UNIVERSIDAD DE CONCEPCION
PROGRAMAS DE POSTGRADO

Facultad de Ciencias Forestales – Programa de Doctorado en Ciencias Forestales

Producción de prepolímeros epoxidados de alto valor agregado para la industria de adhesivos obtenidos desde fracciones funcionalizadas de lignina a través del sistema catalítico quimio-enzimático con lipasa B

(Production of added-value epoxidized pre-polymers for the adhesive industry obtained from functionalized fractions of lignin using a chemo-enzymatic catalytic system with lipase B)

Tesis para optar al grado de Doctor en Ciencias Forestales

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Concepción-Chile
2018

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A mi familia

AGRADECIMIENTOS

Quiero agradecer primero que nada a mi esposa y a mi hijo: a ***Carolina*** por apoyarme en este largo camino con paciencia y amor, y a mi hijo ***Piero*** por ser simplemente lo más maravilloso de la vida.

Agradecer a mis Profesores guías: al ***Dr. Regis Teixeira Mendonça*** por su apoyo académico y orientación durante todo este proceso, y al ***Dr. Steven W. Peretti*** por inspirar este trabajo de investigación, por su disposición, por su corrección y por entregarme las herramientas científicas necesarias durante el transcurso del doctorado.

Quiero agradecer también al Profesor ***Dr. Jaime Rodríguez Gutiérrez***, quien ya no está con nosotros, pero que por muchos años me apoyó y que me inspiro a encantarme con esta área de investigación, sobre lignina y el desarrollo de biomateriales.

A **CONICYT** por otorgarme la beca de Doctorado No 21130772, y también por el apoyo para asistencia a congresos y pasantía al extranjero.

A todo el personal y amigos del **Laboratorio de Recursos Renovables** del **Centro de Biotecnología** de la Universidad de Concepción, por su apoyo y colaboración durante todos estos años.

A la **Facultad de Ciencias Forestales** y a la secretaria de postgrado, ***Sra. Margarita Figueroa***, por las diversas gestiones requeridas en el programa de Doctorado.

También agradecer a ***Ryan Barton*** y a ***Kerri Cushing*** quienes junto al Profesor ***Steven W. Peretti*** me ayudaron en la estadía que realicé en el departamento de Ingeniería Química y Biomolecular de la Universidad Estatal de Carolina del Norte (Raleigh, USA).

Mi Familia se merece algo más que unas líneas, pero desde los más profundo del corazón y en palabras simples quiero agradecer a mis padres ***Marcia y Jaime*** por todo el apoyo y amor incondicional, y a mis hermanas ***Denisse*** y ***Javiera*** junto a sus hermosas familias por su constante amor y preocupación.

Y en especial, quisiera agradecer a todos los que de alguna u otra manera me han apoyado, por ello mis más sinceros agradecimientos a todos mis ***Amigos*** con los que tanto tiempo he compartido y que han estado conmigo en los buenos y malos momentos de la vida.

Finalmente agradecer a la música, y a mi guitarra que siempre me han acompañado. Al Universo, a la Vida y especialmente por todo a ***Dios***.

RESUMEN

La lignina es uno de los componentes de la madera que está disponible en grandes cantidades en la naturaleza. Su estructura química polifenólica ha sido de interés para su estudio, para su valorización y aplicación industrial. Debido a la versatilidad y diversidad química de la lignina, la industria de los biomateriales se ha centrado en su uso como fuente viable de materia prima renovable para la síntesis de nuevos biomateriales. La funcionalización de la lignina es un enfoque prometedor, ya que tiene el potencial de producir productos de alto rendimiento. Las resinas epóxicas son una de las clases más importantes de resinas termoendurecibles, y son ampliamente usadas comercialmente en materiales reforzados, adhesivos, plásticos y otras aplicaciones. Las resinas epóxicas comerciales se sintetizan a partir del bisfenol A (BPA), un compuesto que podría causar serios problemas para la salud humana y que se obtiene de la industria petroquímica. El objetivo general de este trabajo fue desarrollar y optimizar un novedoso sistema catalítico quimio-enzimático para la conversión de lignina organosolv y kraft en productos epoxidados de lignina funcionalizada de alto valor agregado para la industria de resinas adhesivas epóxicas utilizando la enzima lipasa B de *Candida antarctica*, peróxido de hidrógeno y ácido caprílico. En este trabajo, se demostró que la conversión de grupos hidroxilo a anillos oxiranos (grupos epóxidos) alcanzó 90% y 55% después de 12 horas de epoxidación para lignina organosolv y lignina kraft, respectivamente. Se demostró que la lignina organosolv es una materia prima más homogénea a través de un *Mw* más bajo y que es más reactiva que la lignina kraft, como fue determinado mediante el porcentaje de conversión hacia anillos oxiranos. La actividad residual de la enzima lipasa B inmovilizada en el curso de la reacción exhibió desnaturalización transitoria debido a la asociación con el sustrato de lignina así como una desnaturalización irreversible debido a la exposición a peróxido de hidrógeno. La lignina kraft, debido a la mayor recuperación de la actividad enzimática residual, parece ser más adecuada como sustrato para la epoxidación quimio-enzimática. Las resinas epóxicas de lignina funcionalizadas quimio-enzimáticamente y curadas con DDM exhibieron comportamientos comparables a una resina epóxica comercial basada en BPA. Las resinas derivadas de lignina organosolv y kraft exhibieron un peak de degradación térmica entre 350-400 °C, comparable a los 450 °C que se informó para la resina epóxica comercial con BPA. La temperatura de transición vítrea fue inferior (entre 60-70 °C) a la reportada para una resina epóxica comercial (105 ° C). El sistema catalítico quimio-enzimático se optimizó para las ligninas organosolv y kraft como sustratos, en relación a la temperatura de reacción y la concentración de peróxido de hidrógeno para maximizar la generación de anillos oxiranos. Las condiciones óptimas para ambas reacciones que involucraban cualquiera de los sustratos fueron aproximadamente 45 °C y 11.9 M de peróxido de hidrógeno. Estas condiciones dieron como resultado una conversión del 100% para la lignina organosolv; y 74% de conversión para lignina kraft. La lignina funcionalizada tiene aplicaciones potenciales en la producción de resinas adhesivas epóxicas, y el novedoso mecanismo quimio-enzimático desarrollado durante este proyecto representa una ruta verde alternativa para la síntesis de resinas epóxicas desde lignina.

ABSTRACT

Lignin is one of the components of wood that is available in large quantities in nature. Its polyphenolic chemical structure has been of interest for study for its valorization and industrial application. Due to the versatility and chemical diversity of lignin, the biomaterials industry has focused on its use as a viable source of renewable raw material for the synthesis of new biomaterials. Functionalization of lignin is a promising approach, since it has the potential to produce high performance products. Epoxy resins are one of the most important class of thermosetting resins, and are widely used commercially in reinforced materials, adhesives, plastics and other applications. Commercial epoxy resins are synthesized from bisphenol A (BPA), a compound that could cause serious problems to human health and that is obtained from the petrochemical industry. The general objective of this work was to develop and optimize a novel chemo-enzymatic catalytic system for the conversion of organosolv and kraft lignin into epoxidized high-value-added functionalized lignin products for the epoxy adhesive resin industry using the enzyme lipase B from *Candida antarctica*, hydrogen peroxide, and caprylic acid. In this work, it was shown that the conversion of hydroxyl groups to oxirane rings (epoxides) reached 90% and 55% after 12 hours for organosolv and kraft lignin, respectively. Organosolv lignin was shown to be a more homogeneous raw material through a lower *Mw* and more reactive than kraft lignin, as determined through the percentage of conversion to oxirane rings. The residual activity of the immobilized enzyme lipase B in the course of the reaction exhibited transient denaturation due to the association with the lignin substrate as well as irreversible denaturation due to the exposure to hydrogen peroxide. Kraft lignin, due to the higher recovery of residual enzyme activity, seems to be more suitable as a substrate for chemo-enzymatic epoxidation. Lignin epoxy resins functionalized chemoenzymatically and cured with DDM exhibited comparable behaviors to a commercial epoxy resin based on BPA. Organosolv and kraft derived resins exhibited a peak of thermal degradation between 350-400 ° C, comparable to 450 ° C that was reported for the commercial epoxy resin with BPA. The glass transition temperature was lower (between 60-70 °C) to those reported for a commercial epoxy resin (105 °C). The chemoenzymatic catalytic system was optimized for organosolv and kraft lignin as substrates, relative to reaction temperature and the concentration of hydrogen peroxide to maximize the generation of oxirane rings. Optimal conditions for both reactions involving either substrate were found to be approximately 45 °C and 11.9M of hydrogen peroxide. These conditions resulted in 100% conversion for organosolv lignin; and 74% conversion for kraft lignin. Functionalized lignin has potential applications in the production of epoxy adhesive resins, and the novel chemoenzymatic mechanism developed during this project represents a green alternative pathway to epoxy resin synthesis from lignin.

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CAPITULO I: INTRODUCCION

Biorrefinerías del siglo 21

Para 2040, se espera que la producción mundial de biocombustibles aumente entre 55% y 100% en relación con los niveles de 2014 (Conti *et al*., 2014). Lignocelulosa será una materia prima primaria para las biorrefinerías de segunda y tercera generación que generarán este tipo de combustibles. Las biorrefinerías de segunda generación deberán capturar un valor adicional del subproducto de lignina tradicionalmente de bajo valor y alto volumen. La Figura 1 ilustra las ideas recientes sobre el procesamiento de biomasa lignocelulósica, una que incorpora el uso de mayor valor del carbono disponible a través de la modificación de polímeros naturales (celulosa, hemicelulosa, lignina) en materiales valorizados e intermedios químicos.

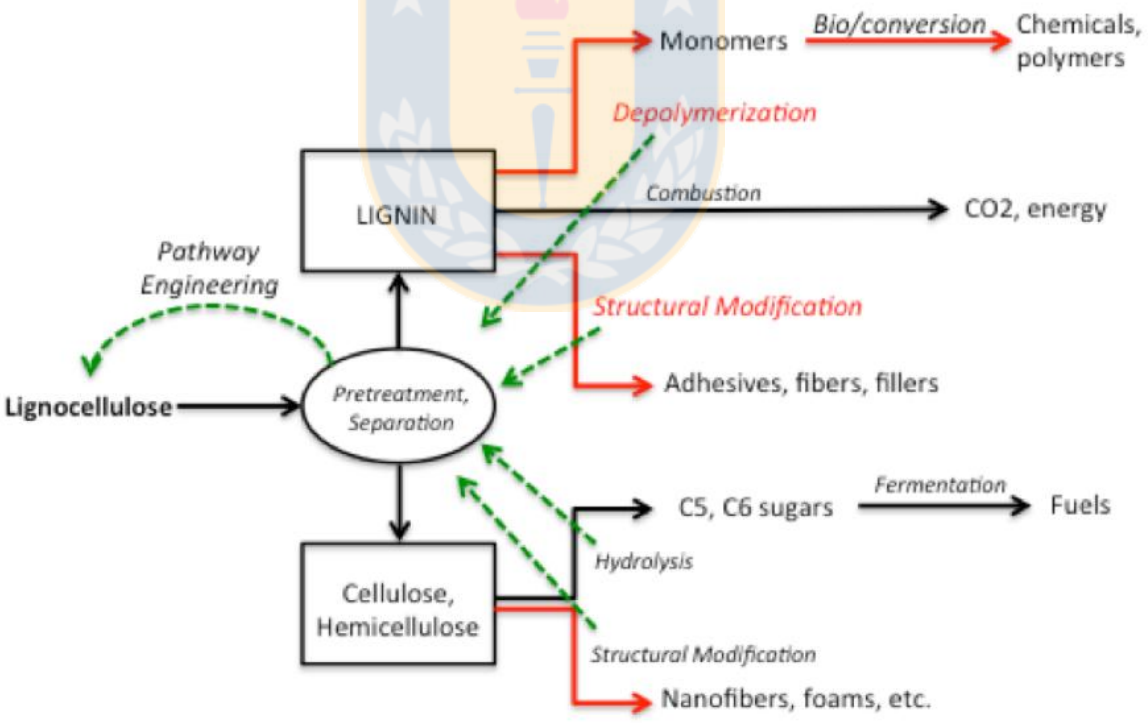

Figure 1: Procesamiento de lignocelulosa para mayor valorización (tomado desde Peretti *et al.* 2015).

El procesamiento del material en una biorrefinería de celulosa de primera generación está indicado por las líneas negras; pretratamiento para separar la lignina de las celulosas, la hidrólisis para generar azúcares, la fermentación de azúcares para producir etanol y la combustión de la lignina. Las líneas rojas representan áreas de esfuerzos de investigación orientados a expandir la cantidad de productos generados a partir de biomasa lignocelulósica. Las líneas verdes discontinuas indican interacciones de retroalimentación establecidas para respaldar esta línea ampliada de productos. La transformación de la lignina en productos de valor agregado podría mejorar en gran medida la productividad y la rentabilidad de la industria lignocelulósica (Kun y Pukanszki, 2017), al tiempo que reduce la dependencia global de las materias primas de combustibles fósiles para producir productos químicos complejos. Hasta la fecha, la industria del papel ha sido el punto objetivo para la conversión de celulosa y hemicelulosas en productos de alto valor (Gall *et al*., 2017).

Productos desde lignina

Debido a su naturaleza fenólica, la lignina ha recibido una gran atención como materia prima sostenible para precursores aromáticos para la síntesis de productos químicos producidos actualmente a partir de materias primas basadas en el petróleo. Una línea emergente de investigación es la despolimerización para formar productos oligoméricos/monoméricos (Zinovyev et al., 2016; Gall et al., 2017). Sin embargo, la lignina es muy resistente al procesamiento químico o bioquímico debido a su alto peso molecular y estructura condensada; su principal uso actual es la generación de energía mediante la quema de licor negro a partir de los procesos químicos utilizados en la industria de la celulosa y el papel. La extracción de lignina de la biomasa lignocelulósica se realiza principalmente con dos propósitos diferentes; caracterización de la cantidad y estructura utilizando métodos analíticos (Guerra *et al*., 2006; Rencoret *et al.,*

2007) o para la obtención de pulpa celulósica para síntesis de papel y derivados (Pandey y Kim, 2011). Durante la fabricación de pulpa, debe eliminarse una gran cantidad de lignina de la lámina media y las paredes celulares para liberar las fibras de madera y permitir que las fibras sean más fibriladas para la producción de papel o la modificación química de la celulosa (Sjöström, 1993). La lignina sólida también se puede convertir en líquidos mediante transformaciones termoquímicas usando licuefacción directa bajo presión de hidrógeno y un catalizador, como por licuefacción o pirólisis, por ejemplo (Pandey y Kim, 2011).

La estructura polifenólica de la lignina confiere carácter antioxidante, biodegradabilidad, alta estabilidad térmica, reactividad y propiedades adhesivas (Naseem et al., 2016). Dada la versátil gama de propiedades de materiales de la lignina, la investigación para desarrollar productos de valor agregado a partir de lignina ha incluido el desarrollo de material bioplástico y fibras de carbono como materiales de alto desempeño, también como polímeros con propiedades adhesivas para la industria de la madera, polímeros para la síntesis de espumas poliuretano y como aditivo en mezclas para conferir características emulsionantes o protectoras (Laurichesse y Averous, 2014; Ragauskas *et al*., 2014; Naseem *et al*., 2016; Zinovyev *et al*., 2016; Kun y Pukanszki, 2017).

Una aplicación prometedora de materiales, utilizando una fracción de lignina de bajo peso molecular, es la síntesis de polímeros termoendurecibles, principalmente los utilizados como adhesivos epóxicos y en la producción de compuestos elastoméricos (El Mansouri *et al*., 2011; Ragauskas *et al*., 2014). El grupo epóxido funcional contiene un anillo oxirano, que es importante en la síntesis de resinas adhesivas, espumas poliuretano y también como precursores de glicoles, carbonilos, alcoholes y derivados de aminas (Chua *et al*., 2012). Aproximadamente el 75% de las resinas epóxicas se producen a partir del éter diglicidílico de bisfenol A (DGEBA), preparado

mediante el acoplamiento de bisfenol A (BPA) y epiclorhidrina (EP). Las resinas y polímeros producidos a partir de ellos generalmente contienen trazas de los reactivos originales. La exposición al BPA es encontrado un problema para los humanos, dada su estructura similar a los estrógenos, que podría causar problemas de salud en el sistema endocrino (Howdeshell *et al*., 1999; Aouf *et al*., 2012). Estos hallazgos recientes han impulsado los esfuerzos para desarrollar epóxidos comerciales libres de BPA y sin epiclorohidrina, que es uno de los objetivos de esta investigación.

Se han estudiado formulaciones de resinas epóxicas utilizando diferentes compuestos del modelo de lignina, específicamente ácido vanílico y ácido gálico (Aouf *et al*., 2012; Aouf *et al*., 2013). Se han propuesto vías alternativas de síntesis de grupos epoxidos en el contexto de tecnologías más limpias. Björkling (1992) describió un método alternativo para epoxidar alquenos, utilizando una lipasa, peróxido de hidrógeno y ácidos grasos. Brevemente, la lipasa cataliza la conversión de un ácido graso en un ácido peroxicarboxílico mediante la transferencia de un átomo de oxígeno del peróxido de hidrógeno al grupo carboxilo del ácido graso. El peroxiácido transporta el oxígeno a un enlace insaturado de otro ácido graso, formando un anillo oxirano y regenerando el ácido graso original (Törnwall *et al*., 2007). Como alternativa al método tradicional de epoxidación de Prilezhaev, el mecanismo quimio-enzimático es a la vez selectivo y amigable con el medio ambiente.

Una posible extensión de este concepto a la lignina requeriría la generación de grupos alilo en la estructura de la lignina. Esto podría lograrse a través de alilación alcalina asistida de los grupos hidroxilo de la lignina, seguido por la epoxidación enzimática del grupo alilo sintetizado quimicamente. El potencial de funcionalización del epóxido ha llevado a preguntas sobre la eficacia de este enfoque a escala industrial (Aouf et al., 2013).

CHAPTER I: INTRODUCTION

21st Century Biorefineries

By 2040, global production of biofuels is expected to rise between 55% and 100% relative to 2014 levels (Conti *et al*., 2014). Lignocellulose will be a primary feedstock for the 2nd and 3rd generation biorefineries generating those fuels. Second generation biorefineries will need to capture additional value from the traditionally low-value, high volume lignin byproduct. Figure 1 illustrates recent thinking about processing lignocellulosic biomass, one that embodies the highest value use of available carbon through the modification of natural polymers (cellulose, hemi-cellulose, lignin) into valorized materials and chemical intermediates.

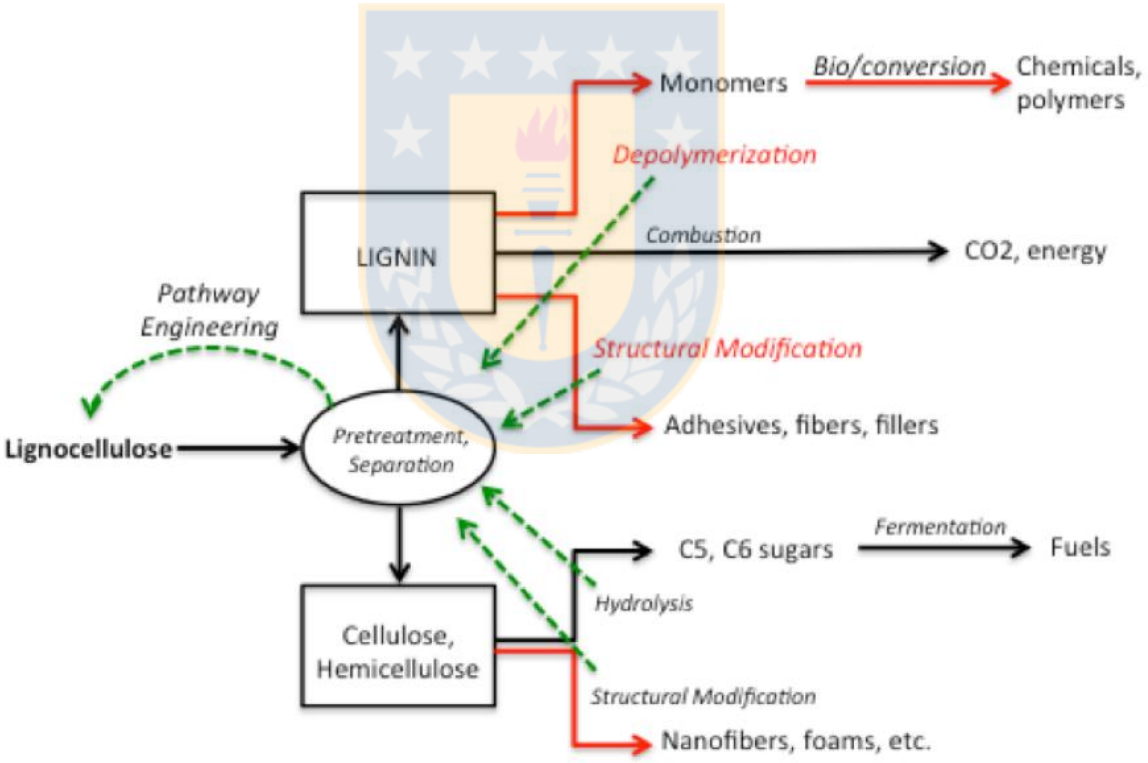

Figure 1: Processing lignocellulose for maximum value (taken from Peretti *et al.* 2015).

Material processing in a first generation cellulosic biorefinery is indicated by the black lines; pretreatment to separate lignin from celluloses, hydrolysis to generate sugars, fermentation of

sugars to ethanol, and combustion of the lignin. The red lines represent areas of research efforts geared towards expanding the number of products generated from lignocellulosic biomass. The dashed green lines indicate feedback interactions established to support this expanded product line. Transforming lignin into value-added products could greatly improve the productivity and profitability of the lignocellulosic industry (Kun and Pukanszki, 2017), while reducing global reliance on fossil fuel feedstocks to produce complex chemicals. To date, the paper industry has been the focal point for conversion of cellulose and hemicelluloses into higher value commodities (Gall *et al*., 2017).

Products from lignin

Due to its phenolic nature, lignin has received great attention as a sustainable feedstock for aromatic precursors for synthesis of chemicals currently produced from fossil-based feedstocks. An emerging line of research is depolymerization to form oligomeric/monomeric products (Zinovyev *et al*., 2016; Gall *et al*., 2017). However, lignin is very resistant to chemical or biochemical processing due to its high molecular weight and condensed structure; its current main use is for energy generation by burning black liquor from the chemical processes used in the pulp and paper industry. Lignin extraction from lignocellulosic biomass is performed primarily for two different purposes; characterization of its amount and structure using analytical methods (Guerra *et al.* 2006; Rencoret *et al.* 2007) or to obtain cellulosic pulp for synthesis of paper and derivatives (Pandey and Kim, 2011). During pulping, a high amount of lignin should be removed from the middle lamella and cell walls to release wood fibers and allow the fibers be further fibrillated for paper production or chemical modification of cellulose (Sjöström, 1993). Solid lignin can also be converted into liquids by thermochemical transformations using direct liquefaction under

hydrogen pressure and a catalyst, by liquefaction or pyrolysis, for instance (Pandey and Kim, 2011).

The polyphenolic structure of lignin confers antioxidant character, biodegradability, high thermal stability, reactivity and adhesive properties (Naseem *et al*., 2016). Given lignin's versatile range of material properties, research to develop value-added products from lignin has included the development of bioplastic material and carbon fibers as high-performance materials, also as polymers with adhesives properties for the wood industry, polymers for the synthesis of polyurethane foams, and as additive in blends to confer emulsifying or protecting characteristics (Laurichesse and Averous, 2014; Ragauskas *et al*., 2014; Naseem *et al*., 2016; Zinovyev *et al*., 2016; Kun and Pukanszki, 2017).

One promising materials application, using a low molecular weight lignin fraction, is in the synthesis of thermosetting polymers, primarily used as epoxy adhesives and in the production of elastomeric compounds (El Mansouri *et al*., 2011; Ragauskas *et al*., 2014). The functional epoxide group contains an oxirane ring, which is important in the synthesis of adhesive resins, polyurethane foams, and also as precursors for glycols, carbonyls, alcohols and amines derivatives (Chua *et al*., 2012). Approximately 75% of epoxy resins are produced from the diglycidyl ether of bisphenol A (DGEBA), prepared by the coupling of bisphenol A (BPA) and epichlorohydrin (EP). Resins and polymers produced from them usually contain trace amounts of the original reactants. BPA exposure is found to be problematic for humans, given its estrogen-like structure that in linked to health problems in the endocrine system (Howdeshell *et al.,* 1999; Aouf *et al*, 2012*)*. These recent findings have prompted efforts to develop commercial epoxides that are BPA-free and epichlorohydrin-free, which is one of the objectives of this research.

Formulations of epoxy resins using different lignin model compounds, specifically vanillic acid and gallic acid, have been studied (Aouf *et al*., 2012; Aouf *et al.,* 2013). Alternative epoxy synthesis pathways have been proposed in the context of green and clean technology. Björkling (1992) described an alternative method to epoxidize alkenes, utilizing a lipase, hydrogen peroxide and fatty acids. Briefly, the lipase catalyzes the conversion of a fatty acid to a peroxycarboxylic acid through the transfer of one oxygen atom from hydrogen peroxide to the carboxyl group of the fatty acid. The peroxyacid transports the oxygen to an unsaturated bond in another fatty acid, forming an oxirane ring and regenerating the original fatty acid (Törnwall *et al*., 2007). As an alternative to the traditional method of Prilezhaev epoxidation, the chemo-enzymatic approach is both selective and environmentally-friendly.

A possible extension of this concept to lignin would require the generation of allyl groups in the lignin structure. This could be accomplished through alkaline-assisted allylation of the hydroxyl groups in lignin, followed by enzymatic of the allyl group. The potential for functionalizing the epoxide has led to questions regarding the efficacy of this approach at an industrial scale (Aouf *et al.,* 2013).

HYPOTHESIS AND OBJECTIVES

This thesis is motivated and guided by the following working hypothesis:

> ***An efficient chemo-enzymatic reaction in two stages, using organosolv and kraft lignin, allyl bromide, and lipase B, will generate epoxidized pre-polymers at high conversion of the hydroxyl groups into oxirane rings. The epoxidized products will have the ability to crosslink for the synthesis of an epoxy adhesive resin with properties similar to that of commercial epoxy resins.***

The resulting ***general objective*** of this thesis is the development of a chemoenzymatic reaction system for the conversion of biomass fractions into epoxidized material. The model system chosen involves organosolv and kraft lignin from *Eucalyptus globulus* and from the Domtar process, respectively, as the biomass fraction. *Candida antarctica* lipase B is the model lipase to be used, and the peroxyacid system chosen involves hydrogen peroxide and caprylic acid.

The general objective gives rise, in turn, to a series of specific objectives, designed to test the working hypothesis and, if successful, achieve the general objective for the project. The ***specific objectives*** formulated for this project are as follows:

1. *Characterization of the chemoenzymatic catalytic process of lignin epoxidation using immobilized lipase B*
2. *Chemical characterization of functionalized epoxidized products obtained through chemoenzymatic processing of Organosolv and Kraft lignin*
3. *Synthesis of an epoxy resin and thermostability evaluation*
4. *Optimization of the reaction conditions of the catalytic system chemo-enzymatic processing of lignin*.

REVIEW OF RELEVANT TECHNOLOGY

Since the overall objective of the process under development is the conversion of lignin into an epoxy resin, it is instructive to review the chemical properties of both the raw materials (lignin) and the desired product.

Structure of lignin

What is lignin? Lignin is one of the three main components of lignocellulose, and represents the second largest source of carbon on earth after cellulose (Abdel-Hamid *et al*., 2013; Abdelaziz *et al*., 2016). Cellulose microfibrils constitute a branched network between lignin and hemicelluloses. It has been suggested that there could be strong covalent linkages between phenolic compounds and carbohydrates (Limayem and Ricke, 2012).

Lignin is a cross-linked aromatic macromolecular network that constitutes roughly 15-30% of the dry weight of the plant cell, varying according to the species of wood (Doherty *et al*., 2011; Abdelaziz *et al*., 2016). It acts as a cementing agent for the polysaccharides, providing mechanical strength and support for plant growth (Henricksson, 2009). Lignin also serves a barrier against microbial degradation of wood by bacteria, molds and fungi (Doherty *et al*., 2011; Xu *et al*., 2014; Abdelaziz *et al*., 2016). An example of a lignin macromolecule and major intermolecular linkage types is shown in Fig. 2. Lignin is the most abundant aromatic polymer in nature and is biodegraded predominantly to single-ring aromatics (Laurichesse and Averous, 2014; Abdelaziz *et al*., 2016; Zinovyev *et al*., 2016).

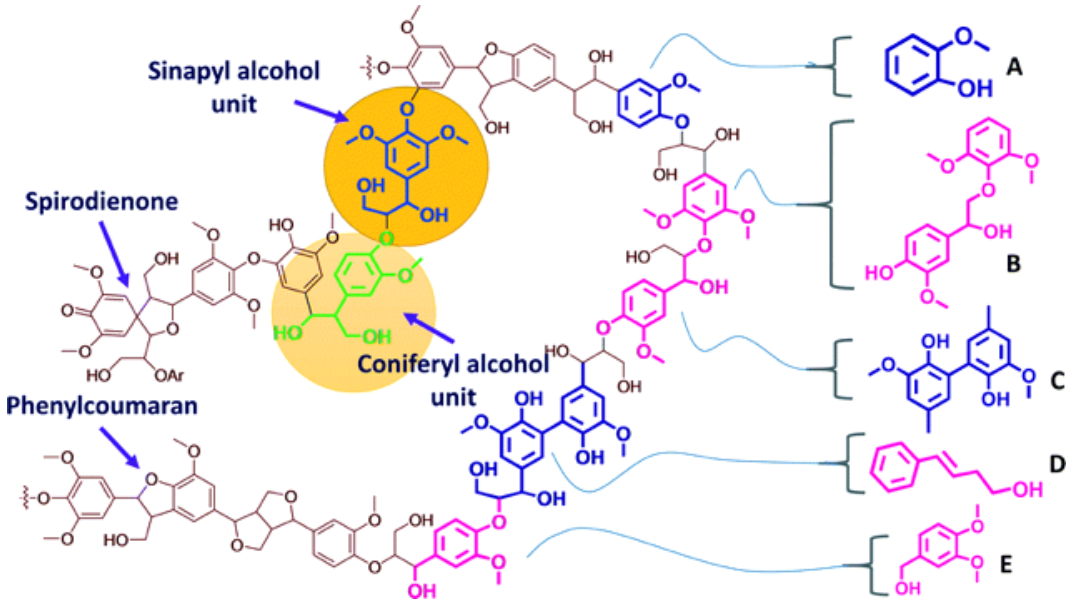

Figure 2. Schematic depiction of lignin showing various linkages and lignin model compounds. Model (A) phenol and methoxy functionalities, (B) a β–O–4 linkage, (C) a 5–5′ linkage, (D) a propyl side chain, and (E) a benzylic group (Reproduced from Dutta and Saha, 2014).

How is lignin synthesized? This macromolecular tridimensional component it is formed mainly for three cinnamyl phenilpropanoid alcohols monomers as it is shown in Figure 3, also knowed as monolignols: *p*-coumaryl alcohol, conyferyl alcohol, and sinapyl alcohol (Abdelaziz *et al*., 2016: Kun and Pukanszki, 2017; Gall *et al*., 2017). The main difference between the monolignols is the presence and placement of methoxy groups on the phenolic ring (Doherty *et al*., 2011; Abdelaziz *et al*., 2016). The p-hydroxyphenyl or H-unit have does not have a methoxy group, the guaiacyl or G-unit, has a methoxy group in the position of the third carbon of the phenolic ring, and the syringyl or S-unit has two methoxy groups, one in the 3 position and one in the 5 position (Abdelaziz *et al*., 2016).

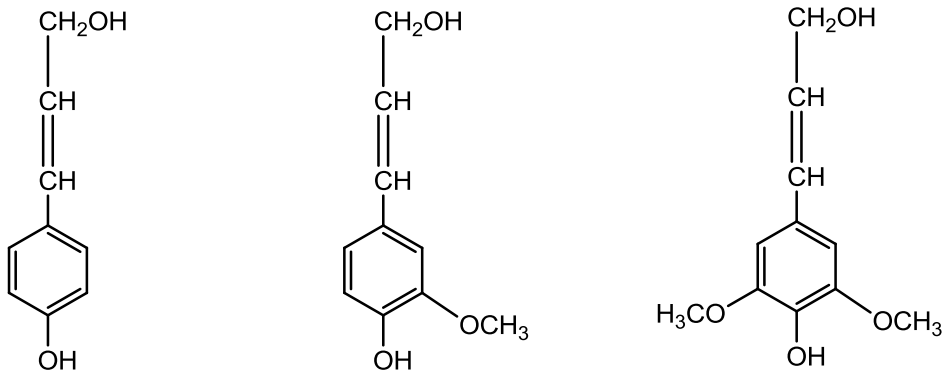

Figure 3. Structures of lignin units, (A): *p*-coumaryl alcohol, (B): conyferylic alcohol, and (C): sinapylic alcohol.

Lignin biosynthesis begins in the shikimate pathway (Abdelaziz et al., 2016), where cinnamic acid is formed from phenylalanine, catalyzed by the enzyme phenylalanine lyase (PAL). Cinnamic acid is then converted to the monolignols (Doherty *et al*., 2011; Abdel-Hamid *et al*., 2013; Abdelaziz *et al*., 2016). Lignins from softwood and hardwood contain primarily G and G-S units, respectively, and the herbaceous species contain mainly G-S units, with a small proportion of H unit (Abdel-Hamid *et al*, 2013; Li and Zheng, 2017).

The polymerization chain reaction of the monomeric structures of lignin occurs through the reaction of two radicals that forms a dimer across an aryl ether β-O-4 linkage. In a similar fashion, branched polymeric structures are formed in reactions involving dimers, trimers, tetramers and oligomers. Linkages in lignin can be classified into two main groups: C-C linkages and C-O ether linkages, including β-O-4, α-O-4, 5-5, β-5, 4-O-5, β-1 and β- β (Figure 4B). The most abundant linkage found in most forms of lignin is the β-O-4 linkage (C-O linkage) (Abdel-Hamid *et al*, 2013). A proposed model of a macromolecular chemical structure of lignin and the principal interlinkages are shown in the Figure 4.

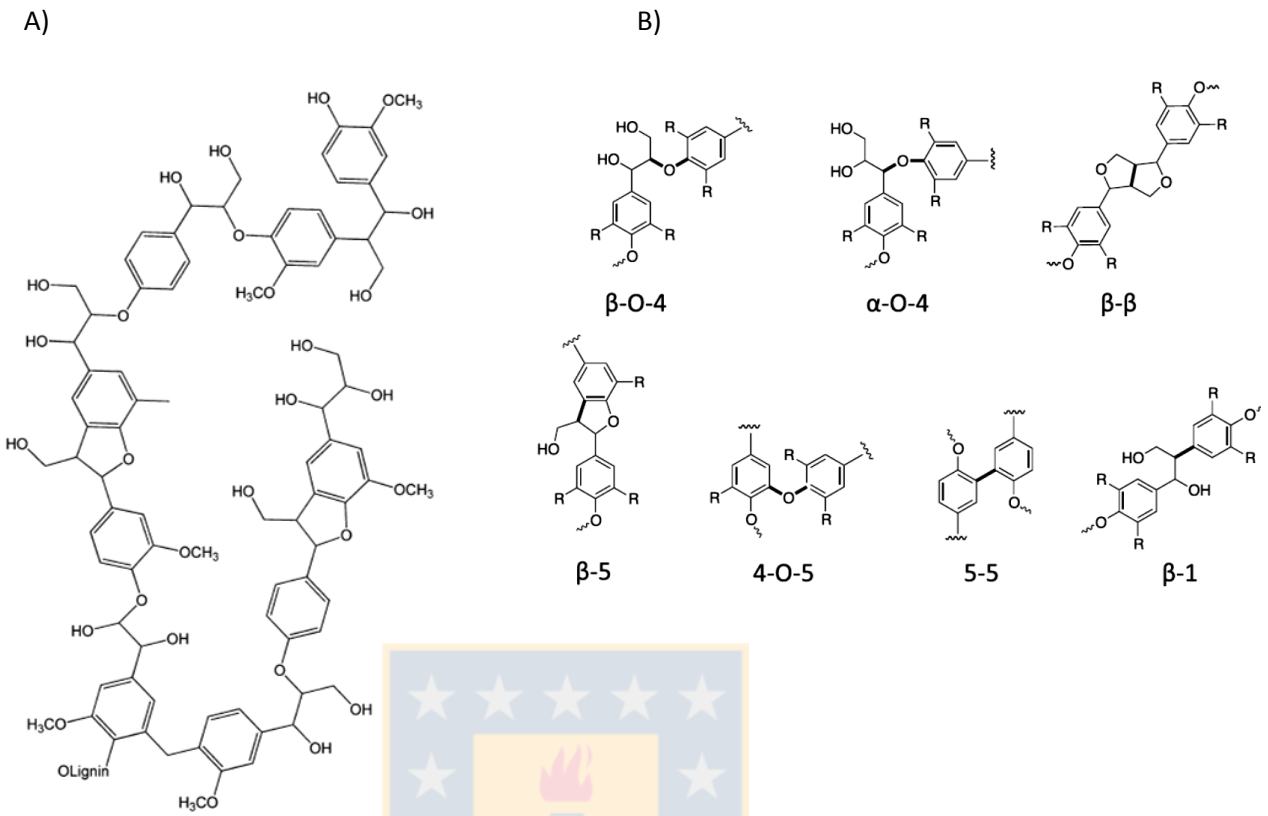

Figure 4. A) Representative fragment of the proposed structure of lignin (Xu *et al*., 2014), and B) Common lignin linkages (Abdel-Hamid *et al*., 2013).

The lignin component of woody plant species is considered a composite that could be useful in the production of liquid fuels and in the synthesis of several chemicals with added value. In the production of second-generation biofuels, the largest technical hurdle is separating the cellulose from the matrix of lignin-hemicellulose. A recent study describes efforts to develop new efficient methods in this decoupling, and thereby convert this industrial bioprocess in a sustainable and viable economically biorefinery, even if the cellulose contain wastes (Doherty *et al.,* 2011).

When lignin is obtained from the woody feedstock, due to the different techniques of segregation and conditioning, the resulting lignin exhibits diverse chemical and physical characteristics.

How is lignin isolated? Chemical delignification processes remove lignin from the plant cell wall for pulp or biofuels production, generating residual lignin with varied molar mass and chemical characteristics. The cleavage of aryl-ether linkages in lignin occurs during pulping processes such as Kraft (an alkaline process) and sulfite (which is generally undertaken with pH varying between 2 and 12) treatment, generating lignophenols containing residual sulfur (Biermann, 1993). Delignification processes using organic solvents (organosolv) generate sulfur-free lignin. Pretreatment processes used for fractionation of lignocellulosic biomass such as autohydrolysis and steam explosion, generated more highly condensed lignins with higher molecular weight compared to the other processes (Shevchenko *et al.,* 1999; Sannigrahi *et al*., 2008, 2010). Significant characteristics of the kraft and organosolv lignin are summarized next.

Kraft lignin: The Kraft process uses sodium hydroxide (NaOH) and sodium sulfide (Na_2S) to fragment and solubilize lignin. The process is carried out between 150-180°C for 1-5 h (Biermann, 1993). Lignin is recovered from kraft black liquors by precipitation after acidification of the medium to pH 3-4. Elemental analysis shows that the purified lignin contains organic elements up to 97% (Nagy *et al.*, 2010). Zhu (2014) showed that the molar mass of recovered lignin can vary according to the pH and temperature applied during recovery, and that it can have Mw values ranging from 10,000 to 15,000 Da, and Mn from 4,000 to 8,000 Da. Phenolic groups are also important for lignin reactivity and presented values between 0.8 and 1.2 OH/aromatic ring.

Organosolv lignin: Organosolv processes use organic solvents such as alcohols, ketones, and organic acids, as delignifying agents. Recently, organsolv processes gained new importance as an alternative for fractionation of lignocellulosic biomass in a biorefinery concept, due to the economic necessity for multiple co-products and particularly a high-quality lignin fraction with potential for several industrial applications (Pan *et al*., 2005). In contrast to lignin produced by other chemical processes, organosolv lignin is sulfur-free and of low molecular weight (El Hage *et al*., 2009; Vallejos *et al.*, 2011). The organosolv lignin process mainly attacks the α-ether linkages and thus retains relatively larger amounts of β-O-4 linkages (McDonough, 1993).

Depending on the type of pretreatment, the size and molecular weight of the polymers and oligomers obtained from the lignin could vary widely, resulting in the observation that lignin from different pretreatment schemes can exhibit diverse behavior and physicochemical properties (Doherty *et al.,* 2011; Lange *et al.,* 2013;). These properties could be affected by the treatment conditions such as temperature, pressure, solvent, and pH. The main goal of an extraction method is to produce a lignin that has high purity in a reproducible manner. The methods of extraction from lignin that have been studied most frequently involve cellulytic enzyme lignin (CEL), milled wood lignin (MWL), enzymatic mill acidolysis lignin (EMAL), kraft lignin, sulfite lignin, organosolv lignin, pyrolysis lignin, and steam explosion lignin. Table 1 lists the principal techniques for the extraction of lignin and the typical molecular weight (number-average) that results (Lange *et al,* 2013).

Table 1. Overview comparing the (average) characteristics of various lignin extracts.

(Lange *et al.*, 2013).

Lignin type	Monomer molecular weight [Da]	Number-average molecular weight (*Mn*) [Da]	Polydispersity index
Milled wood lignin [a]	198	2800 - 14200	3.7 - 12.9
Cellulolytic enzyme lignin [b]	187	~1900	5.7 - 6.7
Enzymatic mild acydolisis lignin [b]	187	~2000	~3.0
Kraft lignin [c]	180	1000 - 3000	2.0 - 4.0
Lignosulfonated lignin (softwood) [d]	215 - 254	36000 - 61000	4.0 - 9.0
Lignosulfonated lignin (hardwood) [d]	188	5700 - 12000	4.0 - 9.0
Organosolv lignin [e]	188	>1000	2.4 - 6.4
Pyrolisis lignin [f]	n.d.	300 - 600	2.0 - 2.2
Steam explosion lignin [g]	188	1100 - 2300	1.5 - 2.8

[a] Norway Spruce; [b] Isolated from milled Norway spruce wood; [c] Norway spruce wood; [d] Norway spruce wood and aspen wood as softwood samples, eucalyptus globules wood as hardwood sample; [e] Norway spruce wood; [f] Beech wood; [g] Japanese white birch wood and larch wood.

Epoxy resin

Epoxy resins are synthesized through the reaction of dimers, trimers, oligomers, that contain oxirane rings, usually catalyzed by a curing agent. Epoxy resins have been produced since the 1940s. These resins are thermosetting materials, in which the epoxide groups are reactive toward other chemical groups, mainly hydroxyl groups. The versatile nature of this reactivity, and the number of reactive targets available, results in broad applicability for these resins. The principal applications of epoxy resins are in carbon structures, polyurethanes foams, surface coatings, paints, epoxy glues, fiber composites and composites involving electronic encapsulation (Raquez *et al.*, 2010). Currently, the synthesis of this material is based mainly on the reaction between bisphenol A (BPA) and epichlorohydrin, giving rise to the diglycidyl ether of bisphenol A (DGEBA) (Raquez *et al.*, 2012; Chua *et al*., 2012; Aouf *et al*., 2012) (Figure 5).

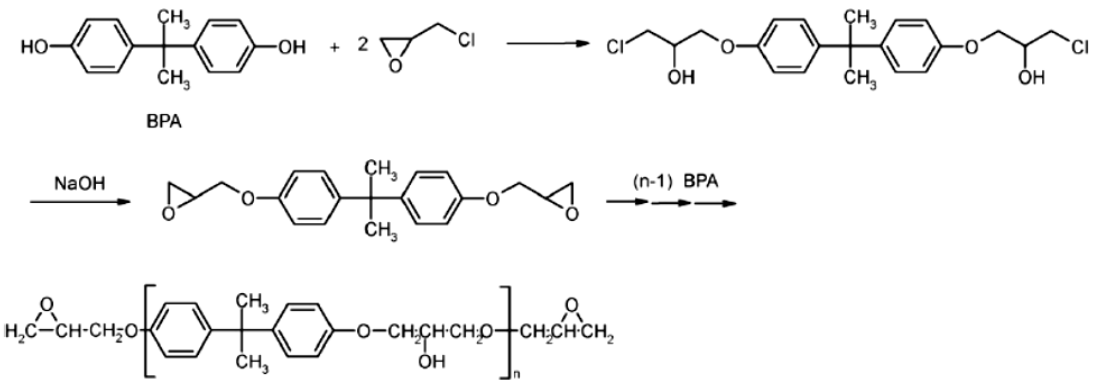

Figure 5. Synthesis of DGEBA (Raquez *et al*., 2010)

During epoxidation reactions, an atom of oxygen is added to carbon-carbon double bonds, generating carbon-oxygen bonds. The epoxidation reaction mechanism is determined by the oxidants and type of catalysts used in the system. Well-studied olefin epoxidation mechanisms used in the synthesis of epoxy resin include: Jacobsen-Katsuki epoxidation (Manganese salen complexes and iron-porphyrin catalyst), Sharpless asymmetric epoxidation, Shi epoxidation, the Prilezhaev reaction (peracid catalyst) and enzymatic epoxidation (Chua *et al.*, 2012).

The two mechanisms most relevant to this project are the Prilezhaev reaction and enzymatic epoxidation. In the Prilezhaev reaction, acetic/formic acid reacts with hydrogen peroxide to generate the peracid (Figure 6A). The bond formed in the peracid is weak, therefore it is expected that this product would adopt "a conformation with intramolecular hydrogen bonds" in solutions. Figure 6B indicates the epoxidation reaction where the mechanism occurs in a single step, with a transition step where the oxygen is transferred to the C=C bond and the proton is shifted in concert.

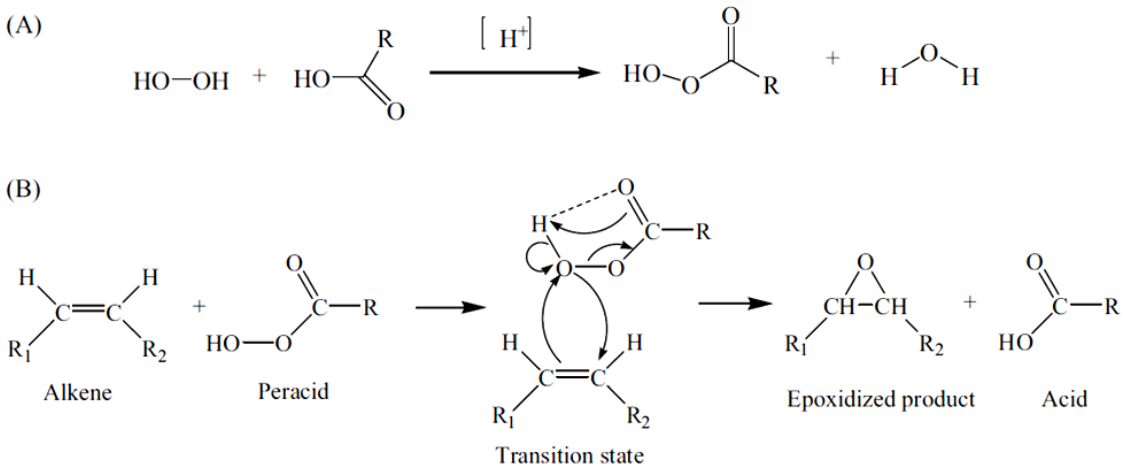

Figure 6. (A) Reaction of formation of peracetic acid from the reaction of hydrogen peroxide with acetic acid in acid catalyst epoxidation. (B) Acid catalyst epoxidation occurs in a single step with a transition state (Chua *et al.*, 2012).

The Prilezhaev reaction is similar to the chemo-enzymatic epoxidation illustrated in Figure 7. In this mechanism, the free fatty acid replaces the role of the strong acid, and the resulting peroxy acid is consumed in the formation of the epoxide structure. The fatty acid is regenerated in this step (Törnwall *et al.*, 2007; Chua *et al.*, 2012). Recent research indicates that lipase is capable of carrying out the epoxidation of alkene analogues from plant oils. Lipases utilize a variety of triglycerides as substrate. Immobilized lipase B from *Candida antarctica* (Novozyme 435) has been used in chemo-enzymatic systems using hydrogen peroxide as the oxidant agent (Chua *et al.*, 2012; Aouf *et al*., 2012). *Candida antarctica* lipase B (Novozyme 435; CalB) has demonstrated capability to epoxidize allylated gallic acid and vanillic acid with hydrogen peroxide in the presence of caprylic acid (Figure 7).

Vanillic and gallic acid have structural motifs that are also present in lignin: hydroxyl groups attached to aromatic rings directly or as part of a primary carboxyl structure. Recognition of this similarity gave rise to the fundamental concept of this thesis: ***Lipase could be the foundation of a novel chemo-enzymatic treatment for functionalizing oxirane rings on lignin for the synthesis of epoxy resin.***

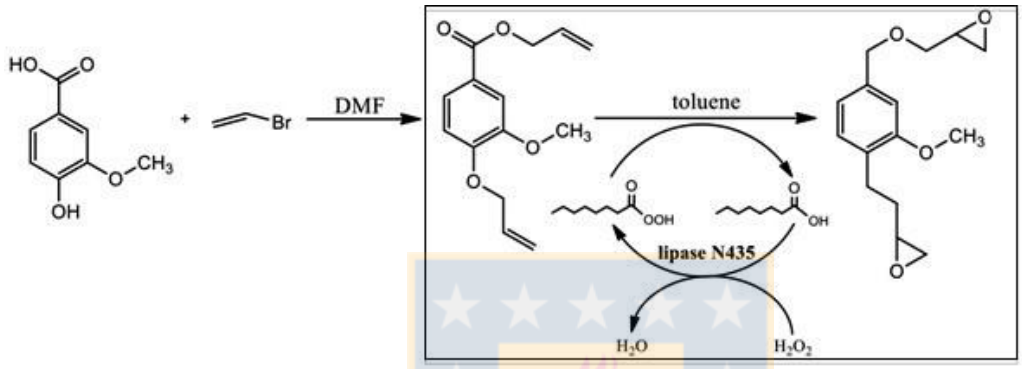

Figure 7. Chemo-enzymatic epoxidation of vanillic acid. The two step functionalization of the two phenolic OH groups are indicated, including the alkaline assisted allylation, and then the enzymatic epoxidation by Novozym 435 using toluene as solvent (Vásquez-Garay *et* al., 2018, Modified from Aouf *et al*., 2012).

Chapter 2 presents the results of proof of concept studies. Vanillic acid, kraft lignin and organosolv lignin, used as starting materials, were subjected to allylation to replace hydroxyl groups with allylic carbons. Immobilized *Candida antarctica* lipase B (CalB), available commercially as Novozym435, was used with caprylic acid and hydrogen peroxide to generate peroxycaprylic acid, which subsequently donated oxygen atoms to the allylic carbons, forming oxirane rings. The vanillic acid reaction was essentially performed as a control, since its epoxidation had been reported previously, as had chemoenzymtic epoxidation of unsaturated fatty acids. Successful epoxidation of both types of lignin prompted further investigation into the

stability of the enzyme under relatively harsh reaction conditions. Results of those studies indicate that inactivation is mediated by two mechanisms; an irreversible denaturation and a reversible adsorption of material derived from the lignin.

Chapter 3 presents the results of material characterization studies and production optimization efforts. Having established that the chemoenzymatic process would effectively epoxidize different lignin types, it was necessary to evaluate the utility of the resulting resins for adhesive formulation. Of particular importance are the thermochemical properties of the resins. The thermal stability and the glass transition temperature of the resins were evaluated using thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). In conjunction with these studies, an optimization analysis was performed to determine the conditions under which the maximal epoxidation of lignin was possible, recognizing that the time-temperature history of the resulting resin could play an important role in the ultimate structure of the resin and its physical properties.

In the final chapter, the implications for these results for the processing of lignin into epoxy adhesive resins are reviewed, and a preliminary economic analysis is presented.

CHAPTER II

Chemoenzymatic lignin valorization: production of epoxidized pre-polymers using *Candida antarctica* lipase B[1]

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[1]This chapter has been published as Enzyme and Microbial Technology 112 (2018) 6–13

Abstract

An innovative chemoenzymatic catalytic system for functionalizing lignin from *Organosolv* and *Kraft* pulping processes to obtain oxirane rings was investigated. Novozym435 (immobilized *C. antarctica* lipase B: CalB) was used to catalyze the peroxidation of caprylic acid to peroxycaprylic acid, which in turn reacted with unsaturated C-C bonds to form the oxirane ring. The conversion of OH groups to oxirane rings (epoxides) reached 90% and 55% after 12 h for the two processes, respectively. The residual enzyme activity over the time course of the reactions indicated transient denaturing due to association with the lignin substrate (10 – 50%) as well as irreversible denaturation due to exposure to hydrogen peroxide. Functionalized lignin has potential applications in the production of epoxy adhesive resins, and chemoenzymatic synthesis represents a "greener" pathway to this synthesis.

Keywords: Lignin modification; Chemoenzymatic epoxidation; Epoxy resins; *Candida antarctica* lipase B.

1. Introduction

The economic viability of biorefineries to produce fuels from biomass is greatly enhanced by the conversion of biomass fractions that are not involved in the biofuel production, such as lignin, into value-added co-products (Cardona and Sanchez, 2007; Doherty *et al*., 2011). Several strategies for the generation of valuable co-products have been evaluated (Kaylen *et al*., 2000; Chen and Qiu, 2010; Limayem and Ricke, 2012). The results indicate that producing ethanol as a single product is not economically feasible, whereas when furfural is a co-product, the process becomes viable economically (Cardona and Sanchez, 2007), highlighting the importance of co-product valorization for biorefinery sustainability. The most effective way to achieve optimal co-product synthesis is to incorporate co-product processing requirements into the facility design process. In that way, economic and environmental criteria for fuel and co-product synthesis can be applied directly to biorefinery design (Chen and Qiu, 2010; Doherty *et al*., 2011).

Lignin offers a significant product opportunity. It can be converted into high-value products including carbon fiber, engineering plastics and thermoplastic elastomers, polymeric foams and membranes, epoxy resins, and a variety of fuels and chemicals that are currently being made by petroleum derivatives (Doherty *et al*., 2011; Lange *et al*., 2013; Ragauskas *et al*., 2014; Ferdosian *et al*., 2016). Lignin depolymerization, the first step in many of these conversion scenarios, is challenging, given the different bond strengths of the ether (C-O-C) and carbon-carbon (C-C) linkages, and the tendency for low-molecular-weight species to undergo condensation reactions. Because of the heterogeneity of low molecular weight species and the diversity of functional lignin, degradation products are difficult to upgrade (Lange *et al*., 2013; Ragauskas *et al*., 2014).

One potential product from low molecular weight lignin fractions is epoxy resin (Doherty *et al*., 2011; El Mansouri *et al*., 2011; Asada *et al*., 2015; Ferdosian *et al*., 2015; Ferdosian *et al*., 2016). The functional epoxide group contains an oxirane ring, which is important in the synthesis of adhesive resins, polyurethane foams, glycols, carbonyls, alcohols and amine derivatives (Raquez *et* al., 2010; Chua *et* al., 2013; Asada *et al*., 2015; Ferdosian *et al*., 2015; Ferdosian *et al*., 2016). Approximately 75% of epoxy resins contain the diglycidyl ether of bisphenol A (DGEBA), prepared by the coupling of bisphenol A (BPA) and epichlorohydrin (EP) (Raquez *et* al., 2010; Aouf *et* al., 2012; Asada *et al*., 2015). Since reactions rarely go to completion, resins and polymers usually contain trace amounts of the original reactants. BPA exposure is problematic for humans due to its estrogen-like structure that can disrupt the endocrine system (Howdeshell *et* al., 1999; Aouf *et* al., 2012; Huang *et* al., 2012; Asada *et al*., 2015). These recent findings have prompted efforts to develop commercial epoxides that are BPA-free, which is one objective of the present research.

Synthesis of oxirane rings using lignocellulosic and phenolic compounds, specifically vanillic acid and gallic acid, have been studied (Kudanga *et* al., 2010; Aouf *et* al., 2012; Aouf *et al.*,2013; Asada *et al*., 2015; Ferdosian *et al*., 2015; Ferdosian *et al*., 2016). Given that epichlorohydrin and other phenolic substrates used in epoxy synthesis are generally derived from petroleum, the approach taken in this work represents a green and clean technology. Other green technologies proposed to incorporate polyphenolic compounds into epoxides include a chemical alkylation step and a subsequent enzymatic alkene epoxidation. This has not been commercialized because of the attendant safety issues and the risk of defunctionalizing the epoxide (Aouf *et al*., 2013). Björkling (1992) described an alternative method to produce epoxides from alkenes (Figure 1; in box). Small quantities of fatty acids were used to study the epoxidation reaction cascade catalyzed by lipase (Moreira and Nascimento, 2007; Aouf *et al*., 2012). Enzymatic formation of

peroxy-carboxylic acid from hydrogen peroxide and carboxylic acid is the first step in the cascade (Méndez-Sánchez *et al*., 2014). The peroxy acid subsequently reacts with the unsaturated C-C double bond in the fatty acid, forming the epoxide and regenerating the original carboxylic acid (Törnvall *et al*., 2007; Chua *et al.*, 2012; Méndez-Sánchez *et al*., 2014).

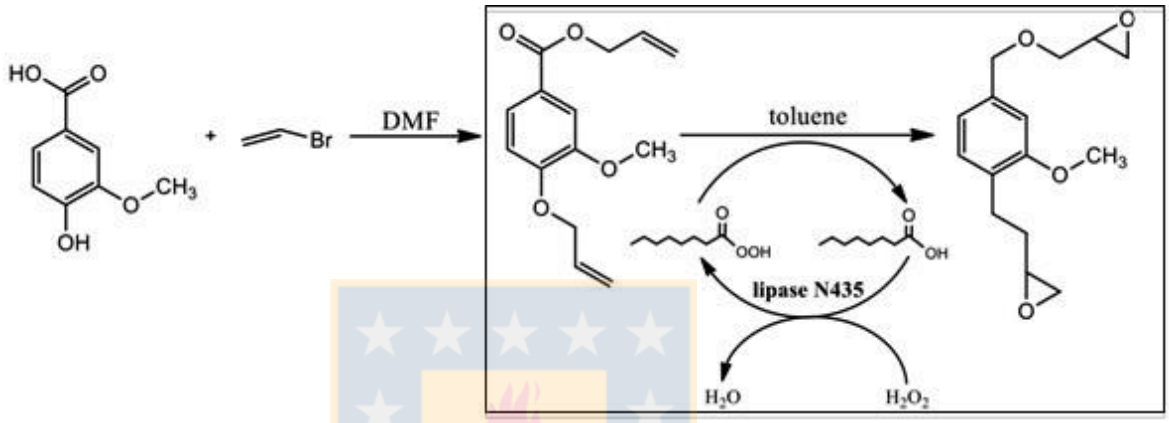

Figure 1. Chemoenzymatic epoxidation of vanillic acid. The functionalization of the two phenolic OH groups is indicated, followed (in box) by the lipase-catalyzed chemo-enzymatic reaction cycle generating peroxy acids from carboxylic acid and subsequent Prilezhaev epoxidation.

Table 1 presents different chemoenzymatic and chemical mechanisms for epoxidation, including systems using immobilized *Candida antarctica* lipase B. The enzymatic systems were evaluated between 25^{o}C and 50^{o}C for reaction times up to 48 h. The ultimate conversion achieved appears to depend on the specific reactions evaluated, and generally are comparable to results achieved using a chemical reagent such as *m*-chloroperoxybenzoic acid (mCPBA).

CalB's ability as part of a chemoenzymatic system to catalyze diverse reactions, such as epoxidation, lactonization and desymmetrization, has been investigated extensively (Méndez-Sánchez *et al*., 2014; Méndez-Sánchez *et al*., 2014b; González-Marínez *et al*., 2015). Some results

for example chemoenzymatic epoxidation of alkenes using CalB compared to the chemoenzymatic by a *Rhizomucor miehei* lipase to form epoxides using limonene and (s)-carvone was studied, finding optimal parameters to condition a good yield in the epoxide production in both cases (Méndez-Sánchez *et al*., 2014).

Table 1. Comparison between chemoenzymatic and chemical mechanisms reported using CalB, mCPBA and N435.

Catalyst	Peroxyacid	Solvent	Product	Conversion %	Conditions	Source
CalB		EA	α-cyano epoxides	85	3 h, 50˚C	[23]
		EA	Azoxybenzenes	74	1 h, 25˚C	[24]
		Toluene	Oleic acid epoxides	20	0.5 h, 50˚C	[25]
CalB Nanoflower		Toluene	Oleic acid epoxides	97	0.5 h, 50˚C	[25]
None	mCPBA	DCM	Vanillic acid epoxides	62	24 h, 25˚C	[14]
	mCPBA	DCM	Gallic acid epoxides	68	24 h, 25˚C	[14]
	mCPBA	EA	Azoxybenzenes	66	1 h, 25˚C	[24]
N435		EA	α-cyano epoxides	91	3 h, 50˚C	[23]
		EA	Azoxybenzenes	94	1 h, 25˚C	[24]
		Toluene	Methyl oleate epoxides	69	6 h, 37˚C	[26]
		Toluene	Vanillic acid epoxides	87	48 h, 40˚C	[14]
		Toluene	Gallic acid epoxides	89	48 h, 40˚C	[14]
		Toluene	Oleic acid epoxides	95	0.5 h, 50˚C	[25]
	Caprylic	Toluene	Vanillic acid epoxides	60	12 h, 40˚C	This work
	Caprylic	Toluene	Organosolv lignin epoxides	90	12 h, 40˚C	This work
	Caprylic	Toluene	Kraft lignin epoxides	55	12 h, 40˚C	This work

It is proposed that this technology could be effectively adapted to lignin functionalization through the conversion of phenolic hydroxyl groups to oxirane ring structures, creating an epoxy resin precursor. Allylation chemistry effectively activates the phenolic hydroxyl structures, creating the carbon-carbon double bonds necessary for oxirane ring formation. It is important to obtain lignin fractions (from Organosolv or Kraft processes) containing the largest number of available phenolic hydroxyl groups, and to understand the relationship between pretreatment technologies and phenolic hydroxy content. It is also hypothesized that lower lignin molecular weight would make these phenolic hydroxyls more accessible for the allylation reaction (Aouf *et al*., 2012), and the subsequent epoxidation. This work characterizes the activity of the proposed chemo-enzymatic catalytic system towards fractions of Organosolv and Kraft lignin. The model system chosen involves functionalized (allylated) lignin, immobilized *Candida antarctica* lipase B (CalB; commercial preparation is Novozym 435 (N435)), hydrogen peroxide and caprylic acid. Of particular interest is the reactivity of different lignin fractions (Organosolv and Kraft) that could affect the rate and degree of epoxidation achievable.

2. Materials and Methods

2.1 Reagents

The Organosolv pretreatment of *Eucalyptus globulus* woodchips was performed in a 5-gallon reactor (Parr reactor 4889) in the presence of a 50/50 (v/v) ethanol/water mixture at a temperature of 200°C for 6 min. Lignin was recovered through precipitation with 3% H_2SO_4 and dried in an oven at 40°C for 72 h prior to fractionation. Fractionated lignin was analyzed by GPC and used in epoxidation studies. The Kraft lignin was obtained from the Domtar process (BioChoice lignin) (Hu *et al.,* 2016). The standard epoxidation reaction mixture contained a source of phenolic hydroxyl groups (vanillic acid (> 98%), Organosolv lignin or Kraft lignin),

allyl bromide (99%), caprylic acid (98%), immobilized *Candida antarctica* lipase B (CalB) (Novozym435; N435), dimethylformamide (DMF) from Sigma Aldrich, and toluene (99.8%) from Fisher Chemical.

2.2 Allylation

A 500 mL three-necked flask equipped with a magnetic stirring bar was charged with 100 mL of DMF and 2.86 g of vanillic acid (17 mmol) or ~ 3 g of lignin. The solution was cooled with an ice bath and 4.7 g of potassium carbonate (34 mmol) was added. After 5 min, 2.9 mL of allyl bromide (97 % w/v) was added dropwise over 5 min. The solution was stirred for 30 min at 0°C and then at 25°C for 48 h. Water (100 mL) was added and the aqueous phase was extracted with 3× 100 mL of ethyl acetate. The organic phase was washed with 100 mL of brine, then dried over $MgSO_4$ and vacuum concentrated. The concentrate was used for the epoxidation studies.

2.3 Epoxidation

Chemo-enzymatic epoxidation reactions were performed in 500 mL three necked flasks equipped with a magnetic stirrer. In the first step, ~ 3 g of allylated phenolic compound, and 2.7 mL of caprylic acid (99 % w/v) were dissolved in 170 mL of toluene. Then, immobilized lipase (Novozym435; 20% wt% relative to the weight of substrate) was added and the mixture heated to 40°C. In the second step, 3.85 mL of hydrogen peroxide (30% v/v) was added over 5 h. All syntheses were carried out for 48 h. When epoxidation was completed, the biocatalyst was removed from the reaction mixture by filtration, the filtered reaction mixture was washed with 2 × 100 mL of water, and the aqueous phase was extracted with 3 × 150 mL of ethyl acetate. The organic phase was dried over $MgSO_4$ and vacuum concentrated. The crude product obtained was stored for further analysis.

2.4 Enzymatic activity assays

Lipase activity was quantified using the p-nitrophenyl butyrate (pNPB) (Sigma Aldrich) hydrolysis reaction. An emulsion of substrate (1-4 mM in 50 mM potassium phosphate buffer, pH 6.5, with 0.5% Triton-X 100) was added to a final catalyst loading of 20 mg dw/mL. Samples with immobilized N435 were gently agitated in a temperature-controlled water bath during the assay. The bath was maintained at 30°C for assays using pNPB, and the absorbance of the supernatant at 400 nm was measured using a UV-Visible spectrophotometer (Shimadzu UV-1650 PC). The maximum observed absorbance within 10 min was recorded. The concentration of p-nitrophenol was calculated based on the absorbance and standard curves prepared in appropriate buffer.

2.5 Molecular weight analysis

Gel permeation chromatography (GPC) was used to determine the number-average (Mn) and weight-average (Mw) molecular weights, and the polydispersity (Mw/Mn) of the lignin. GPC analysis was performed using a Waters (UV/visible Detector 2489) instrument. Three Waters Styragel columns (HR1, HR3, and HR5) were employed, with a flow rate of 0.6 mL/min. All lignins and vanillic acid were acetylated before the analysis in order to enhance their solubility in THF. Polystyrene standards in the molecular weight range of 790 to 1.86 x 10^4 g/mol were used for calibration. EMPOWER GPC software was used for data processing.

2.6 Hydroxyl group quantification and characterization

^{31}P NMR analysis, as described by Norambuena *et al*. (2016), is capable of quantifying and categorizing (aliphatic, carboxyl and phenolic) the hydroxyl group content of the different fractions of lignin and vanillic acid. This assay was performed using a Bruker spectrometer at a frequency 400 MHz with a delay time of 25 s at 25°C. Chloroform-d6 was used as a solvent.

2.7 Oxirane number measurement

ASTM standard D1652–04 protocol was used to quantify the oxirane number of epoxidized lignin and vanillic acid, based on the methodology proposed by Jay (1964). Samples were taken during the reaction (0, 6, 12, 24, 30, 36 and 48 h). Samples (1.3 g) were transferred into a beaker and maintained under constant agitation. Then, 10 mL of chloroform was added, followed by the addition of 10 mL of 20% tetraethylammonium bromide reagent and 6 to 8 drops of crystal violet indicator solution. The resulting solution was titrated with 0.1 *N* perchloric acid reagent to a green end point. The titration volume of perchloric acid reagent was used to calculate the weight percent epoxide using the following equation:

$$\boldsymbol{E = (F\ x\ V)/We}$$

where $\boldsymbol{F}$ is the perchloric acid reagent factor; $\boldsymbol{V}$ is the volume of perchloric acid reagent used in the titration (mL), and $\boldsymbol{We}$ is the weight of epoxy resin specimen used (g). The perchloric acid reagent factor (F) is calculated using the following formula,

$$\boldsymbol{F = (Wd\ x\ Eph)/V}$$

where *Wd* is the potassium hydrogen phthalate standard used (g); Eph is the epoxide of the potassium hydrogen phthalate standard used (normally 21.05% w/w); and V is the volume of perchloric acid reagent used in the titration (mL).

3. Results and Discussion

3.1 Chemoenzymatic epoxidation

3.1.1 Vanillic acid

Vanillic acid is a simple phenolic derivative of wood that was utilized as a substrate in order to gain insight into the epoxidation reaction mechanisms. It has two hydroxyl groups per molecule, one phenolic and one carboxylic, as indicated in Figure 1. These hydroxyl groups were completely allylated in the presence of allyl bromide and potassium carbonate. Subsequently, the allyl groups underwent olefin peroxidation in a reaction with a peroxycarboxylic acid that was generated by lipase-catalyzed perhydrolysis. Epoxidation of allylated vanillic acid, using caprilic acid, hydrogen peroxide, and N435, was characterized by the rate and extent of generation of the oxirane rings. Quantification was accomplished by titration of the oxirane rings and comparison with the initial vanillic acid concentration and the number of possible oxirane rings per vanillic acid molecule (=2) (Figure 2a). The oxirane ring concentration, expressed as a percentage of the theoretical maximum, reaches a final value of approximately 62% within the first 12 hours of reaction, after which the concentration does not change significantly. This result was unexpected, since all of the double bonds were easily accessible to the peroxyacid. Conversions as high as 90% of the theoretical maximum were expected, as reported previously for vanillic acid epoxidation (Aouf *et al.*, 2012). Accordingly, it is speculated that there may be some kind of undesired absorption between vanillic acid and N435, resulting in a lower yield due to a loss of activity of the enzyme.

3.1.2 Lignin

Calculating the percentage of epoxidation of lignin required the total number of hydroxyl groups present, with results indicated in Table 2. Kraft lignin has a higher number of hydroxyl groups (~4.3 mmol/g lignin) groups relative to Organosolv lignin (~2.3 mmol/g lignin) on a mass

basis. This implies a potential to create more oxirane rings, leading to a material with greater adhesive strength, correlated to the higher number of epoxy-mediated linkages. Epoxidation of both types of lignin was accomplished using lipase N435. The results are presented in Figure 2, based on titration of the oxirane rings and the assumption that all of the phenolic hydroxyl groups present underwent allylation. The Organosolv lignin, which had a lower level of hydroxyl groups per unit mass, exhibited the highest level of conversion, achieving approximately 90% conversion (Fig. 2b) versus 55% for Kraft lignin (Fig. 2c).

The molar mass distributions before and after epoxidation are shown in Table 3. Starting with vanillic acid, a small decrease in molecular weight is measured. This, and the absence of any higher molecular weight material on the GPC, suggests that there was no dimerization of vanillic acid epoxide. This ruled out the possibility that cross reactivity between rings was responsible for the relatively low level of oxirane rings measured. The limited final epoxidation of vanillic acid shown in Figure 2a is therefore likely due to enzyme inactivation, a possibility that was subsequently investigated.

Despite containing nearly 50% fewer hydroxyl groups per unit mass relative to Kraft lignin (Table 2), essentially an equal number of oxirane rings were formed with Organosolv and Kraft lignin (Fig 2). Results displayed in Table 3 indicated disparate molar mass distributions for the two lignin types, with Mw of ~6550 g/mol for the Kraft lignin compared to ~1600 g/mol for the Organosolv lignin. While the difference in conversions appears to be inversely correlated to lignin molecular weights, given the fact that approximately the same number of oxirane rings was formed for each lignin, it is equally plausible that the enzyme is inactivated during the course of the reaction. Since vanillic acid has a negligible molecular weight relative to either lignin and yet still exhibited low conversion, enzyme inactivation was investigated.

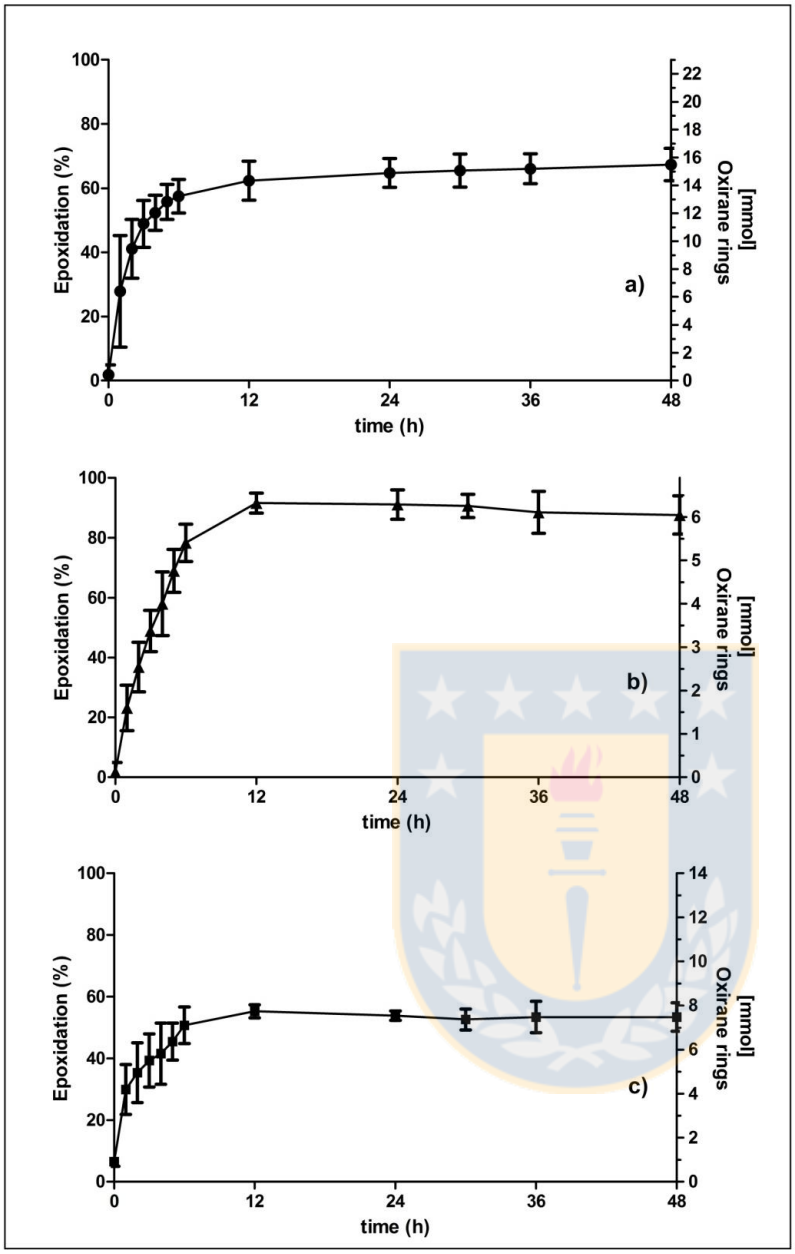

Figure 2. Chemoenzymatic epoxidation of (a) vanillic acid, (b) Organosolv lignin and (c) Kraft lignin in presence of lipase N435.

Table 2. Hydroxyl structures in lignins from Organosolv and Kraft processes determined by ^{31}P-NMR.

Lignin sample	Aliphatic-OH (mmol/g lignin)	Total phenolic-OH (mmol/g lignin)	COOH (mmol/g lignin)	Total OH (mmol/g lignin)
Organosolv	0.03	2.26	n.d.	2.29
Kraft	2.11	1.63	0.53	4.27

n.d. not detected.

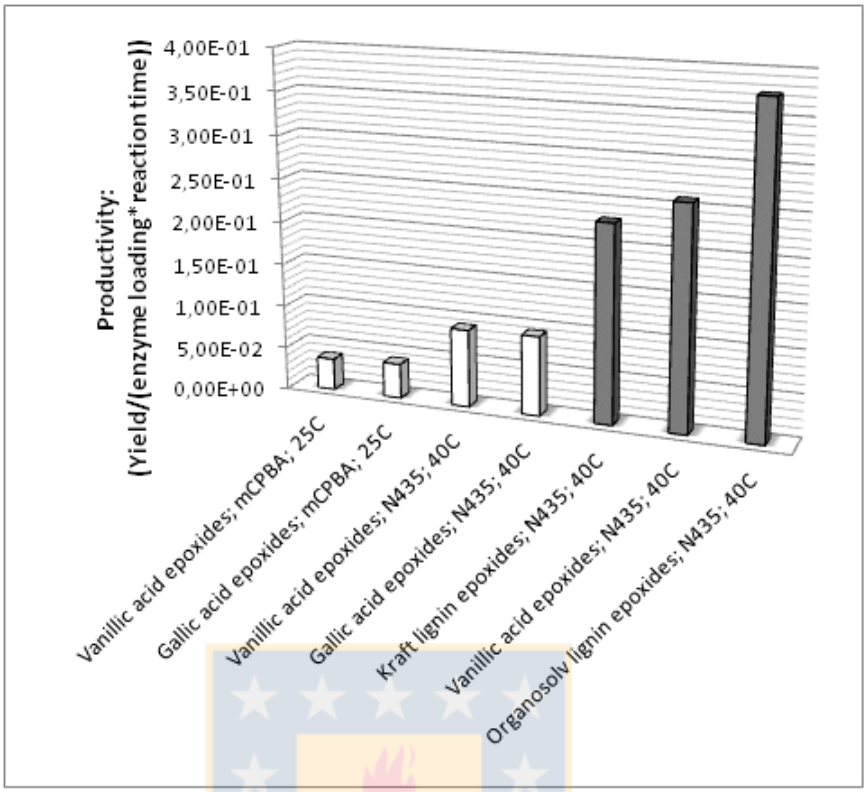

Figure 3. A comparison of epoxidation system productivity for lignin or lignin model compounds. Results from this work indicated in gray bars.

3.2 Enzyme stability

To investigate the stability of the lipase in these reaction systems, immobilized enzymes from batch epoxidation reactions were harvested at different time points and the residual enzymatic activity was quantified using the pNPB hydrolysis reaction immediately upon sampling, as well as following 48 h of soaking in phosphate buffer (Figure 4). The samples analyzed immediately upon sampling (darkened area) indicate that enzyme activity declines steadily during the course of the reaction. The rate of decay of activity appears to depend on the substrate, as indicated by the gradual decline of activity with vanillic acid (Fig. 4a) and the more rapid decline in the presence of the lignin substrates (Figs. 4b and 4c). The recovery of activity following washing also indicates that the vanillic acid reaction system causes a lower degree of

irreversible activity loss than do the lignin systems. Curiously, the Kraft lignin, which exhibited lower conversion than the Organosolv lignin, led to a lower permanent loss of activity than did the Organosolv lignin. The hypothesis that an interaction (absorption or covalent reaction) between the reactants (vanillic acid, lignin) and enzyme contributes to the activity loss was investigated.

Immobilized enzyme (lipase N435) was exposed to different reaction conditions. The control, involving the reaction solution without additional vanillic acid or lignin, indicated a rapid and dramatic decrease in enzyme activity, attributed to the presence of relatively high levels of hydrogen peroxide throughout the exposure time (Figure 5). The residual activity of enzyme after exposure to reaction solutions containing a phenolic substrate without hydrogen peroxide and caprylic acid is presented in Figure 6. The vanillic acid generated minor transient loss of activity that was fully restored following washing in sodium phosphate buffer (pH 6.5). The Kraft lignin caused a much greater transient loss of activity than did the vanillic acid, though essentially all of the activity was recovered upon washing. Exposure to Organosolv lignin resulted in the greatest loss of activity, both transiently and permanently.

Table 3. Molecular weight distribution of vanillic acid, Organosolv and Kraft lignins before (control) and after (epoxy) chemoenzymatic reaction.

Sample		Mn	Mw	Mw/Mn
Vanillic acid	Control	205	216	1.1
	Epoxy	176	183	1.0
Organosolv lignin	Control	1011	1649	1.0
	Epoxy	1108	1682	1.3
Kraft lignin	Control	1924	6821	3.5
	Epoxy	1383	6447	4.7

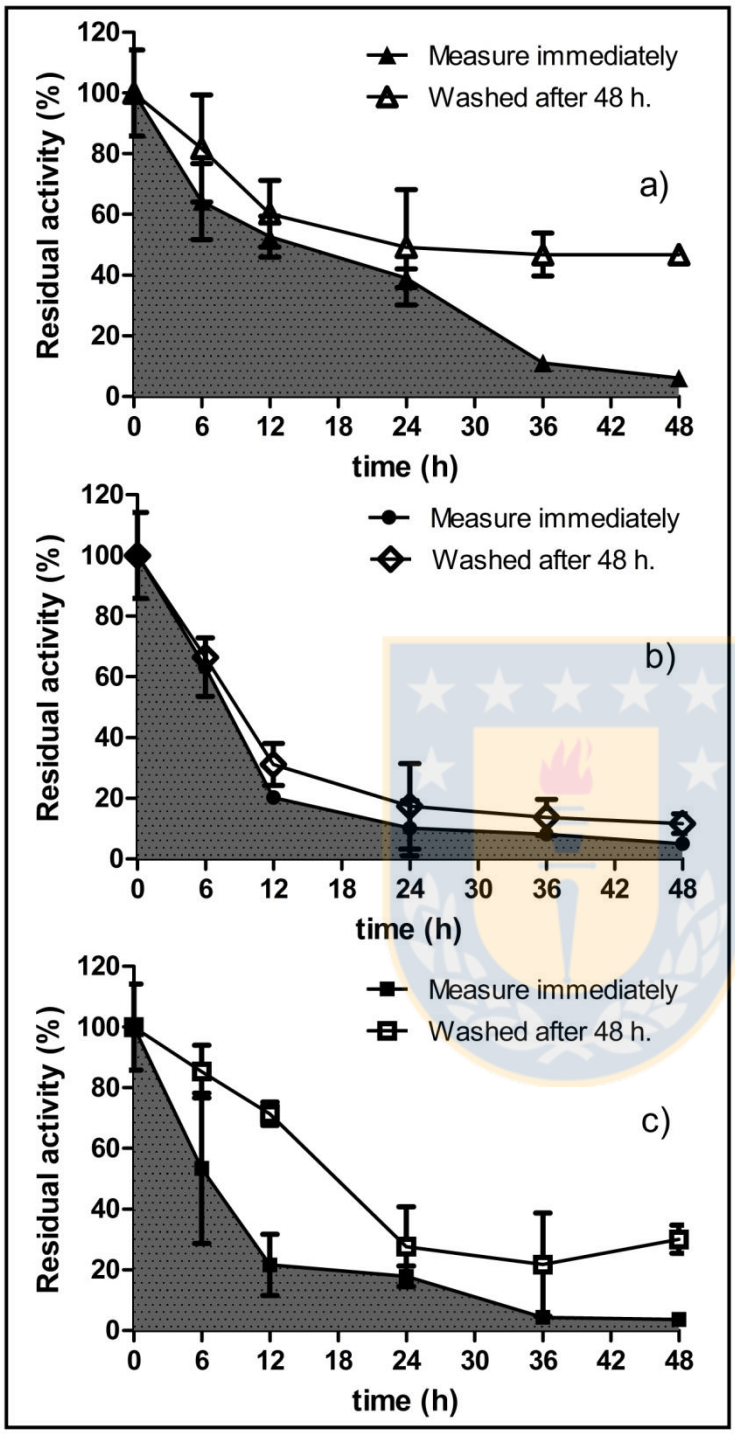

Figure 4. Residual activity of N435 during epoxidation of (a) allylated vanillic acid, (b) allylated organosolv lignin and (c) allylated kraft lignin. Closed symbols denote the immediately assayed samples and the open symbols represent the washed enzyme samples.

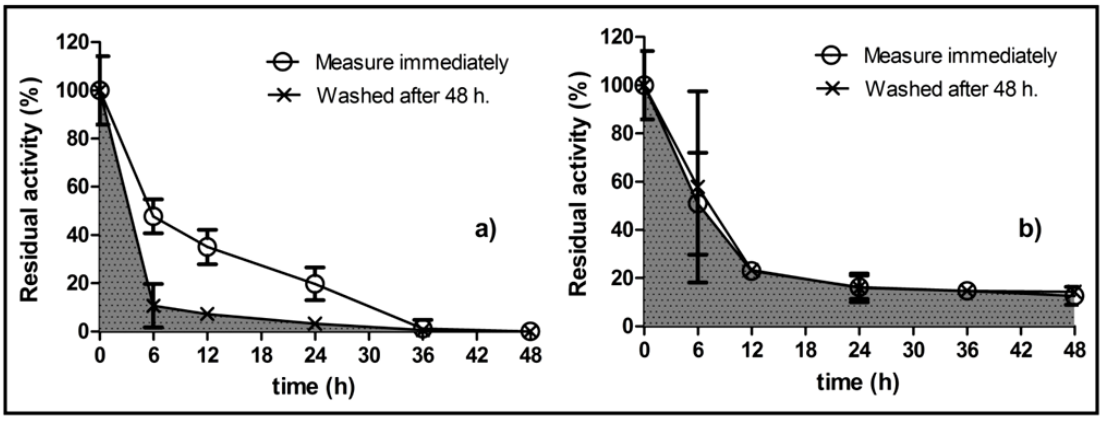

Figure 5. Control experiments with lipase B without the presence of phenolic compounds: a) residual activity of N435 only in presence of hydrogen peroxide and b) residual activity of N435 in the presence of caprylic acid and hydrogen peroxide.

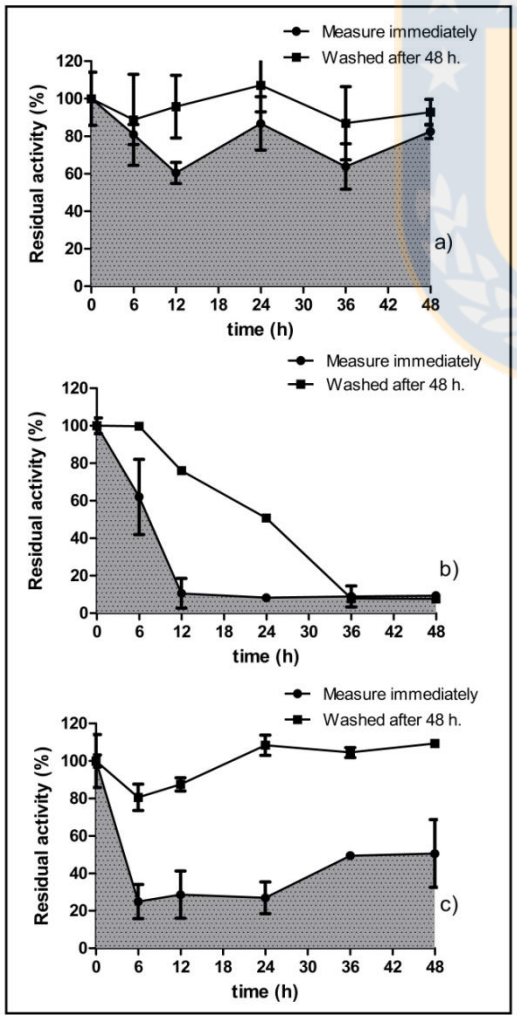

Figure 6. Residual activity of N435 in presence of phenolic compounds and in the absence of caprylic acid and hydrogen peroxide. Results are for (a) allylated vanillic acid, (b) allylated organosolv lignin and (c) allylated kraft lignin.

3.3 Implications of the Results

Chemoenzymatic epoxidation has proven to be an efficient mechanism to generate oxirane rings using lipase B (N435) for in situ generation of peroxyacids. The activity and stability of this chemoenzymatic system for coupled peroxidation/epoxidation reactions was evaluated. Comparable work (Aouf *et al.*, 2012) involves the epoxidation of vanillic and gallic acid by reaction with peroxyacids, either added as reagents (m-chloroperoxybenzoic acid: mCPBA) or generated in situ by a Novozym435/caprylic acid/H_2O_2 chemoenzymatic system (peroxycaprilic acid). As indicated in Table 1, it is difficult to compare the effectiveness of our chemoenzymatic systems since they report conversions only at 48 h, whereas our reaction systems reached essentially >98% of their 48-h conversion within the first 12 h of reaction. The primary focus of this work was on lignin compounds, and it clearly establishes that the phenolic compounds contained in Organosolv and Kraft lignins can be effectively epoxidized chemoenzymatically. Figure 3 indicates that our system is relatively efficient in this epoxidation, and that the Organosolv lignin low Mw substrate is highly reactive in this system.

Chemical epoxidation (mCPBA) exhibits a lower efficiency, needing higher relative reagent loading to achieve reasonable conversions. However, these reactions were performed at 25°C instead of 40°C, so again, it is difficult to make a direct comparison of performance.

Catalyst deactivation was quantified, and appears to be accountable for much of the observed differences in the initial rates of epoxidation of the different lignin types, and the final conversions achieved. Several potential mechanisms could cause the observed deactivation, including enzyme denaturation, loss of enzyme from the support, and blocking of the enzyme or active site due to substrate binding. Mechanistic studies of CalB deactivation in solutions with differing concentrations of H_2O_2 identified broken disulfide bridges at 0.2 – 2.0 M H_2O_2 (Törnvall *et al*., 2010), as well as, protein unfolding. Similar studies involving incubation at 40 ˚C for 3 h in

2.0M H_2O_2 resulted in structural changes (random coils denoting denaturation) and oxidation of susceptible amino acids (Törnvall *et al.*, 2007, 2010). Those results are relevant; in this study, aliquots of H_2O_2 were added to the reaction mixture every 30 min for 5 h, raising the H_2O_2 concentration from 0.6M to 6M.

Free enzyme in solution was reported to have lost 75% of its activity after 1 h and 100% after 3 h at 40˚C in 0.2M H_2O_2; polypeptide cleavage products were observed after 3 h Törnvall *et al.*, 2010). Immobilized CalB (N435) lost significant activity when incubated in H_2O_2 solutions at 22˚C, with the impact strongly dependent on concentration, as indicated by Hernandez (2011). It was also reported that the half-life of N435 at 40˚C was 33 h in 6 M H_2O_2 and 2.2 h in 12 M H_2O_2 (Törnvall *et al.*, 2007).

There are different end points for the irreversible loss of enzyme activity for each substrate evaluated in this study. One postulates that the immobilized enzyme can be treated as two separate populations – one that is susceptible to irreversible denaturation, and a second that is stable. The half-life of the susceptible enzyme activity can be calculated using the following analysis:

$$\boldsymbol{E_{total}(t) = E_{st} + E_{sus}(t)}$$

$$\boldsymbol{\frac{dE_{total}}{dt} = -kE_{sus}}$$

$$\boldsymbol{E_{total}(t) = E_{st} + E_{sus}(0)e^{-kt} = E_{st} + E_{sus}(0)e^{-(tln(2)/t_{1/2})}}$$

Using the washed activity data in Fig. 4 and 5b to determine the fraction of total enzyme that is stable for each substrate, values of the enzyme activity half-life were calculated to be between 5 and 7 h. These apparent half-lives are significantly lower than the value of 33 h reported for 6M H_2O_2. The lower functional half-lives calculated suggest that a mechanism other than denaturation

is contributing to the reduced enzyme activity. Comparison between Figures 4 and 6 indicates that the apparent immediate enzyme deactivation observed for the reaction systems can almost be approximated by a linear contribution between denaturation and adsorption to the catalyst pellet. In the cases of vanillic acid and Kraft lignin, the substrate adsorption proves to be nearly completely reversible upon washing with phosphate buffer, so the ultimate (i.e. post-washing) deactivation is dominated by H_2O_2/peroxyacid-mediated enzyme denaturation, indicated in Fig. 5. The binding of the Organosolv lignin is less reversible, and it ultimately dominates the response for that reactive system.

4. Conclusions

Immobilized lipase effectively catalyzed the peroxyacid-mediated epoxidation of Kraft and Organosolv lignins, with yields as high as 90% based on the assumption of 100% allylation of hydroxyl groups. The yield appears to be limited by the degree of irreversible denaturation of the enzyme, caused by extended exposure to hydrogen peroxide. Some loss in enzyme activity observed was reversible, caused by adsorption of the substrate to the catalyst pellet. Enzyme deactivation was complete within 12 – 24 h, and adsorption to the substrate afforded additional enzyme stability. Kraft lignin, due to the greater retention of residual enzyme activity relative to Organosolv lignin, appears to be a more suitable lignin substrate for chemoenzymatic epoxidation. Characterizing the adhesive properties of resins formed from these epoxidized lignins is needed before a definitive substrate selection can be made.

Acknowledgements

This work was performed in the Laboratory of Renewable Resources of the Biotechnology Center, Universidad de Concepción, Chile and at the North Carolina State University, EE.UU. F. Vásquez-Garay was supported by CONICYT (Doctoral grant No. 21130772) and lipase N435 was kindly donated by Novozyme.

CHAPTER III

Lignin epoxide resin characterization and response surface optimization of chemoenzymatic epoxidation of lignins using *Candida antarctica* lipase B

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This chapter is being prepared for submission as Enzyme and Microbial Technology

Abstract

Epoxy resins are used in the synthesis of a variety of end products, most significantly adhesives and thermosetting polymers. As an alternative to petroleum-based production, the use of modified lignin as a raw material for adhesive resins was evaluated. Epoxy resins based in Organosolv and Kraft lignin modified by chemo-enzymatic epoxidation were generated and important thermal properties were measured. The lignin-based resins were cured with 4,4'-diaminodiphenyl methane (DDM). The thermal stability of the resulting lignin-based epoxy resins were studied using thermogravimetric analysis (TGA).The curing behavior process of the lignin-based epoxy resins was studied using differential scanning calorimetry and the glass transition temperature (*Tg*) was determined by this methodology. The lignin-based epoxy resins after curing displayed lower and similar thermal stability than the commercial epoxy resin, exhibiting TGA_{50} values between 350 – 400 °C. The behavior of the lignin-based epoxy resins was comparable to the commercial epoxy resin and superior to lignin-based resins that were epoxidized usingthe epichlorohydrin. The optimal conditions resulting for chemoenzymatic production of the resins using Novozym435 were 45°C and 11.9M of temperature and hydrogen peroxide concentration, respectively. The results presented in this investigation suggest its potential as an adhesive epoxy resin, but more understanding of the resin structure is necessary to understand the mechanism giving rise to the observed properties.

Keywords: Optimization; Lignin functionalization; Chemoenzymatic epoxidation; Epoxy resins; *Candida antarctica* lipase B.

1. Introduction

Approximately 75% of epoxy resins are produced from the diglycidyl ether of bisphenol A (DGEBA), prepared by the coupling of bisphenol A (BPA) and epichlorohydrin (EP) (Raquez *et al.*, 2010; Aouf *et al*., 2012; Chua *et al.*, 2012). Resins and polymers produced from them usually contain trace amounts of the original reactants, in this case, BPA. BPA exposure is found to be problematic for humans, given its estrogen-like structure that could cause health problems in the endocrine system (Howdeshell *et al.*, 1999; Vanderberg *et* al., 2007; Geens *et* al., 2011; Aouf *et al*, 2012; Huang *et al*., 2012; Maćczak *et al*., 2015*)*. These recent findings have prompted efforts to develop commercial epoxides that are BPA-free (Howdeshell *et al.*, 1999; Baye *et* al., 2005; Aouf *et al*, 2012; Aouf *et al*, 2013; Kim and Sharma, 2012).

Lignins have been investigated for use as a substitute for phenol in the formulation of phenolic adhesives (El Mansouri *et al*., 2006; Jing *et al*., 2015; Kalami *et al*., 2017; Ma *et al*., 2018). The hydroxyl groups present in the polyphenolic structure of lignin are proposed as sites for epoxidation. One novel development utilizing lower molar mass lignin was the synthesis of thermostable epoxy resins used mainly as adhesives in the production of elastomeric compounds (Ragauskas et al., 2014).

Synthesis of epoxy structures from aromatic hydroxyl groups have been studied using lignin model compounds (Kudanga *et al*., 2010; Aouf *et al*., 2012; Aouf *et al*., 2013; Asada *et al*., 2015; Ferdosian *et al*., 2015; Ferdosian *et al*., 2016). Recently, this group (Vásquez-Garay *et al*., 2018) evaluated the chemoenzymatic epoxidation of the hydroxyl groups of lignins derived from organosolv and kraft pretreatment processes. As much as 90% of the lignin hydroxyl groups (phenolic, aliphatic and carboxyl) were converted to oxirane rings under mild conditions. These encouraging results motivated efforts to determine the performance of resins generated from lignin

that was epoxidized in this fashion. The suitability of the resulting material as an epoxy resin was represented by the response of the material to thermal processing with a curing agent, particularly the glass transition temperature and thermal stability. Comparison with the performance of a commercial epoxy resin (Araldite GZ50) will indicate whether the proposed chemoenzymatic process for bio-based epoxy resin synthesis, based on green technology and free of petroleum derivatives, resulted in a material with performance on par with commercial epoxy resins.

In addition to determining the performance of lignin epoxy resins, the conditions for maximal lignin epoxidation were investigated, since previous the time-temperature history and exposure to hydrogen peroxide are likely to have an impact on the properties of the modified lignin. It is proposed that temperature and hydrogen peroxide concentration significantly affect the conversion. A Circumscribed Central Composite (CCC) experimental design, with star points, was utilized to determine the significance of these variables. The primary response variable was the epoxidation percentage. This was quantified through oxirane ring titration and is reported as a percentage of the total possible number of oxirane rings, based on the assumption that all hydroxyl groups have been allylated and could be converted to epoxides.

2. Materials and Methods

2.1 Reagents

As described in Vásquez-Garay *et al.* (2018) organosolv lignin was recovered following pretreatment, and kraft lignin was obtained from the Domtar process. The standard epoxidation reaction mixture contained a source of phenolic hydroxyl groups (lignin), allyl bromide (99%), caprylic acid (98%), immobilized *Candida antarctica* lipase B (CalB) (Novozyme 435), dimethylformamide (DMF) from Sigma Aldrich, and toluene (99.8%) from Fisher Chemical.

Epichlorohydrin and the curing agent 4,4'-diaminodiphenyl methane (DDM), used for resin preparation prior to thermal stability, were purchased from Sigma Aldrich.

2.2 Experimental Design

Variables deemed having significant influence on the course and outcome of the epoxidation reactions were reaction temperature and hydrogen peroxide concentration. To understand the impact of these variables on the epoxidation reaction, a Circumscribed Central Composite (CCC) with star points for two factors was utilized. The critical variables and ranges were temperature (20°C – 60°C) and H_2O_2 (1.8 M – 10.2 M). The time of reaction was constant at 12 h. Eleven experiments were performed; the specific conditions are showed in the table 1. Response surface methodology was use to identify optimal conditions for oxirane ring formation. The software used for the analysis and modelling was Modde 7.0 (Umetrics).

Table 1. Experimental Design for epoxidation reaction optimization of Organosolv/Kraft lignin using N435 lipase B.

Experiment	XT	X[H_2O_2]	T (°C)	[H_2O_2] M
N1	-1	-1	20	1.8
N2	+1	-1	60	1.8
N3	-1	+1	20	10.2
N4	+1	+1	60	10.2
N5	-1.68	0	11.7	6
N6	+1.68	0	68	6
N7	0	-1.68	40	0
N8	0	+1.68	40	11.9
N9	0	0	40	6
N10	0	0	40	6
N11	0	0	40	6

2.3 Allylation

Lignin allylation was accomplished as described in Vásquez-Garay *et al*. (2018). Briefly, DMF was added to lignin, the solution was cooled with an ice bath and potassium carbonate added. After five minutes, allyl bromide was added and the mixture stirred for 48 h. Water was added and the aqueous phase was extracted with ethyl acetate. The organic phase was washed with brine, then dried over $MgSO_4$ and vacuum concentrated. The concentrate was used for the epoxidation studies. All reactions were run with excess allyl bromide for extended periods to allow for complete allylation of the hydroxyl groups present in the lignin samples.

2.4 Epoxidation

Chemo-enzymatic epoxidation reactions were performed in 100 mL three necked flasks equipped with a magnetic stirrer. In the first step, ~ 0.54 g of allylated lignin, and 0.47 mL of caprylic acid (99 % w/v) were dissolved in 100 mL of toluene. Then, immobilized lipase (Novozym 435; 20% wt% relative to the weight of lignin) was added and the mixture heated to temperature for each experiment. In the second step, the specific quantity of hydrogen peroxide (30% v/v) was added over 5 h to achieve the concentrations specified in Table 1. When epoxidation was completed, the biocatalyst was removed from the reaction mixture by filtration, the filtered reaction mixture was washed with 2 × 100 mL of water, and the aqueous phase was extracted with 3 × 150 mL of ethyl acetate. The organic phase was dried over $MgSO_4$ and vacuum concentrated. The crude product obtained was stored for oxirane ring measurement.

2.5 Enzymatic activity assays

The lipase activity was quantified using the p-nitrophenyl butyrate (pNPB) (Sigma Aldrich) hydrolysis reaction. An emulsion of substrate (1-4 mM in 50 mM potassium phosphate

buffer, pH 6.5, with 0.5% Triton-X 100) was added to a final catalyst loading of 20 mg dw/mL. Samples with immobilized N435 were gently agitated in a temperature-controlled water bath during the assay. The temperature was maintained at 30°C and the absorbance of the supernatant at 400 nm was measured using a UV-Visible spectrophotometer (Shimadzu UV-1650 PC). The maximum observed absorbance within 10 minutes was recorded. The concentration of p-nitrophenol was calculated based on the absorbance and standard curves prepared in an appropriate buffer (Vásquez-Garay *et al*., 2018).

2.6 Oxirane number measurement

ASTM standard protocol D1652–04 was used to quantify the oxirane number of epoxidized lignin, based on the methodology proposed by Jay (1964). Samples were taken at intervals during the reaction (0, 3, 6, 9 and 12 h). Samples were transferred into a beaker and maintained under constant agitation. Then, 15 mL of dichloromethane was added, followed by the addition of 10 mL of 20% tetraethylammonium bromide reagent and 6 to 8 drops of crystal violet indicator solution. The resulting solution was titrated with 0.1 *N* perchloric acid reagent to a green end point. The titration volume of perchloric acid reagent was used to calculate the weight percent epoxide, as described previously (Vásquez-Garay *et al.*, 2018). The degree of epoxidation, reported as the percent epoxidation, was calculated by normalizing the oxirane number relative to the total number of available hydroxyl groups in the untreated lignin, as determined by NMR (Vásquez-Garay *et al*., 2018).

2.7 Resin preparation

Eight epoxy resins, indicated in Table 2, were synthesized and cured in the presence of DDM. Six utilized enzymatically-epoxidized lignin representing the maximum (EM) and

minimum (Em) epoxide conversion products for each lignin type (OL, KL) generated in this study (see Table 4). Four resins (ROL-EM, ROL-Em, RKL-EM, RKL-Em) were cured with DDM. Two other resins (ROL-EMEp, RKL-EMEp) reacted the maximal epoxidation lignin with epichlorohydrin prior to curing. Two control resins (ROL-Ep, RKL-Ep) reacted untreated lignin with epichlorohydrin prior to curing. The last two resins synthesized involved adding untreated Organosolv or Kraft lignin to the corresponding quantity of 20% (w/w) of NaOH, and the blend was stirred continuously for 30 min before addition of epichlorohydrin (1/10; L/EPC). The conditions of reaction used were 70°C for 3h (El Mansouri *et al.*, 2011).

Reaction with epichlorohydrin: Resins were added to a corresponding quantity of 20% (w/w) of NaOH, then the mixture was stirred continuously for 30 min. After that time, epichlorohydrin was added to achieve a specific mass ratio (1/10; L/EPC). This mixture was reacted for 3h at 70°C (El Mansouri *et al.*, 2011). The resins obtained were then cured in the presence of DDM.

Curing: The method described in Ferdosian *et al.* (2015) was used to cure the resins. Briefly, the resins were heated to 80˚C to lower the viscosity. A stoichiometric amount of preheated DDM was added (1:1 active amine hydrogen to oxirane ring), based on the known degree of epoxidation of the chemo-enzymatically processed lignin. The mixture was held at 80˚C for 1 min to assure sufficient dispersal of the DDM, then quenched in an ice bath.

Table 2. Formulations of epoxy resins synthesized.

Resin name	Oxirane ring (mmol/g resin)	L/EPC *	DDM (mmol/g resin)	DDM (g)
ROL-Em	0.76	-	0.4	0.08
ROL-EM	2.42	-	1.2	0.24
RKL-Em	1.38	-	0.7	0.14
RKL-EM	3.58	-	1.8	0.35
ROL-EMEp	2.42	1:10	1.2	0.24
RKL-EMEp	3.58	1:10	1.8	0.35
ROL-Ep (Control)	2.29	1:10	1.1	0.23
RKL-Ep (Control)	4.27	1:10	2.1	0.42

*Mass ratio between lignin and epichlorohydrin

2.8 Thermogravimetry Analysis (TGA) and Differential Scanning Calorimetry Analysis (DSC)

The thermal stability of the epoxy lignin resins was analyzed using TGA and DSC. DSC was used to study the curing process of lignin epoxy resins through determination of the glass temperature (*Tg)*. The analysis was carried out in a Mettler Toledo DSC1. Resin samples (10 mg each) were subjected DSC thermal cycle, heated from 25 to 300 °C at 10 °C/min. DSC analyses were conducted under N_2 flowing at 60 mL/min.

The thermal degradation analyses (TGA) were performed using a TG 1000 (TA Instruments) to characterize the mass loss of resin samples as a function of temperature. The resin samples, approximately 5 mg, were heated from 30 to 550 °C at 10°C/min under N_2 flowing at 100 mL/min. The T_g of the cured biobased epoxy resins were indicated by the inflection point of the stepwise transition (Ferdosian *et al.*, 2016). The onset curing temperature

(T_{onset}), the peak curing temperature (T_p), and the end (T_{end}) curing temperature were also determined.

3. Results and Discussion

3.1 Thermal stability and glass transition temperature of epoxy lignin resins

3.1.1 Thermogravimetric analysis (TGA)

The thermogravimetric (TGA) profiles of cured epoxy resins are show in figure 1. The thermal stabilities of the resins were determined by TGA and DTGA analyses. The rates of weight loss of different resins were measured and compared to a commercial resin. Four lignin samples were analyzed; the maximally and minimally epoxidized for each lignin (ROL-EM, ROL-Em, RKL-EM, RKL-Em). These were compared to lignin that were epoxidized using only epichlorohydrin.

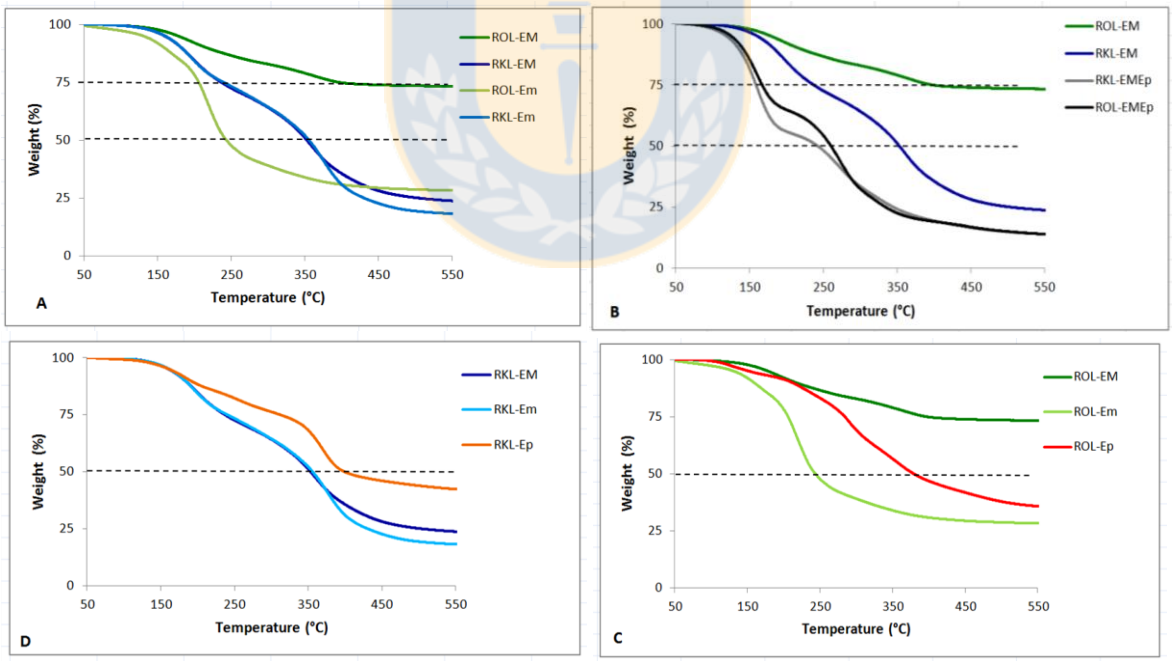


Figure 1. TGA profiles of cured epoxy resins: A) resins synthesized by chemo-enzymatic mechanism using organosolv and kraft lignins, B) resins synthesized with maximal chemo-enzymatic epoxidation yields vs lignin epoxy resins synthesized using EPC, C) organosolv epoxy resins synthesized by chemo-enzymatic mechanism compared against Organosolv epoxy resin

synthesized with EPC, and D) kraft epoxy resins synthesized by chemo-enzymatic mechanism compared against kraft epoxy resin synthesized with EPC.

It is apparent from figures 1A and 1B that organosolv lignin that has undergone maximal chemoenzymatic epoxidation is the most thermally-stable resin, outperforming all kraft-derived resins. Perhaps that should not be surprising given the fact that kraft lignin exhibits much lower chemo-enzymatic epoxidation than does organosolv lignin, so there are more reactive groups (allyl and hydroxyl) present to potentially contribute to thermal degradation for kraft lignin. What was unexpected was that reaction with epichlorohydrin, the traditional reagent used in commercial epoxidation, decreased the thermal stability of enzymatically epoxidized organosolv lignin, as indicated in figure 1B.

The results for kraft lignin are somewhat contradictory. As indicated in figure 1B, reacting epoxidized kraft lignin with epichlorohydrin reduces the thermal stability. Conversely, figure 1D indicates that kraft lignin that has been epoxidized by epichlorohydrin is more thermally stable than that generated by chemoenzymatic epoxidation.

Table 3 summarizes the thermal degradation of the resins and provides a comparison with the performance of a commercial epoxy resin. Of particular interest are the glass transition temperatures (T_g) for the resins, when they go from a glassy (brittle) state to a rubbery state. T_g is representative of the degree of crosslinking present in the polymer, with lower values indicating polymers capable of being processed at lower temperature, but also losing rigidity at lower temperatures. Resins derived from kraft lignin uniformly had higher T_g values than resins derived from organosolv lignin. Furthermore, with the exception of the RKL-EMEp resin, T_g values were lower than a representative commercial epoxy resin.

Table 3. Curing and thermal degradation behavior of epoxy resins

Resin name	T_g	T_{onset}	T_p	T_{end}	TGA_{50}	$DTGA_{max}$***
ROL-Em	56	40	87	152	240	220
ROL-EM	58	40	98	158	>395*	195
RKL-Em	75	41	101	143	355	200
RKL-EM	67	41	100	139	355	200
ROL-EMEp	58	39	98	152	255	155
RKL-EMEp	165	112	203	250	233	160
ROL-Ep (Control)	70	41	107	160	383	126
RKL-Ep (Control)	60	40	81	124	360	180
DGEBA-DDM**	105	76.8	160	260	450	405

*weight loss determined to 25%
** commercial epoxy resin information (Araldite GZ540x90, DGEBA) cured with DDM (Ferdosian *et* al., 2016).
*** results showed in appendix I.

TGA_{50} values are a second indication of thermal stability. Commercial epoxy resin loses 50% of its weight by 450°C (Table 3). A similar thermal degradation is observed from Kraft lignin with maximal or minimal chemoenzymatic epoxidation (RKL-EM and RKL-Em), with a TGA_{50} value of 355 °C, which is lower than that for commercial epoxy resin. The TGA_{50} results indicate that resin generated from kraft lignin is higher than resins derived from organosolv lignin. This could be due to higher concentration of hydroxyl groups (converted to the oxirane rings) per mass unit, and that this lignin is more condensed relative to organosolv lignin and hence has a higher cross-linking density. Ferdosian *et al*., (2016) suggests that lower thermal stability of biobased epoxy resins produced from lignin could be due to a lower epoxy index, indicating less cross-linking.

There is one anomalous result in table 3; the ROL-EM sample, that maintained 75% of initial sample mass through 395°C and beyond. It will be necessary to repeat these analyses in

order to establish the validity of this result, and if validated, to identify the mechanism by which such unique thermal stability is achieved.

3.2 Chemoenzymatic epoxidation

Having established that the resins generated using chemoenzymatic epoxidation exhibit thermal stabilities similar to commercial resins, it is important to identify the most effective conditions for the production of these resins.

A two-factor analysis was used to determine the conditions giving rise to the maximum degree of epoxidation (the response variable) for organosolv and for kraft lignin. The reaction temperature and hydrogen peroxide concentration were varied, while the reaction time was held constant at 12h. Table 4 indicates the reaction conditions for each experiment, as well as the resulting extent of epoxidation and the specific oxirane ring content of the resulting material.

Table 4. Chemoenzymatic lignin epoxidation results in each experiment and productivity of oxirane rings based per 1 g of lignin per 1 g of immobilized N435 lipase.

Experiment	Conditions T/H_2O_2	Lignin Epoxidation (%)		Oxirane rings [mmol/g lignin*g N435]	
		Organosolv	Kraft	Organosolv	Kraft
1	20°C/1.8 M	39	35	4.4	8.0
2	60°C/1.8M	63	56	7.1	13.0
3	20°C/10.2M	43	43	8.7	10.1
4	60°C/10.2M	99	59	10.0	13.9
5	12°C/6M	34	29	3.8	6.9
6	68°C/6M	41	39	4.0	9.0
7	40°C/0.06M	45	48	5.1	8.6
8	40°C/12M	103	76	12.1	17.9
9	40°C/6M	80	64	9.1	15.1
10	40°C/6M	73	63	8.2	14.8
11	40°C/6M	78	62	8.7	14.6

This data allows the generation of an equation for each lignin type that relates the response variable (Y; % epoxidation) to the reaction temperature (X_1) and hydrogen peroxide concentration

(X_2). For each lignin type, the relationship yielding the best fit to the data had the following form: $\mathbf{Y = a + bX_1 + cX_2 + d(X_1)^2}$. Using the Modde software package resulted in the parameter values shown in Table 5.

Table 5: Parameter values for the dependence of epoxidation percentage on reaction temperature and hydrogen peroxide concentration, and corresponding ANNOVA parameter values.

Lignin type	a	b	c	d	p value	F value	R^2
Organosolv	76.95	11.23	15.31	-18.49	0.007	9.9	0.90
Kraft	62.67	6.33	6.39	-14.55	0.000	26.0	0.94

The resulting equations can be used to generate response surfaces for each lignin type. These surfaces are illustrated in Figure 2.

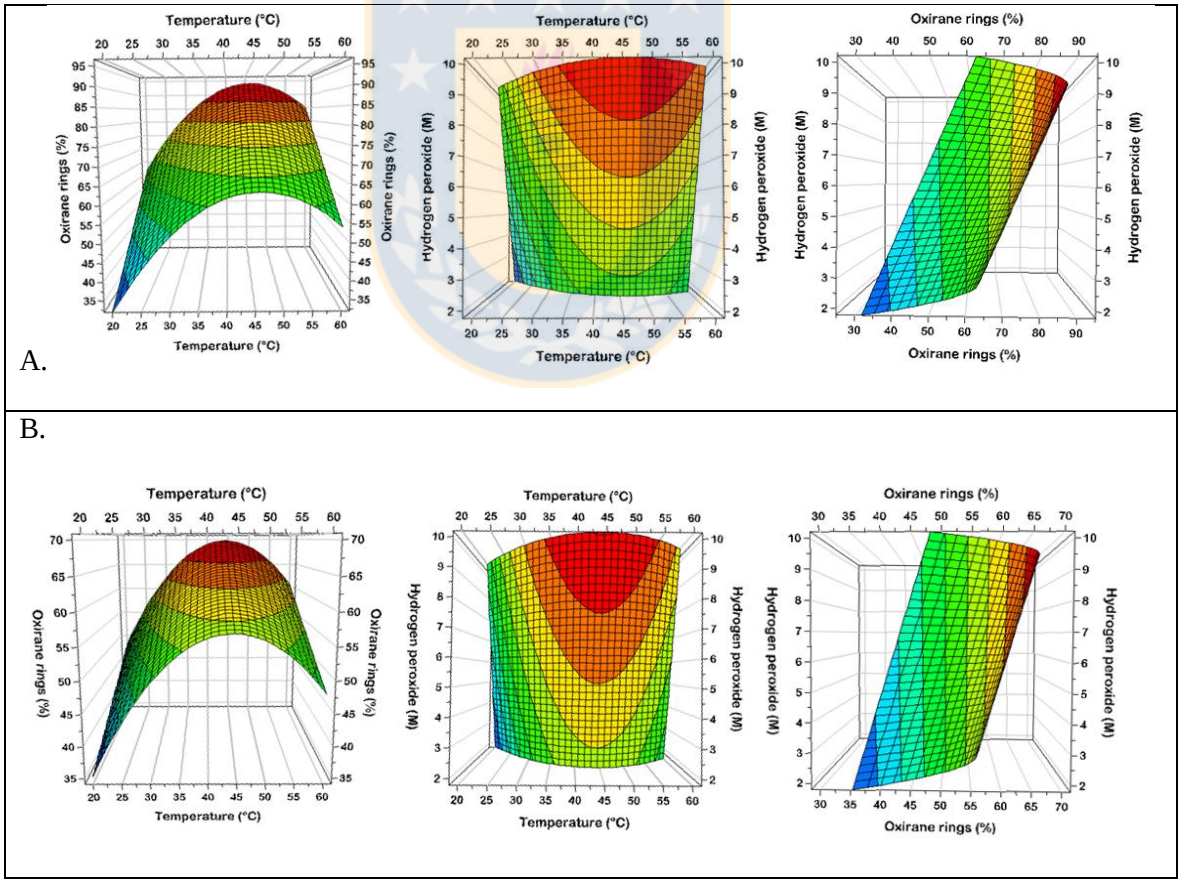


Figure 2. Response surfaces for A) Organosolv and B) Kraft lignin chemoenzymatic epoxidation oxirane ring yield.

The oxirane ring yield using organosolv lignin was measured at a maximum (~100%) when the reaction is operated at a temperature of 40˚C with a hydrogen peroxide concentration of 12M. For kraft lignin, the highest oxirane ring yields measured (76%) were achieved under the same reaction conditions. These results are close to those based on the response surface correlations, which are shown in table 6.

Table 6. Optimal reaction conditions for chemoenzymatic epoxidation of Organosolv and Kraft lignin.

Variable	Epoxidized lignin	
	Organosolv	Kraft
Temperature (°C)	45	44
Hydrogen peroxide [M]	11.9	11.9
Oxirane rings predicted (%)	101	72
Oxirane rings obtained (%)	98.4	74.3

3.3 Residual activity study

Enzyme deactivation is a significant issue in the presence of hydrogen peroxide and will have a major influence on the economic viability of the process, as well as the actual operating conditions in the reactor. The residual activity of N435 is shown in Table 7. These results mirror those reported previously by Vásquez-Garay *et al*., (2018), in that the general trend is that residual activity is maintained to a much higher level for reactions involving Kraft lignin than Organosolv lignin.

Table 7. Residual enzyme activity following chemoenzymatic epoxidation of Organosolv and Kraft lignin.

		Residual Activity (%)	
Experiment	Temp/[H_2O_2]	Organosolv	Kraft
1	20°C/1.8 M	59	40
2	60°C/1.8M	7	32
3	20°C/10.2M	36	10
4	60°C/10.2M	8	6
5	12°C/6M	27	45
6	68°C/6M	5	3
7	40°C/0.06M	46	61
8	40°C/12M	16	58
9	40°C/6M	17	67
10	40°C/6M	9	63
11	40°C/6M	7	71

3.4 Implication of the results

Lignin can undergo condensation reactions when exposed to chemicals or elevated temperature. Since the chemoenzymatic process of lignin epoxidation involves alkaline-assisted allylation, as well as extended (12h) exposure to hydrogen peroxide, it is important to coordinate studies of lignin epoxide resins with material synthesis optimization. The thermochemical analyses of epoxy resins from both organosolv and kraft lignin indicate that the glass transition temperatures for both lignins do not change significantly as a result of chemoenzymatic epoxidation, and generally remain approximately 30˚C lower than a representative commercial resin.

The small difference in *Tg* between resins derived from organosolv versus kraft lignin could be the result of the unreacted hydroxyl groups that exist in kraft lignin that are essentially absent in the processed Organosolv lignin. The hydroxyl groups could be promoting the curing process via etherification and homopolymerization reactions (Ferdosian *et al.*,2016). This would

increase the cross-linking density of the kraft-derived resin, thereby requiring higher temperatures to begin the glass to rubbery transition. Ferdosian *et al*., (2016) reported similar behavior.

The TGA_{50} values of kraft resins were about 355 ˚C. Comparable results were reported by Jablonskis *et al.,* (2018) for a 10% lignin-substituted BPA-based epoxy resin using functionalized kraft lignin. The TGA_{50} reported for that blend was 370°C. Using organosolv lignin, we observed a TGA_{50} close to 380°C.

In one resin, higher thermal stability was observed; the ROL-EM resin exhibited an apparent TGA_{50} close to 395°C. The behavior of ROL-EM shown in figure 1 is qualitatively different from all of the other chemoenzymatic resins, derived from either kraft or organosolv lignin, so further verification of this behavior is needed. It is important to note that Asada *et al*., (2015) carried out the synthesis of an epoxy resin using lignin from *Eucalyptus globules* and reported 25% weight loss at approximately 390 °C. As with the ROL-EM resin synthesized in this study, the lignin used by Asada *et al*., (2015) was organosolv in nature (methanol soluble), with low Mw (1300 – 1400 vs. ~1650 for ROL-EM), and derived from *Eucalyptus globulus*.

4. Conclusions

Organosolv and kraft lignin that was epoxidized chemoenzymatically gave rise to epoxy resins, after curing, that exhibited thermal properties lower or equivalent in some cases, superior in one case, to those of a representative commercial resin. The superior performance observed is consistent with behavior reported in the literature for resins synthesized from similar starting material. While the mechanism by which this thermal stability arises deserves further investigation, what has been clearly established is that the chemoenzymatic epoxidation process can be operated with high efficiency while simultaneously producing material suitable for at least

partial substitution for BPA-derived resins. Earlier studies suggested that for reasons of enzyme stability, kraft lignin was a preferred substrate for chemoenzymatic epoxidation. The thermal stability results presented here suggest that organosolv lignin may give rise to the superior resin.

Acknowledgements

This work was performed in the Laboratory of Renewable Resources of the Biotechnology Center, Universidad de Concepción, Chile in collaboration with the Department of Chemical Biomolecular Engineering of North Carolina State University, EE.UU and Leitat Chile Foundation, Chile. F. Vásquez-Garay was supported by CONICYT (Doctoral grant No. 21130772) and lipase N435 was kindly donated by Novozymes.

The authors declare no conflicts of interest.

CAPITULO IV: Conclusiones generales

Esta investigación estableció varios resultados significativos:

1. Es posible catalizar la epoxidación mediada por peroxiácidos de lignina organosolv y lignina kraft usando lipasa inmovilizada (CalB), con rendimientos que son significativos desde una perspectiva comercial (entre 60 - 90%) y con tasas de productividad que son 2x - 3x más alto de lo que se reportó anteriormente.

2. Las enzimas involucradas sufren una desactivación principalmente reversible con lignina Kraft, indicando que con ingeniería de proteínas adicional, sería posible desarrollar un sistema enzimático más robusto.

3. Las resinas resultantes del procesamiento de lignina exhiben estabilidad térmica y temperaturas de procesamiento que son similares, y posiblemente superiores, a las resinas epóxicas comerciales.

4. Las resinas se pueden sintetizar rápidamente en condiciones de procesamiento moderadas.

5. La lignina organosolv demostró ser una materia prima más homogénea determinada a través de su *Mw* inferior. También demostró ser más reactivo que la lignina kraft como se determina a través del porcentaje de epoxidación total en condiciones óptimas.

6. El grado de reversibilidad de la inhibición enzimática fue mucho mayor para la lignina kraft que para la lignina organosolv.

Estos resultados tienen implicaciones para los esfuerzos por desarrollar un proceso quimio-enzimático económicamente viable para la síntesis de resinas epóxicas. Las resinas sintetizadas presentan comportamientos comparables a las que se producen con una resina comercial basada en BPA en presencia de un agente de curado, como lo indican la temperatura de transición vítrea y la degradación térmica. Debido a que el BPA es un derivado del petróleo, es posible reemplazarlo con lignina quimio-enzimáticamente epoxidada (lignina-QEE), reduciendo o posiblemente eliminando la necesidad de derivados del petróleo en la fabricación de resina epóxicas.

Las resinas de lignina-QEE muestran una estabilidad considerable a altas temperaturas. A través del estudio del proceso de curado y con las temperaturas de transición vítrea (*Tg)* medidas, podemos concluir que las resinas de lignina-QEE son resinas termoendurecibles con características de procesamiento similares a las de una resina epóxica comercial. Se necesitan más estudios para establecer las características adhesivas de las resinas sintetizadas.

Con respecto al uso de una enzima inmovilizada tal como la lipasa B (N435) en un proceso de conversión de lignina, fue posible optimizar las condiciones de reacción basándose en las variables de temperatura y la concentración de peróxido de hidrógeno (H_2O_2). Como se esperaba, el proceso enzimático tiene sensibilidad a altas temperaturas y bajas temperaturas. La concentración de H_2O_2 resultó ser una variable importante en el sistema quimio-enzimático, dando como resultado concentraciones adecuadas entre 10 y 12 M. Sin embargo, sería importante establecer y comprender todos los cambios estructurales y químicos que la lignina epoxidada podría experimentar a altas concentraciones de H_2O_2. También es importante establecer la función catalítica que desempeña el ácido graso saturado utilizado en este mecanismo quimio-enzimático y determinar alternativas que puedan optimizar el mecanismo de reacción.

Al considerar la implementación comercial de un proceso basado en enzimas, es importante evaluar un proceso competitivo en términos de costo para la síntesis de una resina adhesiva epóxica. Para la resina lignina-QEE que se sintetiza a través de un proceso quimio-enzimático, el costo de producción se establece principalmente por los costos de la lignina (US $700/ton) (Hodásová et al., 2015) y la enzima inmovilizada (US $12,000/ton) (Eby y Peretti, 2015a). El uso de enzimas es del 20% (p/p) en relación con la lignina, por lo tanto el costo de una resina epóxica sintetizada quimio-enzimáticamente, sin reciclado enzimático, sería de al menos US $3000/ton aproximadamente, ignorando todos los otros costos del proceso. Si esto se compara con el costo de los principales reactivos de una resina epóxica comercial convencional (DGEBA) basada principalmente en BPA y epiclorohidrina, que tiene un costo aproximado de US $ 3.000/ton (Hodásová et al., 2015), podría competir en términos de costo para ingresar al mercado de resinas epóxicas basadas en BPA para diferentes aplicaciones.

Claramente, la reducción del costo de la enzima tendría un tremendo impacto en el costo de las resinas epóxicas de lignina sintetizadas quimio-enzimáticamente. Recientemente, Eby y Peretti (Eby y Peretti, 2015a, 2015b) informaron la síntesis de un biocatalizador enzimático, basado en la funcionalización de superficie de levadura (YSD) de CalB, que fue capaz de catalizar la síntesis de una variedad de ésteres a tasas similares a las logradas por Novozyme435. Este biocatalizador, que también es capaz de llevar a cabo la síntesis quimio-enzimática de epóxidos usando lignina/H_2O_2/ácido caprílico (resultados no mostrados), se estimó que costaría US $5860/ton (Eby y Peretti, 2015a), que tiene en cuenta el costo del cultivo de la levadura y la liofilización. Usando el mismo 20% (p/p) en relación con la lignina, el proceso quimio-enzimático podría reducirse a US $ 1.900/ton de resina.

Para concluir, la lignina funcionalizada tiene aplicaciones potenciales en la producción de resinas adhesivas epóxicas. El novedoso mecanismo quimio-enzimático establecido en esta tesis representa una vía "más verde" y ofrece la posibilidad de reemplazar el BPA por la lignina para la síntesis de estas resinas epóxicas.

CHAPTER IV: General conclusions

This research established several significant results:

1. It is possible to catalyze the peroxyacid-mediated epoxidation of organosolv and kraft lignin using immobilized lipase (CalB), with yields that are significant from a commercial perspective (between 60 – 90%), and at productivity rates that are 2x – 3x higher than previously reported.

2. The enzymes involved undergo primarily-reversible deactivation with Kraft lignin, indicating that with additional protein engineering, a robust enzyme system is possible to be developed.

3. The resins resulting from such processing exhibit thermal stability and processing temperatures that are similar, and possibly superior, to commercial epoxy resins.

4. The resins can be synthesized rapidly under mild processing conditions.

5. Organosolv lignin was shown to be a more homogeneous raw material determined through its lower Mw. It also proved to be more reactive than kraft lignin as determined through the percentage of total epoxidation under optimal conditions.

6. The degree of reversibility of enzyme inhibition was much higher for kraft lignin than for organosolv lignin.

These results have implications for efforts to develop an economically-viable chemoenzymatic process for the synthesis of epoxy resins. The resins synthesized presented behaviors comparable to those that occur with a commercial resin based on BPA in the process of curing, as indicated by the glass transition temperature and thermal degradation. Since BPA is a petroleum derivative, it is possible to replace it with chemoenzymatically-epoxidized lignin (CEE-lignin), reducing or possibly eliminating the need for petroleum in epoxy resin manufacture.

The CEE-lignin resins exhibit considerable stability at high temperatures. Through the study of the curing process and with the glass transition temperatures (*Tg*) measured, we can conclude that CEE-lignin resins are thermosetting resins with processing characteristics similar to a commercial epoxy resin. Further studies are needed to establish the adhesive characteristics of the resins.

Regarding the use of an immobilized enzyme such as lipase B (N435) in a lignin conversion process, it was possible to optimize the reaction conditions based on temperature and concentration of hydrogen peroxide. As expected, the enzymatic process has sensitivity to high temperatures and low temperatures. The concentration of hydrogen peroxide turned out to be an important variable in the chemo-enzymatic system, resulting in optimal concentrations between 10 and 12M. However, it would be important to establish and understand all the structural and chemical changes that epoxidized lignin could undergo at high concentrations of H_2O_2. It is also important to establish the catalytic function played by the saturated fatty acid used in this chemoenzymatic mechanism, and to determine alternatives that can optimize the reaction mechanism.

When considering commercial implementation of an enzyme-based process, it is important to evaluate a competitive process in terms of cost for the synthesis of an adhesive epoxy resin. For CEE-lignin resin that is synthesized through a chemo-enzymatic process, the production cost is established mainly by the costs of lignin (US $700/ton) (Hodásová *et al*., 2015) and the immobilized enzyme (US $12,000/ton) (Eby and Peretti, 2015a). Enzyme usage is 20 % (w/w) in relation to lignin, therefore the cost of an epoxy resin synthesized chemo-enzymatically, without enzyme recycle, would be at least US $3000/ton approximately, ignoring all other processing costs. If this is compared with the cost of the main reagents of a common commercial epoxy resin (DGEBA) based mainly on BPA and epichlorohydrin, which costs approximately US $3,000/ton (Hodásová *et al*., 2015), it could compete in terms of cost to enter the market of epoxy resins based on BPA for different applications.

Clearly, reducing the cost of the enzyme would have a tremendous impact on the cost of lignin epoxy resins synthesized chemoenzymatically. Recently, Eby and Peretti (Eby and Peretti, 2015 a, 2015b) reported synthesis of an enzymatic biocatalyst, based on yeast surface display (YSD) of CalB, that was capable of catalyzing the synthesis of a variety of esters at rates similar to those achieved by Novozym435. This biocatalyst, which is also capable of carrying out the chemoenzymatic synthesis of epoxides using lignin/H_2O_2/caprylic acid (results not shown), was estimated to cost US $5860/ton (Eby and Peretti, 2015a), which takes into account the cost of yeast cultivation and freeze drying. Using the same 20 % (w/w) in relation to lignin, the chemoenzymatic process could be reduced to US $1,900/ton of resin.

To conclude, functionalized lignin has potential applications in the production of epoxy adhesive resins. The novel chemoenzymatic mechanism established in this thesis represents a "greener" pathway to synthesis of these resins, and offers the potential to replace BPA with lignin as the main precursor for the synthesis of epoxy resins.

REFERENCES

Abdelaziz O. Y., Brink D. P., Prothmann J., Ravi K., Sun M., García-Hidalgo J., Sandahl M., Hulteberg C.P., Turner C., Lidén G., Gorwa-Grauslund M. F., Biological valorization of low molecular weight lignin, Biotechnology Advances. 34 (2016) 1318 – 1346.

Abdel-Hamid A.M., Solbiati J.O., Cann I.O, Insights into lignin Degradation and its Potential Industrial Application, Advances in Applied Microbiology. 82 (2013), Chapter one.

Aouf C., Lecomte J., Villenueve P., Dubreucq E., Fulcrand H., Chemo-enzymatic functionalization of gallic and vanillic acids: Synthesis of bio-based epoxy resins prepolymers, Green Chemistry. 14 (2012) 2328 – 2336.

Aouf C., Nouailhas H., Fache M., Caillol S., Boutevin B., Fulcrand H., Multi-functionalization of gallic acid. Synthesis of a novel bio-based epoxy resin, European Polymer Journal. 49 (2013) 1185 – 1195.

Asada C., Basnet S., Otsuka M., Sasaki C., Nakamura Y., Epoxy resin synthesis using low molecular weight lignin separated from various lignocellulosic materials, International Journal of Biological Macromolecules. 74 (2015) 413 – 419.

Baye T., Becker H.C., von Witzke-Ehbrecht S., *Vernonia galamensis*, a natural source of epoxy oil: variation in fatty acid composition of seed and leaf lipids, Industrail Crops and Products. 2 (2005) 257-261.

Biermann C.J. (1993) Essentials of pulping and papermaking (Academic Press).

Björkling F., Frykman H., Godtfredsen S.E., Kirk O., Lipase Catalyzed Synthesis of Peroxycarboxylic Acids and lipase Mediated Oxidations, Tetrahedron. 48 (1992) 4587 – 4592.

Cardona C., Sánchez O., Fuel ethanol production: Process design trends and integration opportunities, Bioresource Technology. 98 (2007) 2415 – 2457.

Chen H., Qiu W., Key technologies for bioethanol production from lignocelluloses, Biotechenolgy Advances. 28 (2010) 556 – 562.

Chua S., Xu X., Guo Z., Emerging sustainable technology for epoxidation directed toward plant oil-based plasticizers, Process Biochemistry. 47 (2012) 1439 – 1451.

Conti J., Holtberg P., Napolitano S., Schaal A.M., International Energy Outlook 2014 (US Energy Information Administration). (2014), (DOE).

Doherty W., Mousavioun P., Fellows C., Value-adding to cellulosic ethanol: Lignin polymers, Industrial Crops and Products. 33 (2011) 259 – 276.

Dutta S.W., Wu K.C.-W., Saha B., Emerging strategies for breaking the 3D amorphous network of lignin, Catalysis Science and Technology. 4(11) (2014) 3785-3799.

Eby J.M., Peretti S. W., Characterization, performance, and applications of a yeast surface display-based biocatalyst, RSC advances. 5 (2015a) 19166 – 19175.

Eby J.M., Peretti S. W., Performance in synthetic applications of a yeast surface display-based biocatalyst, RSC advances. 5 (2015b) 30425 – 30432.

El Hage R., Brosse N., Chrusciel L., Sanchez C, Sannigrahi P., Ragauskas A., Characterization of milled wood lignin and ethanol organosolv lignin from miscanthus, Polymer Degradation and Stability. **9**(10) (2009) 1632-1638.

El Mansouri N. E., Salvadó J., Structural characterization of technical lignins for the production of adhesives: Application to lignosulfonate, kraft, soda-anthraquinone, organosolv, and ethanol process lignins, Industrial Crops and Products. 24 (2006) 8 – 16.

El Mansouri N.E., Yuan Q., Huang F., Synthesis and characterization of kraft lignin-based epoxy resins, Bioresources. 6 (2011) 2492 – 2503.

Ferdosian F., Yuan Z., Anderson M., Xu C., Sustainable lignin-based epoxy resins cured with aromatic and aliphatic amine curing agents: Curing kinetics ant thermal properties, Thermochimica Acta. 618 (2015) 48 – 55.

Ferdosian F., Zhang Y., Yuan Z., Anderson M., Xu C., Curing kinetics and mechanical properties of bio-based epoxy composites comprising lignin-based epoxy resins, European Polymer Journal. 82 (2016) 153 – 165.

Gall D. L., Ralph J., Donohue T. J., Noguera D. R., Biochemical transformation of lignin deriving valued commodities from lignocellulose, Current opinion in Biotechnology. 45 (2017) 120 – 126.

Geens T., Goeyens L., Covaci A., Are potential sources for human exposure to bisphenol-A overlooked?, International Journal of Hygiene and Environmental Health. 214 (2011) 339-347.

González-Martínez D., Rodríguez-Matta M., Méndez-Sánchez D., Gotor V., Gotor-Fernández V., Lactonization reactions through hydrolase-catalyzed peracid formation. Use of lipases for chemoenzymatic Baeyer-Villiger oxidations of cyclobutanones, Journal of Molecular Catalysis B: Enzymatic. 114 (2015) 31 – 36.

Guerra A., Filpponen I., Lucia L., Argyropoulos D., Comparative evaluation of three lignin isolation protocols for various wood species. Journal of Agricultural and Food Chemistry 54(26) (2006) 9696 – 9705.

Henriksson G., Lignin. Wood Chemistry and Biotechnology, Pulp and Paper Chemistry and Technology, ed Ek MG, G; Henriksson, G (De Gruyter, Berlin). (2009)Vol 1.

Hernandez K., Fernandez-Lafuente R., Lipase B from *Candida Antarctica* immobilized on octadecyl Sepabeads: A very stable biocatalyst in the presence of hydrogen peroxide, Process Biochemistry. 46 (2011) 873 – 878.

Hodásová L., Jablonský M., Skulcová A., Ház A., Lignin, Potential Products and Their Market Value, Wood Research. 60(6) (2015) 973 – 986.

Howdeshell K.L., Hotchkiss A.K., Thayer K.A., Vanderbergh J.G., vom Saal F.S., Environmental toxins: Exposure to bisphenol A puberty, Nature. 401 (1999) 763 – 764.

Hu Z., Du X., Liu J., Chang H-M., Jameel H., Structural Characterization of Pine Kraft Lignin: BioChoice Lignin vs Indulin AT, Journal of Wood Chemistry and Technology. 36 (2016) 432 – 446.

Hua X., Xing Y., Zhang X., Enhanced Promiscuity of Lipase-Inorganic Nanocrystal Composites in the Epoxidation of Fatty Acids in Organic Media, ACS Applied Materials & Interfaces. 8 (2016) 16257 – 16261.

Huang Y.Q., Wong C.K.C., Zheng J.S., Bouwman H., Barra R., Wahlström B., Bisphenol A (BPA) in China: A review of sources, environmental levels, and potential human health impacts, Environment International. 42 (2012) 91 – 99.

Jablonskis A., Arshanitsa A., Arnatouv A., Telysheva G., Evaluation of Ligno Boost™ softwood kraft lignin epoxidation as an approach for its application in cured epoxy resins, Industrial Crops and Products. 112 (2018) 225 – 235.

Jay R.R., Direct titration of epoxy compounds and aziridines, Analytical Chemistry. 36 (1964) 667 – 668.

Jing Z., Lihong H., Bingchuan L., Caiying B., Puyou J., Yonghong Z., Preparation and characterization of novolac phenol-formaldehyde resins with enzymatic hydrolysis lignin, Journal of the Taiwan Institute of Chemical Engineers. 54 (2015) 178 – 182.

Kalami S., Arefmanesh M., Master E., Nejad M., Replacement 100 % of phenol in phenolic adhesive formulations with lignin, Journal of Applied Polymer Science. 134 (2017) 4124.

Kaylen M., Van Dyne D.L., Choi Y-S., Blasé M., Economic feasibility producing ethanol from lignocellulosic feedstocks. Bioresource Technology. 72 (2000) 19 – 32.

Kim J.R., Sharma S., The development and comparison of bio-thermoset plastics from epoxidized plant oils, Industrial Crops and Products. 1 (2012) 485-499.

Kudanga T., Prasetyo E.N., Sipilä J., Nyanhongo G., Guebitz G.M., Chemo-enzymatic functionalization of lignocelluloses materials using oxiranes, Process Biochemistry. 45 (2010) 1557 – 1562.

Kun D., Pukanszki B., Polymer/lignin blends: Interactions, properties, applications, European Polymer Journal. 93 (2017) 618 – 641.

Lange H., Decina S., Crestini C., Oxidative upgrade of lignin. Recent routes reviewed, European Polymer Journal. 49 (2013) 1151 – 1173.

Laurichesse S., Avérous L., Chemical modification of lignins: Towards biobased polymers, Progress in Polymer Science. 39 (2014) 1266 – 1290.

Li X., Zheng Y., Lignin-enzyme interaction: Mechanism, mitigation approach, modeling and research prospects, Biotechnology Advances. 35 (2017) 466 – 489.

Limayem A., Ricke S.C., Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects, Progress in Energy and Combustion Science. 38 (2012) 449 – 467.

Ma R., Guo M., Zhang X., Recent advances in oxidative valorization of lignin, Catalysis Today. 302 (2018) 50 – 60.

Maćczak A., Bukowska B., Michalowicz J., Comparative study of the effect of BPA and its selected analogues on hemoglobin oxidation, morphological alterations and hemolytic changes in human erythrocytes. Comparative Biochemistry and Physiology, Part C. 176 – 177 (2015) 62 – 70.

McDonough T.J., The Chemistry of Organosolv Delignification. TAPPI Journal 76(8) (1993) 186-193.

Méndez-Sánchez D., Ríos-Lombardía N., Gotor V., Gotor-Fernández V., Chemoenzymatic epoxidation of alkenes based on peracid formation by a *Rhizomucor miehei* lipase-catalyzed perhydrolysis reaction, Tetrahedron. 70 (2014) 1144 – 1148.

Méndez-Sánchez D., Ríos-Lombardía N., García-Granda S., Montejo-Bernardo J., Fernández-González A., Gotor V., Gotor-Fernández V., Lipase-catalyzed desymmetrization of *meso*-1,2-diaryl-1,2-diaminoethanes, Tetrahedron: Asymmetry. 25 (2014) 381 – 386.

Moreira M., Nascimento M., Chemo-enzymatic epoxidation of (+)-3-carene, Catalysis Communications. 8 (2007) 2043 – 2047.

Nagy M., Kosa M., Theliander H., Ragauskas A.J., Characterization of CO2 precipitated Kraft lignin to promote its utilization. Green Chemistry 12 (2010) 31 – 34 .

Naseem A., Tabasum S., Zia K. M., Zuber M., Ali M., Noreen A., Lignin-derivatives based polymers, blends and composites: A review, International Journal of Biological Macromolecules. 93 (2016) 296 – 313.

Norambuena M., Vidal C., Carrasco L., Reyes P., Parra C., Contreras D., Teixeira Mendonça R., Optimization of Experimental Variables to Modify Lignin from *Eucalyptus globules* under Alkaline Catalysis, BioResources. 11(1) (2016) 1828 – 1842.

Pan X.J., Arato C., Gilkes N., Gregg D., Mabee W., Pye K., Xiao Z.Z., Zhang X., Saddler J., Biorefining of softwoods using ethanol organosolv pulping: Preliminary evaluation of process streams for manufacture of fuel-grade ethanol and co-products, Biotechnology and Bioengineering. 90(4) (2005) 473 – 481.

Pandey M.P., Kim C.S., Lignin Depolymerization and Conversion: A Review of Thermochemical Methods, Chemical Engineering and Technology. 34(1) (2011) 29 – 41.

Peretti S. W., Barton R., Teixeira Mendonça, R. 2015. Lignin as Feedstock for Fibers and Chemicals. Chapter 7, No 43, RSC Green Chemistry (ed), Cambridege, UK, pp132-165.

Ragauskas A.J., Beckham G.T., Biddy M.J., Chandra R., Chen F., Davis M.F., Davison B.H., Dixon R.A., Gilna P., Keller M., Langan P., Naskar A.K., Saddler J.N., Tschaplinski T.J., Tuskan G.A., Wyman C.E., Lignin valorization: Improving Lignin Processing in the Biorefinery, Science. 344(6185): 1246843.

Raquez J.M., Deléglise M., Lacrampe M-F., Krawczak P., Thermosetting (bio)materials from renewable resources: A critical review, Progress in Polymer Science. 35 (2010) 487 – 509.

Rencoret J., Marques G., Gutierrez A., Ibarra D., Li J., Gellerstedt G., Santos J.I., Jimenez-Barbero J., Martinez A.T., del Rio J.C., Structural characterization of milled wood lignins from different eucalypt species, Holzforschung. 62(5) (2008) 514-526.

Sannigrahi P., Ragauskas A.J., Miller S, Effects of two-stage dilute acid pretreatment on the structure and composition of lignin and cellulose in loblolly pine, Bioenergy Research. 1(3) (2008) 205 – 214.

Sannigrahi P., Miller S.J., Ragauskas A.J., Effects of organosolv pretreatment and enzymatic hydrolysis on cellulose structure and crystallinity in Loblolly pine. Carbohydrate Research 345(7) (2010) 965 – 970.

Shevchenko S.M., Beatson R.P., Saddler J.N., The nature of lignin from steam explosion/ enzymatic hydrolysis of softwood: structural features and possible uses, scientific note. Applied Biochemistry and Biotechnology. 77-79 (1999) 867 – 876.

Sjöström E., Wood Chemistry: Fundamentals and Applications (Academic Press), (1993).

Törnvall U., Orellana-Coca C., Hatti-Kaul R., Adlercreutz D., Stability of immobilized *Candida antarctica* lipase B during chemo-enzymatic epoxidation of fatty acids, Enzyme and Microbial Technology. 40 (2007) 447 – 451.

Törnvall U., Hedström R., Hatti-Kaul R., Stability and activity of lipases on exposure to oxidative environments, Journal of Biotechnology. 150 (2010) 351.

de Torres M., Jiménez-Osés G., Mayoral J.A., Pires E., Evaluation of several catalytic systems for the epoxidation of methyl oleate using H_2O_2 as oxidant, Catalysis Today. 195 (2012) 76 – 82.

Vallejos M., Felissia F.E., Curvelo A.S., Zambon M.D., Ramos L., Area M.C., Chemical and physico-chemical characterization of lignins obtained from ethanol-water fractionation of bagasse, BioResources. 6(2) (2011) 1158 – 1171.

Vanderberg L.N., Hauser R., Marcus M., Olea N., Welshons W.V., Human exposure to bisphenol A (BPA), Reproductive Toxicology. 24 (2007) 139-177.

Vásquez-Garay F., Teixeira Mendonça R., Peretti S., Chemoenzymatic lignin valorization: Production of epoxidized pre-polymers using Candida antarctica lipase B, Enzyme and Microbial Technology. 112 (2018) 6-13.

Xu C., Arancon R. A., Labidi J., Luque R., Lignin depolimerization strategies: towards valuable chemicals and fuels, Chem. Soc. Rev. 43 (2014) 7485 – 7500.

Yang F., Zhang X., Li F., Wang Z., Wang L., Chemoenzymatic synthesis of α-Cyano Epoxides by a Tandem-Knoevenagel-Epoxidation Reaction, European Journal of Organic Chemistry. (2016) 1251 – 1254.

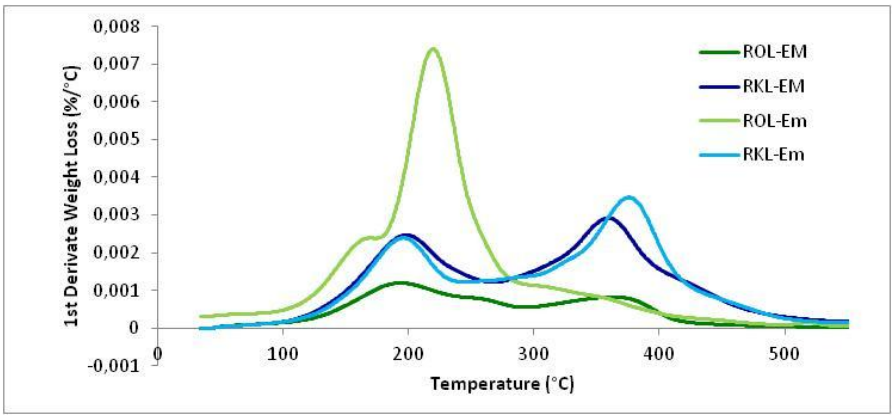
Yang F., Wang Z., Zhang X., Jiang L., Li Y., Wang L., A Green Chemoenzymatic Process for the Synthesis of Azoxybenzenes, ChemCatChem. 7 (2015) 3450 – 3453.

Zhu W., Westman G., Theliander H., Investigation and Characterization of Lignin Precipitation in the LignoBoost Process, Journal of Wood Chemistry and Technology 34(2) (2014) 77 – 97.

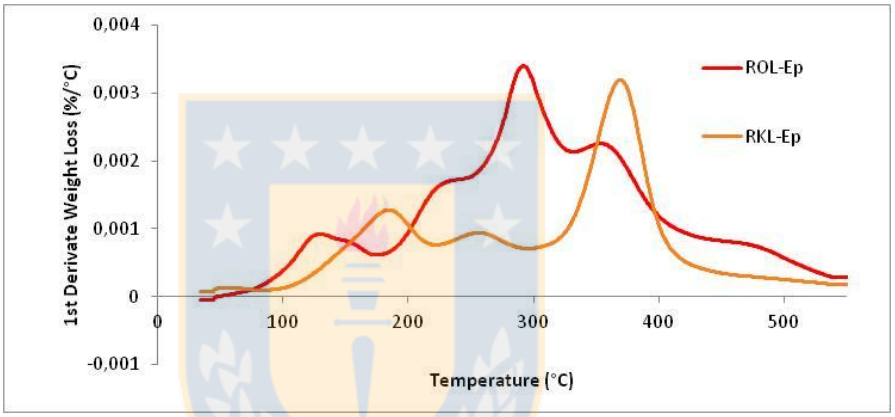
Zinovyev G., Sumerskii I., Kortner P., Sulaeva I., Rosenau T., Potthast A., Molar mass-dependent profiles of functional groups and carbohydrates in Kraft lignin, Journal of Wood Chemistry and Technology. 0 (2016) 1 – 13.

APPENDIX I

A)



B)



C)

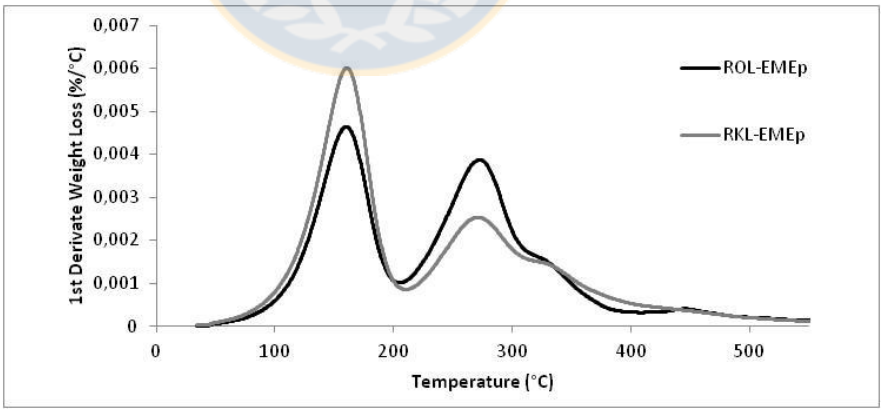


Figure 1. First derivative thermogravimetric (DTG) curves of all biobased resins determined by TGA. A) biobased resins by chemoenzymatic mechanism: ROL-EM, ROL-Em, RKL-EM and RKL-Em; B) unmodified lignins reacted with EPC: ROL-Ep and RKL-Ep; C) Resins ROL-EM and RKL-EM reacted with EPC.