Heterologous expression of a fungal lignin peroxidase in *Pichia pastoris*

By

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Summary

Considerable research has been being devoted to seek cost-effective and environmentally friendly alternatives to replace fossil fuel-based resources for energy and other essential products. Lignocellulosic biomass consisting of cellulose, hemicellulose and lignin presents the most abundant and sustainable resource for this purpose. Lignin, the non-polysaccharide constituent of lignocellulose, consists of p-coumaryl, coniferyl and sinapyl alcohol monomers that can be exploited as a source of aromatic compounds for conversion to phenol derivatives and vanillin, among others. A consortium of enzymes is required for its degradation, including lignin peroxidase, manganese peroxidase, versatile peroxidase and laccase. There is a particular interest in the development of recombinant microorganisms capable of producing these enzymes for the cost-effective conversion of technical lignins to high-value bioproducts.

The poor secretion and complex protein structure of lignin peroxidase have limited the production thereof in native hosts as well as heterologous expression systems such as *Escherichia coli* and *Saccharomyces cerevisiae*. The yeast *Pichia pastoris* is a good alternative host for heterologous protein expression and has been used to produce many enzymes for industrial applications due to its high recombinant protein titres, inexpensive cultivation methods, Generally Regarded As Safe (GRAS) status and clear-cut downstream processes. Molecular biology and process engineering have been implemented to produce various recombinant enzymes in *P. pastoris* in significant amounts.

In this study, three synthetic *Phanerochaete chrysosporium* lignin peroxidase (*lip*H8) genes were expressed in *P. pastoris* DSMZ 70382 under the control of the constitutive P_{GAP} and inducible P_{AOX1} promoters. The codon sequence for the native (*LiP_Nat*) gene was optimised using two different indices, yielding *LiP_CAI* and *LiP_CBI*. Under the transcriptional control of the constitutive P_{GAP} promoter, no recombinant lignin peroxidase was detected. In shake flask cultivations, the activity levels of the recombinant lignin peroxidases encoded by the *LiP_Nat*, *LiP_CBI* and *LiP_CAI* genes under the control of the methanol-inducible P_{AOX1} promoter, peaked at 597, 679 and 1169 U/L, respectively. High cell-density fermentations in a 14-L bioreactor with the best strain, *P. pastoris* pJ901[LiP_CAI], resulted in a 3.3-fold increase in lignin peroxidase activity (3818 U/L). The rLiPH8 constituted 68% of the total protein in the cell-free supernatant and displayed optimal activity on veratryl alcohol at 25 °C and pH 3.

This study demonstrated the successful production of a recombinant fungal lignin peroxidase in *P. pastoris* and indicated that high cell-density fermentations can be used to increase rLiPH8 production in P. pastoris. The codon-optimised *LiP_CAI* and *LiP_CBI* genes resulted in higher extracellular LiP activity in *P. pastoris* than the *LiP_Nat*. The results provide a starting point for the optimisation of heterologous production of a fungal lignin peroxidase in *P. pastoris* at both the molecular and bioprocess level. The cost-effective production of enzymes such as lignin peroxidase is essential for the enzymatic upgrade of industrial lignin, as well as bioremediation applications (i.e. treatment of water). The high levels of enzyme activity coupled with the low amount of endogenous proteins obtained in P. pastoris, will minimize downstream processing (concentration and purification of rLiPH8) and may allow for direct application of the cell-free supernatant.

Opsomming

Aansienlik baie navorsing word aan koste-effektiewe en omgewingsvriendelike alternatiewe vir fossielbrandstofgebaseerde hulpbronne vir energie en ander noodsaaklike produkte gewy. Lignosellulose biomassa, bestaande uit sellulose, hemisellulose en lignien, bied die mees oorvloedige en volhoubare hulpbron vir hierdie doel. Lignien, die nie-polisakkaried bestanddeel van lignosellulose, bestaan uit p-kumariel-, koniferiel- en sinapielalkohol subeenhede wat as 'n bron van aromatiese verbindings vir omskakeling na fenolderivate en vanillien gebruik word. 'n Konsortium van ensieme word vir die afbraak daarvan benodig, insluitende lignienperoksidase, mangaanperoksidase, veelsydige peroksidase en lakkase. Daar bestaan veral 'n groot aanvraag vir die ontwikkeling van 'n rekombinante mikro-organisme wat hierdie ensieme kan produseer vir die koste-effektiewe omskakeling van tegniese ligniene tot hoë-waarde bioprodukte.

Die swak sekresie en komplekse proteïenstruktuur van lignienperoksidase het tot dusver die produksie daarvan in natuurlike gashere sowel as in heteroloë uitdrukkingstelsels soos *Escherichia coli* en *Saccharomyces cerevisiae* beperk. Die gis *Pichia pastoris* is 'n goeie alternatiewe gasheer vir heteroloë proteïenuitdrukking en is reeds gebruik om baie ensieme vir industriële toepassings te produseer danksy sy hoë rekombinante proteïentiters, goedkoop kweking, Algemeen-Veilige status en eenvoudige stroomaf prosesse. Molekulêre biologie en prosesingenieurswese is toegepas om verskeie rekombinante ensieme in *P. pastoris* in beduidende hoeveelhede te produseer.

In hierdie studie, is drie sintetiese *Phanerochaete chrysosporium* lignienperoksidase (*lip*H8) gene in *P. pastoris* DSMZ 70382 onder beheer van die konstitutiewe P_{GAP} en induseerbare P_{AOX1} promotors uitgedruk. Die kodonvolgorde van die natuurlike (*LiP_Nat*) geen is met behulp van twee verskillende indekse ge-optimiseer, wat *LiP_CAI* en *LiP_CBI* gelewer het. Onder die transkripsionele beheer van die konstitutiewe P_{GAP} promotor is geen rekombinante lignienperoksidase waargeneem nie. In skudfles-kulture het rekombinante lignienperoksidase wat deur die *LiP_Nat-, LiP_CBI- en LiP_CAI*-gene onder beheer van die metanol-induseerbare P_{AOX1}-promotor gekodeer is, ensiemaktiwiteit van onderskeidelik 597, 679 en 1169 U/L bereik. Hoë seldigtheid-fermentasies in 'n 14 L bioreaktor met die beste ras, *P. pastoris* pJ901[LiP_CAI], het 'n 3.3-voudige toename in uitdrukkingsvlakke (3818 U/L) gelewer. Die

rLiPH8 het 68% van die totale proteïen in die selvrye bostand verteenwoordig en het optimale aktiwiteit op veratrilalkohol by 25 °C en by pH 3 getoon.

Hierdie studie het die suksesvolle produksie van ’n rekombinante swam-lignienperoksidase in *P. pastoris* getoon en aangedui dat hoë seldigtheid-fermentasies rLiPH8 produksie in *P. pastoris* kan verhoog. Die kodon-geoptimiseerde *LiP_CAI* en *LiP_CBI* gene het hoër ekstrasellulêre LiP-aktiwiteit as die *LiP_Nat* geen in *P. pastoris* gelewer. Hierdie studie bied ’n vertrekpunt vir die optimisering van heteroloë produksie van ’n swam-lignienperoksidase in *P. pastoris* op beide molekulêre en bioprosesseringsvlak. Die koste-effektiewe produksie van ensieme soos lignienperoksidase is noodsaaklik vir die ensiematiese opgradering van industriële lignien, asook bioremediëringstoepassings (bv. waterbehandeling). Die hoë vlakke van ensiemaktiwiteit tesame met die lae hoeveelheid endogene proteïene wat in *P. pastoris* verkry is, sal stroomaf prosessering (konsentrasie en suiwering van rLiPH8) vergemaklik en mag moontlik die direkte gebruik van die selvrye bostand toelaat.

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List of Abbreviations

2G Second generation

AA2 Auxiliary Activity Family 2

BMGY Buffered Glycerol-complex Medium

BMMY Buffered Methanol-complex Medium

CAI Codon Adaptation Index

CBI Codon Bias Index

DO Dissolved Oxygen

DCW Dry Cell Weight

DNA Deoxyribonucleic acid

EC Enzyme Commission number

ER Endoplasmic Reticulum

FDA Food and Drug Administration

GRAS Generally Regarded As Safe

H_2O_2 Hydrogen peroxide

HRP Horseradish peroxidase

LB Luria-Bertani medium

LCC Lignin-carbohydrate complexes

LiP Lignin peroxidase

LiPH8 Lignin peroxidase H8 isozyme

MnP Manganese peroxidase

Nat Native

NCBI National Centre for Biotechnological Information

PAGE Polyacrylamide Gel Electrophoresis

P_{AOX1} Alcohol oxidase 1 promoter

PCR Polymerase Chain Reaction

P_{GAP} Glyceralehyde-3-phosphate dehydrogenase promoter

PTM Post Translational Modifications

PTM_1 *Pichia* Trace Metal salts

rpm Revolutions per minute

SC Synthetic Complex media

SDS Sodium Dodecyl Sulphate

tRNA Transfer Ribonucleic acid

UPR Unfolded protein response

VA Veratryl alcohol

VP Versatile peroxidase

YPD Yeast Extract Peptone Dextrose

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Chapter 1

General Introduction, Aims and interest of the study

Introduction, Aims and Interest of the Study

1.1. Introduction

Lignocellulosic biomass is the most abundant natural and renewable feedstock available on earth to produce bio-products such as biomaterials (composites), chemicals and bioenergy (biofuels, electricity) that can help develop and sustain many economies globally (Iqbal *et al*., 2013; Shahzadi *et al*., 2014; Rambo *et al*., 2015). Lignocellulosic biomass is primary derived from plant material that is nonedible and is composed of a mixture of carbon-based biopolymers that have distinctive characteristics (Kumar *et al*., 2009). These biopolymers are mainly cellulose, hemicellulose and lignin. The establishment of biorefineries where all the different components of lignocellulosic biomass are utilised in an integrated manner may maximise the transformation of lignocellulosic biomass into multiple products, thus improving the economic feasibility of biorefineries (Cannatelli and Ragauskas, 2016).

Pulp and paper mills and second generation (2G) biofuel industries utilise only the carbohydrate portions of lignocellulosic biomass, while producing massive quantities of lignin (greater than 50 million tons per annual globally) that are currently underutilised (Norgren and Edlund, 2014; Abdelaziz *et al*., 2016). At present, most of these industrial or technical lignins are burnt on site to generate steam and electricity, which have low market value (Eudes *et al*., 2014). The diversion of some of these technical lignins to the production of commodities with a higher market value than energy will diversify the product portfolio and increase the revenue of these industries. Examples of these products (listed from higher to lower market value) include phenols (phenol resins and derivatives), carbon fibres and activated carbon, biofuels and cement additives.

Despite the potential of lignin, current applications only account for 2% of the technical lignin that is generated (Demirel, 2017). Hence, there is a quest for the development of efficient technologies for better valorisation of technical lignins. This minimal market is mainly due to its inert nature, lignin heterogeneity, impurities (carbohydrates, nitrogen, sulphur), toxicity, smell and colour problems. There are, however, several properties of technical lignins that can be modified in order to improve their performance during the conversion technology (Sena-Martins *et al*., 2008).

The use of enzyme technology for lignin modifications represents a more sustainable process given its specificity and mild conditions required compared to thermochemical and chemical processes (Liew *et al*., 2011). Moreover, the use of enzymes for the transformation of lignin may improve the quality of desired products, reduce production costs, reduce wastes, enhance energy and water savings and provide safer working conditions than thermochemical and chemical methods, which use severe alkaline, acidic and extreme conditions (Islam *et al*., 2013).

In nature, basidiomycetes such as the well characterised white-rot fungus *Phanerochaete chrysosporium* and some bacterial species, are capable of degrading the highly recalcitrant lignin biopolymer and lignin residues by secreting an assortment of extracellular enzymes known as ligninolytic enzymes, ligninases and oxidoreductases (Dashtban *et al*., 2010; Gasser *et al*., 2012). Microbial or biological delignification is effectively achieved by the synergistic effect of major ligninolytic enzymes such as lignin peroxidase, manganese peroxidase, versatile peroxidase and laccase, together with various accessory enzymes.

The potential of these enzymes in the biorefinery sector is not limited to lignin modification, since these enzymes can also be applied for detoxification of pretreatment liquors, which would in turn improve the yield of biofuels or other products. Other industries where these enzymes hold great potential are the pulp and paper and the textile industries for paper whitening and the degradation of polymeric dyes, respectively (Pant and Adholeya, 2007). Pure lignin and manganese peroxidase preparations have been reported to oxidize xenobiotics, which can be exploited in the bioremediation sector (Conesa *et al*., 2002) In food and feed and cosmetics industries, purer enzyme preparations of lignin peroxidase could potentially be used to clarify juice (Sánchez-Leija *et al*., 2016), produce vanillin for flavouring and the treatment of hyperpigmentation, thus diversifying the application of this enzyme (dos Santos Barbosa *et al*., 2008).

Among the lignin modifying enzymes, this thesis focuses on the fungal lignin peroxidase (LiP). LiP (EC 1.11.1.14) is an extracellular heme-containing enzyme that belongs to the CAZy family AA2 (Auxiliary Activity Family 2). LiP carries out several oxidative reactions using hydrogen peroxide as electron acceptor. Unlike laccases, LiP catalyses the oxidation of both non-phenolic and phenolic substrates and has a very high redox potential. Of interest is the ability of this enzyme to catalyse both the depolymerisation and polymerisation of phenolic compounds by oxidative coupling of the phenoxy radicals (Mäkelä *et al*., 2015). This ability

can be exploited to improve the properties of technical lignins to make them more suitable for certain applications, such biocomposites (Sena-Martins, 2008).

Despite the potential of LiP, there are limited studies on the successful production of this enzyme from white rot fungi or other expression hosts. To our knowledge, there is currently no commercial LiP available at a cost that will be industrially feasible. The secretion of fungal LiP by native producers is hampered by numerous factors such as low secretion levels, nutrient or nitrogen-limiting conditions, long cultivation periods, limited heme availability, inactivation by H_2O_2 and high shearing forces in fermenters (Conesa, Cees, *et al*., 2000). It is thus imperative to use alternative expression systems such as yeast to produce LiP. Yet, the complexity of this enzyme's tertiary structure renders heterologous expression challenging with low levels of catalytically active enzyme being secreted. An appropriate expression host should be able to carry out specific posttranslational modifications such as glycosylation, heme assembly and sulphur-bridge formation (Lambertz *et al*., 2016a). The methylotrophic yeast *Komagataella* (*Pichia*) *pastoris*, referred to as *Pichia pastoris* in this thesis, is routinely utilised specifically for its ease of genetic manipulation and improved eukaryotic post-translation modification. It displays rapid growth rates to obtain very high cell densities and secretes few endogenous proteins, resulting in an extracellular medium enriched in the recombinant protein of interest, simplifying the downstream processing.

Successful production of recombinant proteins in many expression systems, including P. pastoris, is dependent on the gene and enzyme itself, but is also influenced by other factors such as type of strain (Wang & Wen, 2009), secretion signal, choice of promoter, gene copy number and optimal codon selection (Mellitzer *et al*., 2014). Despite the successful expression and secretion of various heterologous proteins in P. pastoris, there is a lack of efficient secretion signals for this yeast. The α-mating factor (α-MF) pre-pro-leader from *Saccharomyces cerevisiae* has been used for the effective expression of most proteins in P. pastoris, although the native secretion signal is preferred for some fungal enzymes (Heiss *et al*., 2015). A higher gene copy number (integrated in the genome) generally correlates positively with productivity, but it generally reaches a plateau at 5 to 8 copies in the case of extracellular proteins. Further increases do not result in further improvements or may even be detrimental for production levels.

In terms of promoters, high secretion levels have been correlated with the use of the strong and tightly regulated inducible alcohol oxidase 1 promoter (P_{AOX1}), which requires methanol for

induction (Cos *et al*., 2006). Different LiP isoforms have been expressed in *Pichia* sp. under the control of P_{AOX1} with different yields. For example, a multi-copy recombinant *P. pastoris* strain has been constructed for the production of LiPH2, but resulted in only 15 U/L of recombinant LiP (Wang and Wen, 2009). In another study conducted by Wang *et al*. (2004), LiPH8 activity levels of 932 U/L were obtained for *Pichia methanolica* under the transcriptional control of the inducible alcohol oxidase (*AUG1*) promoter. The authors were able to enhance expression levels by 2-fold by replacing the native secretion signal with the α-MF pre-pro-leader peptide. The constitutive glyceraldehyde-3-dehydrogenase promoter (P_{GAP}) proved to be a better promoter than P_{AOX1} for heterologous protein production due to its relative ease to use, since it does not require methanol for induction and can utilise a diverse choice of carbon sources (Bohlin *et al*., 2006). To our knowledge, there has not been any reports on the expression of LiP under the control of *GAP* promoter.

It has been suggested that codon usage controls the rate of translation elongation to ensure optimum co-translational protein folding processes (Shen *et al*., 2016). Some native genes have rare codons or non-optimal codons that could reduce the tRNA pool and often decreases the translational efficiency of a desired protein (Mazumder *et al*., 2016). Codon optimisation is a technique routinely employed to overcome bottlenecks caused by rare codons and is used to improve production levels in expression systems such as *P. pastoris* (Tanaka *et al*., 2014). Partial optimization of N-terminal codons of some heterologous genes expressed in *P. pastoris* increased the yield several fold compared to genes employing unfavourable codons (Zhao *et al*., 2014). To our knowledge, the impact of codon optimization on the expression levels of LiP in *P. pastoris* has not been studied yet.

1.2. Aims and Interest of the Study

In this study, the *P. chrysosporium* LiPH8 isozyme was selected as the target protein as it is the most extensively studied and characterised fungal lignin peroxidase. Based on the previous premises, the general aim of this study was to produce LiPH8 in *P. pastoris* strain DSMZ-70382 at levels that would facilitate downstream processing and ideally allow for its direct application on technical lignins. To achieve this aim, the following specific objectives were identified:

1. Contrast the efficacy of the constitutive (*GAP* promoter) and inducible (*AOX1* promoter) expression of LiPH8 using two patent-free vectors.
2. Elucidate the impact of codon optimisation on the production levels of LiPH8.
3. Selection of the best transformants based on enzyme activity in shake-flasks for production at larger scale in 14-litre bioreactors.
4. Enzyme characterization and stability in terms of temperature and pH.

This thesis provides a starting point for the optimisation of heterologous production of a fungal lignin peroxidase in *Pichia pastoris* at both the molecular and bioprocess level. The cost-effective production of enzymes such as lignin peroxidase will provide an essential tool for the enzymatic up-grade of industrial lignins.

This thesis consists of several chapters, including a literature review relevant to the study (Chapter 2), the research carried out to meet the aim and specific objectives of the study (Chapter 3), and final conclusions and future recommendations (Chapter 4). These chapters are followed by an addendum that includes a description of different enzymatic assay methods for lignin peroxidase. Chapter 3 is presented in manuscript format for submission to the Journal of Biotechnology.

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Chapter 2

Literature Review

Literature Review

2.1. Bioeconomy

Humans have been dependent on fossil fuel based resources for energy and manufacturing products as early as the Industrial Revolution (Furukawa *et al*., 2014). Ever since, human dependence on fossil fuels has drastically increased, leading to massive demands and limited supply, which resulted in socio-economic issues such as increased oil prices, global warming, climate change and conflicts between nations. Fossil fuels are finite and non-renewable, hence it is imperative to devote research to seeking sustainable and renewable energy and chemical resources. This will support the massive global demands attributed mainly by industrial processes, transportation and household electricity that have led to the world-wide energy crisis (Lange *et al*., 2010). This shift from non-renewable to renewable resources is not only focused on bio-energy, but also bio-chemicals and bio-materials.

Several countries and organisations such as the European Commission (EU), US, Canada, Australia, Sweden, Finland and South Africa are in support of the bio-economy strategy, which is focused on developing and sustaining economies using biological resources (Lewandowski, 2015). In a bio-economy, biological resources derived from plants, animals and microbes are used to produce materials, chemicals and more essentially, energy (McCormick and Kautto, 2013; Pätäri *et al*., 2016). The aim of the bio-economy is to provide economic growth while simultaneously guaranteeing resource security and efficiency through smart and sustainable utilisation of renewable biological resources and simultaneously decrease the dependence on fossil fuel based resources (Cristóbal *et al*., 2016).

The South African Bio-economy Strategy is an initiative of the Department of Science and Technology (DST) that seeks to integrate a variety of biotechnological processes that can be applied in biorefineries (discussed in section 2.2.) for the production of various bio-products (DST, 2013). The objective of this strategy is to significantly contribute to the country's economy by 2030 through establishing industries that create bio-based products, job opportunities and novel technologies. Furthermore, it seeks to support and prioritise research, development and innovation in a variety of biological processes while improving water and waste management practises to support the "green economy'. The bio-economy has the

potential to provide benefits to industries such as pulp and paper, sugar and textile by creating new businesses opportunities. However, this requires innovative technologies to be established.

Industries such as the pulp and paper and sugar mills lack versatility and depend on the production of a single product (e.g. paper and sugar). Moreover, these industries also face pressure to reduce their environmental footprint. With that mentioned, the bio-economy can provide innovative technologies to expand their product portfolio by utilising by-products produced in these industries for other value-added products, while assisting in waste management and reducing production costs that may advance the performance of many economic sectors (DST, 2013)

The establishment of a bio-economy in the industrial sector involves the utilisation of microorganisms and biological catalyst (enzymes) in the production and manufacturing of bio-energy, bio-chemicals and bio-materials. In addition, the use of biological methods for re-cycling, re-use and retrieval of waste emitted by industries is included. However, there are several challenges that still need to be addressed for the bio-economy strategy to be feasible on a large scale. Significant investment and support from industries, academia and government are necessary. Therefore, cross-linking interdisciplinary collaboration between sciences and engineering is needed to ensure scaled-up technologies and bridging the gap between scientific applications to industrial practicability (DST, 2013). A life-cycle assessment (LCA) of the bio-economy must be taken into consideration to analyse all the benefits and shortcomings to make correct and appropriate policies (Menon and Rao, 2012).

2.2. Biorefinery

A biorefinery is a facility or a group of facilities that combine processes and equipment for the conversion of biomass to commodities such as fuels, power, chemicals, textiles, lumbers, polymers and other high value-added products (Naik *et al*., 2010; Balan *et al*., 2014). As illustrated in Figure 2.1 biomass is transported to a biorefinery where it is dismantled using separation technologies that integrate physical, chemical, thermochemical and biological processes into different types of biopolymers. Thereafter, the biopolymers can be converted into their simple building blocks such as oligosaccharides, monosaccharides, monolignols, proteins and triglycerides, to produce essential products such as biofuels, bio-products, bio-chemicals and energy (Cherubini, 2010; Naik *et al*., 2010). Depending on the type of the feedstock, the composition of the biopolymers differs (Table 2.1). The efficient implementation

of biomass conversion in biorefineries is hindered by the cost of pretreatment methods and the integral exploitation of the three main components of lignocellulosic biomass. The development of cost-effective biotechnological approaches for lignin valorisation can assist in adding revenue in biorefineries by developing new lignin derived products with a high market value.

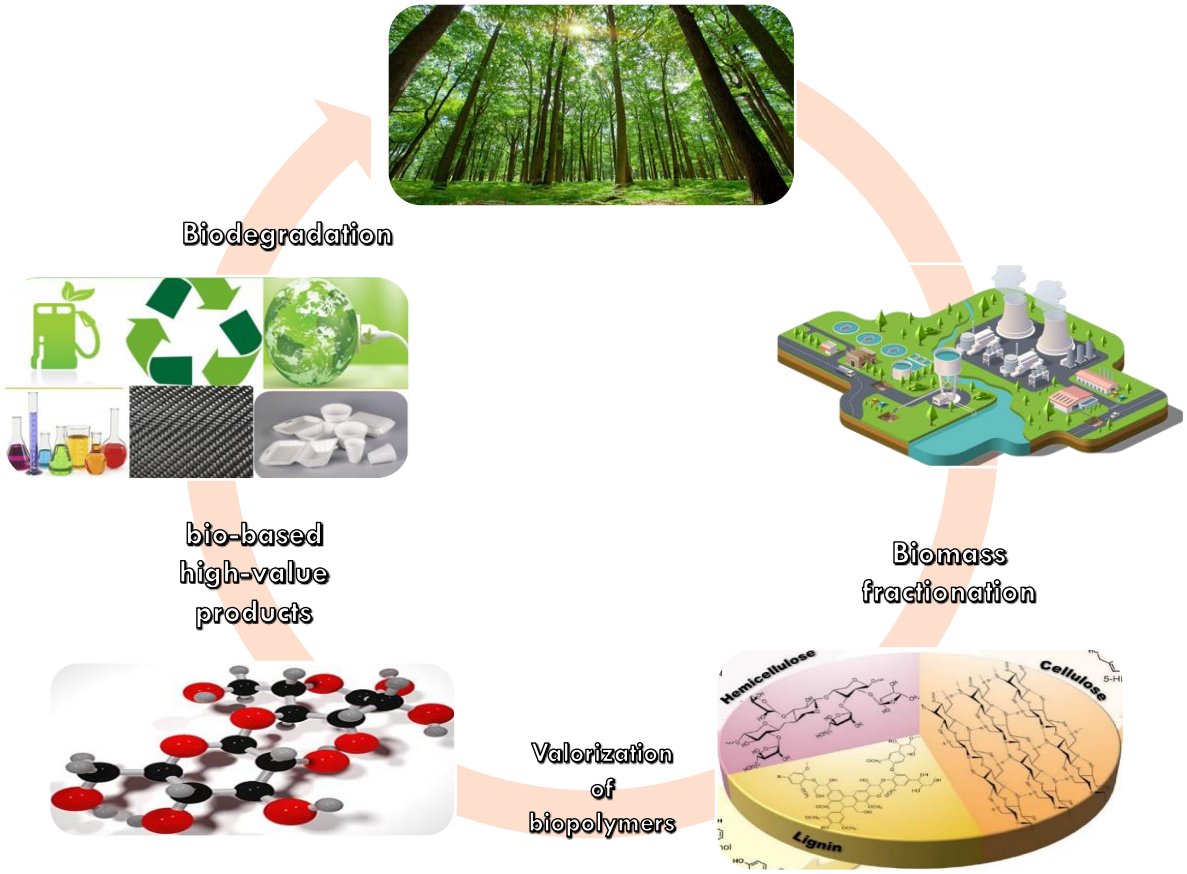

Figure 2.1: Schematic representation of a biorefinery process and the production of bio-products using lignocellulosic biomass as substrates (adapted from Strassberger *et al.*, 2014).

Table 2.1: Composition of lignocellulosic biopolymers in different lignocellulosic materials

Feedstock	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Hardwood	40-55	24-40	18-25	Iqbal *et al*., 2013
Softwood	45-50	25-35	25-35	Malherbe and Cloete, 2002
Grasses	25-40	35-50	10-30	Iqbal *et al*., 2013
Agriculture waste	25-45	20-30	6-25	Isikgor and Becer, 2015
Municipal organic waste	21-64	5-22	3-28	Biswas *et al*., 2015

2.3. Lignocellulosic biomass compositions and structure

Lignocellulosic biomass is derived from plant material and consists of a matrix of organic biopolymers, primarily cellulose, hemicellulose and lignin (Figure 2.2), as well as other components such as proteins, pectin, lipids and inorganic matter (ash). Lignocellulose is the most abundant carbon source after fossil fuel resources and is the only feedstock available in quantities that can effectively replace fossil fuel resources for the production of energy and other essential products (Baeyens *et al*., 2015; Rambo, 2015). A large proportion of this renewable resource is either unexploited or recycled in ecosystems. Lignocellulosic biomass is an attractive feedstock as it does not compete with food security, it is renewable and sustainable, environment benign, provides energy security, decrease biomass decay in forests and offers economic development, more especially for developing countries (Balan *et al*., 2014).

2.3.1. Cellulose

Cellulose is the most abundant biopolymer found in nature and is the major constituent of lignocellulosic biomass (Lynd *et al*., 2002). Furthermore, approximately half of the available organic carbon on earth exists in the form of cellulose (Isikgor and Becer, 2015). Cellulose is composed of linear unbranched repeating disaccharide cellobiose units held together by hydrogen bonds (Figure 2.2). In industries such as pulp and paper and second generation (2G) biofuels, the cellulose portion of lignocellulosic biomass is the primary source for producing value-added products such as paper and bioethanol. The effective utilisation of cellulose for the production of high-value secondary platform chemicals such as ethanol, glycerol, xylitol and butanediol, is hindered by the lack of efficient pretreatment technologies and the high cost of cellulases (Lynd *et al*., 2005; Zhao *et al*., 2017).

2.3.2. Hemicellulose

Contrary to cellulose, hemicellulose is a heterogeneous branched polysaccharide biopolymer made up of five and six carbon atom monosaccharides and small quantities of sugar acids. (Shahzadi, 2014). The network of cross-linked fibres in plants is attributed by the binding of hemicellulose, pectin and cellulose (Sorek *et al*., 2014). In contrast to cellulose, hemicellulose lacks crystallinity due to its branched structure, which enables it to be easily hydrolysed into

simple fermentable monomeric sugars. However, hemicellulose still presents a challenge for biofuel production due to the side chains that are covalently linked to lignin, forming macromolecular structures denoted as lignin-carbohydrate complexes (LCCs), and the inability of *Saccharomyces cerevisiae* to efficiently ferment monosaccharides such as xylose and mannose to ethanol (Zhao *et al.*, 2012).

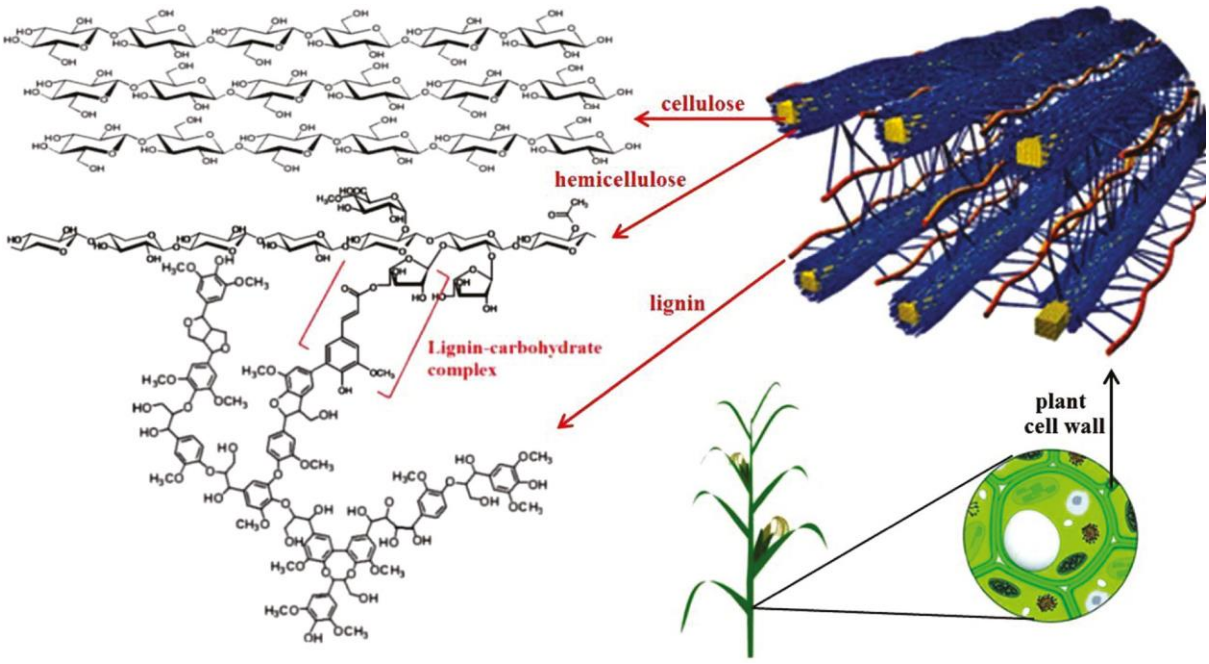

Figure 2.2: Schematic representation of lignocellulosic biomass and the composition of the major biopolymers (Volynets *et al.*, 2017).

2.3.3. Lignin

Lignin is an aromatic non-polysaccharide constituent of lignocellulosic biomass and the second most abundant source of organic carbon after cellulose, accounting for one third of the total organic carbon available on earth (Linger *et al.*, 2014; Xu *et al.*, 2014). Lignin is made up of simple aromatic building blocks collectively known as monolignols (p-coumaryl, conifer and sinapyl alcohol) that form an intricate heterologous crosslinked biopolymer (Figure 2.3A) that lacks a defined primary structure (Wang *et al.*, 2013). These monomers are synthesised by free-radical polymerisation catalysed by plant peroxidases and plant laccases, resulting in dimers joined by C-O-C ether and C-C linkages (Figure 2.3B), forming a complex amorphous branched biopolymer that is highly recalcitrant (Pandey and Kim, 2011). The β-O-4 ether linkages are the dominant linkages (depending on the type of plant) and can account for

approximately 50-62% of the total linkages present in lignin (Table 2.2) (Figueiredo *et al.*, 2018; Guadix-Montero and Sankar, 2018).

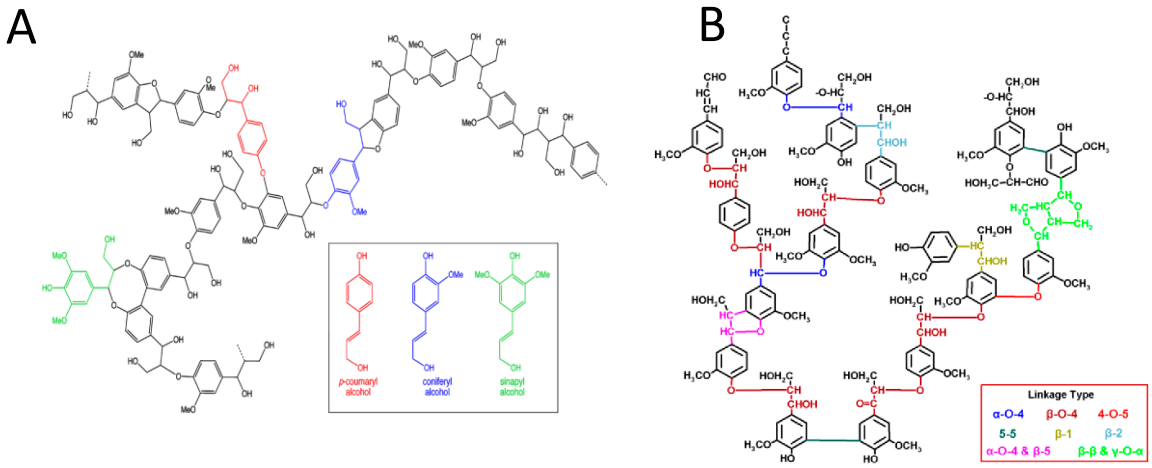

Figure 2.3: Schematic illustration of (a) lignin structure with the different monolignols (b) the different types of ether and C-C linkages present in lignin (Zhang *et al.*, 2017; howaboutlignin.blogspot.com/2013/07/lignin-structure.html accessed January 2018).

Table 2.2: The predominant lignin linkages present in hardwood and softwoods

Inter unit linkage	Hardwood (%)	Softwood (%)	Reference
β-O-4	60-62	35-50	Guadix-Montero, 2018
β–β	3-16	0-3	Strassberger *et al.*, 2014
α–O–4	6-8	6-8	Strassberger *et al.*, 2014
5–5	0-4.5	9-11	Pandey and Kim 2011
4-O-5	2-7	4-8	Axelsson *et al.*, 2012
β-5	0-6	9-12	Santos *et al.*, 2013
β-1	1-7	7-10	Abdelaziz *et al.*, 2016

The main functions of lignin in plants is to provide structural support, rigidity and resistance against pathogens and other environmental stresses (Zhao *et al.*, 2012; Amin *et al.*, 2017). The ability of vascular plants to occupy most of the terrestrial land and survive against pathogens for centuries is associated with the presence of lignin (Welker *et al.*, 2015). Nevertheless, in nature there is a highly specialised group of microorganisms called basidiomycetes (discussed

in section 2.3) as well as a few members of bacteria and insects (termites) that can degrade lignin. From a bio-economy perspective, lignin is the richest source for value-added aromatic compounds. However, due to its complex structure and recalcitrance, this biopolymer is still underexploitede, accounting for less than 2% of the production of value-added products (Figueiredo, 2018).

Industrial applications of technical lignins

The pulp and paper industry is the major source of industrial lignin, producing globally more than 50 million tons (Norgren, 2014) of technical lignins per annum as by-products during the pulping process (Iqbal *et al*., 2013). It is estimated that approximately 225 million tons of technical lignins will be generated in 2G biofuel industries by 2030 (Cotana *et al*., 2014). Technical/industrial lignins (including lignosulfonates, kraft lignin, soda lignin and organosolv lignin) are by-products generated from pulping processes as well as biofuel production. Technical lignins are mostly burnt on-site to produce energy (Eudes, 2014), despite their potential economic value as illustrated in Figure 2.4.

The potential value-added products that can be obtained from industrial lignin include (but are not limited to) vanillin, phenol derivatives, carbon fibres and cement additives (Figure 2.4).For example, Borregaard in Norway produces about 500 000 tons of lignin annually, but only about 1500 tons are used for the production of high-value products such as vanillin (Meister, 2016) (http://www.biorefine2g.eu/images/WS-Stockholm/2-BioREFINE-2G_Workshop_EUBCE2017_Stockholm_Tjosas_Borregaard_web.pdf) (accessed September 2018). The utilisation of lignin to produce value-added products depends on the type of lignin and the quality of lignin (Figure 2.4). Lignin is converted into simpler aromatic compounds through either thermochemical, chemical or biological/enzymatic methods and each is governed by the nature or type of lignin (Tang *et al*., 2015).

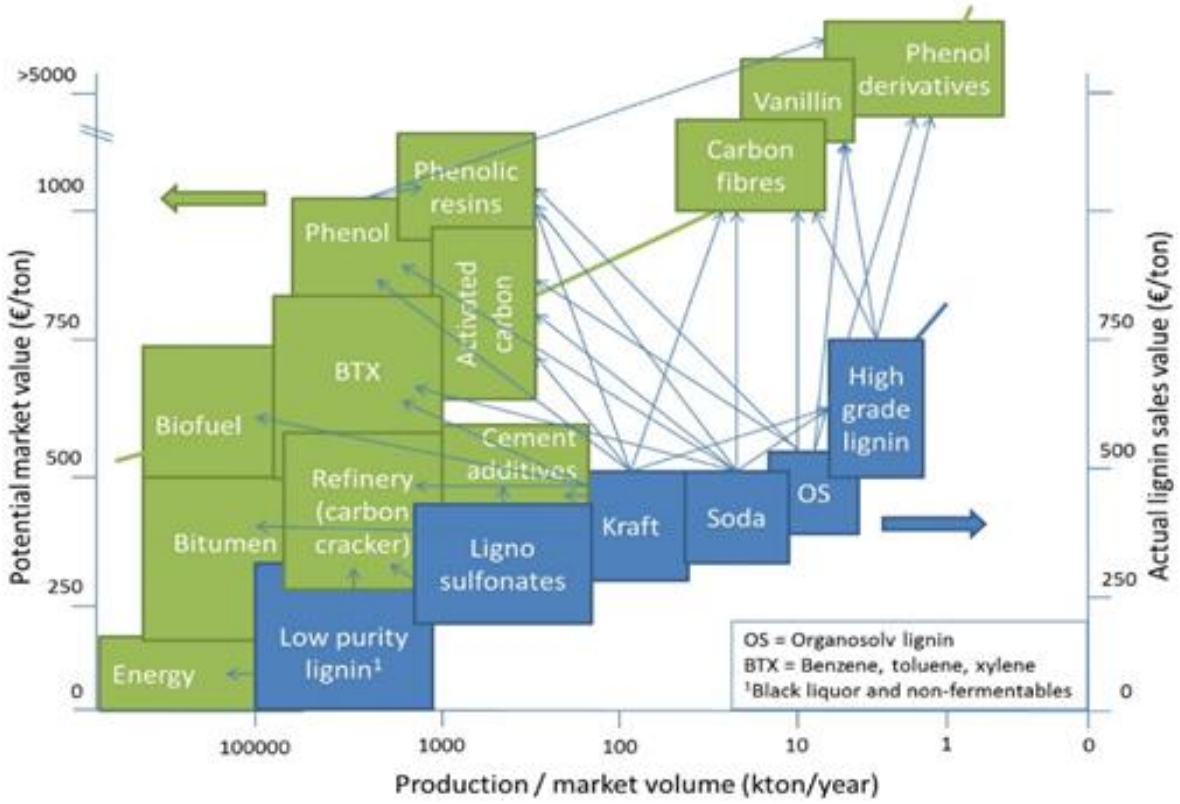

Figure 2.4: Annual lignin production versus the utilization of lignin and the potential market value. Potential value-added products (green boxes) and industrial lignin (blue boxes) (Gosselink, 2015).

2.4. Biological lignin degradation

In nature, lignin is decomposed into simpler aromatic compounds by highly specialised group of fungi known as basidiomycetes belonging to the phylum Basidiomycota as well as bacteria (Janusz *et al*., 2013). These microorganisms are naturally found in forests or in areas where fallen trees and degradation of plants occurs, which is essential for the recycling of carbon and other nutrients in many ecosystems (Furukawa *et al*., 2014). However, biological degradation by these microorganisms is a rather slow process. The phylum Basidiomycota is divided into three groups, i.e. white-rot, brown-rot and soft-rot fungi, with the white-rot fungi being the most effective lignin degraders compared to brown and soft-rot fungi as well as bacteria (Hammel and Cullen, 2008). Hence, most biological lignin degradation studies focus on white-rot fungi and their enzymatic machinery.

White-rot fungi degrade lignin by secreting a consortium of oxidative enzymes and mediators that depolymerise the inert lignin biopolymer. These microorganisms decompose lignin through the action of heme-containing peroxidases, laccases as well as accessory enzymes

resulting in the production of H_2O and CO_2. As a result, the wood displays a whitish colour and a fibrous texture hence the name "white-rot" (Figure 2.5). Enzymes produced by white-rots are considered to be more efficient than bacterial enzymes (Lambertz *et al*., 2016).

Figure 2.5: The presence of white-rot fungi (circled) on stumps of *Eucalyptus* spp (Costa *et al*., 2017).

Many white-rot fungi such as *P. chrysosporium, Trametes versicolor, Heterobasidion annosum, Irpex lacteus* to mention a few, degrade lignin by simultaneously attacking lignin, cellulose and hemicellulose. On the other hand, white-rots such as *Pleurotus ostreatus, Phlebia radiate* and *Coriolus versicolor*, preferentially attack lignin in a selective manner to yield enriched cellulose and hemicellulose molecules and these white-rots are referred to as selective lignin degraders (Dashtban, 2010; Abdel-Hamid *et al*., 2013). Selective lignin degraders possess great biotechnological applications in industries such as pulp and paper mills for bio-pulping, where the removal of lignin is vital to keep cellulose and hemicellulose intact (Dashtban, 2010). The basidiomycete *P. chrysosporium* was the first white-rot fungi whose genome was sequenced and the most extensively studied basidiomycete due to its ability to produce more complete enzyme complexes required for lignin degradation compared to other white-rots (Singh and Chen, 2008; Ayuso-Fernández *et al*., 2017).

The effective lignin degrading machinery of white-rots primarily consists of CAZy family AA2 (Auxiliary Activity Family 2) and AA1_1 enzymes to mineralise lignin (Figure 2.6) (Levasseur *et al.*, 2013). The AA2 consists of heme-containing peroxidases, namely lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and versatile peroxidase (VP, EC 1.11.1.16). The subfamily AA1_1 consists of laccase (EC 1.10.3.2), which are blue copper oxidases. The LiP, MnP and VP peroxidases are classified as class II fungal secreted peroxidases that belong to the family of non-animal peroxidases (Pollegioni *et al.*, 2015). These strong biocatalysts use H_2O_2 as an electron acceptor during their multi-step oxidation of a broad range of substrates such as lignin, lignin model compounds and phenolics.

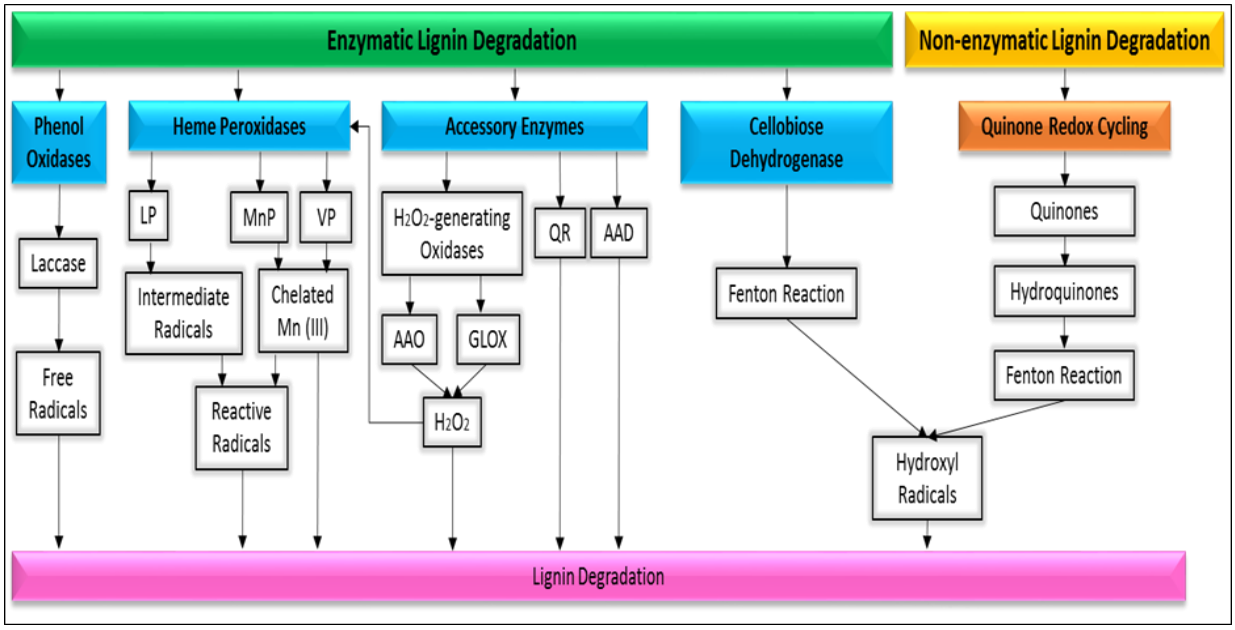

Figure 2.6: Schematic illustration of enzymes produced by basidiomycetes that are involved in lignin degradation (Dashtban *et al.*, 2010).

Laccases are multi-copper oxidases and are the only non-peroxidases of the major ligninolytic enzymes. Fungal laccases exist both as intracellular and extracellular enzymes, but the majority of these are extracellular (Kunamneni *et al.*, 2007). During the oxidation of substrates by laccases, the reduction of atmospheric oxygen to H_2O occurs simultaneously (Fisher and Fong, 2014). Basidiomycetes also secrete a group of enzymes known as auxiliary enzymes, such as cellobiose dehydrogenase (EC 1.1.99.18), quinone reductases (EC 1.6.5.5) as well as H_2O_2 -generating oxidases such as aryl-alcohol oxidase (EC 1.1.3.7) (Figure 2.6) (Dashtban *et al.*, 2010; Pollegioni *et al.*, 2015).

2.5. Lignin peroxidase

Lignin peroxidase, also known as diarylpropane peroxidase or ligninase I, was first reported in 1983 by Tien and Kirk in the basidiomycete *P. chrysosporium* and was the first ligninolytic enzyme to be isolated (Fisher and Fong 2014). White-rots such as *P. chrysosporium, T. versicolor, P. radiata* and many others secrete LiP as a secondary metabolite during nutrient (nitrogen and/or carbon) limiting conditions, making it an inducible enzyme. In the well-characterised white-rot *P. chrysosporium*, LiP is encoded by multiple genes and exits as numerous isozymes (H1, H2, H6, H7, H8 and H10) (Tien and Kirk, 1988; Farrell *et al*., 1989). This enzyme is characterised as an extracellular heme-containing glycoprotein with a molecular weight of 38 to 42 kDa, a theoretical pI of 3.3 to 4.7 and optimum pH of 3 to 4.5 depending on the isozyme (Piontek *et al*., 2001; Wong, 2009; Plácido and Capareda, 2015). The *P. chrysosporium* LiP is secreted with veratryl alcohol (VA; 3,4-dimethoxybenzyl), a natural phenolic substrate of LiP that plays a pivotal role in the reaction mechanism of LiP (Hammel and Cullen 2008).

Lignin peroxidase is a globular glycoprotein composed of α-helices (eight major and eight minor) and relatively few β-sheets located in the C-terminal domain (Martínez, 2002). Structurally, LiP shares a high homology with other class II peroxidases and contains heme, calcium and manganese residues that are conserved in all heme-containing peroxidases. The heme group (forming part of the catalytic domain) is situated in the interior part of the protein, enfolded between the proximal and distal domains (Figure 2.7) (Piontek *et al*., 2001). The enzyme contains eight cysteine residues, resulting in the formation of four disulphide bridges (Martínez, 2002; Wong, 2009).

The ability of LiP to oxidize non-phenolic substrates such as β-O-4 lignin model compounds, VA and many more with high redox potentials, is attributed to the presence of the tryptophan (Trp171) (in the LiPH8 isozyme) residue located on the surface of the enzyme (Figure 2.7) via long-range electron transfer (Plácido and Capareda, 2015; Pham *et al*., 2016; Ayuso-Fernández *et al*., 2017). This residue is absent in other heme-containing peroxidases. The pentacoordinated Fe(III) iron porphyrin ring of LiP situated in the active site is one electron deficient, which also contributes to the oxidization of substrates with high redox potentials (Plácido and Capareda, 2015).

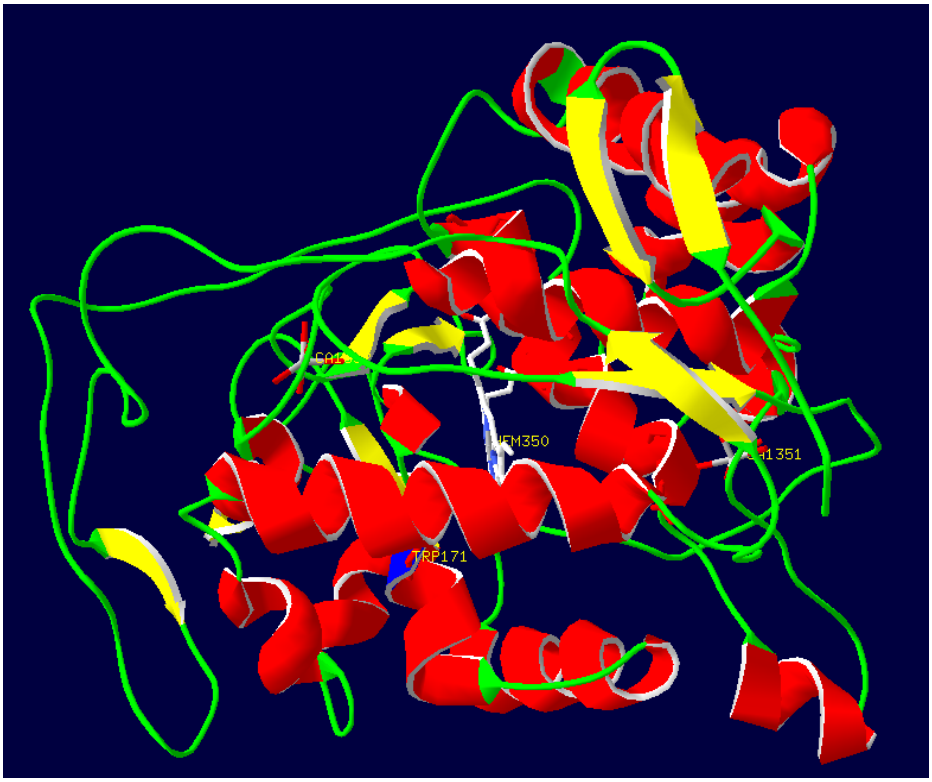

Figure 2.7: Crystallographic ribbon diagram of the *P. chrysosporium* LiPH8 protein depicting the α-helices and β-sheets as red coils and yellow arrows respectively. The heme group represented in grey and blue side chains. Trp171 is represented in blue.

The catalytic mechanism of LiP occurs in multiple steps (Figure 2.8A) and is facilitated by the interaction of the catalytic active site and heme residue with H_2O_2 to form an intermediate. The first reaction involves the reduction of H_2O_2 by native LiP, resulting in a 2ē oxidation of the enzyme by H_2O_2, yielding H_2O and compound I, which occurs as a porphyrin radical (Wong, 2009). The second reaction entails two subsequent 1ē reduction of compound I by a reducing substrate (such as VA), resulting in compound II. The second 1ē reduction returns compound II back to native LiP by receiving a second electron from the reducing substrate, resulting in the complete oxidation of the enzyme (Datta *et al*., 2017). This transfer of electrons occurs in an unspecific manner, resulting in a cation radical $VA^{\bullet+}$ (Martínez *et al*., 2005). The formation of $VA^{\bullet+}$ cation radicals has been reported to play a pivotal role in the protection of enzyme against inactivation by excess H_2O_2 (Mao *et al*., 2010).

Remarkably, LiP catalyses both phenolic and non-phenolic aromatic units of lignin in the absence or presence of mediators (Figure 2.8B). Other major ligninolytic enzymes such as laccases require mediators for the oxidation of non-phenolic lignin units. Since the heme residue of LiP is embedded between the proximal and distal domain, the interaction between

the heme residue and some substrates is hampered. It is suggested that VA partakes as a diffusible redox mediator and facilitates the oxidation of such substrates (Mäkelä, 2015).

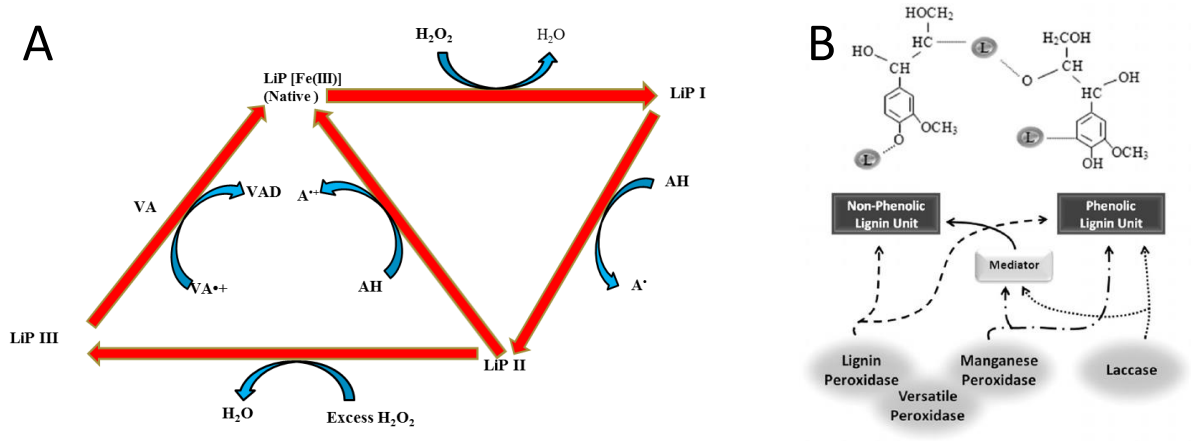

Figure 2.8: An illustration of the (A) catalytic mechanism of LiP (adapted from Datta *et al.*, 2017) and (B) the oxidation of phenolic and non-phenolic lignin monomers by LiP and ligninolytic enzymes (Moreno *et al.*, 2015).

This study focuses particularly on the *P. chrysosporium* lignin peroxidase (LiPH8). The first ligninolytic enzyme to be discovered in the extracellular fluid of *P. chrysosporium* was characterized as an enzyme that requires H_2O_2 for its activity, and a molecular size of 42 kDa, which corresponds to the size of LiPH8 isozyme. Although the *P. chrysosporium* LiPH8 isozyme has been shown to have relatively lower catalytic properties towards VA compared to other isozymes (Table 2.3), it has been ascribed as the major isozyme (Kirk *et al.*, 1986; Farrell, 1989). According to Tien and Kirk (1983), the LiPH8 lignin peroxidase is the dominant isozyme secreted by the white-rot *P. chrysosporium* under optimal growth conditions (Tien and Kirk 1988). The first crystallographic structure of LiP was reported as early as 1993 using the LiPH8 isozyme (Poulos *et al.*, 1993). Furthermore, the LiPH8 is the most extensively studied and characterised isozyme (Table 2.3) and most recombinant LiP production studies focus on the LiPH8 isozyme (discussed in section 2.7).

Table 2.3: The catalytic properties of *P. chrysosporium* LiP isozymes determined on VA.

Isozyme	Specific activity (U/mg)	Km (µM)	Kcat (s^{-1})	Reference
H1	-	86	3.2	Farrel *et al*., 1989
	7.24	-	-	Tien and Kirk, 1988
	-	22.4	207.1	Rothschild *et al*., 1997
H2	-	250	8.33	Farrel *et al*., 1989
	16.4	-	-	Tien and Kirk, 1988
	-	246.6	17.3	Rothschild *et al*., 1997
	-	210	76	Tuisel *et al*., 1989
H6	-	122	6.58	Farrel *et al*., 1989
	5.5	-	-	Tien and Kirk, 1988
	-	166.5	18.8	Rothschild *et al*., 1997
H7	-	483	5.46	Farrel *et al*., 1989
	3.28	-	-	Tien and Kirk, 1988
H8	-	89	1.32	Farrel *et al*., 1989
	7.6	-	-	Tien and Kirk, 1988
	-	165.7	8.1	Rothschild *et al*., 1997
	-	123.7	2.31	Pham *et al*., 2014
	-	92.8	22.9	Sollewij-Gelpke *et al*., 1999
	-	102.9	19.9	Sollewijn-Gelpke *et al*., 2002
	-	-	22.9	Doyle *et al*., 1998
	-	79.3	16.2	Ayuso-Fernández *et al*., 2017
H10	-	190	2.78	Farrel *et al*., 1989
	2.1	-	-	Tien and Kirk, 1988
	18	206.3	8.7	Rothschild *et al*., 1997

- Properties not indicated

2.6. Biotechnological applications of LiP

Considerable research has been devoted to LiP over the past three decades due to its broad spectrum of potential industrial and biotechnological applications that are ascribed to its non-specificity towards a wide range of substrates, making it an economically valuable enzyme (Conesa *et al*., 2000; Wen *et al*., 2010; Maciel *et al*., 2010; Abdel-Hamid *et al*., 2013). Despite its array of potential biotechnological applications, the use of LiP for industrial application is hindered by high production costs and the instability of the enzyme. There is thus a great need for the production of stable LiP at cost-effective prices for this enzyme to be applied in industries such as pulp and paper mills, 2G biofuels, food and feed, pharmaceuticals as well as a bioremediation agent for diverse industrial sectors (Asgher *et al*., 2014; Oliveira *et al*., 2015).

Thermochemical and chemical methods for the depolymerisation of lignin are not recommended as they produce toxic compounds and employ harsh conditions. There is therefore a huge demand for "greener" and cheaper alternatives to these methods, such as the use of LiP given its delignification capabilities in various industries. Furthermore, the use of biological methods for residual lignin treatment may provide safer working conditions in pulp and paper mills and 2G biofuel industries.

Biopulping is the process of using lignin degrading microorganisms or their enzymes to degrade the lignin present in the cell walls of wood that renders the wood softer for the production of paper (López *et al*., 2017). This process is highly efficient as it removes wood extractives to improve paper strength and quality, and aids in the reduction of energy requirements for the pulp processing (Liew, 2011; Asgher, 2014). The treatment of wood chips using white-rot fungi that secrete LiP and MnP has been proposed as an alternative to mechanical and chemical pulping (Maciel *et al*., 2010). Another possible application of LiP in pulping is the replacement of chlorine during the bleaching process, which is done to achieve a certain level of brightness in pulps. In pulp and paper mills, residual lignin is removed from the pulp by using chemical bleach that contains contaminating chlorine and other chlorinate compounds. The use of LiP for biopulping and biobleaching could reduce costs by reducing harsh pulping conditions and substituting expensive chlorine containing chemicals (Harada *et al*., 2016).

The ability of LiP to oxidize lignin and lignin analogous structures has supported its potential applications in 2G biofuel industries (Moreno *et al*., 2015). Delignification processes should occur at low temperatures to preserve the polysaccharides, which favours biocatalysts such as

LiP that optimally work at low temperatures (Zeng *et al*., 2014). Although LiP shows potential in this industry, there is limited documentation of the successful utilisation of LiP for the production of biofuels and/or its derivatives. Nevertheless, Sahadevan and co-wokers (2016) isolated and purified LiP from a Deuteromycete fungus that degraded lignin into dodecane,1-fluoro (an alkaline hydrocarbon derivative) that is used in biofuels industries. Pinto and co-workers (2012) investigated the influence of three ligninolytic enzymes produced by several white-rots on straw saccharification during fungal pretreatment. They reported that only LiP displayed a significant increase in the saccharification yields during solid-state fermentation of wheat straw (Pinto *et al*., 2012).

In the food and cosmetics industries, LiP plays a pivotal role in the synthesis of aromatic compounds such as vanillin, known as a lucrative product of degraded lignin (Sana *et al*., 2017). Vanillin is used in the flavouring of foods, beverages, confectionaries and ice-creams, as well as a fragrance ingredient in perfumes and other cosmetics products due to its natural rich vanilla aroma (Rana *et al*., 2013). However, only about 1% of vanillin is obtained from natural vanilla due to high market costs (Winter *et al*., 2012; Gallage and Møller, 2015). Synthetic vanillin is obtained mainly from lignin and guaiacol substrates, both through the action catalysed by LiP. However, the conversion of lignin to vanillin is achieved through chemical methods that are discouraged due to hazardous processes. There are studies documenting the production of vanillin using purified LiP or LiP producing white-rot fungi *P. chrysosporium* (Ten Have *et al*., 1998; Dos Santos Barbosa *et al*., 2008). Ten Have *et al*. (1998) reported the conversion of O-acetyl isoeugenol and O-acetyl coniferyl by LiP into alcohol O-acetyl vanillin, whereas Dos Santos Barbosa (2008) reported *P. chrysosporium* producing vanillin using solid state fermentation of green coconut residue.

Commercial dyes (such as azoic dyes, acidic, basic, disperse, oxidative and others) are extensively used in many textile and dyeing industries all over the world. Some of these dyes do not bind to the fibres during the dyeing process, but remain as impurities that are often disposed into water ecosystems (Ngieng *et al*., 2013). According to previous reports, LiP secreted by *P. chrysosporium* has dye decolourising capabilities and mineralises a variety of textile effluents that are recalcitrant (non-biodegradable) (Robinson *et al*., 2001; Husain, 2010). Pant and Adholeya (2007) isolated two fungal strains, namely *Penicillium pinophilum* and *Alternaria gaisen* from the contaminated soil of a distillery effluent site. Both these fungal strains were able to produce active LiP together with MnP and laccase displaying decolorising capabilities (Pant and Adholeya, 2007). Ferreira-Leitão and co-workers (2006) demonstrated

the ability of LiP to decolorise methylene blue, with concentrations of up to 50 mg/mL of methylene that were degraded within 30 min by LiP (Ferreira-Leitão *et al*., 2006). Furthermore, a molar ratio of 1:5 of methylene blue: H_2O_2 resulted in a 90% removal of the colour dye. This enzyme has demonstrated oxidation capabilities of aromatic compounds.

The conversion of lignin into high-value aromatic compounds is hampered by factors such as difficult bond cleavages, physical properties, heterogeneity of lignin and low reactivity (Prasetyo *et al*., 2012; Bugg and Rahmanpour, 2015). The application of LiP together with other ligninolytic enzymes can depolymerise lignin, although commercially available products produced from enzymatically depolymerised lignin remain elusive. Horseradish peroxidase (HRP: EC 1.11.1.7) is a plant peroxidase known to catalyse polymerisation reactions of lignin and analogous compounds, which may help to increase the molecular weight of technical lignins to produce high-value products (Figure 2.9) (Liu *et al*., 2015).

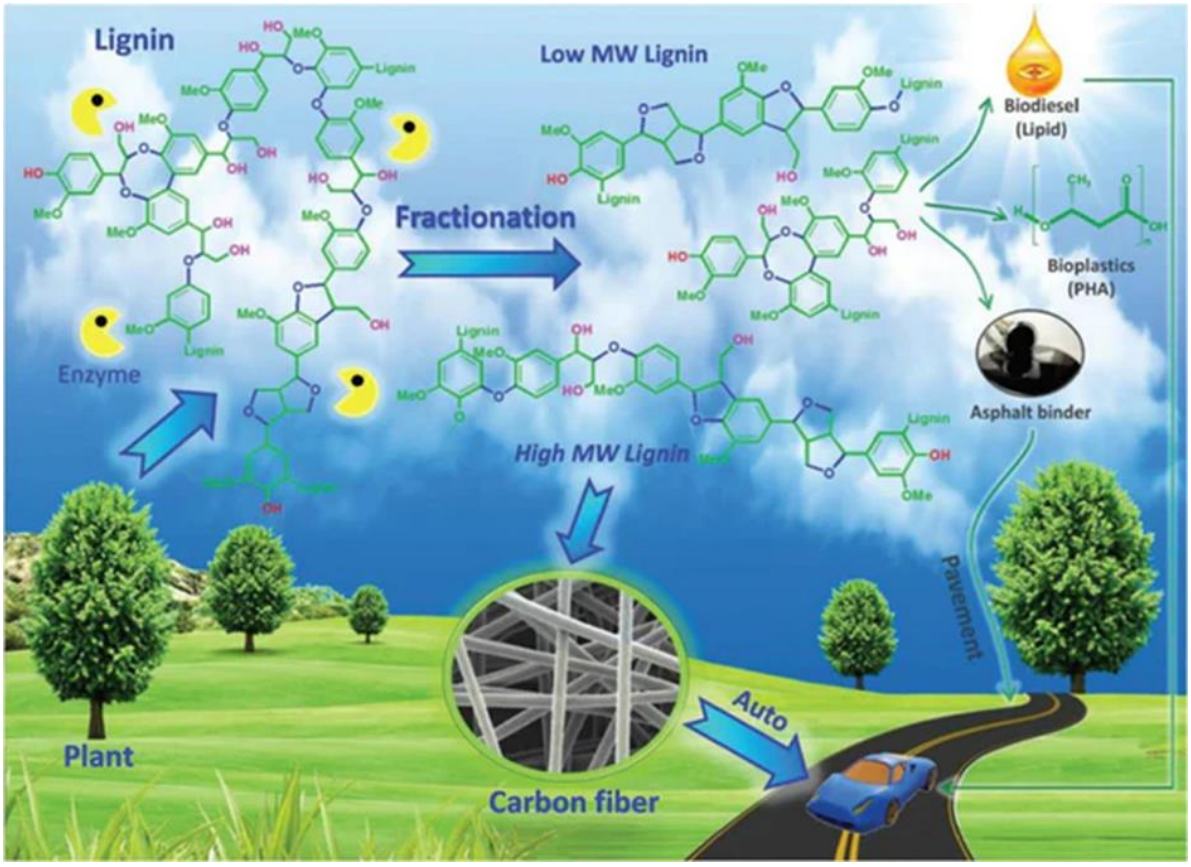

Figure 2.9: Products that can be derived from depolymerized lignin using ligninolytic enzymes such as LiP https://newatlas.com/lignin-waste-carbon-fiber/49533 (accessed September 2017).

Similarly to LiP, HRP requires H_2O_2 for its catalytic activity and the reaction mechanism is hypothesised to be similar (Abdel-Hamid *et al.*, 2013). However, compared to HRP, reports documenting the polymerisation of aromatic compounds by LiP are still lacking. Liu *et al.* (1999) reported the enzymatic polymerisation of polyaniline (a conducting polymer) using HRP, but HRP showed low activity towards aniline and low stability at pH below 4.5 (Chattopadhyay and Mazumdar, 2000). In contrast, lignin peroxidase may be a more suitable candidate to produce aniline since it is optimally active at pH 3 to 4.

2.7. Recombinant protein expression systems

A variety of recombinant proteins of pharmaceutical, industrial and economic importance have been produced using different microbial systems (Idiris *et al.*, 2010). Bacteria have been used to express many proteins, but they have certain drawbacks as expression systems for eukaryotic proteins. For example, some proteins are produced as inactive proteins, localised in inclusion bodies that require complex purification steps and lack post-translational modifications (protein folding, glycosylation, etc.) that most eukaryotic proteins require (Cereghino and Cregg, 2000). It is thus clear that there is a need for alternative expression systems that may address these constraints.

Eukaryotic expression systems such as yeast allow the production of eukaryotic proteins in their biological active native forms and in higher titres (Idiris *et al.*, 2010). Apart from a rapid growth rates and efficient secretion levels, yeasts are favoured over bacteria to produce eukaryotic proteins as they are often not pathogenic and allow high cell density fermentations (Schmidt *et al.*, 2004). Conventional yeast (such as *S. cerevisiae*) and non-conventional yeast (such as *Hansenula polymorpha*, *Kluyveromyces lactis*, *Yarrowia lipolytica* and P. pastoris) are most commonly used to produce recombinant eukaryotic proteins.

The discovery of LiP in *P. chrysosporium* by Tien and Kirk in the 1980s prompted researchers to explore expression systems to produce the enzyme in copious amounts. The filamentous fungi *Aspergillus niger*, baculovirus and *Escherichia coli* are some of the expression systems that have been used to produce recombinant LiP (Johnson and Li, 1991; Doyle and Smith, 1996; Aifa *et al.*, 1999; Conesa *et al.*, 2000). However, only small amounts were detected in the case of *A. niger* and baculovirus, and it was expressed as inactive inclusion bodies in *E. coli*. Other researchers have attempted homologous expression in native producers; Table 2.4

illustrates some of the expression hosts used to produce recombinant LiP as well as the activity levels obtained.

Table 2.4: Production of recombinant lignin peroxidase in different expression systems and the activity obtained using VA and other substrates.

Enzyme	Source	Expression host organism	Substrate (Activity)	Reference
LiPH8	*P. chrysosporium*	Baculovirus	VA (20 U/mg)	Johnson *et al*., 1991
LiPH2	*P. chrysosporium*	*E. coli*	VA (K_m 300 µM)	Johnson *et al*., 1992
LiPH8	*P. chrysosporium*	*E. coli*	VA (39 U/mg)	Doyle and Smith, 1996
LiPH8	*P. chrysosporium*	*E. coli*	VA (K_{cat} 29.7/s)	Doyle *et al*., 1998
LiPH2	*P. chrysosporium*	*E. coli*	VA (K_{cat} 39/s)	Nie *et al*., 1998
LiPH8	*P. chrysosporium*	*P. chrysosporium*	VA (K_{cat} 23.2/s K_m 89.2 µM)	Sollewijn-Gelpke *et al*., 1999
LiPH8	*P. chrysosporium*	*A. niger*	VA (N/D)	Conesa *et al*., 2000
LiPH8	*P. chrysosporium*	*Pichia methanolica*	VA (932 and 1933 U/L)	Wang *et al*., 2004
LiPH2	*P. chrysosporium*	*S. cerevisiae*	2,4-dichlorophenol (K_{cat} 2.23 /min; K_m 359 µM)	Ryu *et al*., 2008
YlpA	*Phanerochaete sordida*	*P. sordida*	VA (0.009 nKat/mL)	Sugiura *et al*., 2009
LiPH2	*P. chrysosporium*	P. pastoris	VA (15 U/L)	Wang and Wen, 2009
LiPH8	*P. chrysosporium*	*P. chrysosporium*	VA (4 U/mL)	Coconi-Linares *et al*., 2014

2.8. *Pichia pastoris* as an expression system

In recent years, the methylotrophic yeast *P. pastoris* has been the preferred expression system to produce many intracellular and extracellular proteins that are applied in diverse sectors (Macauley-Patrick *et al*., 2005; Lin-Cereghino *et al*., 2013). *Pichia pastoris* has been

reclassified to the genus *Komagataella* based on differences in partial sequences of 18S and 26S rRNAs between *P. pastoris* and other methylotrophic yeast (Kurtzman, 2005). In this study, we use the name *P. pastoris* to keep consistency with previous reports in literature. It was discovered in the 1970s by Phillip Petroleum and used for the production of the animal feed additive single cell protein (SCP) (Ahmad *et al*., 2014; Byrne, 2015) in high cell density fermentation bioreactors using the tightly regulated alcohol oxidase promoter (P_{AOX1}). A decade later, *P. pastoris* was used as host system for heterologous expression of numerous proteins with significant importance in biorefineries, such as lignin modifying enzymes (Figure 2.10).

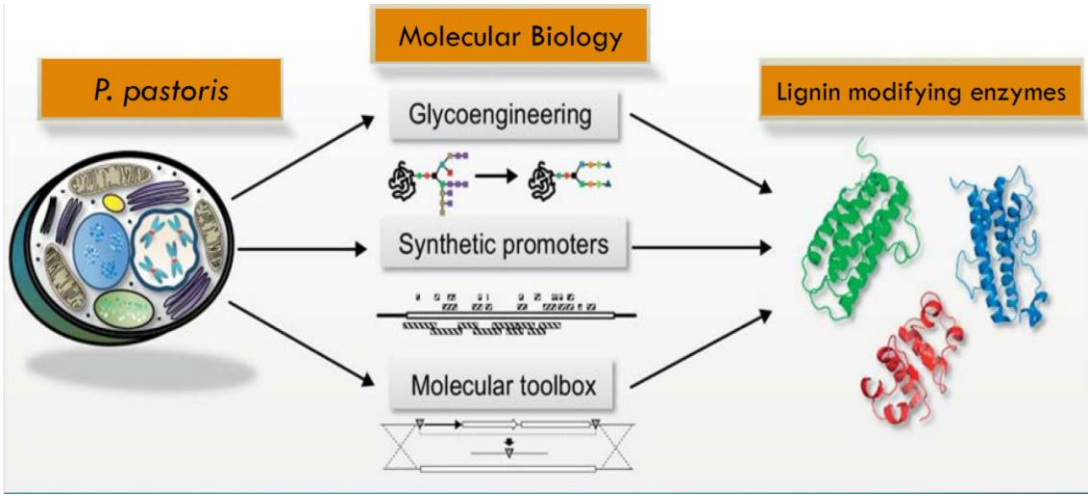

Figure 2.10: Schematic illustration of *P. pastoris* as a biotechnological tool for the production of proteins (adapted from Vogl and Glieder, 2013).

2.8.1. Challenges encountered in *P. pastoris* for recombinant protein production

One of the main advantages of using *P. pastoris* to produce recombinant proteins is its ability to grow to high cell densities, which sometimes correlate with high protein yields. However, other cellular materials such as extracellular or cell-bound proteases may also increase proportionally to biomass (Zhang *et al*., 2007) and result in proteolytic degradation or a decrease in the protein yield or biological activity. Sinha *et al*. (2005) reported that protease levels are lower in glycerol cultivation conditions than when methanol is used as the sole carbon source. Furthermore, proteolytic activity increases with induction time under methanol inducing conditions (Sinha *et al*., 2005).

Pichia pastoris has diverse promoters to choose from, both inducible and constitutive, that allows for high protein yields. Some of these are not tightly regulated and transcription is therefore not tightly controlled. Moreover, the overexpression of recombinant proteins may sometimes lead to misfolded proteins, resulting in physiological and metabolic burdens on the cell. Although there are many optimisation strategies that can be employed to address some of these challenges in P. pastoris, the biggest dilemma most researchers are faced with, is choosing the best combination of genetic elements such as promoters, secretion signals and selection markers, as well as choosing the best cultivation conditions for optimal protein production levels (Figure 2.11).

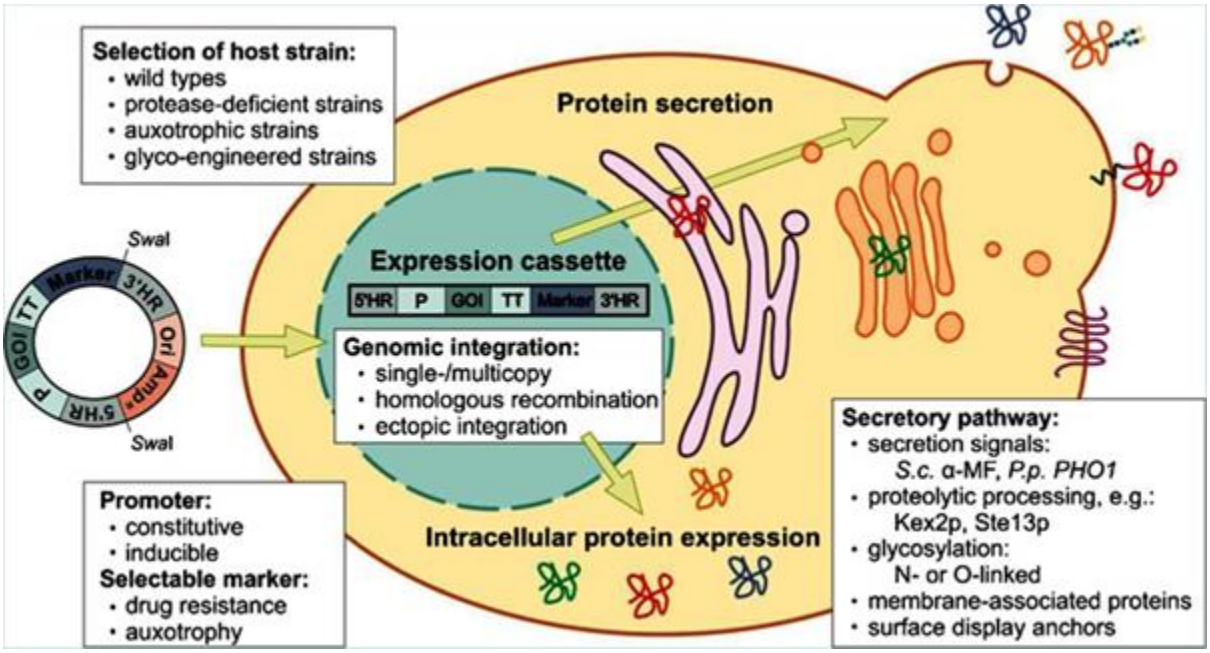

Figure 2.11: Characteristics that enable *P. pastoris* to be the ideal host for recombinant protein expression (Ahmad *et al.*, 2014).

2.8.2. Promoters

The success of *P. pastoris* as an expression system has been highly associated with the methanol-inducible P_{AOX1}, which is strongly and tightly regulated and permits high yields of recombinant proteins (Zahrl *et al.*, 2017). The P_{AOX1} promotes the transcription of the alcohol oxidase 1 gene. Under the transcriptional control of the P_{AOX1}, cells are initially grown in glycerol to allow biomass production (P_{AOX1} is repressed), and then transcription of the gene of interest is induced with methanol (P_{AOX1} is de-repressed) (Vogl and Glieder, 2013). Before

methanol induction, there are none or minute traces of recombinant protein. This may be advantageous when co-expressing transcription factors such as chaperones before the protein of interest is produced (Ahmad *et al*., 2014). Similar to P_{AOX1}, the P_{AOX2} also requires methanol for induction, but the P_{AOX2} is relatively weaker for the transcription of recombinant proteins (Vogl and Glieder, 2013). Furthermore, P_{AOX1} is preferred should the protein of interest exhibit cytotoxic effects on the cell.

The P_{AOX1} has several benefits, but also has disadvantages that may require alternative promoters. For example, the use of methanol is discouraged for producing certain food products, additives and/or pharmaceutical products as it is toxic and derived from a petrochemical derivative (Waterham *et al*., 1997). Induction with methanol needs to be done with precaution as *P. pastoris* cells are sensitive to high methanol concentrations. Methanol is oxidized into formaldehyde and H_2O_2, which may initiate cellular stress that can be detrimental to the cell (Krainer *et al*., 2012). In addition, methanol is highly flammable and is not recommended for large-scale usage and storage. Methanol may evaporate in shake flask cultivations, which complicates the reproducibility of protein yields (Byrne, 2015). *Pichia pastoris* grows relatively slower in methanol than in glucose or glycerol. Therefore, in high cell density fermentations in bioreactors, the glycerol-feed batch phase is carried out before the induction phase. As this may prolong cultivation periods before the production of the desired protein and requires process handling, it may not be suitable for industrial applications where time is a crucial factor.

To overcome some of these bottlenecks, the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (P_{GAP}) is the most preferred alternative. The P_{GAP} was discovered by Waterham and co-workers (1997) for producing proteins at comparable yields to that of the P_{AOX1} using glucose as the carbon source (Cereghino and Cregg, 2000). The P_{GAP} is encoded by the glyceraldehyde-3-phosphate dehydrogenase (*GAP)* gene in *P. pastoris* (Li *et al*., 2007). The P_{GAP} requires simpler protein production conditions since there is no need to change cultures from one carbon source to another for induction. Despite its advantages, the P_{GAP} is not suitable if the protein of interest is toxic to the cell. Although other promoters have been used in P. pastoris, including P_{ENO1}, P_{FLD1}, $P_{ET9,}$ P_{GK1}, the P_{AOX1} is still the most extensively used promoter for heterologous protein production in *P. pastoris*. For more information on *P. pastoris* promoters please refer to the review of Vogl and Glieder, 2013.

2.8.3. Secretion signals

Secretion signals or signal peptides play a pivotal role in the secretion of newly synthesised extracellular proteins (Govindappa *et al*., 2014; Tanghe *et al*., 2015). The secretion efficiency of secretion signals differs and is protein and strain dependent (Massahi and Çalik, 2015). For example, Wang *et al*. (2004) demonstrated that LiP production levels were increased 2-fold when the native signal peptide was replaced with that of the *S. cerevisiae* α-mating factor (α-MF) in P. pastoris. Some proteins may prefer the use of the native secretion signal, while others prefer alternative secretion signals for the correct cleavage and secretion in the expression host. Most proteins produced in *P. pastoris* are expressed under the control of the native signal peptide, the endogenous *P. pastoris* acid phosphatase (*PHO*1), the *S. cerevisiae* invertase (SUC2) or the α-MF signal sequences (Table 2.5)

Table 2.5: Secretion signals used to facilitate extracellular expression of recombinant proteins in *P. pastoris*.

Secretion signal	Source	Supplier	Reference
α-mating factor pre-sequence	*S. cerevisiae*	Life Technologies™ / PichiaPink™	Brake *et al*., 1984
acid phosphatase	P. pastoris	Life Technologies™	Laroche *et al*., 1994
Glucoamylase signal sequence	*Aspergillus awamori*	PichiaPink™	Aleshin *et al*., 1992
Serum albumin signal sequence	*Homo sapiens*	PichiaPink™	Dugaiczyk *et al*., 1982
Inulinase presequence	*Kluyveromyces maxianus*	PichiaPink™	Chung *et al*., 1996
Invertase signal sequence	*S. cerevisiae*	PichiaPink™	Perlman *et al*., 1982
killer-toxin-secretion-signal	*K. lactis*	PichiaPink™	Baldari *et al*., 1987

Among these, the α-MF is still the most commonly used for high protein yields, and is sometimes used as benchmark to compare the efficiency of novel secretion signals in *P. pastoris* (Liang *et al*., 2013; Lin-Cereghino, 2013; Chahal *et al*., 2017). Commercial vectors available from PichiaPink™ have several secretion signals to direct recombinant protein secretion in P. pastoris.

2.8.4. Co- and post-translational modifications

Pichia pastoris can carry out complex co- and post-translation modifications (PTM) processes, such as glycosylation, protein folding, signal peptide cleavage and disulphide bridge formation that most eukaryotic proteins require to be secreted in their biological active form. Glycosylation is predicted to be the most important PTM as it plays an important role in determining the structural configuration, function and stability of a protein (Delic *et al*., 2013; Vogl *et al*., 2013). Compared to *S. cerevisiae,* glycosylation is less extensive in *P. pastoris* as it generally does not hyperglycosylate recombinant proteins (Daly and Hearn, 2005; Teh *et al*., 2011). Glycosylation has been reported to have several benefits, such as improved thermostability and biocatalytic activity. In fact, there are a number of reports asserting the effects of glycosylation in *P. pastoris* (Zou *et al*., 2013; Yang *et al*., 2015; Gündüz Ergün and Çalık, 2016; Niu *et al*., 2018). Pérez de los Santos *et al*. (2016) reported that invertase A and B produced in *P. pastoris* displayed improved catalytic properties due to glycosylation. Bovine enterokinase light chain (bEK_l) has been produced in *P. pastoris* and reported that glycosylation had an influence on the enzymatic activity and improved the thermostability of the enzyme (Wang *et al*., 2018). However, this is not universal, but rather protein dependent (Li *et al*., 2007).

2.8.5. Upregulation of transcriptional factors

There are many strategies that can be employed to enhance protein yields in P. pastoris, such as the co- or individual expression of helper factors involved in processes such as unfolded protein response (UPR). For example, Hac1 and Kar2, (binds to misfolded proteins in the secretory pathway) or Pdi (involved in the isomerisation of disulphide bonds) are some of the strategies that researchers have employed to increase protein yields (Guerfal *et al*., 2010; Vanz *et al*., 2014; Yu *et al*., 2017). Ruth *et al*. (2014) overexpressed the activator of ferrous transport (Aft1), which is involved in the regulation of secretory and carbohydrate metabolism in P.

pastoris, and reported a 2.5-fold increment of the *Sphingopyxis sp.* carboxylesterase in bioreactor cultivations (Ruth *et al*., 2014). However, the overexpression of complex proteins in *P. pastoris* may exert physiological burdens on the cell (Yu *et al*., 2017). Huangfu *et al*. (2014) reported the co-overexpression of the *TPX* gene (involved in oxidative stress response in P. pastoris) increased the yields of the reporter protein β-glucuronidase from *Penicillium purpurogenum* by approximately 2.5-fold in P. pastoris.

2.8.6. Codon optimisation

Codon optimisation is one of the many techniques that may be employed to increase protein yields in *P. pastoris* (Mellitzer *et al*., 2014). The genetic code is composed of 20 amino acids encoded by 64 different codons, signifying each amino acid is encoded by more than one codon (except for methionine and tryptophan). Synonymous codons refers to dissimilar codons that encode the same amino acid (Mazumder *et al*., 2016). It has been established that some codons are favoured compared to synonymous codons during the coding of a gene into a protein (Behura and Severson, 2013). Synonymous codons are used at different frequencies between species and the identity of frequent and rare codons for different amino acids differ between species (Hershberg and Petrov, 2008; Angov, 2011). Some organisms have a strong codon usage bias, while others use different synonymous codons at similar frequencies. Codon optimisation is the introduction of preferred codons over the native ones that will encode the same amino acid sequence of the expression host. It is hypothesised that *P. pastoris* has a non-random pattern of synonymous codon usage and is biased towards a subset of codons.

Codon usage bias is an important factor to consider when expressing genes in a foreign host system. Moreover, some native genes employ rare codons that may decrease the efficiency of translation or in some cases, disengage the translation machinery in *P. pastoris* (Xu *et al*., 2016). It is thus important that the optimisation of a gene sequence is based on the codon usage of the expression host system. The replacement of these rare codons with synonymous ones may improve translation effectively, leading to increasing protein titres.

Different genes display variations in codon usage bias, which depends on numerous aspects such as gene length, gene composition (GC content), expression levels and mRNA stability (Behura and Severson, 2013). There are several reports documenting the expression of codon optimised genes (from diverse native hosts) in *P. pastoris* that outperformed their native counterparts by several folds (Hu *et al*., 2013; Li *et al*., 2014; Zhao *et al*., 2014; Tian *et al*.,

2016; Xu *et al*., 2016). Numerous studies have reported increased production levels of recombinant proteins due to codon optimization (Table 2.6). Tu *et al*., (2016) reported that bovine interferon-α (bIFN-α) was produced at 3-fold higher levels from the optimised gene compared to the wild type using the same gene copy number in P. pastoris. Similarly, P-glycoprotein (Pgp), a membrane protein used to elute a wide range of hydrophobic compounds out of cells, was produced at approximately three times higher levels using the opt-Pgp compared to the native gene in *P. pastoris* (Bai *et al*., 2011).

The OptimumGene™ algorithm by GenScript® Biotech Co. is one of the most commonly used algorithms for codon optimization of genes in P. pastoris. It is based on the codon adaptation index (CAI), where a CAI value of 1 or close to 1 indicates a strong adaptation to the host and great improvement in terms of gene expression levels in *P. pastoris* (Zhou, 2016). Qiao *et al*. (2017) reported the increase of the CAI of the *Galactomyces geotrichum* lipase I (GGl I) gene from 0.72 to 0.85 for the native and optimised versions, respectively, enhanced the production levels of GGl 1 in P. pastoris. The native gene (GGl I-wt) yielded no detectable activity, while the optimized gene (GGl I-op) gave 150 U/mL (Table 2.6). The α-amylase gene from *Bacillus licheniformis* was codon optimised to adjust the CAI from 0.74 to 0.86 for the native (*BlAmy*) and optimised (*BlAmy-opt*), respectively in *P. pastoris* (Wang *et al*., 2015). Compared to the *BlAmy*, the *BlAmy-opt* was expressed 2.62-fold higher in bioreactor cultivations after 168 hours of methanol induction.

Table 2.6: Recombinant enzymes produced in *P. pastoris* at higher levels due to codon optimisation by adjusting the CAI.

Strain	Enzyme	CAI increase	Fold increase	Reference
GS115	Xylanase	N/I	37%	Zheng, 2017
GS115	α-Amylase	0.86.	2.62-fold	Wang *et al*., 2015
X-33	Lipase I	0.85	150 U/ml*	Qiao *et al*., 2017
KM71H (aox1:ARG4, arg4)	Endoglucanase	0.91	1.24-fold	Akcapinar *et al*., 2011
X-33	Lipase	0.87	2.2-fold	Zhou *et al*., 2015
X-33	Alpha 1-antitrypsin	0.96	40%	Arjmand *et al*., 2013

*hydrolytic activity of GGl I-opt

Based on the above-mentioned premise, the *P. chrysosporium lip*H8 gene was optimized according to the OptimumGene™ algorithm to investigate its potential effect on protein yields. However, Chung and Lee (2012) argued that codon optimisation that leads to the increment of the CAI might not be ideal, as overexpression of a gene may result in the rapid depletion of tRNAs. This in turn may lead to an unbalanced tRNA pool, resulting in elevated translational errors as tRNA abundance is crucial for regulating translation efficiency (Gustafsson *et al*., 2004).

The codon bias index (CBI) measures the codon usage bias based on the degree to which a gene uses a subset of optimum codons (Uddin, 2017). Similar to the CAI, the CBI uses a scaling factor where a gene with a high codon bias has a CBI value of 1.0 and a gene with a CBI value close to 0 indicates a random choice of codons. Furthermore, a CBI value of less than 0 indicates non-preferred codons (Uddin, 2017). The CBI approach uses codons that are frequently used in forming Watson-Crick base pairs with the anticodon of major tRNA species and not those frequently used in highly expressed genes within that host as in the case of the CAI (Xia, 2007). A drawback of the CBI approach is that there is no excellent foundation when comparing species, since optimal codons encoding for the same amino acid polypeptide in different species are highly likely to be different (Behura and Severson, 2013).

This thesis is framed within a lignin valorization project that aims to develop green technologies, such as biocatalyst-based (lignin peroxidase) processes for the upgrade of lignin-enriched residues. In this study, *P. pastoris* was used to express *lip*H8 encoding genes and investigate the impact of codon optimisation on the expression levels of recombinant LiP according to two different strategies (CAI and CBI) compared to that of the native *P. chrysosporium* gene. *Pichia pastoris* was chosen as the expression host system due to attributes such as high protein yields, simple cultivation methods and simple downstream processes that will assist in the cost-effective production of LiP for the biocatalytic upgrade of technical lignins.

2.9. References

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Chapter 3

Heterologous expression of a fungal lignin peroxidase in *Pichia pastoris*

Heterologous expression of a fungal lignin peroxidase in *Pichia pastoris*

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Abstract

The lack of cost-effective commercial lignin peroxidase (LiP) preparations has limited the potential biotechnological applications of LiP, such as the valorization of technical lignin residues into high value-added bioproducts. This study aimed to produce the *Phanerochaete chrysosporium* LiPH8 isozyme in *Pichia pastoris* in sufficient amounts that would allow direct application of the supernatant. The impact of codon optimisation of the *lip*H8 gene on expression levels was investigated using the transcriptional control of the P_{AOX1} and P_{GAP} promoters. Lignin peroxidase production was not detected in any of the recombinant *P. pastoris* strains when the P_{GAP} promoter was used. When the P_{AOX1} promoter was used, 597, 672 and 1169 U/L of LiP were detected in the best producing strains containing pJ901[LiP_Nat], pJ901[LiP_CBI] and pJ901[LiP_CAI], respectively. Compared to the LiP_Nat, the LiP_CBI and LiP_CAI were expressed at higher levels in shake flasks cultivations. Lignin peroxidase titres using the P_{AOX1} promoter were increased to 3818 U/L in bioreactor fermentations. *P. pastoris* strains using the P_{AOX1} promoter with methanol feeding was more efficient at secreting recombinant LiP compared to *P. pastoris* strains using the P_{GAP} promoter with glycerol feeding. The activity levels obtained in this study exceeded previous reports. The cost-effective production of LiP can provide an essential tool for the enzymatic upgrade of technical lignins.

Keywords: Lignin peroxidase, *Pichia pastoris*, codon optimisation, *AOX1* and *GAP* promoter

3.1. Introduction

Lignin is the most abundant carbon source after cellulose, accounting for approximately 30% of the earth's available organic carbon (Mahmood *et al*., 2016). Lignin consists of aromatic

compounds that could be used for the production of several products including biofuels, biomaterials and biochemicals (Strassberger *et al*., 2014). However, lignin is currently underexploited, at least from a chemical point of view. The existing technologies for lignocellulose processing i.e. pulp and paper mills and second generation (2G) biofuel industries, are focusing on the exploitation of the polysaccharide portion of lignocellulosic biomass (cellulose and hemicellulose), while the remaining lignin (referred to as technical/industrial lignins) is generally burnt on site to produce energy for the plant, which is a low value application ($0.18 /kg) (Abdelaziz *et al*., 2016; Mahmood *et al*., 2016). Only a small proportion (approx. 1.5%) is currently used to produce bio-based products with a higher market value, for example vanillin with an estimated value of $100-200 /kg (Agrawal *et al*., 2014; Welker, 2015; Chen and Wan, 2017). The conversion of lignin, however, is challenging due to its inert, heterogeneous and recalcitrant nature, and in the case of technical/industrial lignins, the presence of impurities (carbohydrates, nitrogen, sulphur), toxicity, smell and colour problems (Vishtal and Kraslawski, 2011).

Lignin valorisation by green technologies such as those based on enzyme bioprocesses, has been identified as a solution for the conversion of technical lignins into high value-added products due to their environmental benign nature and selectiveness towards their substrates (Wang *et al*., 2017). In nature, fungi belonging to basidiomycetes, such as the well characterised *Phanerochaete chrysosporium*, and some bacterial species are capable of degrading lignin by secreting several enzymes denoted as lignin-modifying enzymes, ligninolytic enzymes, ligninases or oxidoreductases (Dashtban *et al*., 2010; Gasser *et al*., 2012). Among the lignin-modifying enzymes (LME), lignin peroxidase (LiP, EC 1.11.1.14) presents great biotechnological potential within a biorefinery and other industrial sectors (cosmetics, food and feed, bioremediation, etc.) (Maciel *et al*., 2010) and it is considered as one of the major oxidoreductases involved in lignin modification and degradation by fungi. This is because fungal LiPs exhibit a broad oxidative activity towards a range of substrates such as lignin, aromatic amines, phenolic and non-phenolic substrates, lignin analogous structures and polymeric substrates with high redox potentials (close to 1.4 V) in the presence of hydrogen peroxide (H_2O_2) (Maciel *et al*., 2010; Fisher and Fong, 2014; Furukawa *et al*., 2014).

Lignin peroxidase and the hybrid versatile peroxidase (EC 1.11.1.16) are the only class II heme-containing peroxidases capable of catalysing both the phenolic and non-phenolic components of lignin, in the presence and absence of mediators. Compared to the other ligninolytic enzymes (manganese peroxidase and laccase) as well as horseradish peroxidase,

LiP has a higher redox potential at its optimum pH (of 3 to 4.5) (Wong, 2009). Of interest is the ability of LiP to catalyse both the depolymerisation and polymerisation of phenolic compounds by oxidative coupling of the phenoxy radicals (Mäkelä *et al*., 2015). This ability can be exploited to improve the properties of technical lignins to make them more suitable for certain applications, such as the production bio-composites (Sena-Martins, 2008).

Enzyme-based processes require the production of large quantities of enzyme in a cost-effective manner. To our knowledge, there is no commercial enzyme preparation of LiP available at a cost that will render its industrial application economically feasible. As a result, many researchers have cloned numerous LiP encoding genes in a variety of expression host systems ranging from *P. chrysosporium, Escherichia coli* to *Aspergillus niger* in attempt to produce the enzyme (Doyle and Smith, 1996; Aifa *et al*., 1999; Sollewijn-Gelpke *et al*., 1999). However, fungal peroxidases are particularly challenging for recombinant production in ample amounts in both native producers and foreign expression host systems. This is mainly due to low secretion titres, inadequate posttranslational modifications, its complex chemical structure (i.e. contains a heme group), improper folding and need for exogenous heme group production (Lambertz *et al*., 2016). The successful production of LiP would thus require an expression system capable of addressing the above-mentioned factors.

Amongst several expression host systems, the non-conventional methylotrophic yeast *Pichia pastoris* has stood out as the ideal expression host system to produce complex proteins such as lignocellulolytic enzymes (Chahed *et al*., 2018). Apart from its advantageous characteristics such as high protein yields, strong promoters and easy cultivation methods in inexpensive media, *P. pastoris* has been conferred the Generally Regarded As Safe (GRAS) status, which significantly expands the use of products derived from it (Vogl *et al*., 2013; Ahmad *et al*., 2014; Spohner *et al*., 2015; Wagner and Alper, 2016). Furthermore, *P. pastoris* can easily be genetically manipulated, has an efficient secretory pathway and performs higher eukaryotic protein post-translational modifications. Since it produces few endogenous proteins, it simplifies downstream processing, making it ideal for industrial applications.

Successful production of recombinant proteins in many expression systems, including P. pastoris, is dependent on the gene and enzyme itself, but is also influenced by other factors such as type of strain (Wang and Wen, 2009), secretion signal, choice of promoter, gene copy number and optimal codon selection (Mellitzer *et al*., 2014). Codon optimisation is one of many techniques that can be applied to enhance protein production levels in P. pastoris. As a result,

numerous studies have reported the expression of codon optimised genes in *P. pastoris* that outperformed native genes in both eukaryotic and prokaryotic hosts (Hu *et al*., 2013; Li *et al*., 2014; Ou *et al*., 2014; Tu *et al*., 2016). Genes employing codons unfavourable to the expression host could lead to the premature termination of transcription and/or translation and consequently, low protein yields (Qiao *et al*., 2017). To our knowledge, the impact of codon optimisation on the expression levels of LiP in *P. pastoris* has not been studied yet.

In this study, recombinant *P. pastoris* strains were constructed that express native and codon optimized genes of *P. chrysosporium* LiP (LiPH8). The native nucleotide sequence was optimised according to two different indexes namely the codon adaptation index (CAI) (Xia, 2007) and the codon bias index (CBI) (Uddin, 2017). The three synthetic *lip*H8 genes (native, CAI and CBI optimised), encoding for the same mature protein, were cloned and expressed in *P. pastoris* DSMZ 70382 under the control of both constitutive (P_{GAP}) and inducible (P_{AOX1}) promoters, and evaluated for functional activity based on the oxidation of veratryl alcohol (VA). The successful production and partial characterisation of recombinant LiP following codon optimisation and high cell-density fermentations in a bioreactor are reported. This study provides a starting point for the optimisation of heterologous production of fungal lignin peroxidase in *Pichia pastoris* at both the molecular and bioprocessing level. The cost-effective production of enzymes such as lignin peroxidases will provide an essential tool for the enzymatic up-grade of industrial lignins.

3.2. Materials and methods

3.2.1. Microorganisms and plasmids

All the microbial strains and plasmids used in this study are presented Table 3.1. *Escherichia coli* DH5α (New England Biolabs [NEB], Midrand, South Africa) competent cells were used as the microbial host for amplification of all the constructs that were evaluated. Competent *E. coli* DH5α cells were prepared as described by Sambrook *et al*. (1989) and *E. coli* transformants were grown at 37 °C in conventional or low salt Luria Bertani (LB) broth (Sigma) supplemented with 100 µg/mL ampicilin, 50 µg/mL geneticin or 25 µg/mL zeocin (low-salt LB). The zeocin and geneticin were acquired from Invitrogen, while the ampicillin was purchased from Roche. The *P. pastoris* strain DSMZ 70382 (CBS704) (DSMZ German Collection of Microorganisms and Cell Cultures) was used as the expression host. *P. pastoris* cells were grown in yeast peptone dextrose (YPD) medium, cell recovery after transformation

with pJ905_LiP was carried out in yeast peptone dextrose sucrose (YPDS) media and plated in YPD agar containing increasing concentrations of zeocin (100, 250, 500 and 1000 µg/mL) (Melford Laboratories Ltd, UK). The recovery after transformation with linearised pJ901_LiP plasmid DNA was conducted in yeast dextrose sucrose (YPS) media and plated in media with increasing concentrations of geneticin (200, 350 and 500 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA).

Table 3.1: Microorganisms and plasmids used in this study

Strain and Plasmid	Relevant genotypes	Reference/Source
Bacteria strain		
Escherichia coli DH5α	*supE44?lacU169 (φ80lacZ?M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook *et al*.1989
Yeast Strains		
Pichia pastoris DSM 70382		Kurtzman, 2011
***P. pastoris* transformants**		
pJ905	*Sh ble* GAP_p*-*$AOX1_T$	This study
pJ905[LiP_Nat]	*Sh ble* GAP_p*-Lip_Nat-* $AOX1_T$	This study
pJ905[[LiP_CAI]	*Sh ble* GAP_p*-Lip_Opt-* $AOX1_T$	This study
pJ905[LiP_CBI]	*Sh ble* GAP_p*-Lip_CBI-* $AOX1_T$	This study
pJ901[LiP_Nat]	*kanMX* $AOX1_p$*-Lip_Nat-* $AOX1_T$	This study
pJ901[LiP_CAI]	*kanMX* $AOX1_p$*-Lip_Opt-* $AOX1_T$	This study
pJ901[LiP_CBI]	*kanMX* $AOX1_p$*-Lip_CBI-* $AOX1_T$	This study
Plasmids		
pJ905*fopA*	*Sh ble* GAP_p*-*$AOX1_T$	Dr Volschenk, Department of Microbiology Stellenbosch University
pJ901*fopA*	*Sh ble* AOX_p*-*$AOX1_T$	
pJ905*lipNat*	*Sh ble* GAP_p*-Lip_Nat-* $AOX1_T$	This study
pJ905*lipCAI*	*Sh ble* GAP_p*-Lip_Opt-* $AOX1_T$	This study
pJ905*lipCBI*	*Sh ble* GAP_p*-Lip_CBI-* $AOX1_T$	This study
pJ901*lipNat*	*kanMX* $AOX1_p$*-Lip_Nat-* $AOX1_T$	This study
pJ901*lipCAI*	*kanMX* $AOX1_p$*-Lip_Opt-* $AOX1_T$	This study
pJ901*lipCBI*	*kanMX* $AOX1_p$*-Lip_CBI-* $AOX1_T$	This study

3.2.2. Optimisation of the *lip*H8 gene

Three different versions of the *lip*H8 gene of *P. chrysosporium* were synthesized and cloned into pUC57 by GenScript® Biotech Co. (Piscataway, New Jersey, USA). The mature *lip*H8 coding sequence fused with the native secretion signal peptide (called *LiP_Nat*) was synthesized according to the native sequence (Genbank M27401.1). This DNA sequence was optimized by GenScript® Biotech Co. according to their OptimumGene™ algorithm for expression in *P. pastoris* (called *LiP_CAI*). The authors also optimised the native *lip*H8 coding

sequence according to the codon bias index (CBI; Carbone *et al*., 2003) strategy to have a CBI value of 0.93 (*LiP_CBI*). These three synthetic *lip*H8 genes contained no introns and several restriction sites were avoided (including *Eco*RI, *Xho*I, *Not*I, *Swa*I, *Xma*JI).

3.2.3. Yeast transformation

For genomic integration of the DNA constructs linearised DNA constructs of plasmids pJ905_LiP and pJ901_LiP (*Swa*I at 25 °C for 1 h) were used to transform *P. pastoris* competent cells. Yeast transformations were performed as described by Lin-Cereghino *et al*. (2005) using 0.2 cm electroporation cuvettes and a BioRad system (GenePluserXcell TM, Bio- Rad, Hercules, CA, USA) at 1.4 kV, 200 Ω and 25 µF using. Selective YPD/YD plates were supplemented with increasing concentrations of either zeocin (YPD-100-1000 µg/mL) or geneticin (YD-G418) (350-500 µg/mL). Untransformed *P. pastoris* and cells transformed with the receiving plasmid (pJ905 and pJ901) were used as negative controls.

Single colonies representing transformants were grown overnight in 5 mL YPD and plated on YPD agar for 72 h. The putative transformants were again subjected to selective pressure to confirm the stability of the construct integration. Single colonies of the respective transformants and controls were grown in 96 deep-well plates of double strength synthetic complex media (2×SC; 3.4 g/L yeast nitrogen base without amino acid and ammonium sulfate, 2.6 g/L amino acid pool without uracil, 10 g/L ammonium sulfate and 20 g/L glucose) containing the corresponding selective marker (100-200 µg/mL zeocin or 350-500 µg/mL geneticin) for 48 hours at 30°C in a shaking incubator and the absorbance at 600 nm was measured. Transformants that did not grow under these conditions, were discarded.

Genomic DNA of the transformants was isolated using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, California, USA) and used as template to confirm construct integration by polymerase chain reaction (PCR). PCR reactions were carried out in a Perkin Elmer Gene Amp® PCR System 2400 using Taq DNA polymerase (New England Biolabs) as per the manufacturer's recommendations. Primers that anneal to the *GAP*1 locus for pJ905_LiP constructs (forward primer 5'ACGCATGTCATGAGATTATTGG 3') or the *AOX*1 locus for pJ901_LiP constructs (forward 5'GACTGGTTCCAATTGACAAGC 3') were used in combination with a primer specific to the *AOX1* terminator (reverse primer 5'GCAAATGGCATTCTGACATCC 3').

3.2.4. Construction of the expression plasmids

All three synthetic LiP genes were retrieved from the pUC57 vector by restriction digestion with *EcoR*I and *Xho*I and directionally cloned into the pJexpress IP-free vectors pJ905 and pJ901 (*P. pastoris* shuttle vectors) (ATUM, previously known as DNA 2.0, USA) (Figure 3.1). All DNA manipulations were performed according to standard protocols (Green and Sambrook, 2012). T4 DNA ligase and restriction enzymes were purchased from New England Biolabs (Ipswich, Massachusetts, USA) and used as instructed by the supplier. DNA was visualised and purified from 0.8% agarose gels using the Zymoclean™ Gel Recovery Kit (Zymo Research, California, USA), following manufactures instructions. All the constructs were confirmed by restriction digest (*Hind*III and *Xba*I for pJ905_LiP; *Pvu*II and *Pst*I for J901_LiP) and Sanger sequencing (Central Analytical Facilities, Stellenbosch University).

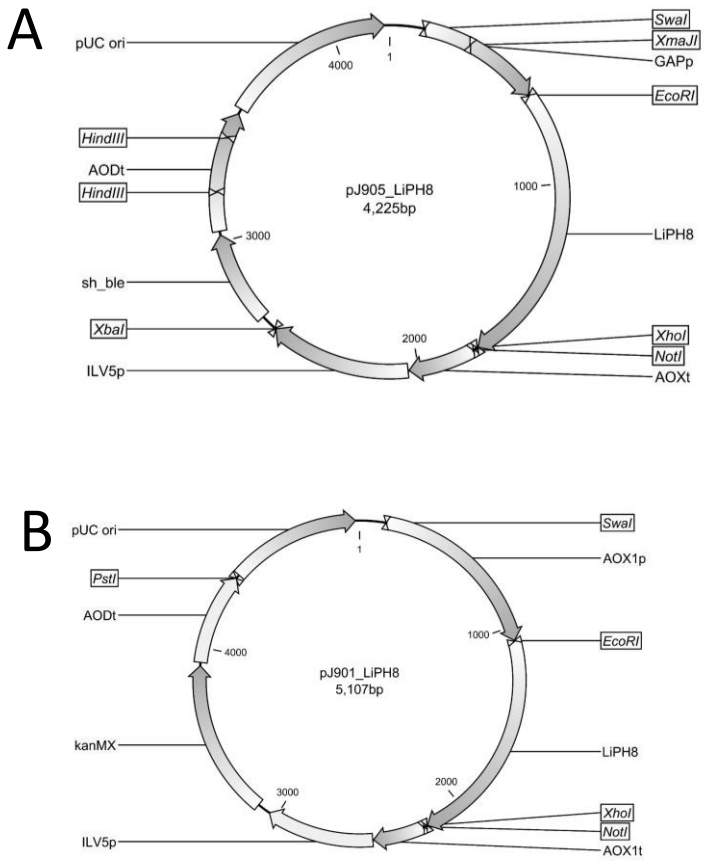

Figure 3.1: Schematic representation of the plasmids constructed for the expression of LiP. (A) pJ905_LiP constructs under the constitutive control of the P_{GAP} promoter and (B) pJ901_LiP constructs under the methanol-inducible P_{AOX1} promoter.

3.2.5. Screening of recombinant strains

Pre-inoculums of *P. pastoris* strains were prepared in triplicate by inoculating single colonies from the YPD agar plates into 50 ml of buffered glycerol complex medium (BMGY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34 % yeast nitrogen base [YNB], 4×10−5% biotin, 1 % glycerol) supplemented with 1% casamino acids in 250 mL baffled shake flasks and incubated at 30°C and 200 rpm for approximately 16 hours. When a final absorbance of 2-6 at 600 nm was reached, the cells were harvested by centrifuging at 3 000 × g for 5 mins at room temperature, the supernatants were decanted and the cells were re-suspended in fresh 50 mL BMGY media in 250 mL baffled shake flasks to an absorbance of 1.0 at 600 nm and returned to the incubator. The same protocol was also followed for the pJ901_LiP transformants but BMGY was substituted with Buffered Methanol-complex Medium (BMMY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% YNB without amino acids, 0.5% methanol and 0.002 % biotin) and supplemented with 1% casamino acids.

The wild-type *P. pastoris* and the *P. pastoris* strains transformed with linearised pJ905 or pJ901 plasmid DNA were used as negative controls. The cultivations were carried out for 72 h and samples were withdrawn every 24 h. Directly after sampling, sterile glycerol to a final concentration of 1% (v/v) was added *P. pastoris* (pJ905_LiP) cultures in baffled shake flasks. Induction was maintained by adding sterile methanol to a final concentration of 0.5% (v/v) daily directly after each sampling in *P. pastoris* (pJ901_LiP) fermentations. Samples were centrifuged at 13 200 rpm for 2 min to separate the cells from the supernatant, which was used for enzyme activity determination and/or SDS-PAGE analysis. The strain that exhibited the highest level of LiP activity was selected for large-scale fermentations.

3.2.6. Fermentation of recombinant *P. pastoris* in 14-L bioreactor

All fermentations parameters were set and monitored according to the Invitrogen *Pichia* fermentation process protocol (Invitrogen, 2013).

Glycerol-batch phase

High cell density fermentation was carried out in a 14-L bioreactor (BioFlo110, New Brunswick Scientific Co, Inc, NBS). Single colonies of the best producing strain were precultured in test tubes containing 4 mL of BMGY medium and grown overnight at 30 °C.

Thereafter, the overnight culture was diluted in a 2 L shake flask containing 400 mL BMGY medium and grown at 30 °C at 200 rpm for 18 h. The shake flask preculture was used to inoculate 3.6 L fermentation basal salts medium (BSM) consisting of 26.7 mL/L H_3PO_4 (85%), 0.93 g/L $CaSO_4 \cdot 2\ H_2O$, 18.2 g/L K_2SO_4, 14.9 g/L $MgSO_4 \cdot 7\ H_2O$, 4.13 g/L KOH and 40.0 g/L glycerol (100%) used for bioreactor cultivations. *Pichia* Metal Trace salts (PTM_1) comprising of 6 g/L CuSO4·5 H2O, 0.08 g/L NaI, 3 g/L $MnSO_4 \cdot H_2O$, 0.5 g/L $CoCl_2$, 20 g/L $ZnCl_2$, 0.02 g/L H_3BO_3, 0.2 g/L $Na_2Mo_4 \cdot 2\ H_2O$, 65 g/L $FeSO_4 \cdot 7\ H_2O$, 0.2 g/L biotin and 5 ml/L H_2SO_4 supplemented with sterile 4.35 mL PTM_1 trace salts per L of fermentation medium.

Glycerol-fed batch phase

Batch cultures were grown for approximately 24 h until the glycerol was consumed, indicated by an increase in the dissolved oxygen (DO) to 100%. The glycerol fed-batch phase was initiated by the addition of 50 % w/v glycerol solution containing 12 mL PTM_1 trace salts per litre and the feeding rate was adjusted to 18.15 mL/h/L. Once the carbon source (glycerol) became a limitation (indicated by a spike in the DO), the glycerol fed-batch was terminated.

Induction phase

The methanol induction fed-batch phase was initiated by the addition of 0.12 mL/min of 100 % methanol supplemented with 12 mL/L PTM_1 trace salts per litre of methanol using a step-wise function feeding rate ranging between 0.12 – 0.71 mL/min to allow the cells to adapt to methanol. Samples were routinely taken to determine the dry cell weight, LiP activity and protein concentration at different time intervals.

3.2.7. Analytical determinations

Biomass

The absorbance at 600 nm was measured using the Biochrom WPA Lightwave™ II UV/Visible spectrophotometer (Biochrom Ltd, Cambourne, Cambridge UK). The dry cell weight per volume was estimated during bioreactor fermentations.

Lignin peroxidase activity

The supernatant from the shake flasks screening and bioreactor fermentations was used to assess the extracellular lignin peroxidase activity. The enzyme activity assay consisted of monitoring the increase of the absorbance due to oxidation of VA into veratraldehyde at a

wavelength of 310 nm (ε_{310} =9,300 M^{-1} cm^{-1}) (xMark^{TM} Microplate Spectrophotometer, Bio-Rad, San Francisco, USA). The reaction mixtures consisted of 120 µL of 125 mM sodium tartrate buffer (pH 3.0), 60 µL of 10 mM VA and 108 µL of supernatant and the assays were initiated by adding 12 µL of 10 mM H_2O_2 and incubated at 25 ºC for 4 min. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmole of VA per min per mL of the supernatant under the specified conditions. Boiled samples of supernatant were used as blank spectrophotometer readings. All assays were performed in biological triplicates.

Optimum temperature and thermal stability

The enzyme activity was investigated at temperatures ranging from 25 to 70 °C under the specified conditions. To determine the LiP temperature stability, the supernatant was incubated at these temperatures for 3 h.

Optimum pH and pH stability

To determine the optimal pH of recombinant LiP, the enzymatic assay was carried out at as described above, but the pH of the buffer was modified to values ranging from 2.5 to 7.0.

Effect of H_2O_2 concentration

The optimal concentration of H_2O_2 was determined by conducting the assay as described above at the H_2O_2 concentrations ranging from 2.5 to 1.0 mM.

Protein concentration

The total protein concentrations of the cell-free culture supernatants were quantified using the Bicinchoninic acid assay (BCA) protein assay reagent kit (Sigma-Aldrich, USA). Bovine serum albumin (BSA) was used as standard.

SDS-PAGE analysis and densitometry

A 10% SDS-PAGE gel was prepared for the visualization of total and recombinant LiP proteins (Sambrook, 1989) in selected shake flask and bioreactor samples. 20 µL of the cell-free supernatants were mixed with 4 µL of denaturing loading buffer (60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14 mM β-mercaptoethanol, 0.1 % bromophenol blue) and boiled for 3 mins at 100 °C. Electrophoresis was carried out in a 1 × Tris/glycine buffer (25 mM Tris/HCL, 250 mM glycine and 0.1% SDS) for approximately 1 h at 150 V. The PageRuler^{TM} protein ladder (ThermoScientific, USA) was used as the size marker. The supernatant of the *P. pastoris* with pJ905 or pJ901 was used as the negative controls. Silver nitrate staining procedure was used for visualisation of the protein bands (Gallagher and Sasse, 2012). The ImageJ® digital

program was used to analyse photographs of the SDS-PAGE from bioreactor fermentations and estimate the concentration of recombinant protein based on the intensity of the corresponding band. Values were expressed as % of the total protein concentration as determined by BCA (see above).

3.3. Results

3.3.1. Codon optimisation of the native *lip*H8 gene

Codon optimisation is one of the many strategies that can be employed to enhance gene expression and protein production in the methylotrophic yeast P. pastoris. In this study, the native *P. chrysosporium* lipH8 gene was codon optimised using two different indices, the codon adaptation index (CAI) and the codon bias index (CBI). Table 2.1 shows that the codon usage of the *LiP_Nat* gene differs significantly from the optimal codons used in P. pastoris. For example, the codons AGA (Arg) and TTG (Leu) were predominant in the optimised genes and highly expressed genes in P. pastoris, as opposed to CGT/CGC (Arg) and CTC (Leu) that dominated in the *LiP_Nat* gene, when the Codon Adaptation Index (CAI) of the *LiP_CAI* gene was increased from 0.53 to 0.83, which a reduced the GC content from 61.91% to 44.32%. The native gene was also optimized based on the CBI algorithm, resulting on an improvement of the CBI (0.93 versus 0.32) and a reduction in the GC content to 51.16 %. Interestingly, while the CAI values of the codon optimised versions (*LiP_CAI* and *LiP_CBI* genes) were similar (0.83 and 0.85), the CBI value of *LiP_CAI* was the lowest among the three genes (0.29). The codon optimized nucleotide sequences, LiP_CAI and LiP_CBI, shared a 77% and 86% nucleotide homology (Table 3.2), respectively, with that of the *LiP_Nat*.

3.3.2. Effect of promoter and codon usage on production of recombinant lignin peroxidase

Extracellular LiP activity was not detected in any of the supernatants from shake flask cultivations with *P. pastoris* strains expressing the *LiP_Nat, LiP_CAI, LiP_CBI* genes under the transcriptional control of the $P_{GAP,}$ nor in the control *P. pastoris* strain (contains "empty" pJ905 plasmid) in all the tested substrates (see Table S1). All the transformed *P. pastoris* strains expressing genes under the transcriptional control of the inducible $P_{AOX1,}$ were initially screened for LiP activity on VA (Figure 3.2A). After 72 h of induction, LiP activity in the transformants

expressing *LiP_Nat* varied between 100 U/L and 597 U/L (average of 246 U/L). The recombinant transformants expressing the *LiP_CBI* varied between 125 U/L and 672 U/L (average of 319 U/L), while the *LiP_CAI* expressing transformants varied between 25 U/L and 1170 U/L (average of 391 U/L) (Figure 3.2A).

Table 3.2: Codon usage frequency for native and optimized *Phanerochaete chrysosporium*_LiPH8 (*Pc*_lipH8) genes (according to CAI or CBI), %GC, homology among the sequences, and calculated codon usage indices. Preferred codons in highly expressed genes of *Pichia pastoris* (*Pp*) are highlighted (Bai *et al*., 2011). The codon usage of the housekeeping gene ARG4 from *Pichia pastoris* is included for comparison.

Amino acid	Codon	% usage				Amino acid	Codon	% usage			
		Pc_lipH8			*Pp_ARG4*			*Pc_lipH8*			*Pp_ARG4*
		Native	CAI	CBI				Native	CAI	CBI	
Ala (40)	GCG	32.5	0.0	0.0	0.0	Leu (25)	**TTG**	4.0	44.0	100.0	47.2
	GCA	15.0	40.0	0.0	15.6		TTA	0.0	0.0	0.0	18.9
	GCT	32.5	60.0	75.0	62.5		CTG	16.0	0.0	0.0	7.5
	GCC	20.0	0.0	25.0	21.9		CTA	0.0	0.0	0.0	11.3
Cys (8)	**TGT**	37.5	50.0	100.0	100.0		CTT	12.0	56.0	0.0	7.5
	TGC	62.5	50.0	0.0	0.0		CTC	68.0	0.0	0.0	7.5
Asp (26)	GAT	30.8	100.0	30.8	56.7	Met (6)	**ATG**	100.0	100.0	100.0	100.0
	GAC	69.2	0.0	69.2	43.3	Asn (14)	AAT	0.0	28.6	0.0	60.0
Gluc (18)	**GAG**	88.9	55.6	88.9	47.4		AAC	100.0	71.4	100.0	40.0
	GAA	11.1	44.4	11.1	52.6	Pro (31)	CCG	29.0	0.0	0.0	8.3
Phe (28)	TTT	3.6	39.3	0.0	72.2		**CCA**	6.5	51.6	71.0	58.3
	TTC	96.4	60.7	100.0	27.8		CCT	29.0	48.4	29.0	25.0
Gly (32)	GGG	0.0	0.0	0.0	14.3		CCC	35.5	0.0	0.0	8.3
	GGA	3.1	59.4	0.0	40.0	Gln (23)	CAG	91.3	52.2	0.0	37.5
	GGT	37.5	40.6	100.0	40.0		**CAA**	8.7	47.8	100.0	62.5
	GGC	59.4	0.0	0.0	5.7	Arg (9)	AGG	0.0	44.4	0.0	11.1
His (8)	**CAT**	0.0	50.0	0.0	50.0		**AGA**	0.0	55.6	55.6	59.3
	CAC	100.0	50.0	100.0	50.0		CGG	0.0	0.0	0.0	7.4
Ile (23)	ATA	0.0	0.0	0.0	7.1		CGA	0.0	0.0	0.0	7.4
	ATT	13.0	78.3	13.0	67.9		CGT	44.4	0.0	44.4	14.8
	ATC	87.0	21.7	87.0	25.0		CGC	55.6	0.0	0.0	0.0
Lys (11)	**AAG**	100.0	100.0	100.0	57.1	Thr (21)	ACG	9.5	0.0	0.0	0.0
	AAA	0.0	0.0	0.0	42.9		ACA	0.0	28.6	0.0	10.7
Ser (23)	AGT	0.0	0.0	0.0	5.4		**ACT**	33.3	42.9	47.6	50.0
	AGC	4.3	0.0	0.0	2.7		ACC	57.1	28.6	52.4	39.3
	TCG	39.1	0.0	0.0	2.7	Val (23)	GTG	8.7	47.8	0.0	19.0
	TCA	0.0	47.8	0.0	16.2		GTA	0.0	0.0	0.0	19.0
	TCT	8.7	52.2	52.2	51.4		**GTT**	13.0	52.2	21.7	47.6
	TCC	47.8	0.0	47.8	21.6		GTC	78.3	0.0	78.3	14.3
Trp (3)	**TGG**	100.0	100.0	100.0	100.0	Stop	TGA	0.0	0.0	0.0	100.0
Tyr (0)	TAT	0.0	0.0	0.0	56.3		TAG	0.0	0.0	0.0	0.0
	TAC	0.0	0.0	0.0	43.8		**TAA**	100.0	100.0	100.0	0.0
GC (%)		61.9	44.3	51.2	41.7	CAI		0.58	0.83	0.85	0.78
Homology (%)		100.00	77.00	86.00	N/A	CBI		0.32	0.29	0.93	0.32

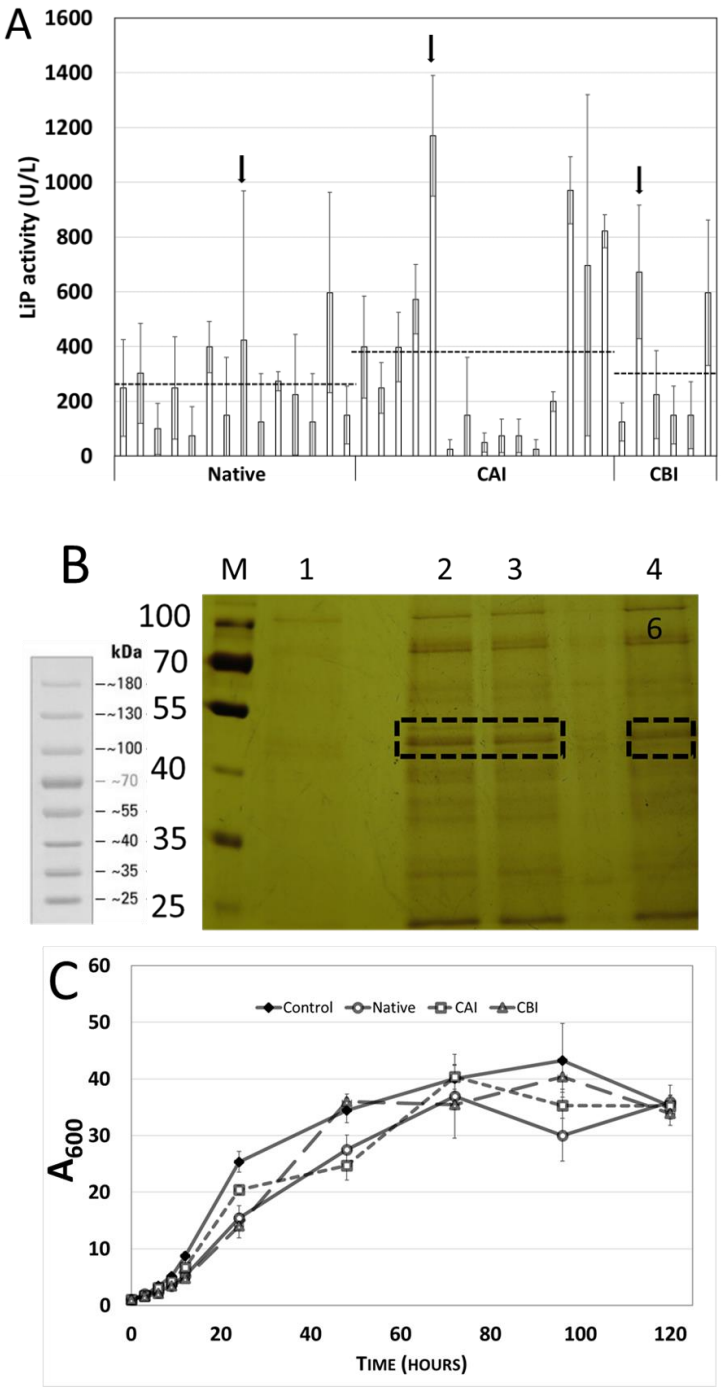

Figure 3.2: Recombinant LiP production in P. pastoris. (A) LiP activity, expressed as U/L, after 72 h of shake flask cultivations under inducing conditions representing the pJ901[LiP_Nat], pJ901[LiP_CAI] and pJ901[LiP_CBI]. Broken horizontal straight lines represent the average of each group of transformants. (B) Silver stained SDS-PAGE gel of the best transformants selected (indicated by arrows) cell-free supernatants of *P. pastoris* expressing LiP. Lane (M) PageRuler Prestained Protein ladder. Lane (1) control strain (empty pJ901), lane (2) [LiP_CAI], lane (3) [LiP_Nat] and lane (4) [LiP_CBI] and (C) biomass production by the different recombinant LiP producing *P. pastoris* strains (diamond) control strain, (square) LiP_Nat, (triangle) LiP_CAI and (asterisks) LiP_CBI. The standard deviation of the three biological repeats are represented by the error bars.

The last samples (72 h) shake flask cultures of the best recombinant LiP strains from each group (native, CAI or CBI optimised) were analysed by SDS-PAGE together with the strains transformed with empty plasmids pJ901 (Figure 3.2B). Samples from the three transformants presented a protein species at about 45 kDa that presumably corresponds with the recombinant LiPH8 (in silica molecular weight of 42 kDa). This protein species was not present in the *P. pastoris* transformed with empty plasmid. The growth patterns of the best recombinant *P. pastoris* strains were evaluated by monitoring the absorbance at 600 nm during the shake flasks (Figure 3.2C). Compared to the wild strain (control), the LiP transformants lagged during the first 24 h (absorbance of 14-20 at 600 nm versus 25 in the control), after which the differences with the control (absorbance of 40 at 600 nm) were reduced (absorbance of 35 to 37 at 600 nm).

3.3.3. Large-scale fermentations in 14-L bioreactor

The best LiP producing *P. pastoris* strain (corresponding to recombinant strain with the linearised pJ901[LiP_CAI]) insert was selected for high cell density fermentations in a 14-L bioreactor. The fermentation consisted of glycerol-batch, a glycerol fed-batch phase for biomass production, and an induction phase with methanol. Figure 3.3A illustrates the time course for enzyme production and dry cell weight (DCW), expressed as volumetric activity of rLiPH8 (U/L) and g/L, respectively, during the induction phase of the fermentation. LiP activity reached a volumetric activity of 672 U/L within the first 3 h of induction and increased to a maximum of 3818 U/L after 96 h of induction, when the maximum DCW of 321 g/L was also reached. The total protein concentration at the end of the induction phase was 1 ± 0.13 g/L. The cell-free supernatant at 96 h of induction were analysed by SDS-PAGE (Figure 3.3B) and densitometry. A protein species of approximately 45 kDa was observed in the culture supernatants of the recombinant *P. pastoris* pJ901[LiP_CAI] strain. Densitometry of the protein LiP_CAI in the supernatant showed a protein concentration of 0.68 g/L that corresponds to 68% of the total extracellular protein.

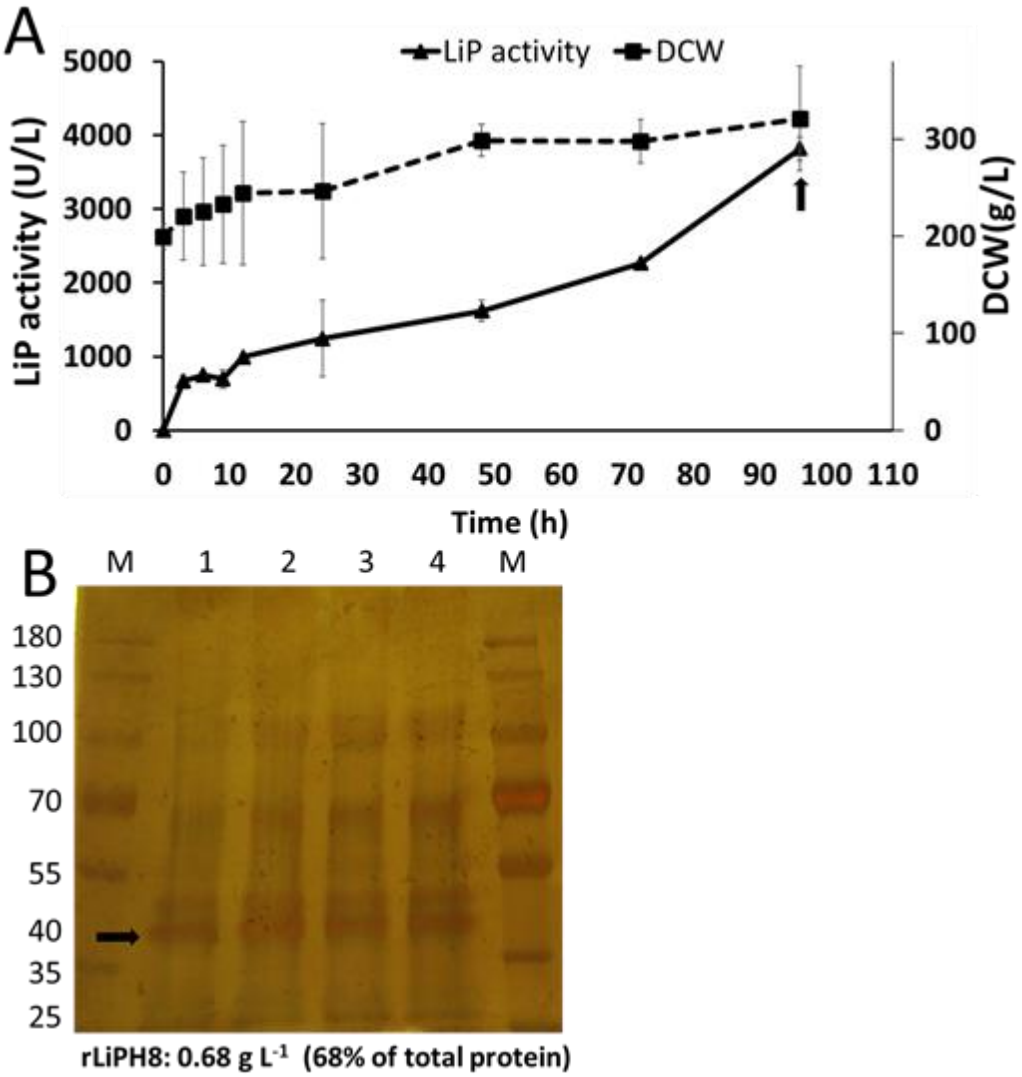

Figure 3.3: Recombinant LiP production in high-cell density fermentations in P. pastoris. (A) LiP volumetric activity (U/L) and biomass in DCW (g /L) during the methanol-induced phase of high-cell density *P. pastoris* LiP_CAI fermentation cultures; (B) silver stained SDS-PAGE gel of cell-free supernatants of *P. pastoris* expressing LiP at 96 hrs. Lane (M) PageRuler Prestained Protein ladder, lane (1 to 4) *P. pastoris* pJ901[LiP_CAI]. The standard deviation of the three biological repeats are represented by the error bars.

3.3.4. Biochemical characterization of recombinant LiP_CAI

The effect of temperature on the enzyme activity of the crude extract of the *P. pastoris* pJ901[LiP_CAI] strain bioreactor fermentations was evaluated at a broad range of temperatures between 25 - 70 °C (Figure 3.4A) after 5 min and 3 h of incubation at the selected temperature. Lignin peroxidase was active between 25 °C and 60 °C both for 5 min and 3 h incubation

periods. The maximum LiP activity of 3818 U/L was obtained at 25 °C for 5 min. The recombinant LiP retained 59.3, 51.5, 30.6 and 22.8% of the maximum activity when the enzymatic assay was conducted at 30, 40, 50 and 60 °C, respectively. No activity was detected at 70 °C. The thermostability of the recombinant LiP_CAI was evaluated at a range of temperatures after 3 h of incubation. The recombinant LiP displayed maximum thermostability at 30 °C, with decreased activity with an increase in temperature. Minimal thermostability was observed at 60 °C.

The optimum pH for the recombinant LiP_CAI in the crude extract was pH 3.0 where it exhibited the maximum activity of 3818 U/L (Figure 3.4B). When the enzymatic assay was conducted at pH 2.5 and pH 4, the recombinant LiP_CAI from the crude extract retained between 55 and 59% of the maximum activity (respectively), asserting its acidic nature. The stability of the enzyme at different pH was also evaluated after an incubation period of 3 h (Figure 3.4B), which is more relevant for an industrial application. Noteworthy, the maximum activity was observed at the lowest pH (2.5).

Hydrogen peroxide concentration is a determining factor in the activity of heme-containing peroxidases such as LiPH8. The optimum activity was observed at 0.4 mM of H_2O_2 (Figure 3.5). Increasing the H_2O_2 concentration further substantially decreased the activity to only 26% and 20% of the maximum activity (3818 U/L) at 0.6 mM and 0.8 mM of H_2O_2 respectively. At 0.2 mM H_2O_2, only about 40% of the maximum activity was obtained.

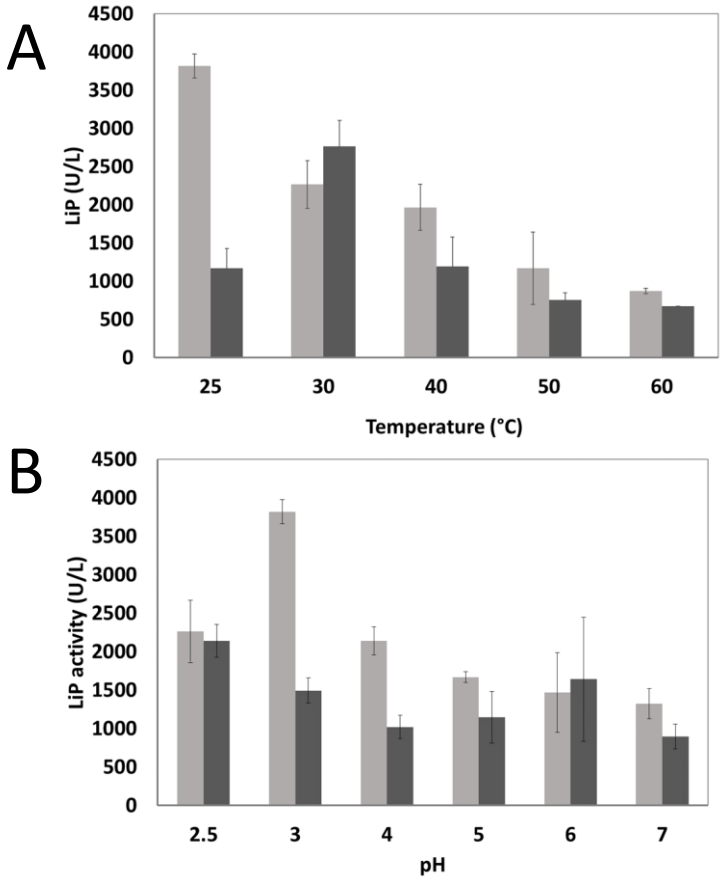

Figure 3.4: Effect of (A) temperature and (B) pH on the recombinant LiP_CAI activity from cell-free supernatant from fermentations in *P. pastoris* after incubation of 5 min (light grey) and 3 h (dark grey). The standard deviation of the three biological repeats are represented by the error bars.

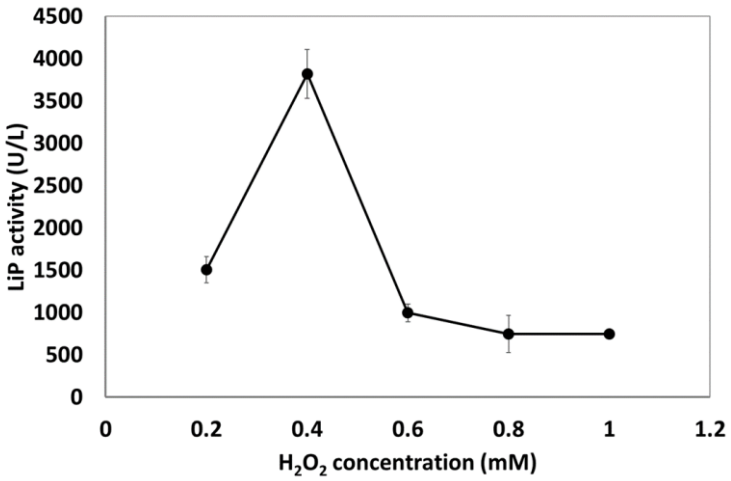

Figure 3.5: Effect of H_2O_2 concentration on the LiP activity on the supernatant containing rLiPH8_CAI The standard deviation of the three biological repeats are represented by the error bars.

3.4. Discussion

Lignin peroxidase has gained increased attention for its potential use in a broad range of industrial and biotechnological applications (Karigar and Rao, 2011). Lignin peroxidase could be used to valorise technical lignins into high-value bioproducts. However, low secretion titers in native producers and limited cost-effective commercial LiP preparations have prohibited its industrial application. Producing LiP and other heme-containing peroxidases in copious amounts has been a challenge, due to the complexity of their chemical structures (Colao *et al*., 2006; Lambertz *et al*., 2016). This study therefore focused on the construction of yeast strains capable of secreting sufficient amounts of LiP, allowing its direct application within lignocellulose biorefineries (upgrade of industrial or technical lignins) and bioremediation. The *P. chrysosporium* LiPH8 isozyme was selected as the target protein as it is the most extensively studied and characterized isozyme (Tien and Kirk, 1988). The impact of codon optimisation and choice of promoter on the yield of recombinant *P. chrysosporium* LiPH8 in *Pichia pastoris* was investigated.

Pichia pastoris has a non-random pattern of synonymous codon usage and is biased towards a subset of codons in highly expressed genes (Table 3.2). The substitution of native codons by these highly used codons may enhance the expression levels of recombinant heterologous proteins in this host. Codon optimisation of native genes by increasing the CAI and adjusting the GC content has been reported to increase protein yields by several folds in *P. pastoris* (Akcapinar *et al*., 2011; Wang *et al*., 2015; Zhou *et al*., 2015; Qiao, 2017). The CAI index uses a scaling factor of 0-1 where a CAI value of >0.8 is regarded as good in terms of gene expression in P. pastoris. Two codon optimisation indexes CAI (commonly used in P. pastoris) and CBI (uses a subset of optimal codons) were employed in attempt to increase the expression of *lip*H8. The CAI value and GC content of the codon optimised genes (Table 3.2) were similar to some of the genes highly expressed in *Pichia pastoris* in the studies mentioned above. The best producing transformants containing the optimised *LiP_CBI* and *LiP_CAI* genes gave about 1.1 and 2-fold higher LiP activity compared to the best producing transformant containing the *LiP_Nat* gene. It is possible that the presence of a subset of none-optimal codons in the *LiP_Nat* gene for the expression in *P. pastoris* could have led to lower LiP activity levels.

In the present study, it was demonstrated that the P_{AOX1} was a better promoter to produce extracellular LiP at shake flask level compared to the P_{GAP} (Figure 3.2A and B). There were only detectable amounts of LiP activity during shake flask cultures with transformants under

the control of the inducible P_{AOX1}. This finding is in line with the study of Kim *et al*. (2009) on the expression of *Coprinus cinereus* peroxidase (CIP) in P. pastoris, where the P_{AOX1} - outperformed the P_{GAP} both in terms of extracellular CIP activity and protein yields. The P_{GAP} is a constitutive promoter that essentially doesn't require induction and therefore transcription of the desired gene is a continuous process. A possible reason for the inefficiency of the P_{GAP} promoter to control expression of LiPH8 could be the accumulation of the rLiPH8 in the endoplasmic reticulum (ER) and activation of the unfolded protein response (UPR) to eliminate the misfolded proteins (Damasceno *et al*., 2012; Vanz, 2014; Roth *et al*., 2018).

Based on the results obtained in this study, the codon optimised genes were expressed in *Pichia pastoris* at higher levels compared to the native gene under the control of the P_{AOX1} (Figure 3.2A). The *P. pastoris* pJ901[LiP_CAI] transformants gave the best LiP activity on average followed by the pJ901[LiP_CBI] transformants and then the pJ901[LiP_Nat] transformants (Figure 3.2A). This may also have been due to differences in gene copy number between the best producing transformants. Therefore, gene copy number studies should be conducted to rule out this possibility. There variation in LiP activity levels between the transformants from the same gene is hypothesised to be due to methanol evaporation in shake flask cultures which is required for induction. The introduction of foreign LiP genes had exerted some metabolic burden on the yeast indicated by the lag phase (over the first 24 h), but growth patterns after that were similar to the control strain (Figure 3.2C).

The extracellular LiP obtained from the best pJ901[LiP_Nat] and pJ901[LiP_CBI] transformants under the control of the P_{AOX1} were both higher than that of the multicopy strain reported by Wang and Wen (2009) (15 U/L), but lower than the 932 U/L reported by Wang *et al*. (2004). Furthermore, two pJ901[LiP_CAI] strains produced higher amounts of recombinant LiP than to 932 U/L reported by Wang *et al*. (2004). However, the authors reported that LiP expression levels were increased by 2-fold when the native secretion signal was replaced with that of the *S. cerevisiae* α-mating factor (α-MF), resulting in higher levels than levels obtained in this study at shake flask level. In the present study, only the native secretion signal was used for the secretion of LiP and perhaps the use of alternative secretion signals such as the α-MF secretion signal might enhance production yields.

With better control of cultivation conditions, bioreactor fermentations generally allow the cells to grow to high densities and considerably higher recombinant protein production levels are

obtained compared to shake flask cultivations (Kastilan *et al*., 2017; Shang *et al*., 2017). To our knowledge, this is the first report on the expression of *P. chrysosporium* LiPH8 isozyme through codon optimisation and high-cell density fermentations in bioreactors in P. pastoris. The high cell density fermentations in a 14-L bioreactor of the best producing strain in shake flasks, led to a maximum activity of 3818 U/L (Figure 3.3A), 3.3-fold increase in LiP production levels relative to shake flask cultures. Similar values have been reported using the native host fungus *P. chrysosporium*, but it required much longer incubation periods (12 days) and downstream purification processes (Coconi-Linares *et al*., 2014). Noteworthy, recombinant LiP accounted for the majority (more than 60%) of the total secreted protein (Figure 3.3B).

There is an on-going quest for the production of lignocellulolytic enzymes such as lignin peroxidase for the conversion of lignocellulosic biomass into high value-added bio-products. However, apart from the lack of cost-efficient commercial enzyme preparations for industrial applications, challenges such as harsh process conditions limit the application of these enzymes (Martínez *et al*., 2017). Therefore, there is a need that these vital biocatalysts must be robust and thermostable. The purification and biochemical characterisation of a native LiP from *P. chrysosporium* displaying good activity and stability has been conducted (Zeng *et al*., 2013). In this study, the optimum temperature profile of LiP_CAI (Figure 3.4A) was similar to that of Zeng *et al*. (2013), but the purified native LiP exhibited maximum activity at 30 °C under standard assay conditions as opposed to 25 °C for the crude. The purified LiP from *P. chrysosporium* was also more thermostable than to the recombinant LiP_CAI reported in this study, retaining more than 50% of its maximum activity after 24 h incubation at 50 °C. In contrast, only 20 % of the maximum activity was retained when the crude extract from LiP_CAI was incubated at the same temperature for 3 h.

The optimum pH of the recombinant LiP_CAI (Figure 3.4B) was pH 3, which is in accordance with that of the purified native LiP from *P. chrysosporium* (Zeng *et al*., 2014). Surprisingly, after 3 h of incubation at pH 3, only about 40% of the maximum activity was maintained. This was different from what was observed by Zeng *et al*. (2014), where the purified native LiP maintained about 95% of the optimum activity after 24 h of incubation. The similarities and differences in the biochemical properties might have been caused by different expression hosts, cultivation media and purity of the enzyme or maybe by the impact of different glycosylation patterns. Noteworthy, an increase in the H_2O_2 concentration above 0.4 mM negatively impacted

the enzymatic activity of the enzyme which is consistent with literature reports (Ansari *et al*., 2016). It would be of great interest to evaluate the stability of recombinant LiP on different H_2O_2 concentrations for prolong periods.

3.5. Conclusions

This is the first study to attempt to produce recombinant LiP using native and codon optimised genes under the transcriptional control of a constitutive (P_{GAP}) and inducible (P_{AOX1}) in *Pichia pastoris*. Based on the findings, the P_{AOX1} was a better promoter than to the P_{GAP} for directing the production of Pc_LiPH8. This is also the first study to report the high level expression of recombinant LiP through codon optimisation and high-cell density fermentations. This study further demonstrates how the development of synthetic biology tools can be utilised to enhance protein production levels in P. pastoris. Furthermore, based on the activity levels reached, LiP_CAI could be used directly or at least the downstream processing (purification steps) can be simplified.

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Chapter 4

Conclusions and recommendations for future studies

Conclusions and recommendations for future studies

4.1. General discussion and Conclusions

The conversion of technical lignins produced in lignocellulosic biorefineries (pulp and paper mills, biofuel facilities, etc.) into high-value added products has triggered the interest of many researchers (Bugg *et al*., 2015; Moreno *et al*., 2015; Gall *et al*., 2017). Biological conversions using microbial systems, or their enzymes, constitute a sustainable route for the upgrading of these lignin streams. To evaluate these processes, the search for appropriate expression production systems is of paramount importance to produce enzymes in sufficient quantities for industrial application.

This study has documented the successful expression of the fungal lignin peroxidase LiPH8, from *Phanerochaete chrysosporium*, the first white-rot fungi whose genome was sequenced (Singh and Chen, 2008). The impact of constitutive and inducible promoters and codon optimization on secretion levels of the recombinant LiPH8 (rLiPH8) in the methylotrophic yeast *Pichia pastoris* DSMZ 70382 was evaluated. This is one of the first studies evaluating the P_{GAP}, codon optimization and production at bioreactor scale of fungal lignin peroxidase. The recombinant enzyme from the cell-free supernatant was also partially characterised.

The research findings of this thesis demonstrate that:

1. The P_{AOX1} promoter has proven to be a better promoter compared to P_{GAP} for directing the expression of LiPH8. The synthetic *LiP_Nat*, *LiP_CAI* and *LiP_CBI* genes were successfully expressed in *P. pastoris* under the control of the P_{AOX1} and terminator sequences.
2. Codon optimisation (*LiP_CAI* and *LiP_CBI*) resulted in higher production levels of LiP than from the native *LiP_Nat* gene, with the LiP_CAI strain that presented the highest LiP activity values in shake flasks.
3. Upscaled fermentation of the best *P. pastoris* LiP_CAI strain in a 14-L bioreactor resulted in 3-fold more LiP activity in the supernatant (3,818 U/L bioreactor vs 1169 U/L in shake flask) with the rLiPH8 being the predominant protein in the supernatant (68% of the total protein). The high levels of activity coupled with the low amount of endogenous proteins will minimize downstream processing (concentration and purification of rLiPH8) and may allow for direct application of the cell-free supernatant.

4. The rLiPH8 displayed optimal activity at 25 °C and pH 3, respectively, on veratryl alcohol, but the enzyme exhibited optimal stability at 30°C and pH 2.5, which would be ideal for the treatment of effluents with an acidic pH.

This study provides a starting point for the optimisation of heterologous production of a fungal lignin peroxidase in *Pichia pastoris* at both the molecular and bioprocess level. The cost-effective production of enzymes such as lignin peroxidase will provide an essential tool for the enzymatic up-grade of industrial lignins as well as bioremediation applications (i.e. treatment of water).

4.2. Recommendations for future work

This study has also highlighted some limitations, and the recognition of these should refine future research avenues:

1. Future studies should focus on the influence of different secretion signals (such as the *Saccharomyces cerevisiae* α-mating factor) on the expression of *lip*H8 genes under the transcriptional control of the P_{GAP} and P_{AOX1}. The use of novel promoters should be explored to produce higher levels of extracellular LiP in P. pastoris. Furthermore, the use of dual promoters (*AOX1* and *GAP*) should be investigated to elucidate whether this could help facilitate higher production levels of LiP suitable for industrial applications and overcome slow growth rates experienced in methanol (Parashar and Satyanarayana, 2016).

2. The initial screening should include a larger number of transformants to ensure a robust process and statistically significant data. In addition, the enzyme activity values should ideally be normalized according to gene copy number to ascertain whether this may contribute to the expression levels from the three different constructs.

3. Future studies should also focus on the optimisation of the cultivation media (e.g. the addition of heme) and other fermentation parameters such as temperature and incremental increases in the methanol concentration during feeding, or using methanol with an additional co-substrate (Gmeiner *et al*., 2015; Krainer *et al*., 2015).

4. The application potential of the rLiPH8 for biorefinery and bioremediation applications should be evaluated, for example the detoxification of pretreatment liquors rich in furfurals and 5-hydroxymethyl-furfural, increment of molecular weight of technical lignins (such as sodaAQ lignin or lignosulphonates) by polymerization reactions, or degradation of lignin into monomers in combination with other ligninolytic enzymes.

5. The catalytic performance and stability of the rLiPH8 produced in this study was evaluated only on veratryl alcohol. These studies should be also evaluated on organic solvents that more likely to be used in an industrial set-up using technical lignin as substrate. A deeper biochemical characterization of the rLiPH8 can shed light on the limitations of the enzyme and will guide the design of strategies to modify the protein structure to improve stability (to acid, to H_2O_2 and organic solvents) and the catalytic performance of the enzyme.

4.3. References

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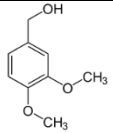
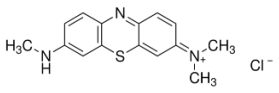
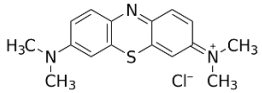
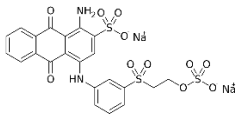
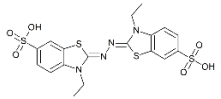
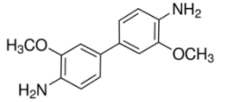
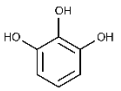
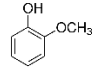
Addendum

Supplementary Information

Addendum: Supplementary Information

Table S1: Enzymatic assays and conditions tested for extracellular lignin peroxidase activity.

Enzymatic assays Lignin Peroxidase								
Substrate	Veratryl alcohol	Azure B	Methylene Blue	Remazol Brilliant Blue R	ABTS (2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)	o-dianisidine	Pyrogallol	Guaiacol
Substrate structure								
ƛ	310 nm	651 nm	664 nm	592 nm	420 nm	460 nm	420 nm	470 nm
Assay conditions								
Substrate	VA 10 mM: 500 µL (2 mM final)	AB 0.032 mM final AB 0.16 mM: 500 µL	MB: 0.04 mM final MB 1.2 mM: 100 µL	RBBR 0.2%: 100 µL	ABTS 0.5 mM: 100 µL (final 0.5 mM)	O-D 1 mM: 100 µL	Pyrogallol solution 50 mg/mL: 320 µL	Guaicol: 2 mM final
Buffer	Sodium tartrate 125 mM: 1000 µL		Sodium tartrate 0.5 mM: 600 µL	Sodium tartrate 50 mM: 250 µL	Phosphate buffer 50 mM (Ph 4)	McIlvaine: 200 µL	Phosphate buffer 100 mM: 320 µL	Sodium tartrate 25 mM final
H_2O_2	2 mM: 500 µL (final 0.4 mM)		2.7 mM: 100 µL (final 0.1 mM)	2 mM: 50 µL	2 mM: 50 µL (final 0.1 mM)	2 mM: 100 µL	0.027 v/v 160 µL	
Sample	500 µL (20%)		2200 µL (73.3%)	600 µL (60%)	600 µL (60%)	600 µL (60%)	100 µL (0.45-0.75 U/mL)-3.3%	0.1 µM enzyme
H_2O	-						2100 µL	
Temperature	Recommended 30 °C							
Time	First 2 minutes							

*McIlvain Buffer can work for all assays. Amount of sample can be adjusted while keeping concentration of the other elements.