	-	-
Sulphuric Acid Industrial Grade	22.9	22.9	22.9	-	-	-
Laccase	4.6	0.9	9.2	9.69	9.69	18.46
Water	1	1	1	0.13	0.13	0.13
Utilities						
Electricity	12.67	12.68	12.69	12.29	12.29	12.29
Total **Labour** Costs	6.37	6.37	6.37	6.37	6.37	6.37
Maintenance (5% of FCI)	2.81	2.92	2.79	2.11	2.11	2.05
Property Insurance and Tax (0.7% of FCI)	0.39	0.41	0.39	0.30	0.30	2.87
Production Cost	74.6	71.1	79.2	54.8	54.8	66.1

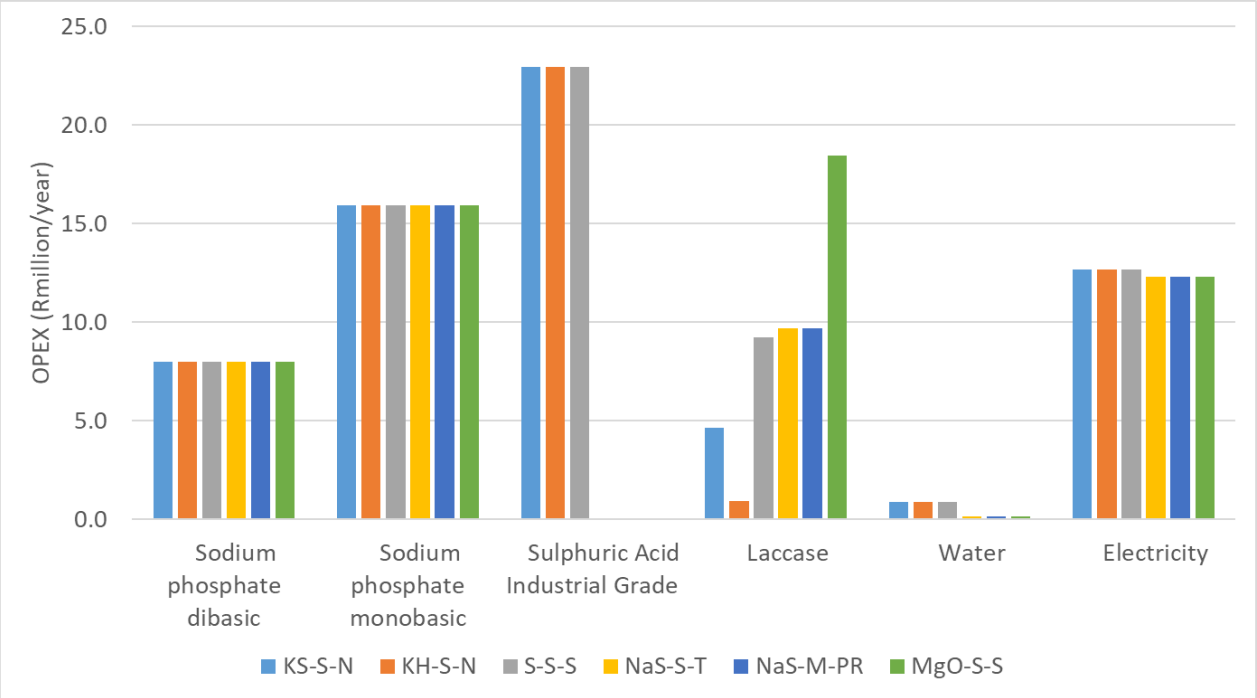

Figure 26: Distribution of the variable operating costs for all the scenarios

5.4.3 Financial Performance of Proposed Plant

The results from the NPV and MRSP analysis of the various scenarios are summarised in Table 32. It is noted that the NPV for all six simulated scenarios are negative. Thus, none of the proposed scenarios are economically feasible. However, the most 'profitable' scenario corresponds to scenarios 4 and 5 – NaS-S-T and NaS-M-PR, respectively, since these scenarios have the least negative NPV (R-57 800 000). The negative NPV is most probably due to the low income generated. Additionally, the operating costs are much more than the income generated, resulting in no profit every year. Thus, the minimum required selling price of the lignin was determined and summarised in Table 32.

Table 32: Probability indicators for the various process plant scenarios

Probability Indicators	**MRSP (R/tonne)**	**NPV (R million)**
Scenario 1 KS-H-N	735 965	-397.5
Scenario 2 KH-S-N	705 126	-372.3
Scenario 3 S-S-S	772 546	-428.0
Scenario 4 NaS-S-T	250 432	-57.8
Scenario 5 NaS-M-PR	250 421	-57.8
Scenario 6 MgO-S-S	291 780	-132.4

NaS-M-PR and NaS-S-T showed the lowest MRSPs of R250 421 and R 250 432 per tonne, respectively. These are nearly identical, owing to the similarity in the process and the optimum operating conditions. MgO-S-S achieved a higher MRSP as more enzymes are required.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

This study investigates the enzymatic valorisation by repolymerising various lignins derived from industrial-spent pulping liquor. The liquor investigated was obtained from various pulping mills in South Africa to give insight into an area of study that was not commonly investigated. The purpose of this study was to compare the various liquors available in SA to determine which showed the greatest potential to be repolymerised to a higher molecular weight lignin by enzyme modification, to be used for higher-value applications. This would then enable further investigation into the lignin and enzymes to eventually implement them industrially. Two enzymes, laccase and lignin peroxidase were investigated for the modifications at various conditions. A handful of studies have investigated the effect enzymes have on lignin and an even less effect on industrial lignins.

6.1 Conclusions

The overall aim of this study was to investigate the potential valorisation of the enzymatic modification of industrial lignin, specifically by increasing the molecular weight of the lignin.

The following conclusions can be drawn from the results determined in Chapter 3 regarding the characterisations of the various industrial lignins:

The compositional results of lignin showed that the Kraft and soda lignin had the lowest ash contents of below ≤ 1.4%. This is mainly attributed to the isolation and purification steps done before the characterisation. The lignosulphonates could not be purified, owing to their high solubility and higher ash contents that were observed between 17% and 32%, indicating higher impurities in the lignosulphonates. These higher impurities could hinder the action of the enzymes.

The structural analysis included determining the FT-IR spectra of the lignin samples. Broad spectra were observed for all the lignin at 3 400 cm^{-1}, with MgO-S-S and S-S-S obtaining the highest intensities. This band is allocated to the hydroxyl groups, which influence the reactivity of lignin. These lignins are expected to perform well as the modification enzymes oxidise the phenolic groups to form radicals.

The results from the phenolic content analysis were comparable to the FT-IR spectra with ranges between 0.95 to 2.23 mmol/g reported. The GPC analysis showed a range of molecular weights between 5 657 to 12 156 g/mol, where the highest molecular weight was observed for KS-S-N, while KH-S-N and S-S-S lignin had the lowest molecular weight. As KS-S-NT has a higher guaiacyl monomer content and more condensed C-C bonds than hardwood and non-wood it results in a higher molecular weight. It is expected that a higher molecular weight lignin is more thermally stable. This was confirmed by the TGA analysis, where KS-S-N was found to be thermally the most stable with a degradation temperature of 434°C. The lignosulphonates had low degradation temperatures, between 276°C and 307°C, owing to the inorganic content causing a catalytic effect and resulting in a lower degradation temperature.

The following conclusions can be drawn from the results in Chapter 4 on the enzymatic modification of lignin:

The results showed that the enzymes laccase and LiP were able to repolymerise the purified lignin and lignosulphonates over a 24-hour period. The highest molecular weight increase was found when S-S-S was treated with LiP at a dosage of 2 U/g lignin and a 5.8-fold molecular weight increase was observed.

As was predicted in Chapter 3, the molecular weight increase of NaS-M-PR was lower than the other lignosulphonates, owing to the high impurities present. S-S-S achieved the highest molecular weight increase which corresponds to Chapter 3, where it had the highest intensity at the peak assigned to the phenolic and aliphatic groups.

Lower molecular weight lignin was found to be more reactive in comparison to higher molecular weight lignin. The lower molecular weight lignin allows for a more efficient interaction with the enzyme. The syringyl units are present in hardwoods and grasses that aid in the repolymerisation action, causing these lignins to perform better than softwoods. A drop in the reaction rate after 4 or 10 hours was because of the micelle-like structure of lignosulphonates, where the enzyme only has access to the phenolic end groups on the surface of the micelle.

The relationship between the decrease in the phenolic content and the increase in the molecular weight was observed only in all the laccase enzymatic modifications. There is a minimum enzyme dosage for LiP to induce repolymerisation. At dosages below 0.067 U/g lignin, there is a high possibility of depolymerisation by LiP, instead of repolymerisation. This was further proven by the phenolic content data which showed that at low LiP dosages (0.067 U/g and lower), an increase in phenolic end groups was observed indicating that depolymerisation occurred. Other experiments showed an increase in molecular weight, while an increase in phenolic content was found for the same dosage of LiP. This indicated that repolymerisation occurred by LiP, oxidising the non-phenolic groups.

It was found that for laccase a high lignin concentration to enzyme dosage ratio causes the inhibition of the enzyme.

Based on the results from the techno-economic analysis in Chapter 5, the following conclusions could be drawn:

Using the lab-scale experimental setup, a process was created for the enzymatic modification of lignins. The optimum enzyme dosages and lignin concentrations determined in Chapter 4, were used in the proposed process. Two processes were designed, one for the soda and Kraft lignin which included a purification step, and the other for the lignosulphonates without the purification step.

It was found that all the scenarios investigated resulted in negative NPV values, indicating that the proposed process is not economically feasible. The MRSPs were determined and for the investor to receive a return on investment the MRSP for NaS-SM-PR is R250 421.

6.2 Recommendations

As all the lignin achieved repolymerisation at almost all of the enzyme dosages, further optimisations are recommended to improve the process to become economically feasible. Investigations that are recommended: decreasing the reaction time of the enzymatic reactions which could be beneficial; the

reuse of the enzymes used in the modification as an area where costs can be saved; methods such as the immobilization of the enzymes; and the non-phenolic groups which can be repolymerised by LiP. Investigation into the conditions which cause the depolymerisation or repolymerisation action of the enzymes would also be beneficial.

Further study should be undertaken into solubilising the Kraft and soda lignin at higher concentrations and a lower pH, which will aid the enzyme in accessing the lignin. The use of surfactants to achieve this could be a possible solution. Higher lignin concentrations are beneficial to produce the treated lignin on a larger scale. Batch pilot plant scale reactions should be carried out to determine the changes in the outcomes owing to scale up. If the solubility issue is not resolved, higher concentrations of the lignosulphonates should be investigated. This would be very beneficial for the techno-economic analysis.

Investigation into alternative purification processes for the Kraft and soda lignin is required – a more environmentally and economically friendly alternative.

In order to apply these treated lignins to higher value applications, the use of these enzymatically-treated lignins should be investigated in various fields, such as carbon fibres, cement additives or polymer blends.

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APPENDIX A: ENZYMATIC EXPERIMENT

6.3 Enzymatic reaction Results

6.3.1 KS-S-N Enzyme modification

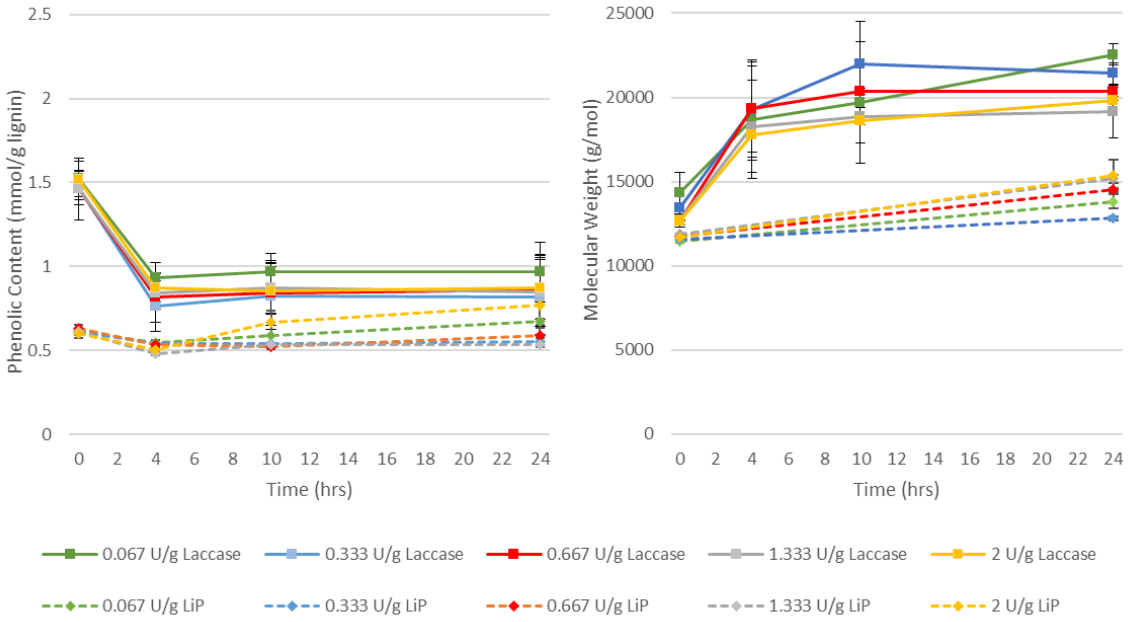

Figure 27: Molecular weight and phenolic content analysis of KS-S-N during enzymatic experiment

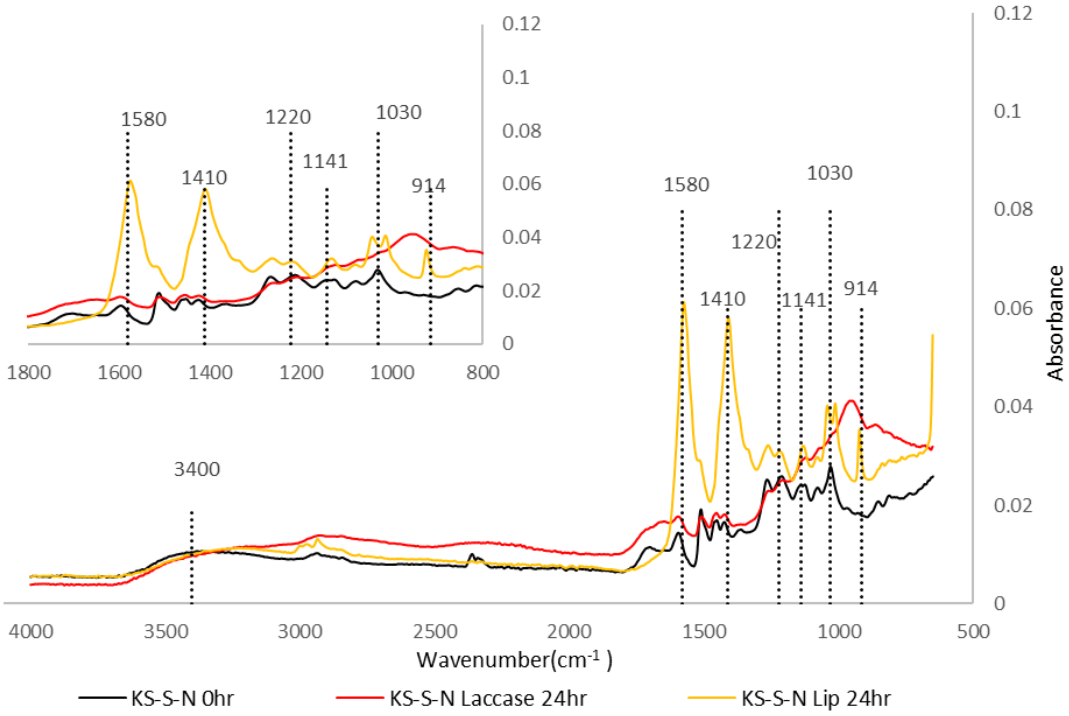

Figure 28: FT-IR Spectra of KS-S-N before and after 24-hour enzymatic experiment – insert enlarges the 'fingerprint' region of the lignin

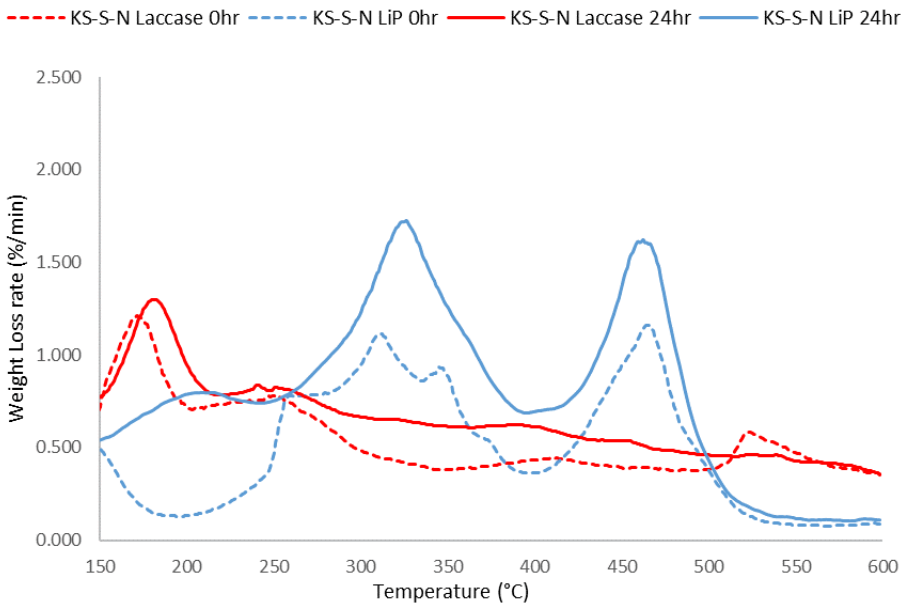

Figure 29: Rate of degradation of KS-S-N obtained before and after 24-hour enzymatic experiment

6.3.2 KH-S-N Enzyme modification

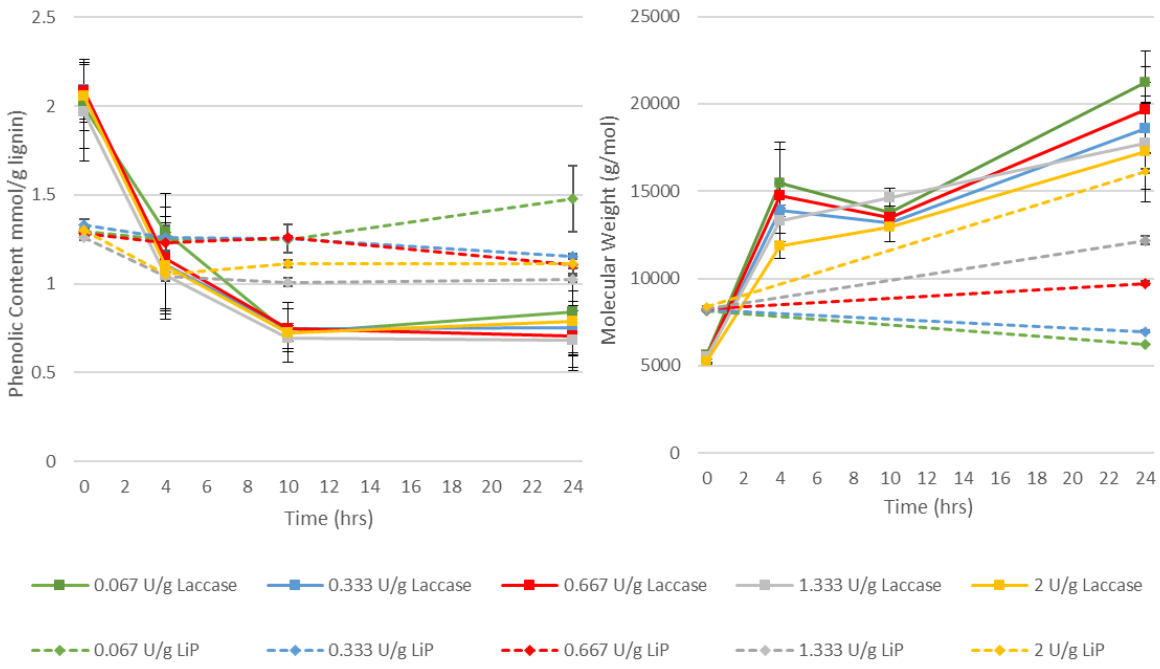

Figure 30: Molecular weight and phenolic content analysis of KH-S-N during enzymatic experiment

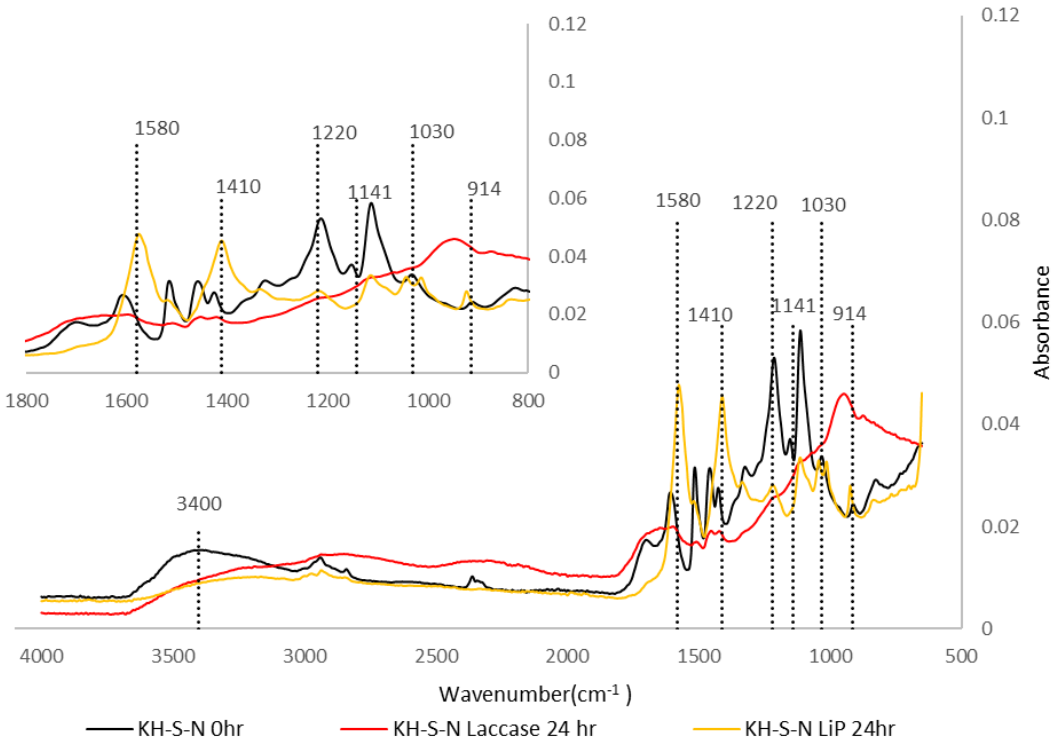

Figure 31: FT-IR Spectra of KH-S-N before and after 24-hour enzymatic experiment – insert enlarges the 'fingerprint' region of the lignin

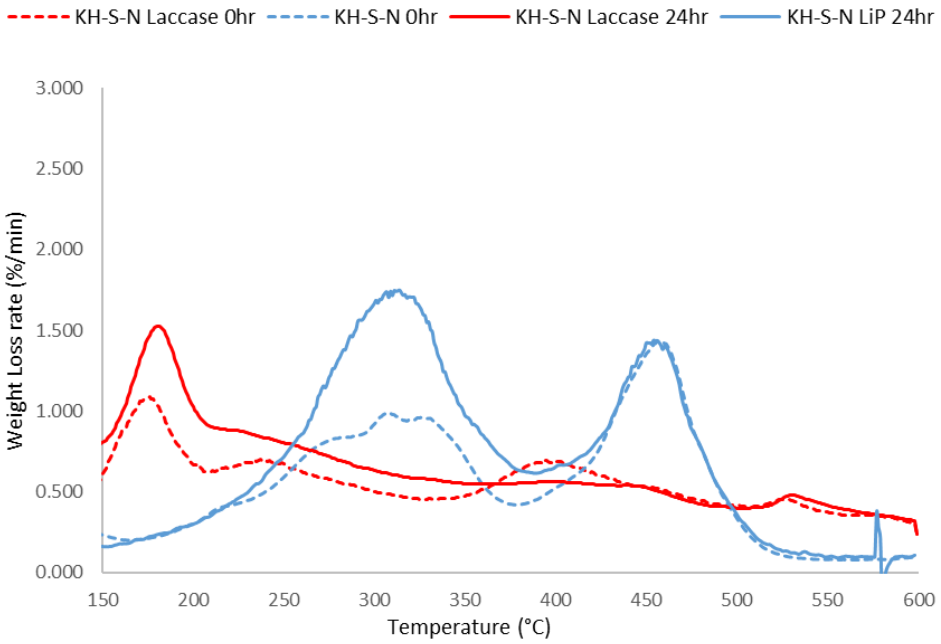

Figure 32: Rate of degradation of KH-S-N obtained before and after 24-hour enzymatic experiment

6.3.3 S-S-S Enzyme modification

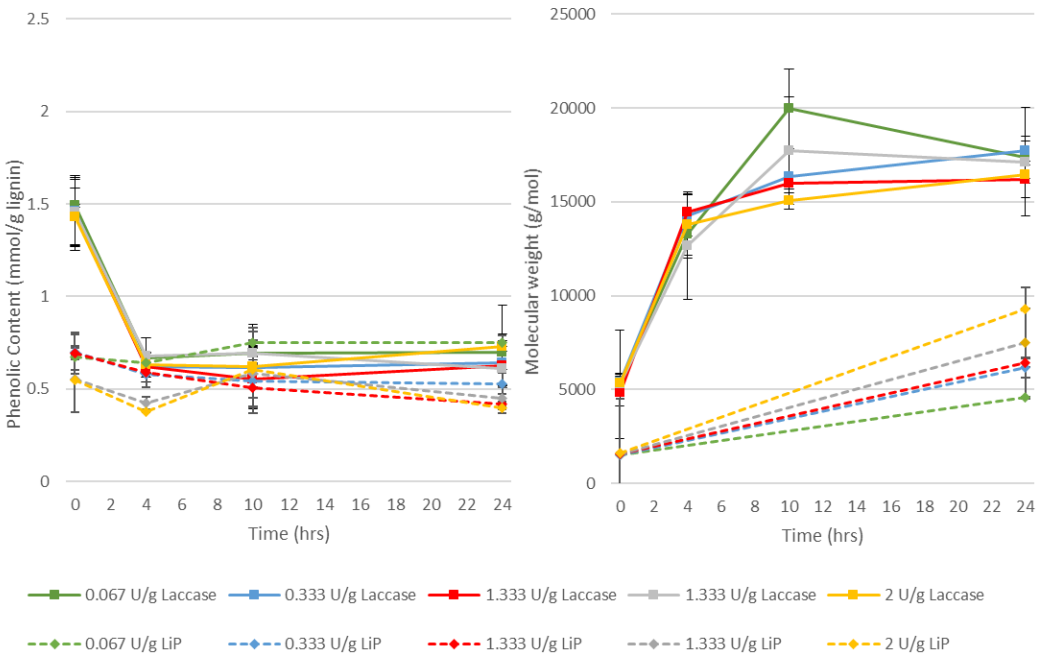

Figure 33: Molecular weight and phenolic content analysis of S-S-S during enzymatic experiment

.

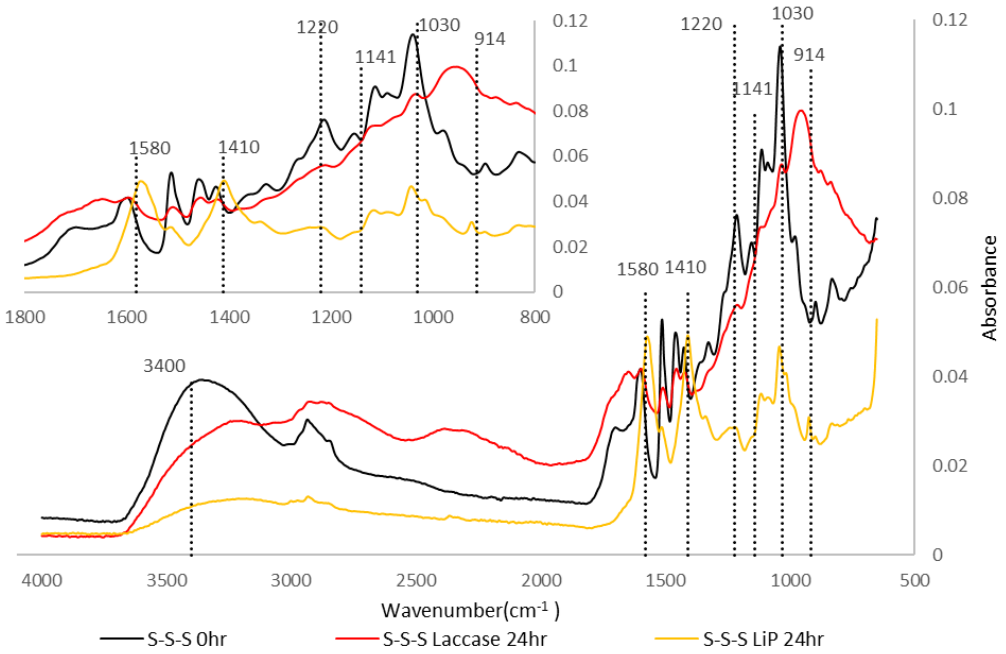

Figure 34: FT-IR Spectra of S-S-S before and after 24-hour enzymatic experiment – insert enlarges the 'fingerprint' region of the lignin

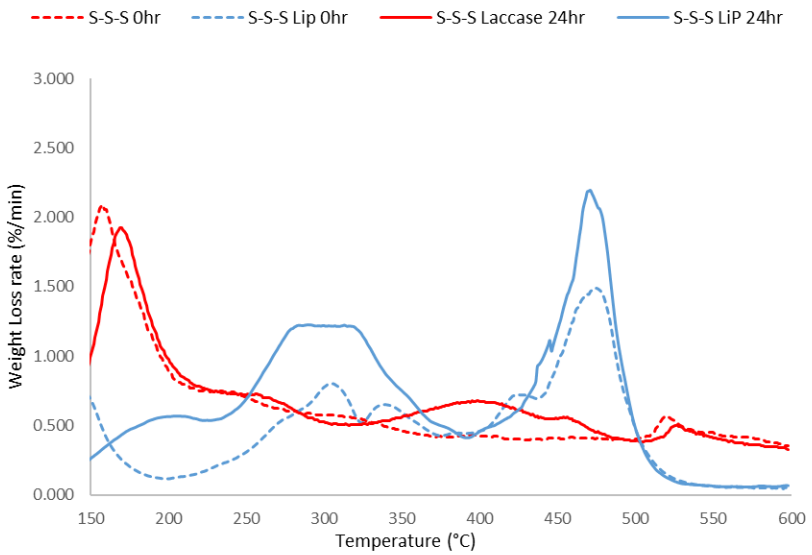

Figure 35: Rate of degradation of S-S-S obtained before and after 24-hour enzymatic experiment

6.3.4 NaS-S-T Enzyme modification

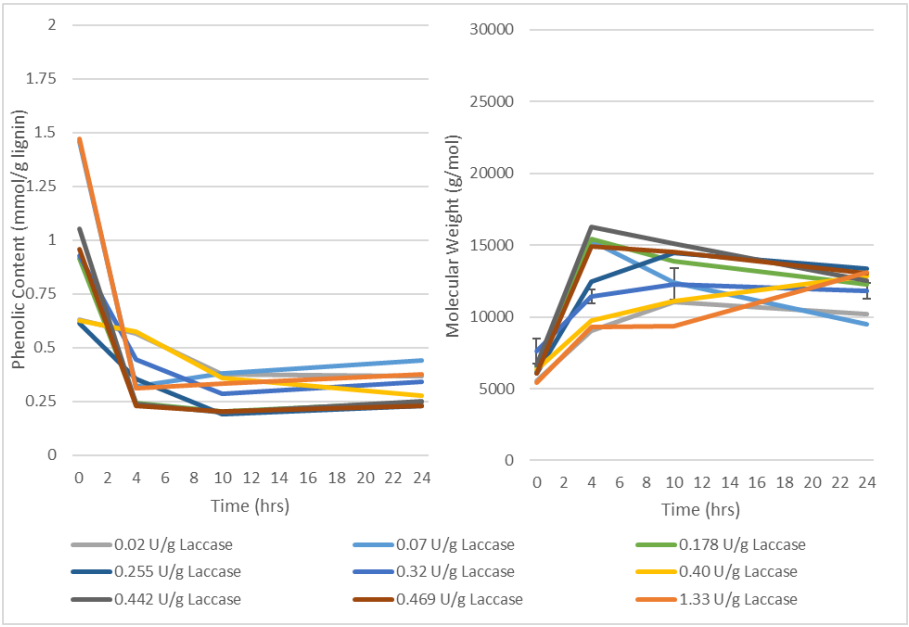

Figure 36: Molecular weight and phenolic content analysis of NaS-S-T during the laccase enzymatic experiment

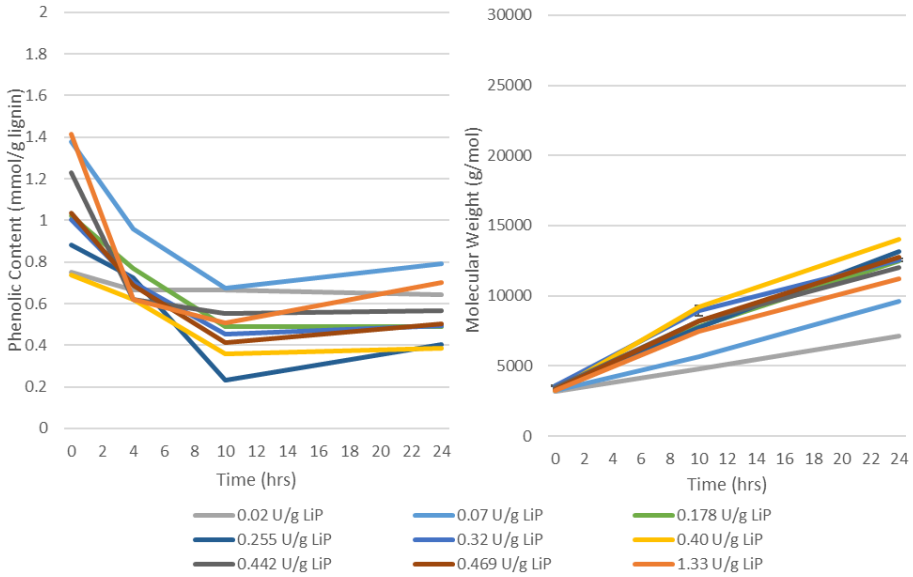

Figure 37: Molecular weight and phenolic content analysis of NaS-S-T during the LiP enzymatic experiment

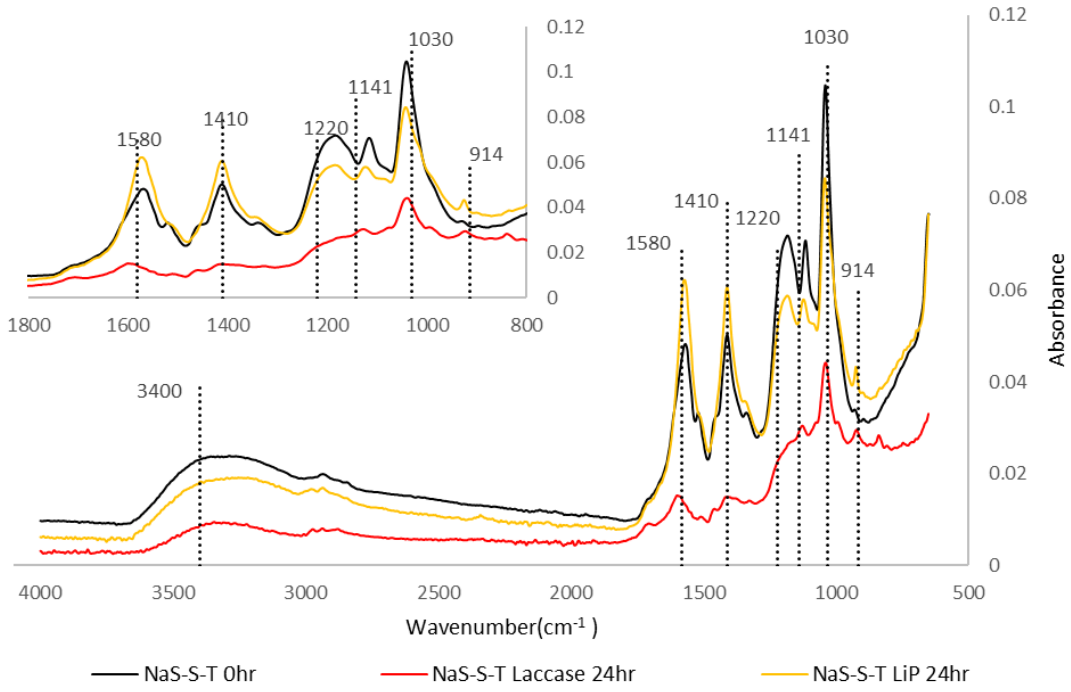

Figure 38: FT-IR Spectra of NaS-S-T before and after 24-hour enzymatic experiment – insert enlarges the 'fingerprint' region of the lignin

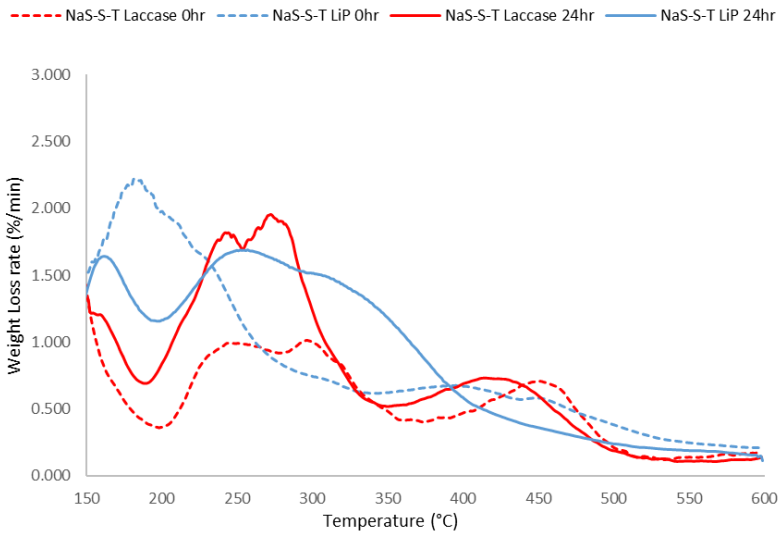

Figure 39: Rate of degradation of NaS-S-T obtained before and after 24-hour enzymatic experiment

6.3.5 NaS-M-PR Enzyme modification

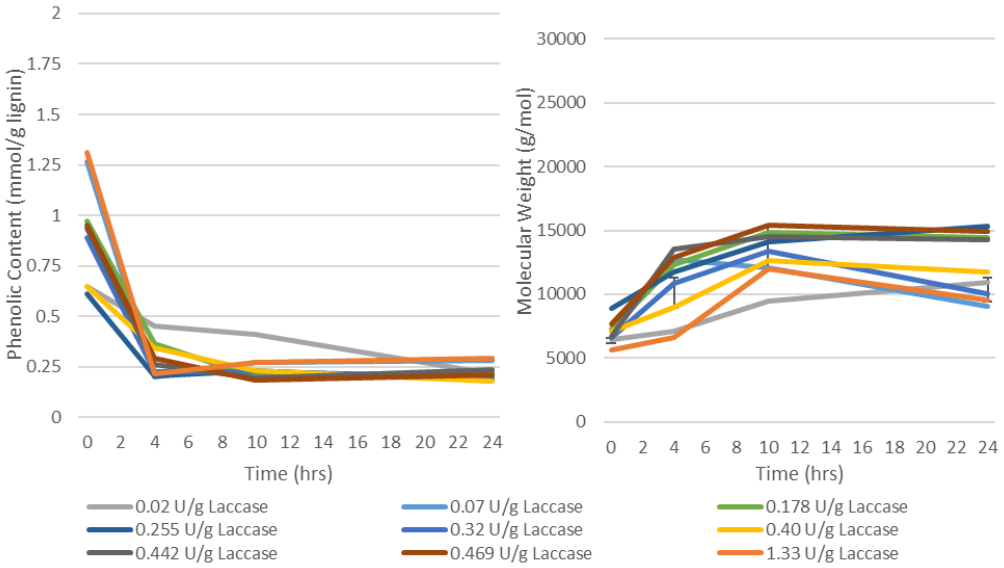

Figure 40: Molecular weight and phenolic content analysis of NaS-M-PR during the laccase enzymatic experiment

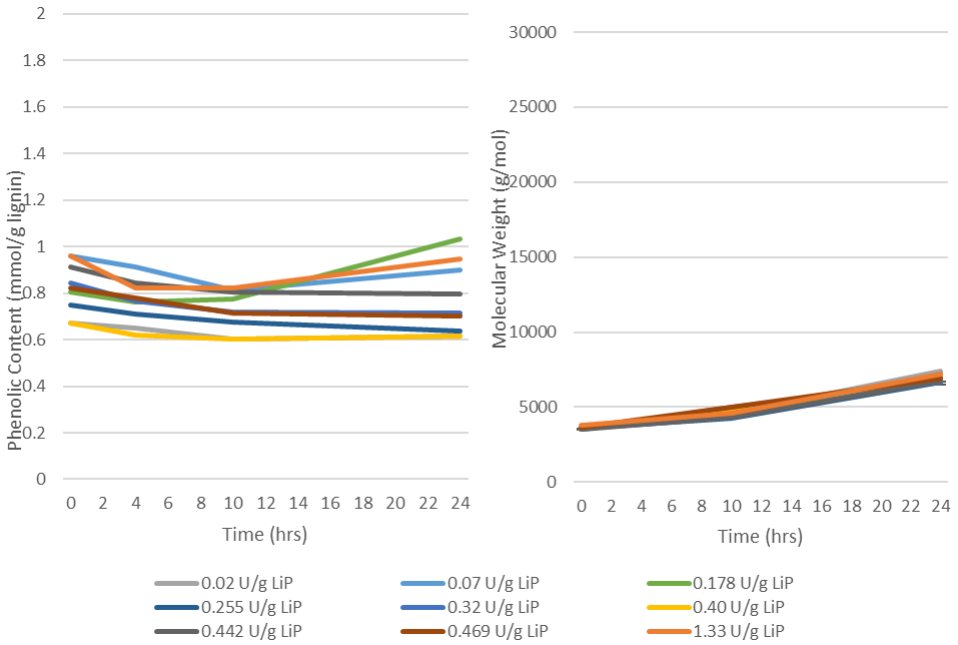

Figure 41: Molecular weight and phenolic content analysis of NaS-M-PR during the LiP enzymatic experiment

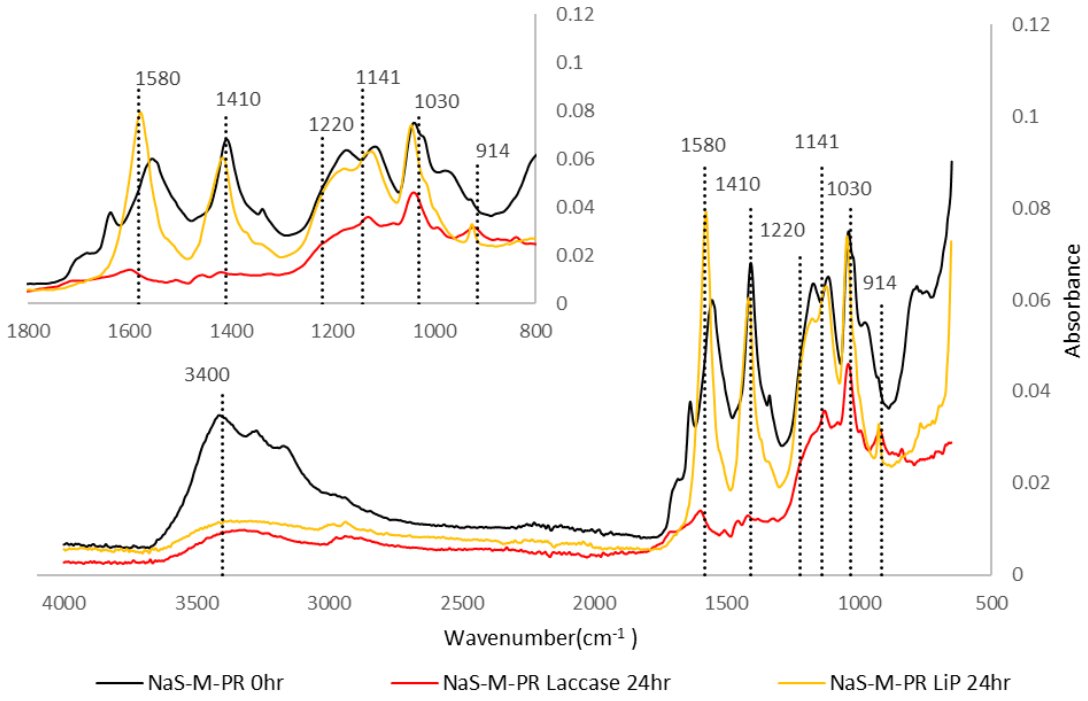

Figure 42: FT-IR Spectra of NaS-M-PR before and after 24-hour enzymatic experiment – insert enlarges the 'fingerprint' region of the lignin

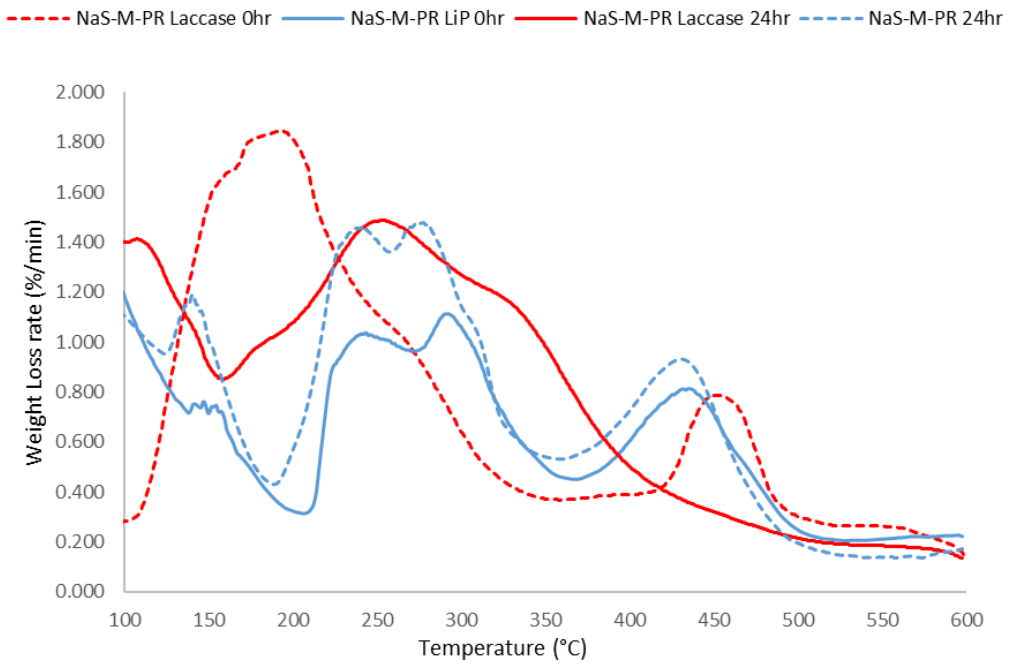

Figure 43: Rate of degradation of NaS-M-PR obtained before and after 24-hour enzymatic experiment

6.3.6 MgO-S-S Enzyme modification

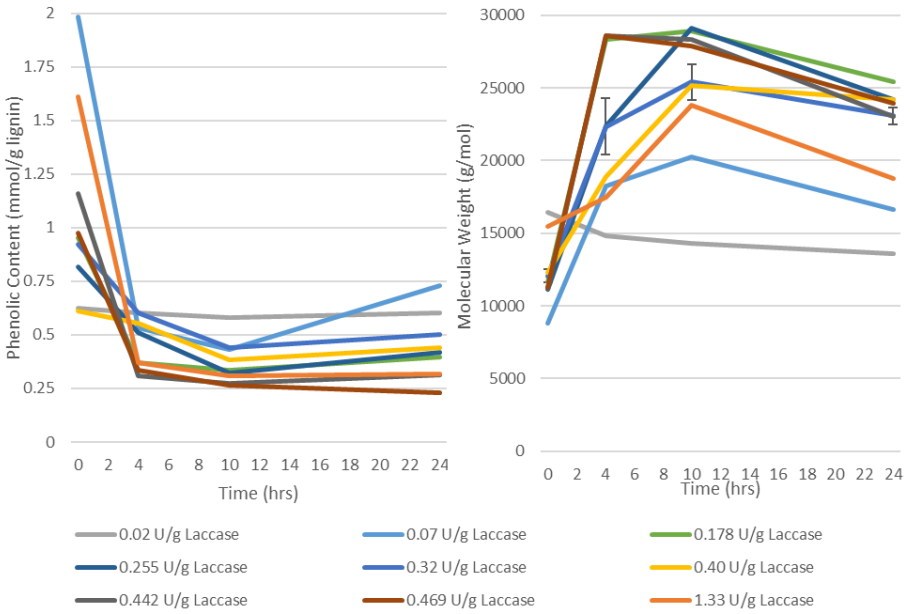

Figure 44: Molecular weight and phenolic content analysis of MgO-S-T during the laccase enzymatic experiment

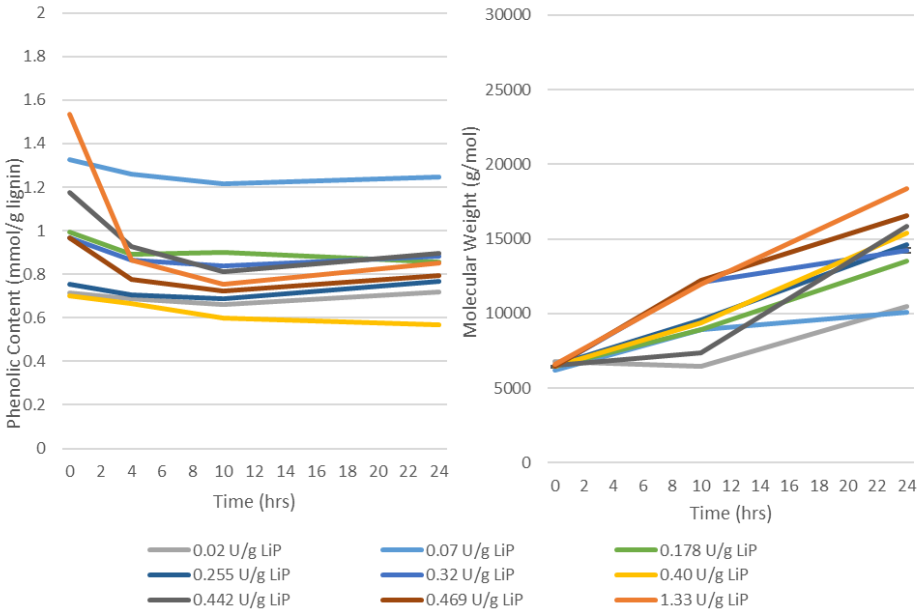

Figure 45: Molecular weight and phenolic content analysis of MgO-S-T during the LiP enzymatic experiment

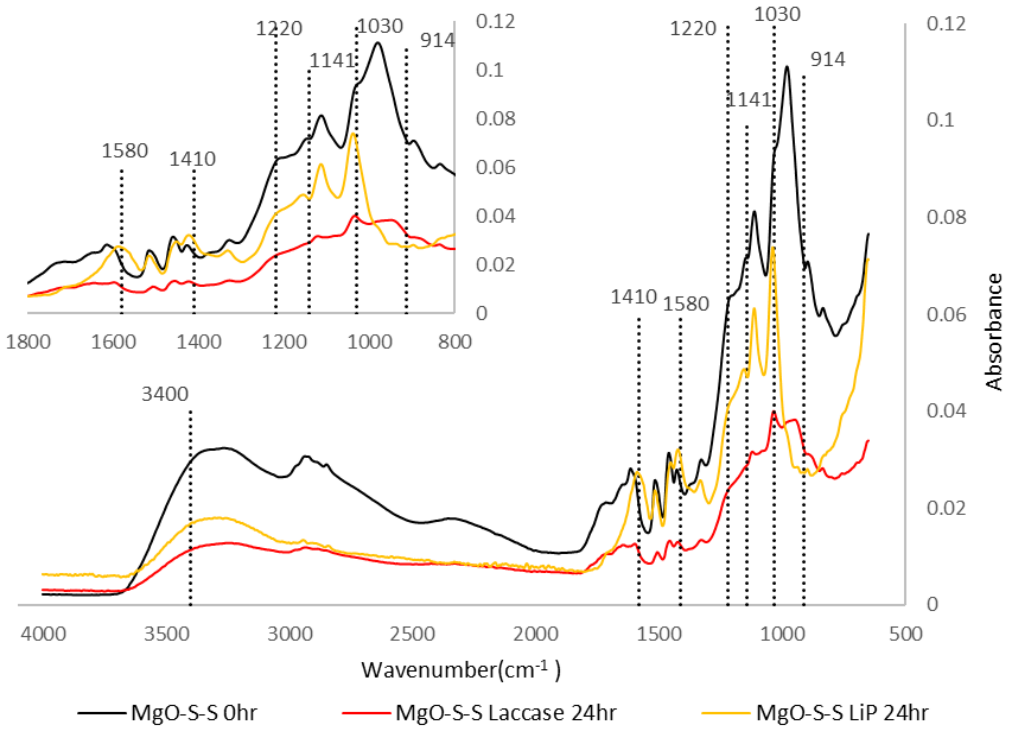

Figure 46: FT-IR Spectra of MgO-S-S before and after 24-hour enzymatic experiment – insert enlarges the 'fingerprint' region of the lignin

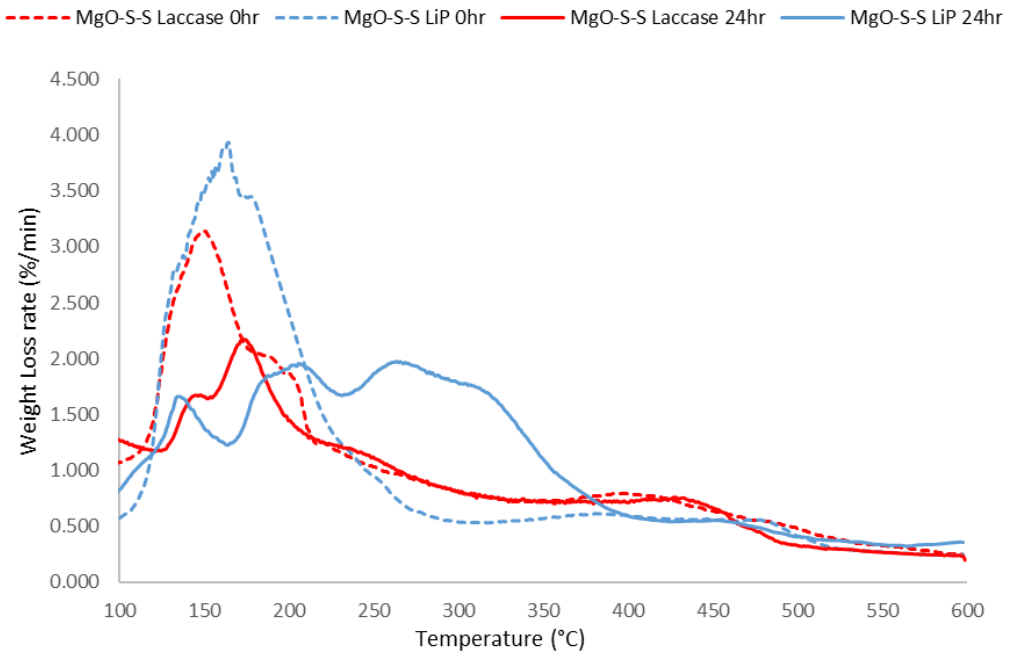

Figure 47: Rate of degradation of MgO-S-S obtained before and after 24-hour enzymatic experiment

6.3.7 Summary Enzymatic reaction results

Table 33: Summarizing the Molecular weight fold increase and the phenolic content decreases after a 24-hour enzyme experiment of the various lignosulphonates

Sample ID	Molecular weight fold increase after 24hr experiment								
	0.02 U/g	0.07 U/g	0.178 U/g	0.255 U/g	0.32 U/g	0.40 U/g	0.442 U/g	0.469 U/g	1.33 U/g
NaS-S-T Laccase	1.84	1.54	1.87	2.21	1.55	2.02	1.87	2.15	**2.42**
NaS-S-T LiP	2.27	2.63	3.46	3.85	3.50	**4.34**	3.48	3.83	3.44
NaS-M-PR Laccase	1.69	1.28	1.98	1.72	1.52	1.66	**2.12**	1.94	1.70
NaS-M-PR LiP	**2.05**	1.89	1.89	1.86	1.88	2.03	1.89	1.88	1.89
MgO-S-S Laccase	0.83	1.88	**2.18**	2.17	1.92	1.97	2.06	2.12	1.21
MgO-S-S LiP	1.54	1.62	2.09	2.23	2.22	2.38	2.41	2.56	**2.79**
	Phenolic content percentage decrease after 24hr experiment								
NaS-S-T Laccase	41.7%	69.8%	73.2%	62.4%	63.1%	55.5%	**76.1%**	75.9%	74.5%
NaS-S-T LiP	14.4%	42.6%	52.0%	54.2%	50.8%	47.9%	53.9%	51.7%	50.4%
NaS-M-PR Laccase	66.7%	77.6%	77.1%	69.3%	77.4%	72.5%	74.8%	**77.8%**	77.7%
NaS-M-PR LiP	8.3%	6.1%	-28.8%	**14.9%**	**14.9%**	8.3%	12.5%	14.7%	1.1%
MgO-S-S Laccase	3.3%	63.2%	58.3%	48.8%	45.6%	27.7%	72.8%	76.3%	**80.4%**
MgO-S-S LiP	-1.2%	5.9%	13.9%	-1.5%	8.8%	18.9%	24%	18.0%	**44.5%**

Table 34: Elemental analysis and estimated empirical formulae after the 24-hour enzymatic experiment

	Elemental Analysis after 24-hour enzyme experiment					Estimated Empirical Formula from Elemental Analysis	
Sample ID	C [%]	H [%]	N [%]	S [%]	O [%]	T=0	T=24
KS-S-N Laccase	31.50	4.68	1.10	1.68	61.05	$C_9H_{10.81}N_{0.01}S_{0.23}O_{3.38}$	$C_9H_{16.03}N_{0.27}S_{0.18}O_{19.84}$
KS-S-N LiP	40.39	6.07	0.32	1.17	52.05		$C_9H_{16.23}N_{0.06}S_{0.10}O_{15.13}$
KH-S-N Laccase	22.64	4.13	0.99	1.42	70.82	$C_9H_{10.23}N_{0.01}S_{0.23}O_{3.54}$	$C_9H_{19.68}N_{0.34}S_{0.21}O_{27.99}$
KH-S-N LiP	44.47	5.96	0.26	1.48	47.83		$C_9H_{14.47}N_{0.05}S_{0.11}O_{13.70}$
S-S-S Laccase	30.57	4.75	1.10	0.61	62.98	$C_9H_{13}N_{0.01}S_{0.07}O_{5.06}$	$C_9H_{16.77}N_{0.28}S_{0.07}O_{20.45}$
S-S-S LiP	42.27	5.79	0.43	0.82	50.69		$C_9H_{14.80}N_{0.08}S_{0.07}O_{14.47}$
NaS-S-T Laccase	25.51	4.41	0.46	4.52	65.11	$C_9H_{16.50}N_{0.03}S_{0.70}O_{12.03}$	$C_9H_{18.65}N_{0.14}S_{0.60}O_{24.69}$
NaS-S-T LiP	27.52	5.77	0.17	3.63	62.91		$C_9H_{22.66}N_{0.05}S_{0.45}O_{22.52}$
NaS-M-PR Laccase	18.24	3.24	0.47	6.23	71.83	$C_9H_{16.44}N_{0.03}S_{1.41}O_{17.45}$	$C_9H_{19.18}N_{0.20}S_{1.15}O_{35.16}$
NaS-M-PR LiP	22.96	4.11	0.15	5.89	66.89		$C_9H_{19.35}N_{0.05}S_{0.87}O_{27.57}$
MgO-S-S Laccase	29.04	4.97	0.46	3.96	61.57	$C_9H_{17.82}N_{0.02}S_{0.56}O_{9.70}$	$C_9H_{18.48}N_{0.12}S_{0.46}O_{21.49}$
MgO-S-S LiP	37.48	5.17	0.22	4.54	52.59		$C_9H_{14.90}N_{0.05}S_{0.41}O_{16.49}$

6.4 Statistical analysis of enzymatic modification results

6.4.1 KS-S-N Statistical Results

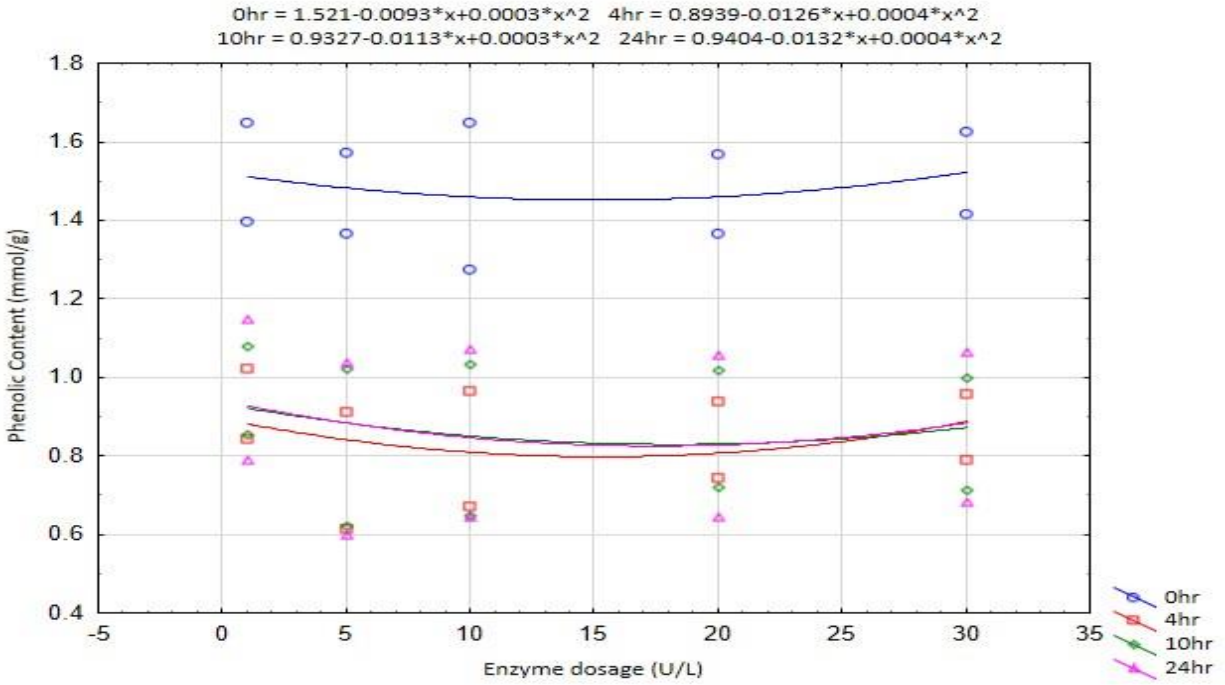

Figure 48: Scatterplot analysis of phenolic content of KS-S-N during the laccase enzymatic experiment

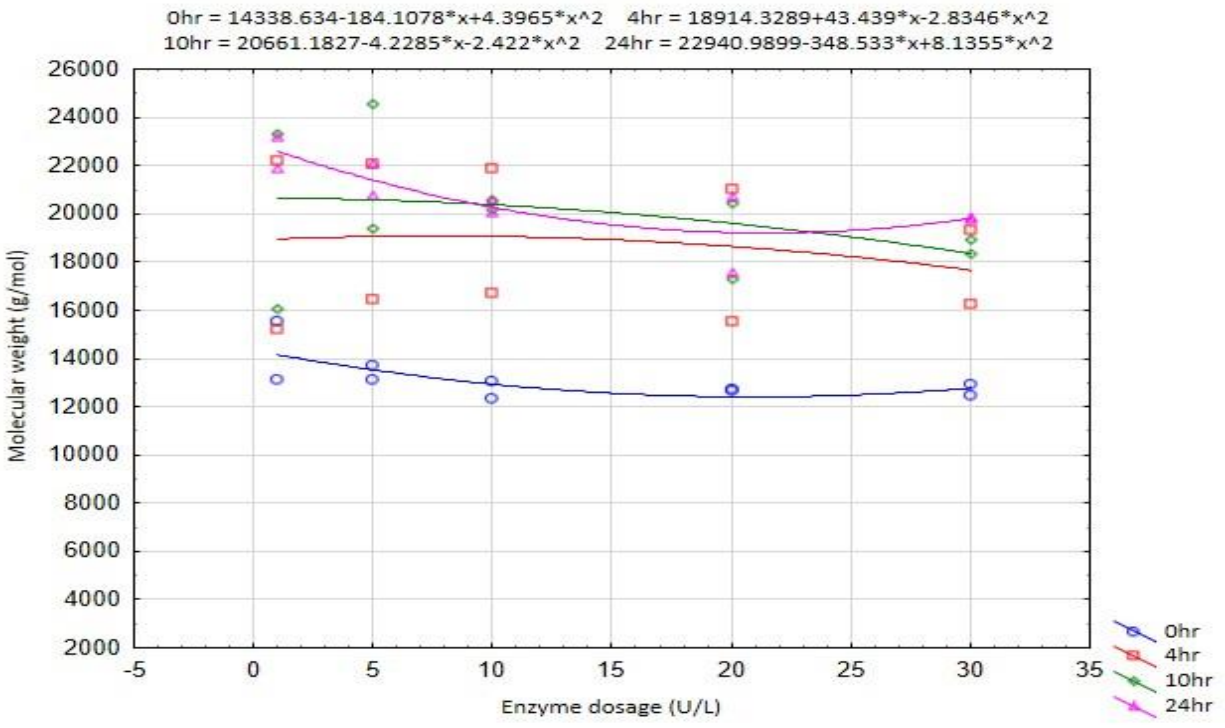

Figure 49: Scatterplot analysis of molecular weight of KS-S-N during laccase enzymatic experiment

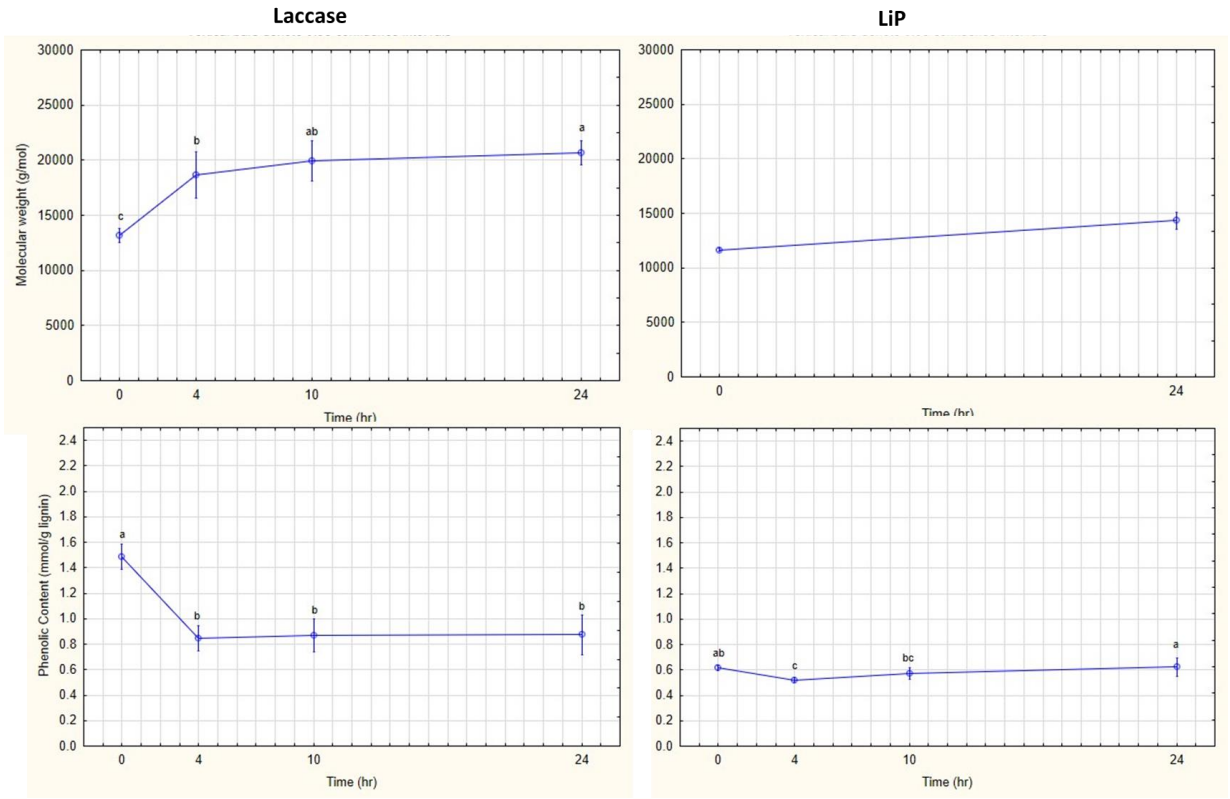

Figure 50: KS-S-N LSD test diagrams

6.4.2 KH-S-N Statistical Results

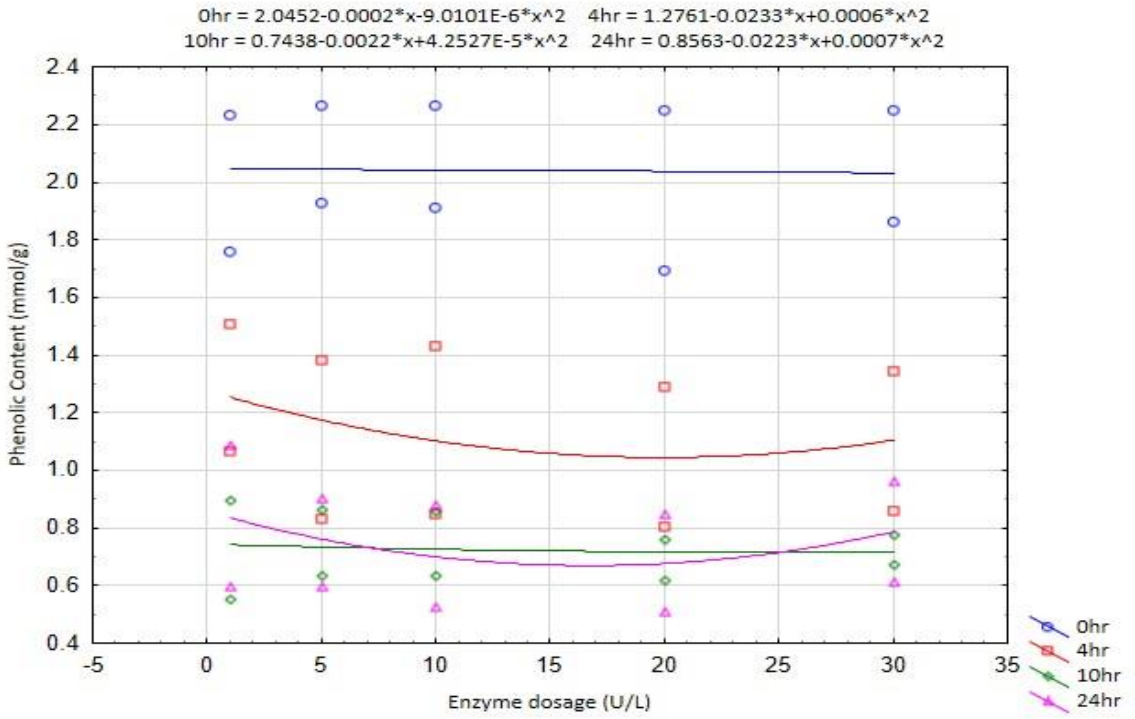

Figure 51: Scatterplot analysis of phenolic content of KH-S-N during the laccase enzymatic experiment

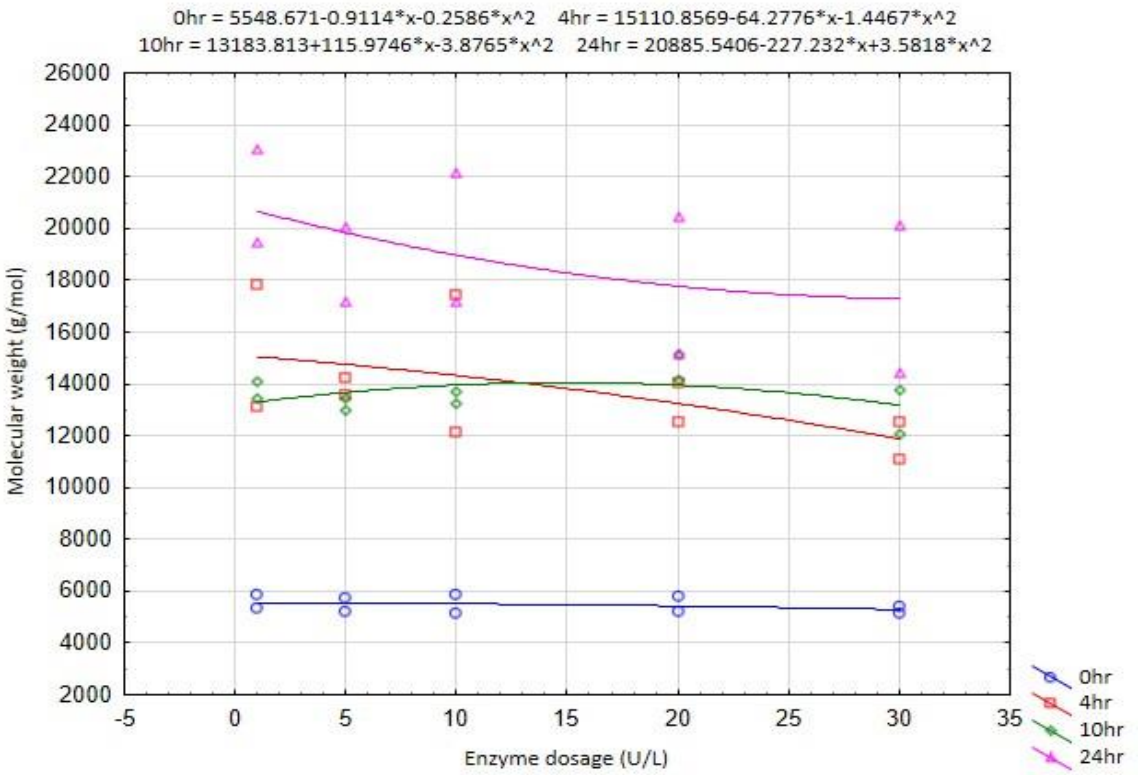

Figure 52: Scatterplot analysis of molecular weight of KH-S-N during laccase enzymatic experiment

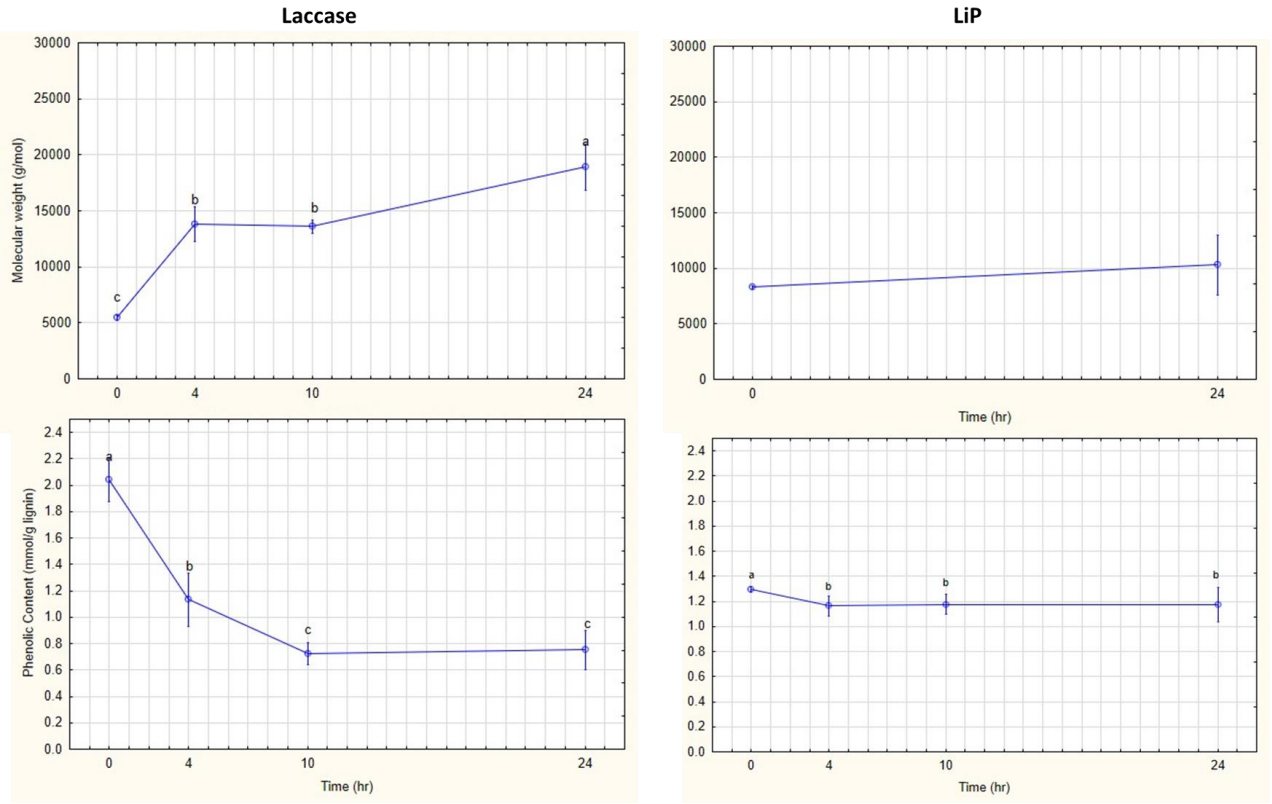

Figure 53: KH-S-N LSD test diagrams

6.4.3 S-S-S Statistical Results

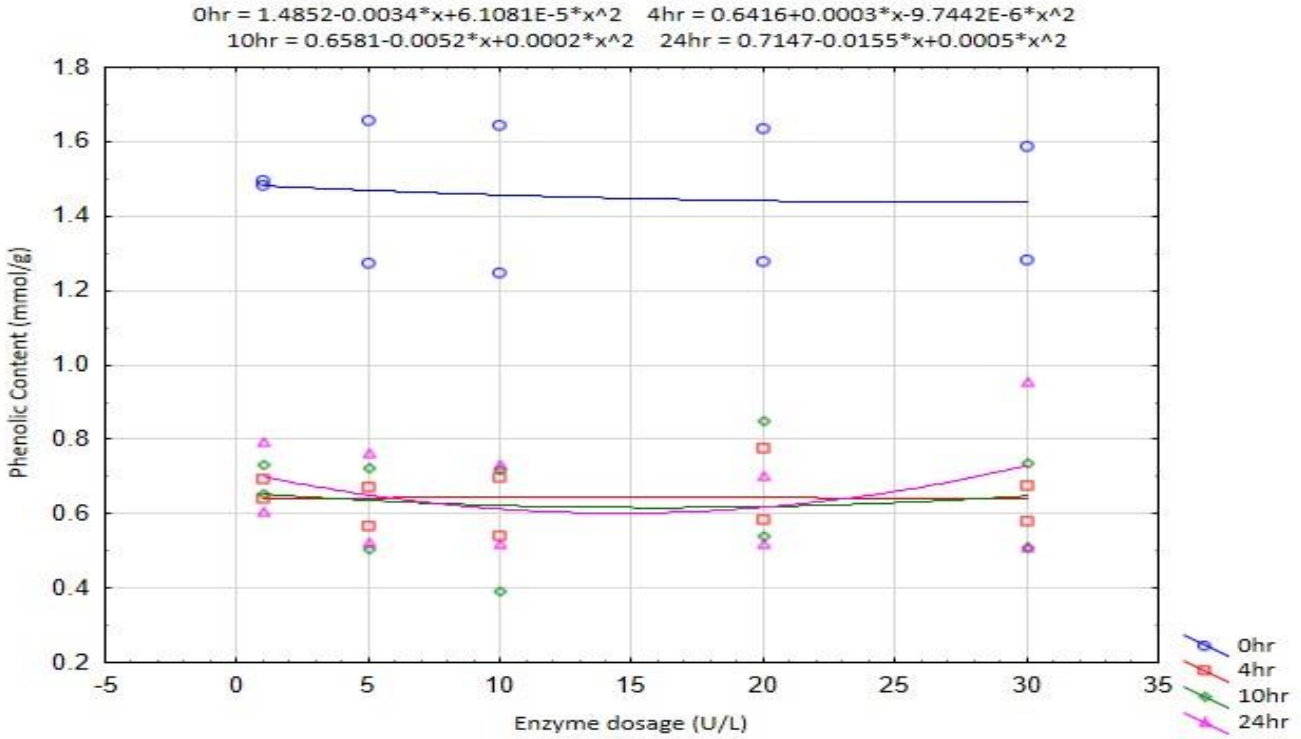

Figure 54: Scatterplot analysis of phenolic content of S-S-S during the laccase enzymatic experiment

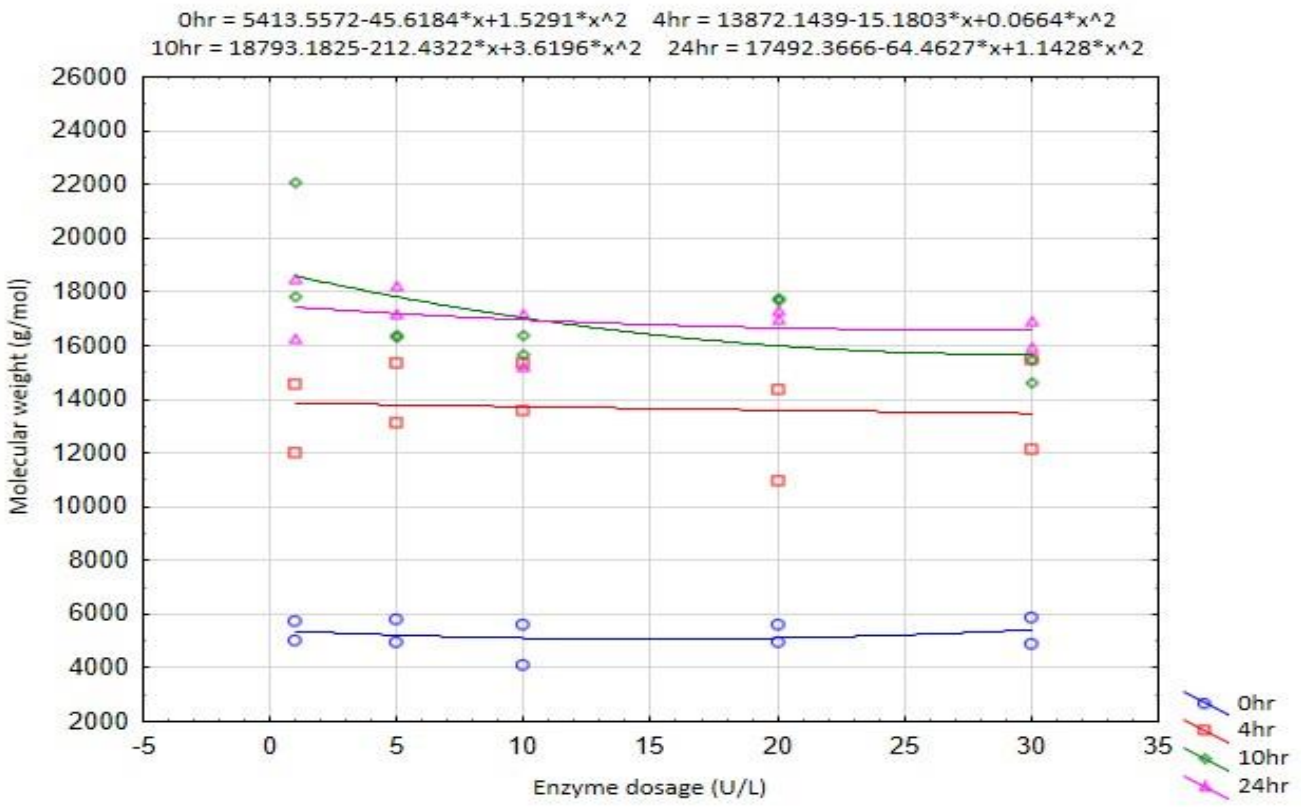

Figure 55: Scatterplot analysis of molecular weight of S-S-S during laccase enzymatic experiment

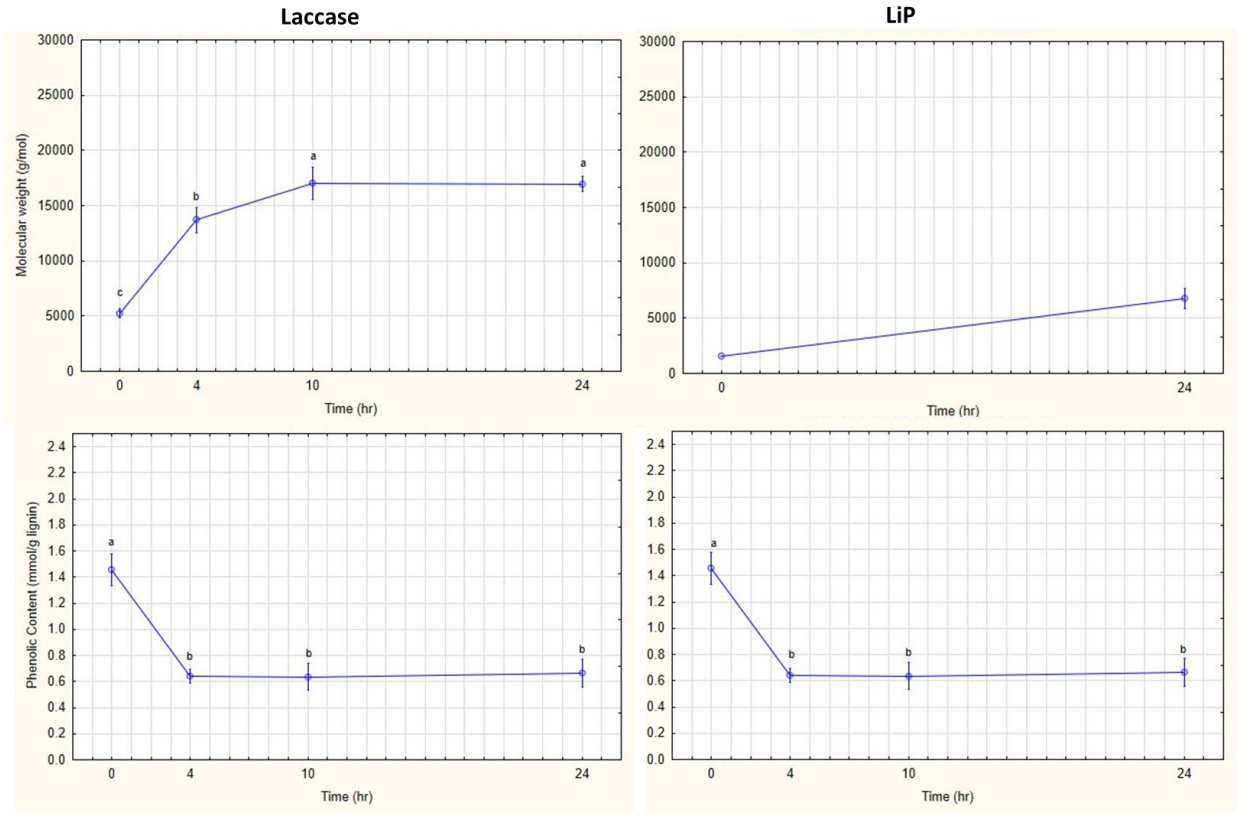

Figure 56: S-S-S LSD test diagrams

6.4.4 NaS-S-T Statistical Results

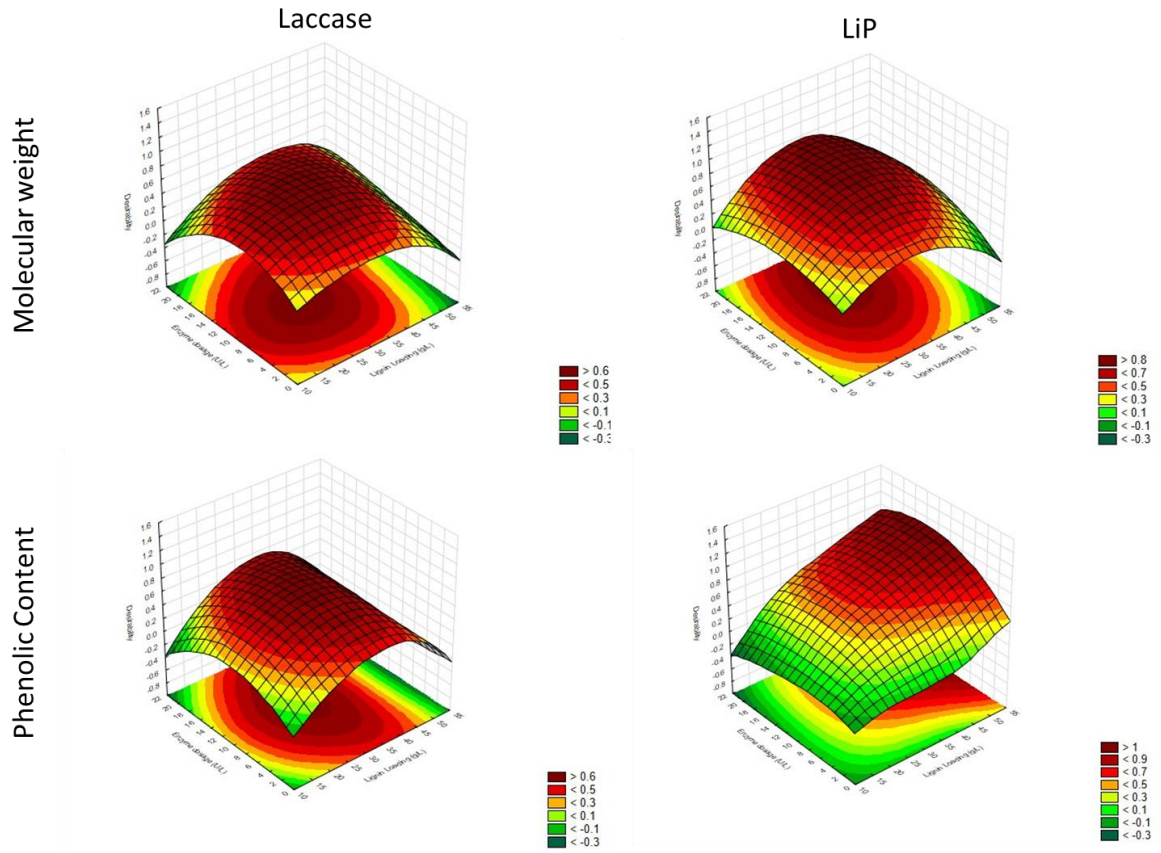

Figure 57: Desirability surface plots for optimum values for the maximization of the molecular weights and the minimization of the phenolic content of NaS-S-T

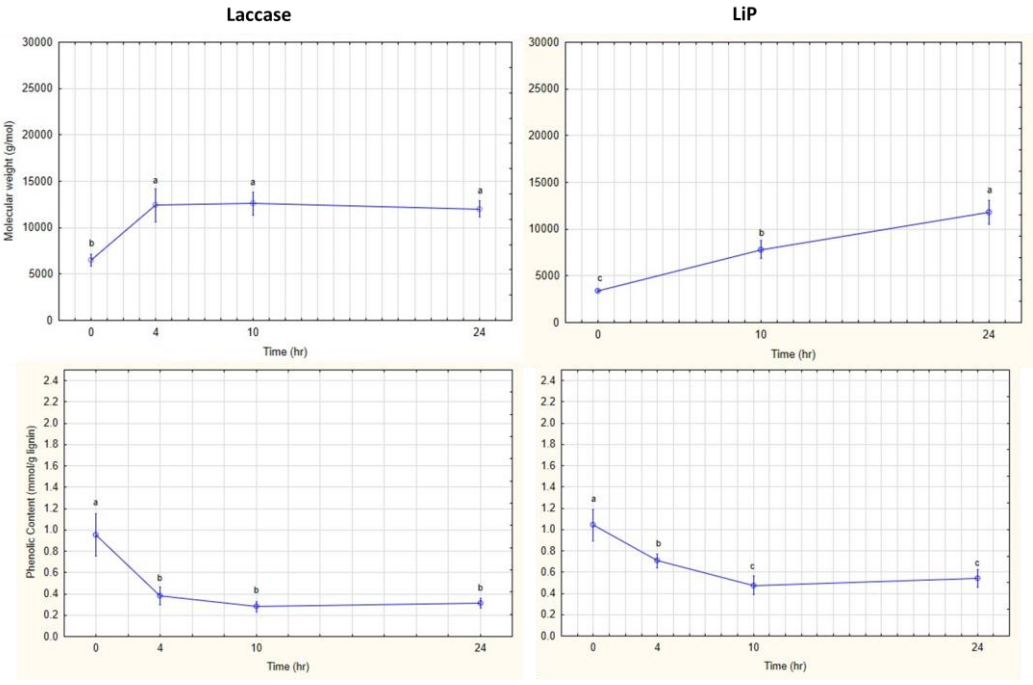

Figure 58: NaS-S-T LSD test diagrams

6.4.5 NaS-M-PR Statistical Results

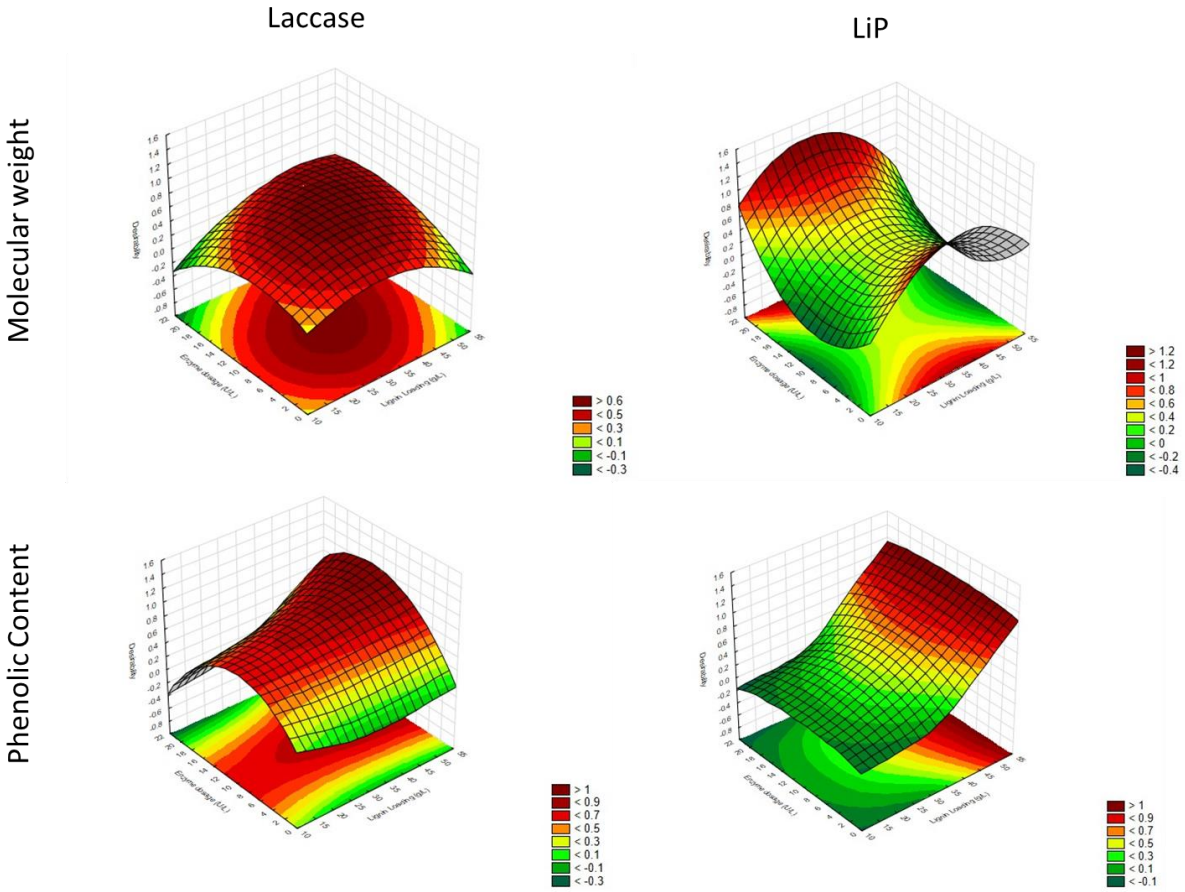

Figure 59: Desirability surface plots for optimum values for the maximization of the molecular weights and the minimization of the phenolic content of NaS-M-PR

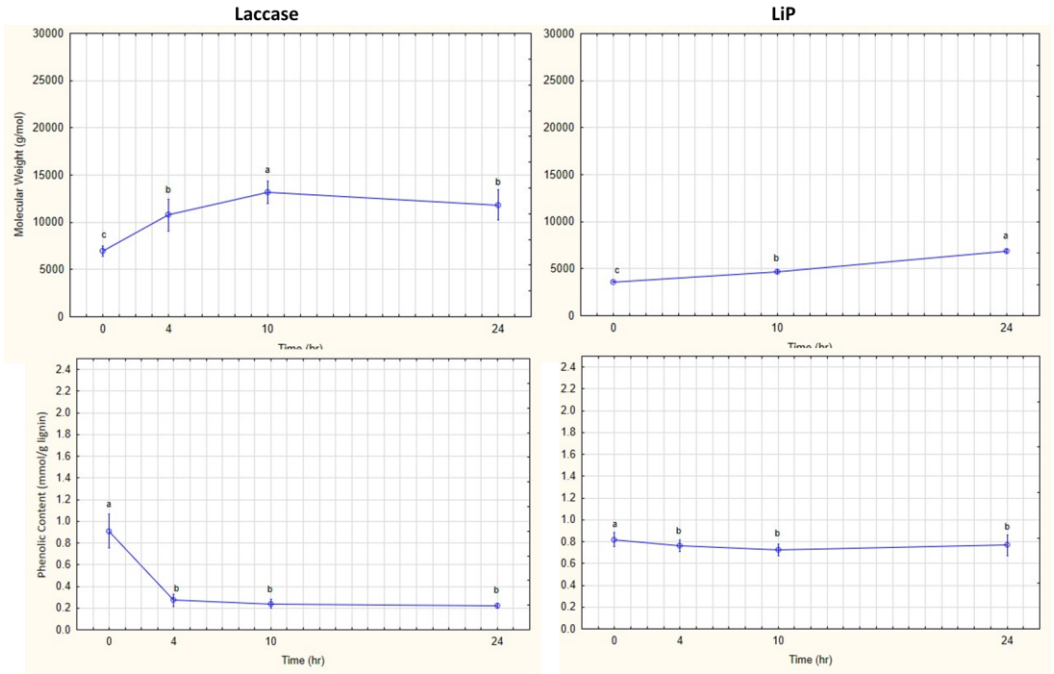

Figure 60: NaS-M-PR LSD test diagrams

6.4.6 *MgO-S-S Statistical Results*

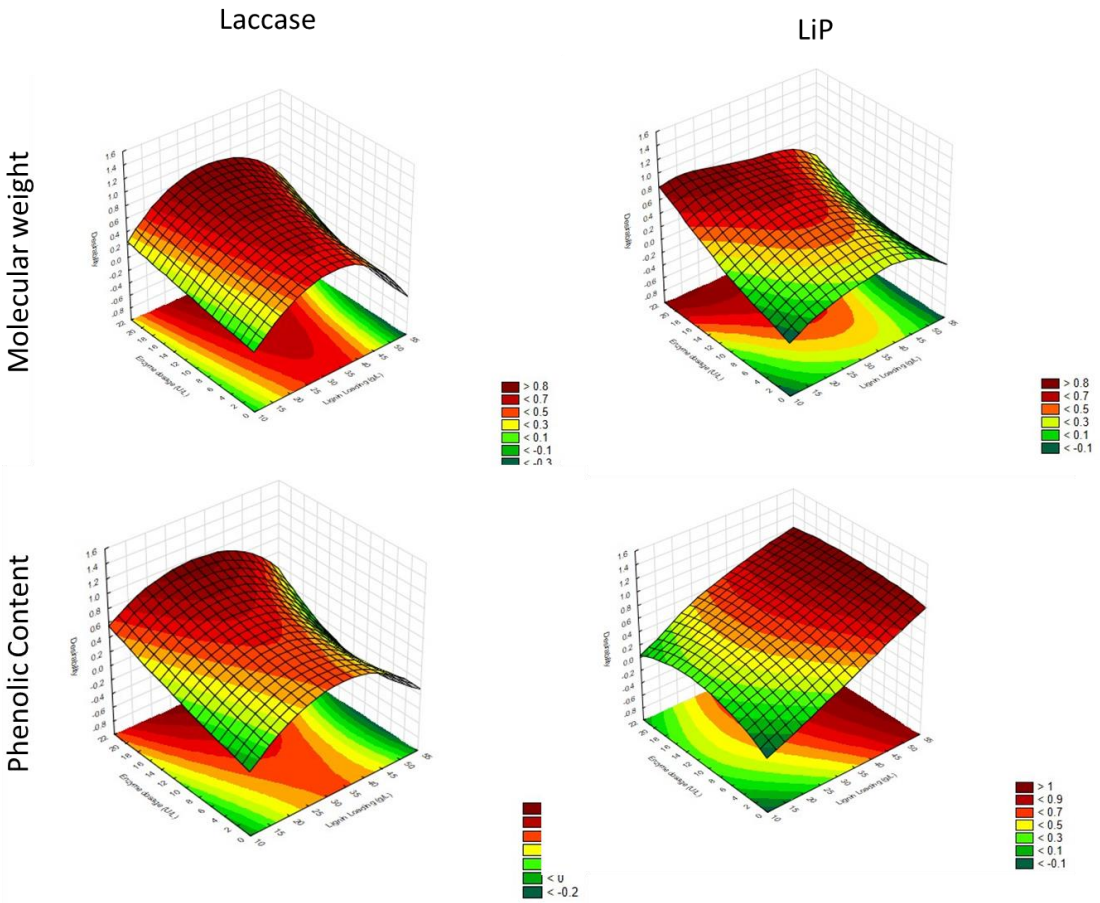

Figure 61: Desirability surface plots for optimum values for the maximization of the molecular weights and the minimization of the phenolic content of MgO-S-S

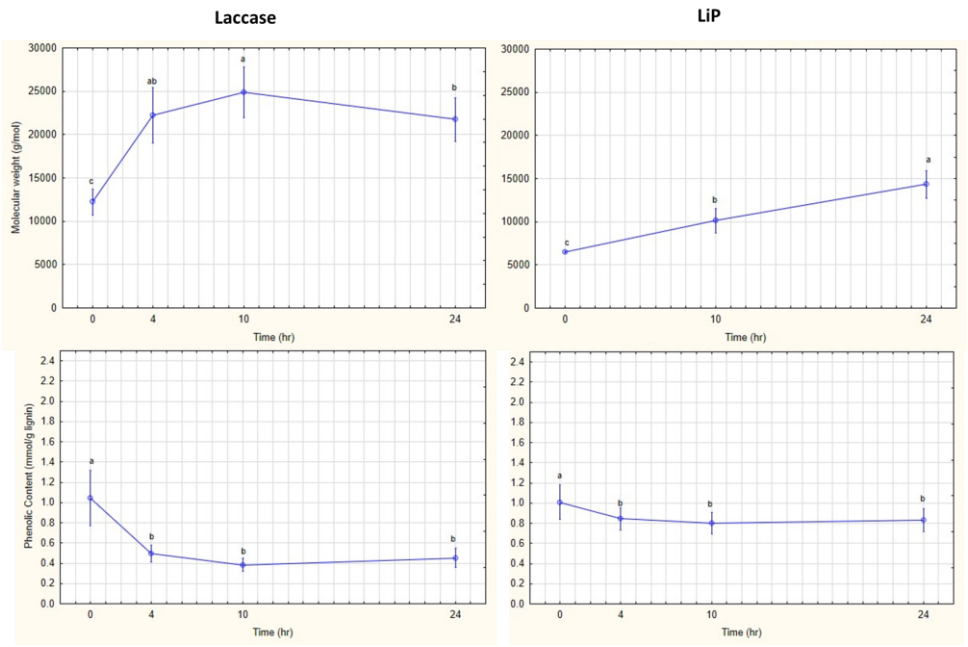

Figure 62: MgO-S-S LSD test diagrams

APPENDIX B: TECHNO-ECONOMIC ANALYSIS

7.1 Equipment Sizing and Specifications

Table 35: Equipment Sizing for all proposed plant Scenarios

Equipment Sizing and Specifications

	Scenario 1 KS-S-N	Scenario 2 KH-S-N	Scenario 3 S-S-S	Scenario 4 NaS-S-T	Scenario 5 NaS-M-Pr	Scenario 6 MgO-S-S
Pumps						
P-100A/B	Kraft liquor	Kraft liquor	Soda Liquor	Na Sulphite liquor	Na Sulphite liquor	Mg Sulphite liquor
Type	Centrifugal	Centrifugal	Centrifugal	Centrifugal	Centrifugal	Centrifugal
Capacity (m3/hr)	0.057	0.064	0.095	0.079	0.142	0.257
Duty (kW)	0.1196	0.1288	0.1675	0.1758	0.2355	0.1570
Inlet flow (kg/hr)	74	80	104	109	146	98
Head (m)	10	10	10	10	10	10
P-101A/B	Water	Water	Water	Water	Water	Water
Type						
Capacity (m3/hr)						
Duty (kW)	9.7711	9.7711	9.7711	0.8270	0.8270	0.8270
Inlet flow (m3/hr)	6.1	6.1	6.1	1.03	1.03	1.03
Head (m)	10	10	10	5	5	5
P-102A/B	Sulphuric Acid	Sulphuric Acid	Sulphuric Acid	Phosphate Buffer	Phosphate Buffer	Phosphate Buffer
Type						
Capacity (m3/hr)						
Duty (kW)	0.2604	0.2604	0.2604	1.0048	1.0048	1.0048
Inlet flow (m3/hr)	0.17	0.17	0.17	1.25	1.25	1.25
Head (m)	5	5	5	5	5	5
P-103A/B	Mixed H2S04 +liq	Mixed H2S04 +liq	Mixed H2S04 +liq	Laccase	Laccase	Laccase
Type						
Capacity (m3/hr)						
Duty (kW)	19.0438	19.0644	19.1569	0.0010	0.0010	0.0018
Inlet flow (m3/hr)	6.31	6.31	6.34	0.001	0.001	0.002
Head (m)	10	10	10	5	5	5
P-104A/B	Water	Water	Water	Treated lignin	Treated lignin	Treated lignin
Type						
Capacity (m3/hr)						
Duty (kW)	0.8270	0.8270	0.8270	2.2147	2.2046	2.2147
Inlet flow (m3/hr)	1.03	1.03	1.03			
Inlet flow (kg/hr)				1377.5	1371.25	1377.5
Head (m)	5	5	5	10	10	10
P-105A/B	Phosphate Buffer	Phosphate Buffer	Phosphate Buffer			
Type						

Capacity (m3/hr)						
Duty (kW)	1.0048	1.0048	1.0048			
Inlet flow (m3/hr)	1.25	1.25	1.25			
Head (m)	5	5	5			
P-106A/B	Sodium hydroxide Soln	Sodium hydroxide Soln	Sodium hydroxide Soln			
Type						
Capacity (m3/hr)						
Duty (kW)	0.0010	0.0010	0.0010			
Inlet flow (kg/hr)	0.00125	0.00125	0.00125			
Head (m)	5	5	5			
P-107A/B	Laccase	Laccase	Laccase			
Type						
Capacity (m3/hr)						
Duty (kW)	0.0005	0.0001	0.0010			
Inlet flow (kg/hr)	0.001	0.000	0.001			
Head (m)	5	5	5			
P-108A/B	Treated Lignin	Treated Lignin	Treated Lignin			
Type						
Capacity (m3/hr)						
Duty (kW)	2.1765	2.1765	2.1765			
Inlet flow (kg/hr)	1354	1354	1354			
Head (m)	10	10	10			

Belt Conveyors

CV-100A/B						
Mass Flowrate (ton/day)	0.496035	0.496035	0.496035	1.157415	0.99207	1.157415
Length (m)	3	3	3	3	3	3
Width (m)	0.5	0.5	0.5	0.5	0.5	0.5
Power (kW)	5.5	5.5	5.5	5.5	5.5	5.5
CV-101A/B						
Mass Flowrate (kg/hr)	110	110	110	110	110	110
Length (m)	3	3	3	3	3	3
Width (m)	0.5	0.5	0.5	0.5	0.5	0.5
Power (kW)	5.5	5.5	5.5	5.5	5.5	
CV-102A/B						
Mass Flowrate (kg/hr)	357	357	357	357	357	357
Length (m)	3	3	3	3	3	3
Width (m)	0.5	0.5	0.5	0.5	0.5	0.5
Power (kW)	5.5	5.5	5.5	5.5	5.5	5.5

Centrifuge

C-100						
Orientation	Horizontal	Horizontal	Horizontal	Horizontal	Horizontal	Horizontal
Diameter (m)	1	1	1	1	1	1

Mixers						
M-100						
Capacity (m3)	100	100	100	30	30	30
Agitator	Propeller	Propeller	Propeller	Propeller	Propeller	Propeller
Residence time (hr.)	24	24	24	-	-	-
M-101						
Capacity (m3)	30	30	30	-	-	-
Agitator	Propeller	Propeller	Propeller	-	-	-
Dryers						
D-100						
Type	Pan	Pan	Pan	Pan	Pan	Pan
Size (m2)	10	10	10	10	10	10
D-101	-	-	-			
Type	-	-	-	Pan	Pan	Pan
Size (m2)	-	-	-	13	14	15
Reaction vessel						
RX-100						
Capacity (m3)	30	30	30	30	30	30
Agitated	Propeller	Propeller	Propeller	Propeller	Propeller	Propeller
Residence time (hr.)	4	4	4	4	4	4
Storage tanks						
Feed Liquor						
Orientation	Vertical	Vertical	Vertical	Vertical	Vertical	Vertical
Capacity (m3)	3	3	3	3	3	3
Removed Flow rate (m3/day)	1.37	1.54	2.27	1.90	3.40	1.58
Residence time (hr.)	24	24	24	24	24	24
Material of Construction	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel
Water Tank						
Orientation	Vertical	Vertical	Vertical	Vertical	Vertical	Vertical
Capacity (m3)	150	150	150	50	50	50
Removed Flow rate (l/day)	170.55	170.55	170.55	24.69	24.69	24.69
Residence time (hr.)	24	24	24	24	24	24
Material of Construction	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel
Sulphuric Acid						
Orientation	Vertical Closed	Vertical Closed	Vertical Closed	-	-	-
Capacity (m3)	5	5	5	-	-	-
Removed Flow rate (l/day)	4140.00	4140.00	4140.00	-	-	-
Residence time (hr)	24	24	24	-	-	-
Material of Construction	Carbon Steel	Carbon Steel	Carbon Steel	-	-	-
Quantity	2	2	2	(Required as a backup)		
Laccase Storage						

Orientation	Vertical Closed	Vertical Closed	Vertical Closed	Vertical Closed	Vertical Closed	Vertical Closed
Capacity (m3)	3	3	3	3	3	3
Removed Flow rate (l/day)	15	3	30	30	30	54
Residence time (hr)	24	24	24	24	24	24
Material of Construction	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel
Sodium phosphate dibasic storage						
Orientation	Vertical	Vertical	Vertical	Vertical	Vertical	Vertical
Capacity (m3)	3	3	3	3	3	3
Removed Flow rate (kg/day)	110	110	110	110	110	110
Residence time (hr)	24	24	24	24	24	24
Material of Construction	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel
Sodium phosphate monobasic storage						
Orientation	Vertical	Vertical	Vertical	Vertical	Vertical	Vertical
Capacity (m3)	3	3	3	3	3	3
Removed Flow rate (kg/day)	357	357	357	357	357	357
Residence time (hr)	24	24	24	24	24	24
Material of Construction	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel	Carbon Steel
Sodium hydroxide Storage						
Orientation	Vertical	Vertical	Vertical	-	-	-
Capacity (m3)	1	1	1	-	-	-
Removed Flow rate (kg/day)	1	1	1	-	-	-
Residence time (hr)	24	24	24	-	-	-
Material of Construction	Carbon Steel	Carbon Steel	Carbon Steel	-	-	-

7.2 Equipment Costs

Table 36: Equipment costing for all processes plant scenarios

Equipment Costs	Scenario 1 KS-S-N			Scenario 2 KH-S-N			Scenario 3 S-S-S		
Equipment	Purchase Cost in 2004	Purchase Cost in 2020	Installation Cost 2020	Purchase Cost in 2004	Purchase Cost in 2020	Installation Cost 2020	Purchase Cost in 2004	Purchase Cost in 2020	Installation Cost 2020
Belt conveyors									
CV-100A/B	R71 463	R96 571.01	R106 228	R71 463	R96 571.01	R106 228	R71 463	R96 571.01	R106 228
CV-101A/B	R71 463	R96 571.01	R106 228	R71 463	R96 571.01	R106 228	R71 463	R96 571.01	R106 228
CV-102A/B	R71 463	R96 571.01	R106 228	R71 463	R96 571.01	R106 228	R71 463	R96 571.01	R106 228
Centrifuges									
C-100	R957 000	R1 293 243.24	R2 974 459	R957 000	R1 293 243.24	R2 974 459	R957 000	R1 293 243.24	R2 974 459
Reaction Vessel									
RX-100	R2 363 471	R3 193 879.18	R6 387 758	R2 363 471	R3 193 879.18	R6 387 758	R2 363 471	R3 193 879.18	R6 387 758
Mixers (Agitators and Tank)									
M-101	R828 472	R1 119 556.92	R1 791 291	R828 472	R1 119 556.92	R1 791 291	R828 472	R1 119 556.92	R1 791 291
M-102	R438 361	R592 379.53	R947 807	R438 361	R592 379.53	R947 807	R438 361	R592 379.53	R947 807
Pan Dryers					R0.00				
D-100	R284 430	R384 364.22	R614 983	R284 430	R384 364.22	R614 983	R284 430	R384 364.22	R614 983
Storage Tanks									
Liquor feed Tank	R76 554	R103 451.36	R268 974	R76 554	R103 451.36	R268 974	R76 554	R103 451.36	R268 974
Water Tank	R967 245	R1 307 088.45	R3 398 430	R967 245	R1 307 088.45	R3 398 430	R967 245	R1 307 088.45	R3 398 430
Sulphuric Acid Tank	R251 359	R339 673.93	R883 152	R251 359	R339 673.93	R883 152	R251 359	R339 673.93	R883 152
Laccase	R92 503	R125 003.73	R325 010	R92 503	R125 003.73	R325 010	R92 503	R125 003.73	R325 010
Sodium phosphate dibasic	R92 503	R125 003.73	R325 010	R92 503	R125 003.73	R325 010	R92 503	R125 003.73	R325 010
Sodium phosphate monobasic	R92 503	R125 003.73	R325 010	R92 503	R125 003.73	R325 010	R92 503	R125 003.73	R325 010
Sodium hydroxide	R47 850	R64 662.16	R168 122	R47 850	R64 662.16	R168 122	R47 850	R64 662.16	R168 122
Pumps									
P-100A/B	R48 706	R65 819.45	R151 385	R47 930	R64 769.77	R148 970	R45 448	R61 416.22	R141 257

P-101A/B	R64 605	R87 304.25	R200 800	R64 605	R87 304.25	R200 800	R64 605	R87 304.25	R200 800
P-102A/B	R42 430	R57 338.38	R131 878	R45 203	R61 084.53	R140 494	R45 203	R61 084.53	R140 494
P-103A/B	R84 522	R114 218.30	R262 702	R55 961	R75 623.47	R173 934	R56 043	R75 733.43	R174 187
P-104A/B	R40 097	R54 184.74	R124 625	R40 097	R54 184.74	R124 625	R40 097	R54 184.74	R124 625
P-105A/B	R40 420	R54 621.72	R125 630	R40 420	R54 621.72	R125 630	R40 420	R54 621.72	R125 630
P-106A/B	R40 740	R55 053.86	R126 624	R40 740	R55 053.86	R126 624	R40 740	R55 053.86	R126 624
P-107A/B	R63 450	R85 742.67	R197 208	R354 311	R478 799.13	R1 101 238	R33 654	R45 477.74	R104 599
P-108A/B	R43 867	R59 279.12	R136 342	R43 867	R59 279.12	R136 342	R43 867	R59 279.13	R136 342
TOTAL			R29 882 469			R31 061 091			R29 620 426

Equipment Costs	Scenario 4 NaS-S-T			Scenario 5 NaS-M-Pr			Scenario 6 MgO-S-S		
Equipment	Purchase Cost in 2004	Purchase Cost in 2020	Installation Cost 2020	Purchase Cost in 2004	Purchase Cost in 2020	Installation Cost 2020	Purchase Cost in 2004	Purchase Cost in 2020	Installation Cost 2020
Belt conveyors									
CV-100A/B	R71 463	R96 571.01	R106 228	R71 463	R96 571.01	R106 228	R71 463	R96 571.01	R106 228
CV-101A/B	R71 463	R96 571.01	R106 228	R71 463	R96 571.01	R106 228	R71 463	R96 571.01	R106 228
CV-102A/B	R71 463	R96 571.01	R106 228	R71 463	R96 571.01	R106 228	R71 463	R96 571.01	R106 228
Reaction Vessel									
RX-100	R2 363 471	R3 193 879.18	R6 387 758	R2 363 471	R3 193 879.18	R6 387 758	R2 363 471	R3 193 879.18	R6 387 758
Mixers (Agitators and Tank)									
M-100	R828 472	R1 119 556.92	R1 791 291	R828 472	R1 119 556.92	R1 791 291	R828 472	R1 119 556.92	R1 791 291
Pan Dryers					R0.00				
D-100	R284 430	R384 364.22	R614 983	R284 430	R384 364.22	R614 983	R112 048	R151 416.21	R242 266
D-101	R284 430	R384 364.22	R614 983	R284 430	R384 364.22	R614 983	R284 430	R384 364.22	R614 983
Storage Tanks									

Liquor feed Tank	R76 554	R103 451.36	R268 974	R76 554	R103 451.36	R268 974	R76 554	R103 451.36	R268 974
Water Tank	R967 245	R1 307 088.45	R3 398 430	R967 245	R1 307 088.45	R3 398 430	R967 245	R1 307 088.45	R3 398 430
Laccase	R92 503	R125 003.73	R325 010	R92 503	R125 003.73	R325 010	R92 503	R125 003.73	R325 010
Sodium phosphate dibasic	R92 503	R125 003.73	R325 010	R92 503	R125 003.73	R325 010	R92 503	R125 003.73	R325 010
Sodium phosphate monobasic	R92 503	R125 003.73	R325 010	R92 503	R125 003.73	R325 010	R92 503	R125 003.73	R325 010
Pumps									
P-100A/B	R45 047	R60 873.70	R140 010	R43 001	R58 109.39	R133 652	R46 007	R62 171.65	R142 995
P-101A/B	R40 097	R54 184.74	R124 625	R40 097	R54 184.74	R124 625	R40 097	R54 184.74	R124 625
P-102A/B	R41 032	R55 448.66	R127 532	R40 420	R54 621.72	R125 630	R40 420	R54 621.72	R125 630
P-103A/B	R33 654	R45 477.74	R104 599	R33 654	R45 477.74	R104 599	R20 670	R27 932.06	R64 244
P-104A/B	R43 956	R59 399.77	R136 619	R43 956	R59 399.77	R136 619	R43 988	R59 442.88	R136 719
TOTAL			R22 436 330			R22 424 479			R21 774 461

7.3 NPV Determinations

Table 37: NPV determination for Scenario 1 KS-S-N and Scenario 2 KH-S-N, respectively

	Capital				Cash Flow			Tax						Discounted Profitability Criteria			
								Depreciation			Payable on Profit						
Year	Plant	Land	Working Capital	Total Capital	Sales	Production Costs	Cash Flow	Plant	Buildings	Total	Profit: Previous Year before Tax	Tax	After Tax Cash Flow	Project Cash Flow	Cumulative Cash Flow	Project Discounted Cash Flow	Project DCFROR
0	-R 56 202 948	R 0	-R 2 810 147	-R 59 013 095	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0	-R 59 013 095	-R 59 013 095	-R 59 013 095	-R 59 013 095
1	R 0	R 0	R 0	R 0	R 17 066 779	-R 52 246 150	-R 35 179 371	R11 240 590	R 0	R 11 240 590	-R 11 240 590	R 0	-R 35 179 371	-R 35 179 371	-R 94 192 467	-R 30 590 758	-R 28 143 497
2	R 0	R 0	R 0	R 0	R 19 504 890	-R 59 709 886	-R 40 204 996	R 11 240 590	R 0	R 11 240 590	-R 46 419 961	R 0	-R 40 204 996	-R 40 204 996	-R 134 397 462	-R 30 400 753	-R 25 731 197
3	R 0	R 0	R 0	R 0	R 21 943 001	-R 67 173 621	-R 45 230 620	R 11 240 590	R 0	R 11 240 590	-R 51 445 585	R 0	-R 45 230 620	-R 45 230 620	-R 179 628 083	-R 29 739 867	-R 23 158 078
4	R 0	R 0	R 0	R 0	R 24 381 112	-R 74 637 357	-R 50 256 245	R 11 240 590	R 0	R 11 240 590	-R 56 471 210	R 0	-R 50 256 245	-R 50 256 245	-R 229 884 327	-R 28 734 171	-R 20 584 958
5	R 0	R 0	R 0	R 0	R 25 388 052	-R 77 719 880	-R 52 331 828	R 11 240 590	R 0	R 11 240 590	-R 61 496 834	R 0	-R 52 331 828	-R 52 331 828	-R 282 216 155	-R 26 018 167	-R 17 148 093
6	R 0	R 0	R 0	R 0	R 26 436 579	-R 80 929 711	-R 54 493 132	R 0	R 0	R 0	-R 52 331 828	R 0	-R 54 493 132	-R 54 493 132	-R 336 709 287	-R 23 558 885	-R 14 285 048
7	R 0	R 0	R 0	R 0	R 27 528 410	-R 84 272 108	-R 56 743 698	R 0	R 0	R 0	-R 54 493 132	R 0	-R 56 743 698	-R 56 743 698	-R 393 452 985	-R 21 332 058	-R 11 900 016
8	R 0	R 0	R 0	R 0	R 28 665 333	-R 87 752 546	-R 59 087 213	R 0	R 0	R 0	-R 56 743 698	R 0	-R 59 087 213	-R 59 087 213	-R 452 540 199	-R 19 315 715	-R 9 913 189
9	R 0	R 0	R 0	R 0	R 29 849 211	-R 91 376 726	-R 61 527 515	R 0	R 0	R 0	-R 59 087 213	R 0	-R 61 527 515	-R 61 527 515	-R 514 067 714	-R 17 489 960	-R 8 258 083
10	R 0	R 0	R 0	R 0	R 31 081 983	-R 95 150 585	-R 64 068 601	R 0	R 0	R 0	-R 61 527 515	R 0	-R 64 068 601	-R 64 068 601	-R 578 136 315	-R 15 836 778	-R 6 879 314
11	R 0	R 0	R 0	R 0	R 32 365 669	-R 99 080 304	-R 66 714 635	R 0	R 0	R 0	-R 64 068 601	R 0	-R 66 714 635	-R 66 714 635	-R 644 850 950	-R 14 339 859	-R 5 730 743
12	R 0	R 0	R 0	R 0	R 33 702 372	-R 103 172 321	-R 69 469 949	R 0	R 0	R 0	-R 66 714 635	R 0	-R 69 469 949	-R 69 469 949	-R 714 320 899	-R 12 984 430	-R 4 773 939
13	R 0	R 0	R 0	R 0	R 35 094 280	-R 107 433 338	-R 72 339 058	R 0	R 0	R 0	-R 69 469 949	R 0	-R 72 339 058	-R 72 339 058	-R 786 659 957	-R 11 757 119	-R 3 976 882
14	R 0	R 0	R 0	R 0	R 36 543 673	-R 111 870 334	-R 75 326 661	R 0	R 0	R 0	-R 72 339 058	R 0	-R 75 326 661	-R 75 326 661	-R 861 986 618	-R 10 645 816	-R 3 312 902
15	R 0	R 0	R 0	R 0	R 38 052 927	-R 116 490 579	-R 78 437 652	R 0	R 0	R 0	-R 75 326 661	R 0	-R 78 437 652	-R 78 437 652	-R 940 424 270	-R 9 639 555	-R 2 759 780
16	R 0	R 0	R 0	R 0	R 39 624 513	-R 121 301 640	-R 81 677 127	R 0	R 0	R 0	-R 78 437 652	R 0	-R 81 677 127	-R 81 677 127	-R 1 022 101 397	-R 8 728 407	-R 2 299 007
17	R 0	R 0	R 0	R 0	R 41 261 005	-R 126 311 398	-R 85 050 393	R 0	R 0	R 0	-R 81 677 127	R 0	-R 85 050 393	-R 85 050 393	-R 1 107 151 790	-R 7 903 383	-R 1 915 165
18	R 0	R 0	R 0	R 0	R 42 965 085	-R 131 528 059	-R 88 562 974	R 0	R 0	R 0	-R 85 050 393	R 0	-R 88 562 974	-R 88 562 974	-R 1 195 714 764	-R 7 156 342	-R 1 595 409
19	R 0	R 0	R 0	R 0	R 44 739 543	-R 136 960 167	-R 92 220 625	R 0	R 0	R 0	-R 88 562 974	R 0	-R 92 220 625	-R 92 220 625	-R 1 287 935 388	-R 6 479 912	-R 1 329 039
20	R 0	R 0	R 0	R 0	R 46 587 286	-R 142 616 622	-R 96 029 336	R 0	R 0	R 0	-R 92 220 625	R 0	-R 96 029 336	-R 96 029 336	-R 1 383 964 725	-R 5 867 419	-R 1 107 143
															NVP	-R 397 532 449	-R 253 814 575

	Capital				Cash Flow			Tax						Discounted Profitability Criteria			
								Depreciation			Payable on Profit						
Year	Plant	Land	Working Capital	Total Capital	Sales	Production Costs	Cash Flow	Plant	Buildings	Total	Profit: Previous Year before Tax	Tax	After Tax Cash Flow	Project Cash Flow	Cumulative Cash Flow	Project Discounted Cash Flow	Project DCFROR
0	-R 55 972 678	R 0	-R 2 798 634	-R 58 771 312	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0	-R 58 771 312	-R 58 771 312	-R 58 771 312	-R 58 771 312
1	R 0	R 0	R 0	R 0	R 17 066 779	-R 49 654 015	-R 32 587 236	R11 194 536	R 0	R 11 194 536	-R 11 194 536	R 0	-R 32 587 236	-R 32 587 236	-R 91 358 548	-R 28 336 727	-R 26 069 789
2	R 0	R 0	R 0	R 0	R 19 504 890	-R 56 747 445	-R 37 242 556	R 11 194 536	R 0	R 11 194 536	-R 43 781 772	R 0	-R 37 242 556	-R 37 242 556	-R 128 601 104	-R 28 160 723	-R 23 835 236
3	R 0	R 0	R 0	R 0	R 21 943 001	-R 63 840 876	-R 41 897 875	R 11 194 536	R 0	R 11 194 536	-R 48 437 091	R 0	-R 41 897 875	-R 41 897 875	-R 170 498 979	-R 27 548 533	-R 21 451 712
4	R 0	R 0	R 0	R 0	R 24 381 112	-R 70 934 307	-R 46 553 194	R 11 194 536	R 0	R 11 194 536	-R 53 092 411	R 0	-R 46 553 194	-R 46 553 194	-R 217 052 173	-R 26 616 940	-R 19 068 188
5	R 0	R 0	R 0	R 0	R 25 388 052	-R 73 863 894	-R 48 475 841	R 11 194 536	R 0	R 11 194 536	-R 57 747 730	R 0	-R 48 475 841	-R 48 475 841	-R 265 528 015	-R 24 101 061	-R 15 884 564
6	R 0	R 0	R 0	R 0	R 26 436 579	-R 76 914 472	-R 50 477 894	R 0	R 0	R 0	-R 48 475 841	R 0	-R 50 477 894	-R 50 477 894	-R 316 005 908	-R 21 822 986	-R 13 232 477
7	R 0	R 0	R 0	R 0	R 27 528 410	-R 80 091 040	-R 52 562 631	R 0	R 0	R 0	-R 50 477 894	R 0	-R 52 562 631	-R 52 562 631	-R 368 568 539	-R 19 760 240	-R 11 023 183
8	R 0	R 0	R 0	R 0	R 28 665 333	-R 83 398 800	-R 54 733 467	R 0	R 0	R 0	-R 52 562 631	R 0	-R 54 733 467	-R 54 733 467	-R 423 302 006	-R 17 892 468	-R 9 182 752
9	R 0	R 0	R 0	R 0	R 29 849 211	-R 86 843 170	-R 56 993 959	R 0	R 0	R 0	-R 54 733 467	R 0	-R 56 993 959	-R 56 993 959	-R 480 295 965	-R 16 201 240	-R 7 649 600
10	R 0	R 0	R 0	R 0	R 31 081 983	-R 90 429 793	-R 59 347 810	R 0	R 0	R 0	-R 56 993 959	R 0	-R 59 347 810	-R 59 347 810	-R 539 643 775	-R 14 669 871	-R 6 372 423
11	R 0	R 0	R 0	R 0	R 32 365 669	-R 94 164 544	-R 61 798 874	R 0	R 0	R 0	-R 59 347 810	R 0	-R 61 798 874	-R 61 798 874	-R 601 442 650	-R 13 283 249	-R 5 308 483
12	R 0	R 0	R 0	R 0	R 33 702 372	-R 98 053 540	-R 64 351 168	R 0	R 0	R 0	-R 61 798 874	R 0	-R 64 351 168	-R 64 351 168	-R 665 793 818	-R 12 027 693	-R 4 422 179
13	R 0	R 0	R 0	R 0	R 35 094 280	-R 102 103 151	-R 67 008 871	R 0	R 0	R 0	-R 64 351 168	R 0	-R 67 008 871	-R 67 008 871	-R 732 802 689	-R 10 890 815	-R 3 683 852
14	R 0	R 0	R 0	R 0	R 36 543 673	-R 106 320 011	-R 69 776 338	R 0	R 0	R 0	-R 67 008 871	R 0	-R 69 776 338	-R 69 776 338	-R 802 579 027	-R 9 861 396	-R 3 068 796
15	R 0	R 0	R 0	R 0	R 38 052 927	-R 110 711 027	-R 72 658 100	R 0	R 0	R 0	-R 69 776 338	R 0	-R 72 658 100	-R 72 658 100	-R 875 237 127	-R 8 929 280	-R 2 556 430
16	R 0	R 0	R 0	R 0	R 39 624 513	-R 115 283 393	-R 75 658 880	R 0	R 0	R 0	-R 72 658 100	R 0	-R 75 658 880	-R 75 658 880	-R 950 896 007	-R 8 085 269	-R 2 129 608
17	R 0	R 0	R 0	R 0	R 41 261 005	-R 120 044 597	-R 78 783 592	R 0	R 0	R 0	-R 75 658 880	R 0	-R 78 783 592	-R 78 783 592	-R 1 029 679 598	-R 7 321 035	-R 1 774 049
18	R 0	R 0	R 0	R 0	R 42 965 085	-R 125 002 439	-R 82 037 354	R 0	R 0	R 0	-R 78 783 592	R 0	-R 82 037 354	-R 82 037 354	-R 1 111 716 952	-R 6 629 038	-R 1 477 854
19	R 0	R 0	R 0	R 0	R 44 739 543	-R 130 165 039	-R 85 425 497	R 0	R 0	R 0	-R 82 037 354	R 0	-R 85 425 497	-R 85 425 497	-R 1 197 142 449	-R 6 002 450	-R 1 231 111
20	R 0	R 0	R 0	R 0	R 46 587 286	-R 135 540 856	-R 88 953 570	R 0	R 0	R 0	-R 85 425 497	R 0	-R 88 953 570	-R 88 953 570	-R 1 286 096 019	-R 5 435 088	-R 1 025 565
															NVP	-R 372 347 414	-R 239 219 160

Table 38: NPV determinations for Scenario 3 S-S-S and Scenario NaS-S-T, respectively

	Capital				Cash Flow			Tax						Discounted Profitability Criteria			
								Depreciation			Payable on Profit						
Year	Plant	Land	Working Capital	Total Capital	Sales	Production Costs	Cash Flow	Plant	Buildings	Total	Profit: Previous Year before Tax	Tax	After Tax Cash Flow	Project Cash Flow	Cumulative Cash Flow	Project Discounted Cash Flow	Project DCFROR
0	-R 55 710 098	R 0	-R 2 785 505	-R 58 495 603	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0	-R 58 495 603	-R 58 495 603	-R 58 495 603	-R 58 495 603
1	R 0	R 0	R 0	R 0	R 17 066 779	-R 55 466 268	-R 38 399 490	R11 142 020	R 0	R 11 142 020	-R 11 142 020	R 0	-R 38 399 490	-R 38 399 490	-R 96 895 093	-R 33 390 861	-R 30 719 592
2	R 0	R 0	R 0	R 0	R 19 504 890	-R 63 390 021	-R 43 885 131	R 11 142 020	R 0	R 11 142 020	-R 49 541 509	R 0	-R 43 885 131	-R 43 885 131	-R 140 780 224	-R 33 183 464	-R 28 086 484
3	R 0	R 0	R 0	R 0	R 21 943 001	-R 71 313 774	-R 49 370 772	R 11 142 020	R 0	R 11 142 020	-R 55 027 151	R 0	-R 49 370 772	-R 49 370 772	-R 190 150 996	-R 32 462 084	-R 25 277 835
4	R 0	R 0	R 0	R 0	R 24 381 112	-R 79 237 526	-R 54 856 414	R 11 142 020	R 0	R 11 142 020	-R 60 512 792	R 0	-R 54 856 414	-R 54 856 414	-R 245 007 410	-R 31 364 333	-R 22 469 187
5	R 0	R 0	R 0	R 0	R 25 388 052	-R 82 510 036	-R 57 121 984	R 11 142 020	R 0	R 11 142 020	-R 65 998 433	R 0	-R 57 121 984	-R 57 121 984	-R 302 129 394	-R 28 399 721	-R 18 717 732
6	R 0	R 0	R 0	R 0	R 26 436 579	-R 85 917 700	-R 59 481 122	R 0	R 0	R 0	-R 57 121 984	R 0	-R 59 481 122	-R 59 481 122	-R 361 610 515	-R 25 715 330	-R 15 592 619
7	R 0	R 0	R 0	R 0	R 27 528 410	-R 89 466 102	-R 61 937 692	R 0	R 0	R 0	-R 59 481 122	R 0	-R 61 937 692	-R 61 937 692	-R 423 548 207	-R 23 284 673	-R 12 989 275
8	R 0	R 0	R 0	R 0	R 28 665 333	-R 93 161 051	-R 64 495 719	R 0	R 0	R 0	-R 61 937 692	R 0	-R 64 495 719	-R 64 495 719	-R 488 043 926	-R 21 083 765	-R 10 820 586
9	R 0	R 0	R 0	R 0	R 29 849 211	-R 97 008 603	-R 67 159 392	R 0	R 0	R 0	-R 64 495 719	R 0	-R 67 159 392	-R 67 159 392	-R 555 203 318	-R 19 090 891	-R 9 013 981
10	R 0	R 0	R 0	R 0	R 31 081 983	-R 101 015 058	-R 69 933 075	R 0	R 0	R 0	-R 67 159 392	R 0	-R 69 933 075	-R 69 933 075	-R 625 136 393	-R 17 286 387	-R 7 509 007
11	R 0	R 0	R 0	R 0	R 32 365 669	-R 105 186 980	-R 72 821 311	R 0	R 0	R 0	-R 69 933 075	R 0	-R 72 821 311	-R 72 821 311	-R 697 957 703	-R 15 652 447	-R 6 255 303
12	R 0	R 0	R 0	R 0	R 33 702 372	-R 109 531 202	-R 75 828 831	R 0	R 0	R 0	-R 72 821 311	R 0	-R 75 828 831	-R 75 828 831	-R 773 786 534	-R 14 172 951	-R 5 210 918
13	R 0	R 0	R 0	R 0	R 35 094 280	-R 114 054 841	-R 78 960 562	R 0	R 0	R 0	-R 75 828 831	R 0	-R 78 960 562	-R 78 960 562	-R 852 747 096	-R 12 833 299	-R 4 340 903
14	R 0	R 0	R 0	R 0	R 36 543 673	-R 118 765 306	-R 82 221 633	R 0	R 0	R 0	-R 78 960 562	R 0	-R 82 221 633	-R 82 221 633	-R 934 968 728	-R 11 620 273	-R 3 616 146
15	R 0	R 0	R 0	R 0	R 38 052 927	-R 123 670 313	-R 85 617 386	R 0	R 0	R 0	-R 82 221 633	R 0	-R 85 617 386	-R 85 617 386	-R 1 020 586 115	-R 10 521 905	-R 3 012 394
16	R 0	R 0	R 0	R 0	R 39 624 513	-R 128 777 897	-R 89 153 384	R 0	R 0	R 0	-R 85 617 386	R 0	-R 89 153 384	-R 89 153 384	-R 1 109 739 499	-R 9 527 356	-R 2 509 445
17	R 0	R 0	R 0	R 0	R 41 261 005	-R 134 096 424	-R 92 835 419	R 0	R 0	R 0	-R 89 153 384	R 0	-R 92 835 419	-R 92 835 419	-R 1 202 574 918	-R 8 626 814	-R 2 090 468
18	R 0	R 0	R 0	R 0	R 42 965 085	-R 139 634 607	-R 96 669 522	R 0	R 0	R 0	-R 92 835 419	R 0	-R 96 669 522	-R 96 669 522	-R 1 299 244 440	-R 7 811 392	-R 1 741 443
19	R 0	R 0	R 0	R 0	R 44 739 543	-R 145 401 516	-R 100 661 973	R 0	R 0	R 0	-R 96 669 522	R 0	-R 100 661 973	-R 100 661 973	-R 1 399 906 413	-R 7 073 046	-R 1 450 692
20	R 0	R 0	R 0	R 0	R 46 587 286	-R 151 406 598	-R 104 819 313	R 0	R 0	R 0	-R 100 661 973	R 0	-R 104 819 313	-R 104 819 313	-R 1 504 725 725	-R 6 404 489	-R 1 208 484
															NVP	-R 428 001 082	-R 271 128 096

	Capital				Cash Flow			Tax						Discounted Profitability Criteria			
								Depreciation			Payable on Profit						
Year	Plant	Land	Working Capital	Total Capital	Sales	Production Costs	Cash Flow	Plant	Buildings	Total	Profit: Previous Year before Tax	Tax	After Tax Cash Flow	Project Cash Flow	Cumulative Cash Flow	Project Discounted Cash Flow	Project DCFROR
0	-R 42 191 888	R 0	-R 2 109 594	-R 44 301 483	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0	-R 44 301 483	-R 44 301 483	-R 44 301 483	-R 44 301 483
1	R 0	R 0	R 0	R 0	R 36 978 020	-R 38 376 088	-R 1 398 068	R8 438 378	R 0	R 8 438 378	-R 8 438 378	R 0	-R 1 398 068	-R 1 398 068	-R 45 699 550	-R 1 215 711	-R 1 118 454
2	R 0	R 0	R 0	R 0	R 42 260 595	-R 43 858 386	-R 1 597 792	R 8 438 378	R 0	R 8 438 378	-R 9 836 445	R 0	-R 1 597 792	-R 1 597 792	-R 47 297 342	-R 1 208 160	-R 1 022 587
3	R 0	R 0	R 0	R 0	R 47 543 169	-R 49 340 685	-R 1 797 516	R 8 438 378	R 0	R 8 438 378	-R 10 036 169	R 0	-R 1 797 516	-R 1 797 516	-R 49 094 857	-R 1 181 896	-R 920 328
4	R 0	R 0	R 0	R 0	R 52 825 743	-R 54 822 983	-R 1 997 239	R 8 438 378	R 0	R 8 438 378	-R 10 235 893	R 0	-R 1 997 239	-R 1 997 239	-R 51 092 097	-R 1 141 928	-R 818 069
5	R 0	R 0	R 0	R 0	R 55 007 447	-R 57 087 172	-R 2 079 725	R 8 438 378	R 0	R 8 438 378	-R 10 435 617	R 0	-R 2 079 725	-R 2 079 725	-R 53 171 822	-R 1 033 991	-R 681 484
6	R 0	R 0	R 0	R 0	R 57 279 254	-R 59 444 872	-R 2 165 618	R 0	R 0	R 0	-R 2 079 725	R 0	-R 2 165 618	-R 2 165 618	-R 55 337 440	-R 936 256	-R 567 704
7	R 0	R 0	R 0	R 0	R 59 644 887	-R 61 899 945	-R 2 255 058	R 0	R 0	R 0	-R 2 165 618	R 0	-R 2 255 058	-R 2 255 058	-R 57 592 499	-R 847 760	-R 472 920
8	R 0	R 0	R 0	R 0	R 62 108 221	-R 64 456 413	-R 2 348 192	R 0	R 0	R 0	-R 2 255 058	R 0	-R 2 348 192	-R 2 348 192	-R 59 940 691	-R 767 628	-R 393 961
9	R 0	R 0	R 0	R 0	R 64 673 291	-R 67 118 463	-R 2 445 172	R 0	R 0	R 0	-R 2 348 192	R 0	-R 2 445 172	-R 2 445 172	-R 62 385 863	-R 695 071	-R 328 185
10	R 0	R 0	R 0	R 0	R 67 344 298	-R 69 890 456	-R 2 546 158	R 0	R 0	R 0	-R 2 445 172	R 0	-R 2 546 158	-R 2 546 158	-R 64 932 021	-R 629 371	-R 273 392
11	R 0	R 0	R 0	R 0	R 70 125 617	-R 72 776 931	-R 2 651 314	R 0	R 0	R 0	-R 2 546 158	R 0	-R 2 651 314	-R 2 651 314	-R 67 583 335	-R 569 882	-R 227 746
12	R 0	R 0	R 0	R 0	R 73 021 805	-R 75 782 619	-R 2 760 814	R 0	R 0	R 0	-R 2 651 314	R 0	-R 2 760 814	-R 2 760 814	-R 70 344 149	-R 516 016	-R 189 722
13	R 0	R 0	R 0	R 0	R 76 037 606	-R 78 912 441	-R 2 874 835	R 0	R 0	R 0	-R 2 760 814	R 0	-R 2 874 835	-R 2 874 835	-R 73 218 984	-R 467 241	-R 158 046
14	R 0	R 0	R 0	R 0	R 79 177 959	-R 82 171 525	-R 2 993 566	R 0	R 0	R 0	-R 2 874 835	R 0	-R 2 993 566	-R 2 993 566	-R 76 212 550	-R 423 077	-R 131 658
15	R 0	R 0	R 0	R 0	R 82 448 008	-R 85 565 209	-R 3 117 200	R 0	R 0	R 0	-R 2 993 566	R 0	-R 3 117 200	-R 3 117 200	-R 79 329 750	-R 383 087	-R 109 677
16	R 0	R 0	R 0	R 0	R 85 853 111	-R 89 099 052	-R 3 245 941	R 0	R 0	R 0	-R 3 117 200	R 0	-R 3 245 941	-R 3 245 941	-R 82 575 690	-R 346 877	-R 91 365
17	R 0	R 0	R 0	R 0	R 89 398 845	-R 92 778 843	-R 3 379 998	R 0	R 0	R 0	-R 3 245 941	R 0	-R 3 379 998	-R 3 379 998	-R 85 955 688	-R 314 089	-R 76 111
18	R 0	R 0	R 0	R 0	R 93 091 017	-R 96 610 609	-R 3 519 592	R 0	R 0	R 0	-R 3 379 998	R 0	-R 3 519 592	-R 3 519 592	-R 89 475 280	-R 284 401	-R 63 403
19	R 0	R 0	R 0	R 0	R 96 935 676	-R 100 600 627	-R 3 664 951	R 0	R 0	R 0	-R 3 519 592	R 0	-R 3 664 951	-R 3 664 951	-R 93 140 231	-R 257 519	-R 52 818
20	R 0	R 0	R 0	R 0	R 100 939 119	-R 104 755 433	-R 3 816 313	R 0	R 0	R 0	-R 3 664 951	R 0	-R 3 816 313	-R 3 816 313	-R 96 956 544	-R 233 178	-R 43 999
															NVP	-R 57 754 621	-R 52 043 112

Table 39: NPV determinations for Scenario 5 NaS-M-PR and Scenario 6 MgO-S-S, respectively

	Capital				Cash Flow			Tax						Discounted Profitability Criteria			
								Depreciation			Payable on Profit						
Year	*Plant*	*Land*	*Working Capital*	*Total Capital*	*Sales*	*Production Costs*	*Cash Flow*	*Plant*	*Buildings*	*Total*	*Profit: Previous Year before Tax*	*Tax*	*After Tax Cash Flow*	*Project Cash Flow*	*Cumulative Cash Flow*	*Project Discounted Cash Flow*	*Project DCFROR*
0	-R 42 160 570	R 0	-R 2 108 029	-R 44 268 599	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0	-R 44 268 599	-R 44 268 599	-R 44 268 599	-R 44 268 599
1	R 0	R 0	R 0	R 0	R 36 978 020	-R 38 379 982	-R 1 401 962	R8 432 114	R 0	R 8 432 114	-R 8 432 114	R 0	-R 1 401 962	-R 1 401 962	-R 45 670 560	-R 1 219 097	-R 1 121 569
2	R 0	R 0	R 0	R 0	R 42 260 595	-R 43 862 836	-R 1 602 242	R 8 432 114	R 0	R 8 432 114	-R 9 834 076	R 0	-R 1 602 242	-R 1 602 242	-R 47 272 802	-R 1 211 525	-R 1 025 435
3	R 0	R 0	R 0	R 0	R 47 543 169	-R 49 345 691	-R 1 802 522	R 8 432 114	R 0	R 8 432 114	-R 10 034 356	R 0	-R 1 802 522	-R 1 802 522	-R 49 075 324	-R 1 185 187	-R 922 891
4	R 0	R 0	R 0	R 0	R 52 825 743	-R 54 828 546	-R 2 002 802	R 8 432 114	R 0	R 8 432 114	-R 10 234 636	R 0	-R 2 002 802	-R 2 002 802	-R 51 078 127	-R 1 145 109	-R 820 348
5	R 0	R 0	R 0	R 0	R 55 007 447	-R 57 092 965	-R 2 085 518	R 8 432 114	R 0	R 8 432 114	-R 10 434 916	R 0	-R 2 085 518	-R 2 085 518	-R 53 163 644	-R 1 036 871	-R 683 383
6	R 0	R 0	R 0	R 0	R 57 279 254	-R 59 450 904	-R 2 171 650	R 0	R 0	R 0	-R 2 085 518	R 0	-R 2 171 650	-R 2 171 650	-R 55 335 294	-R 938 864	-R 569 285
7	R 0	R 0	R 0	R 0	R 59 644 887	-R 61 906 226	-R 2 261 339	R 0	R 0	R 0	-R 2 171 650	R 0	-R 2 261 339	-R 2 261 339	-R 57 596 633	-R 850 121	-R 474 237
8	R 0	R 0	R 0	R 0	R 62 108 221	-R 64 462 953	-R 2 354 732	R 0	R 0	R 0	-R 2 261 339	R 0	-R 2 354 732	-R 2 354 732	-R 59 951 366	-R 769 766	-R 395 059
9	R 0	R 0	R 0	R 0	R 64 673 291	-R 67 125 273	-R 2 451 983	R 0	R 0	R 0	-R 2 354 732	R 0	-R 2 451 983	-R 2 451 983	-R 62 403 348	-R 697 007	-R 329 100
10	R 0	R 0	R 0	R 0	R 67 344 298	-R 69 897 547	-R 2 553 250	R 0	R 0	R 0	-R 2 451 983	R 0	-R 2 553 250	-R 2 553 250	-R 64 956 598	-R 631 124	-R 274 153
11	R 0	R 0	R 0	R 0	R 70 125 617	-R 72 784 316	-R 2 658 699	R 0	R 0	R 0	-R 2 553 250	R 0	-R 2 658 699	-R 2 658 699	-R 67 615 297	-R 571 469	-R 228 380
12	R 0	R 0	R 0	R 0	R 73 021 805	-R 75 790 308	-R 2 768 503	R 0	R 0	R 0	-R 2 658 699	R 0	-R 2 768 503	-R 2 768 503	-R 70 383 800	-R 517 453	-R 190 250
13	R 0	R 0	R 0	R 0	R 76 037 606	-R 78 920 448	-R 2 882 842	R 0	R 0	R 0	-R 2 768 503	R 0	-R 2 882 842	-R 2 882 842	-R 73 266 642	-R 468 542	-R 158 486
14	R 0	R 0	R 0	R 0	R 79 177 959	-R 82 179 862	-R 3 001 904	R 0	R 0	R 0	-R 2 882 842	R 0	-R 3 001 904	-R 3 001 904	-R 76 268 546	-R 424 255	-R 132 025
15	R 0	R 0	R 0	R 0	R 82 448 008	-R 85 573 891	-R 3 125 882	R 0	R 0	R 0	-R 3 001 904	R 0	-R 3 125 882	-R 3 125 882	-R 79 394 428	-R 384 154	-R 109 982
16	R 0	R 0	R 0	R 0	R 85 853 111	-R 89 108 092	-R 3 254 981	R 0	R 0	R 0	-R 3 125 882	R 0	-R 3 254 981	-R 3 254 981	-R 82 649 410	-R 347 843	-R 91 620
17	R 0	R 0	R 0	R 0	R 89 398 845	-R 92 788 257	-R 3 389 412	R 0	R 0	R 0	-R 3 254 981	R 0	-R 3 389 412	-R 3 389 412	-R 86 038 822	-R 314 964	-R 76 323
18	R 0	R 0	R 0	R 0	R 93 091 017	-R 96 620 412	-R 3 529 395	R 0	R 0	R 0	-R 3 389 412	R 0	-R 3 529 395	-R 3 529 395	-R 89 568 216	-R 285 193	-R 63 580
19	R 0	R 0	R 0	R 0	R 96 935 676	-R 100 610 835	-R 3 675 159	R 0	R 0	R 0	-R 3 529 395	R 0	-R 3 675 159	-R 3 675 159	-R 93 243 375	-R 258 236	-R 52 965
20	R 0	R 0	R 0	R 0	R 100 939 119	-R 104 766 062	-R 3 826 943	R 0	R 0	R 0	-R 3 675 159	R 0	-R 3 826 943	-R 3 826 943	-R 97 070 318	-R 233 827	-R 44 122
															NVP	-R 57 759 207	-R 52 031 790

	Capital				Cash Flow			Tax						Discounted Profitability Criteria			
								Depreciation			Payable on Profit						
Year	*Plant*	*Land*	*Working Capital*	*Total Capital*	*Sales*	*Production Costs*	*Cash Flow*	*Plant*	*Buildings*	*Total*	*Profit: Previous Year before Tax*	*Tax*	*After Tax Cash Flow*	*Project Cash Flow*	*Cumulative Cash Flow*	*Project Discounted Cash Flow*	*Project DCFROR*
0	-R 40 945 268	R 0	-R 2 047 263	-R 42 992 531	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0	-R 42 992 531	-R 42 992 531	-R 42 992 531	-R 42 992 531
1	R 0	R 0	R 0	R 0	R 36 978 020	-R 46 269 569	-R 9 291 549	R8 189 054	R 0	R 8 189 054	-R 8 189 054	R 0	-R 9 291 549	-R 9 291 549	-R 52 284 080	-R 8 079 608	-R 7 433 239
2	R 0	R 0	R 0	R 0	R 42 260 595	-R 52 879 508	-R 10 618 913	R 8 189 054	R 0	R 8 189 054	-R 17 480 603	R 0	-R 10 618 913	-R 10 618 913	-R 62 902 993	-R 8 029 424	-R 6 796 104
3	R 0	R 0	R 0	R 0	R 47 543 169	-R 59 489 446	-R 11 946 277	R 8 189 054	R 0	R 8 189 054	-R 18 807 967	R 0	-R 11 946 277	-R 11 946 277	-R 74 849 271	-R 7 854 871	-R 6 116 494
4	R 0	R 0	R 0	R 0	R 52 825 743	-R 66 099 385	-R 13 273 641	R 8 189 054	R 0	R 8 189 054	-R 20 135 331	R 0	-R 13 273 641	-R 13 273 641	-R 88 122 912	-R 7 589 248	-R 5 436 884
5	R 0	R 0	R 0	R 0	R 55 007 447	-R 68 829 289	-R 13 821 843	R 8 189 054	R 0	R 8 189 054	-R 21 462 695	R 0	-R 13 821 843	-R 13 821 843	-R 101 944 755	-R 6 871 899	-R 4 529 141
6	R 0	R 0	R 0	R 0	R 57 279 254	-R 71 671 939	-R 14 392 685	R 0	R 0	R 0	-R 13 821 843	R 0	-R 14 392 685	-R 14 392 685	-R 116 337 440	-R 6 222 355	-R 3 772 956
7	R 0	R 0	R 0	R 0	R 59 644 887	-R 74 631 990	-R 14 987 103	R 0	R 0	R 0	-R 14 392 685	R 0	-R 14 987 103	-R 14 987 103	-R 131 324 543	-R 5 634 207	-R 3 143 023
8	R 0	R 0	R 0	R 0	R 62 108 221	-R 77 714 291	-R 15 606 070	R 0	R 0	R 0	-R 14 987 103	R 0	-R 15 606 070	-R 15 606 070	-R 146 930 613	-R 5 101 652	-R 2 618 264
9	R 0	R 0	R 0	R 0	R 64 673 291	-R 80 923 892	-R 16 250 601	R 0	R 0	R 0	-R 15 606 070	R 0	-R 16 250 601	-R 16 250 601	-R 163 181 214	-R 4 619 435	-R 2 181 119
10	R 0	R 0	R 0	R 0	R 67 344 298	-R 84 266 048	-R 16 921 751	R 0	R 0	R 0	-R 16 250 601	R 0	-R 16 921 751	-R 16 921 751	-R 180 102 965	-R 4 182 798	-R 1 816 959
11	R 0	R 0	R 0	R 0	R 70 125 617	-R 87 746 236	-R 17 620 619	R 0	R 0	R 0	-R 16 921 751	R 0	-R 17 620 619	-R 17 620 619	-R 197 723 584	-R 3 787 433	-R 1 513 600
12	R 0	R 0	R 0	R 0	R 73 021 805	-R 91 370 156	-R 18 348 351	R 0	R 0	R 0	-R 17 620 619	R 0	-R 18 348 351	-R 18 348 351	-R 216 071 934	-R 3 429 438	-R 1 260 889
13	R 0	R 0	R 0	R 0	R 76 037 606	-R 95 143 743	-R 19 106 137	R 0	R 0	R 0	-R 18 348 351	R 0	-R 19 106 137	-R 19 106 137	-R 235 178 072	-R 3 105 281	-R 1 050 371
14	R 0	R 0	R 0	R 0	R 79 177 959	-R 99 073 180	-R 19 895 221	R 0	R 0	R 0	-R 19 106 137	R 0	-R 19 895 221	-R 19 895 221	-R 255 073 293	-R 2 811 765	-R 875 001
15	R 0	R 0	R 0	R 0	R 82 448 008	-R 103 164 902	-R 20 716 894	R 0	R 0	R 0	-R 19 895 221	R 0	-R 20 716 894	-R 20 716 894	-R 275 790 186	-R 2 545 992	-R 728 911
16	R 0	R 0	R 0	R 0	R 85 853 111	-R 107 425 612	-R 21 572 501	R 0	R 0	R 0	-R 20 716 894	R 0	-R 21 572 501	-R 21 572 501	-R 297 362 688	-R 2 305 340	-R 607 212
17	R 0	R 0	R 0	R 0	R 89 398 845	-R 111 862 290	-R 22 463 446	R 0	R 0	R 0	-R 21 572 501	R 0	-R 22 463 446	-R 22 463 446	-R 319 826 133	-R 2 087 436	-R 505 832
18	R 0	R 0	R 0	R 0	R 93 091 017	-R 116 482 203	-R 23 391 186	R 0	R 0	R 0	-R 22 463 446	R 0	-R 23 391 186	-R 23 391 186	-R 343 217 319	-R 1 890 128	-R 421 378
19	R 0	R 0	R 0	R 0	R 96 935 676	-R 121 292 918	-R 24 357 242	R 0	R 0	R 0	-R 23 391 186	R 0	-R 24 357 242	-R 24 357 242	-R 367 574 561	-R 1 711 469	-R 351 025
20	R 0	R 0	R 0	R 0	R 100 939 119	-R 126 302 315	-R 25 363 196	R 0	R 0	R 0	-R 24 357 242	R 0	-R 25 363 196	-R 25 363 196	-R 392 937 757	-R 1 549 698	-R 292 418
															NVP	-R 132 402 008	-R 94 443 351