Constitutive expression of enzymes in *Pichia pastoris* for use in lignin valorisation

by

Cleo Gertrud Conacher

Thesis presented in partial fulfilment
of the requirements for the Degree

of

MASTER OF ENGINEERING
(CHEMICAL ENGINEERING)

in the Faculty of Engineering
at Stellenbosch University

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily attributed to the NRF

Supervisor
Prof. Johann Görgens

Co-Supervisor
Dr. María García-Aparicio

March 2018

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Cleo Conacher

……
Signature

March 2018

…….
Date

Summary

Lignin valorisation is a key breakthrough in developing efficient integrated biorefineries. Enzymatic modification of lignin wastes offers an environmentally benign, energy efficient avenue for lignin upgrading. However, for enzymatic lignin valorisation to be a viable option, large quantities of the selected enzymes are required at minimal cost. Heterologous enzyme production using recombinant *P. pastoris* is a well-studied method for the successful large-scale production of recombinant eukaryotic proteins. Considering the disadvantages associated with methanol-induction, the use of the constitutive P_{GAP} promoter for heterologous protein production in *P. pastoris* is the preferred expression strategy.

Three enzymes of interest for lignin degradation and modification were selected in this study for recombinant enzyme production: glucuronoyl esterase (GE), cellobiose dehydrogenase (CDH) and laccase (LCC). These enzymes were selected based on novelty and their broad range of functions in the lignocellulose biodegradation process. These enzymes are representative of carbohydrate degradation (GE, CDH: Fenton reaction) and lignin degradation and/or modification (LCC).

The aim was to create, and provide fundamental data of, the capabilities of a constitutive *P. pastoris* expression system to produce three enzymes (CDH, LCC, and GE), that are associated with lignin valorisation. This study is rooted in aspects of molecular biology as well as bioprocess engineering.

The DNA 2.0 (USA) pJexpress expression system, which is free of any proprietary restrictions, was used to create the expression constructs. The created recombinant *P. pastoris* strains were screened at shake-flask level, and based on these results, one strain for each enzyme was selected for further studies. The enzyme production process was scaled up to a 14 L bioreactor. A two-stage fermentation strategy, consisting of a batch phase, followed by a constant glycerol fed-batch stage was implemented. The fermentation culture was harvested and concentrated through a two-stage tangential filtration process, after which freezing was evaluated as a possible storage strategy. Glycerol was evaluated as a possible cryoprotectant.

The constant glycerol feed strategy was shown to be effective, returning high biomass and protein yields. Results of the bioreactor fermentations showed similar biomass growth kinetics (μ_{max} = 0.15 – 0.17 h^{-1}) and biomass yields (119.54-136.47 g_{dcw}/L) throughout the fermentation process for the three recombinant enzymes. High titres of recombinant protein were obtained, with the highest being glucuronoyl esterase, at 2778.01 mg/L, followed by cellobiose

dehydrogenase, at 1489.3 mg/L, and lastly laccase, at 778.54 mg/L. The first incidence of constitutive expression of *H. jecorina* glucuronoyl esterase and *N. crassa* cellobiose dehydrogenase is reported here. In addition, the highest yield of constitutively expressed *T. versicolor* laccase *lcc2* is also reported. Although the laccase fermentation returned the lowest productivity, preliminary experiments showed that lowering the fermentation temperature may improve this value by aiding in secretion and/or preventing temperature-related laccase degradation at 30 °C.

No glycerol was accumulated during the glycerol fed-batch stage. A decline in growth rate was observed during the fed-batch stage due to constant feeding rate during biomass growth. However, since constitutive expression is largely growth associated, it may be beneficial to increase the glycerol feed rate in order to maintain the growth rate at values nearer to the maximum growth rate of the recombinant strains (0.15 – 0.17 h^{-1}).

Tangential flow filtration was successfully used to concentrate the cell-free enzyme extracts, where 5.20 – 6.01-fold increases in volumetric activity were obtained, with final volumetric activity yields of 49.57-60.04 %. It was found that glucuronoyl esterase was the most sensitive to volumetric activity loss after refrigeration and freezing, with a decrease of 10.71 % in volumetric activity after freezing at -20 °C. Cellobiose dehydrogenase showed no sensitivity to the freezing process, and laccase activity was successfully preserved with the use of 10 % glycerol as a cryoprotectant.

This study has successfully reported the baseline capabilities of a constitutive *P. pastoris* expression system to produce three enzymes, namely cellobiose dehydrogenase, laccase, and glucuronoyl esterase, associated with lignin valorisation, using a bioreactor fermentation bioprocess. Even though an identical molecular and bioprocess strategy was used, there were significant differences in protein secretion of each enzyme, emphasising the effect of the selected gene on secretion levels in *P. pastoris*. The yields obtained in the current study may be improved at the microbiological level by improving secretion with the use of alternative secretion factors or gene coding sequences. The bioprocess may be improved by optimisation of the glycerol feed, to maintain the growth rate at a value nearer to the maximum growth rate of each strain. Further, preliminary data suggests that lowering the fermentation temperature may be beneficial for laccase production.

Opsomming

Waardetoevoeging van lignien is a belangrike stap tot die ontwikkeling van doeltreffende geïntergreerde bio- raffinaderye. ‘n Moontlike omgewingsbewus, energie doeltreffende oplosing is die ensiematiese degradasie en/of modifikasie van lignienverrykte afval. Vir die ensiematiese omskakling van lignien om ‘n realistiese opsie te wees, groot skaal produksie moet ekonomies haalbaar wees in terme van kostes. Heteroloë produksie van ensieme in rekombinante *P. pastoris* is ‘n bekende metode vir die suksesvolle grootskaalse produksie van rekombinante eukariotiese proteïene. As die nadele wat verband hou met metanol-induseerbare promotors in ag geneem word, is die konstitutiewe P_{GAP} promotor vir heteroloë produksie van proteïen in *P. pastoris* ‘n verkieslike uitdrukking strategie.

Drie ensieme vir lignien afbraak en modifikasie was gekies vir rekombinante ensiem produksie: glucuronoyl esterase (GE), sellobiose dehidrogenase (SDH) en laccase (LCC). Die ensieme was gekies gebaseer op nuwigheid, asook hul wye verskeidenheid van funksies in terme van lignosellulose biodegradasie. Hierdie ensieme verteenwoordig beide koolhidraat degradasie (GE, CDH: Fenton reaksie) en lignien degradasie en/of modifikasie (LCC). Die doel van die studie was om fundamentele data te skep van *P. pastoris* se vermoë om die drie begenoemde ensieme uit te druk onder beheer van die konstitutiewe P_{GAP} promotor, met die oog op lignien waardetoevoeging. Hierdie studie bevat aspekte van molekulêre biologie asook bioproses ingenieurswese.

Die DNA 2.0 (VSA) pJexpress uitdrukking stelsel, wat vry van enige eiendomsregtelike beperkings is, was gebruik om die uitdrukkingskonstrukte te skep. Die rekombinante *P. pastoris* stamme was gekeur op skud-fles vlak, en een gisstam vir produksie van elke ensiem was gekies gebaseer op sekresieproduksie van die ensieme. Ensiem produksie was opgeskaal na 14 L bioreaktors toe, met ‘n twee-stadium fermentasie strategie, wat bestaan uit 'n enkellading fase, gevolg deur 'n konstante gliserolvoerfase. Die fermentasie kultuur is geoes en die supernatant gekonsentreer deur 'n twee-stadium filtrasie proses, waarna vries as 'n moontlike berging strategie geëvalueer was. Gliserol was ook as n moontlike kriobeskermingsmiddel geëvalueer.

Die konstante gliserolvoerfase was doeltreffend, met hoë opbrengste in terme van biomassa en proteïenproduksie. Die bioreaktor fermentasies het soortgelyke biomassa groei-kinetika (μ_{max} = 0.15 – 0.17 h^{-1}) en -opbrengste (119.54-136.47 g_{dcw}/L) getoon regdeur die fermentasie prosese. Hoë vlakke van rekombinante proteïen was verkry, GE het die hoogste vlakke gehad

met 2778.01 mg/L, gevolg deur SDH met 1489.3 mg/L, en laastens LLC met 778.54 mg/L. Hierdie is die eerste studie wat na die konstitutiewe uitdrukking van *H. jecorina* GE *en N. crassa* SDH kyk, asook die hoogste gerapoteerde vlakke van konstitutiewe uitgedrukte *T. versicolor* laccase lcc2. Alhoewel die laccase fermentasie die laagste produktiwiteit teruggekeer het, dui voorlopige eksperimente doorop dat verlaging van die fermentasie temperatuur hierdie waarde kan verbeter, deur temperatuur-verwante degradasie van LCC te verhoed.

Geen gliserol was versamel gedurende die gliserolvoerfase nie. Daar was ’n afname in groeitempo tydens die gliserolvoerfase as gevolg van konstante voedings tempo gedurende biomassa groei. Egter, sedert konstitutiewe uitdrukking grootliks groei geassosieer is, mag dit voordelig wees om die gliserol voedings tempo te verhoog, om die groeitempo so na as moontlik aan die maksimum groeitempo van die rekombinante stamme te hou (0.15 – 0.17 h^{-1}).

Tangensiale vloei filtrasie is suksesvol gebruik om die sel-vrye ensiem ekstrakte te konsentreer, waar 5.20 – 6.01-voud stygings in volumetriese aktiwiteit verkry is, met finale volumetriese aktiwiteit opbrengste van 49.57-60.04 %. Die sensitiefste ensiem na verkoeling en vries was GE met 'n afname van 10.71 % in volumetriese aktiwiteit na bevriesing by -20 ° C. Sellobiose dehidrogenase het geen sensitiwiteit getoon na die vries proses nie, en LCC se aktiwiteit was suksesvol behou met die gebruik van 10% gliserol as 'n kriopreserveermiddel.

Hierdie studie rapporteer op 'n *P. pastoris* uitdrukking stelsel, gebaseer op die konstitutiewe P_{GAP} promotor, en die stelsel se produksie vermoë in ‘n bioreaktor fermentasie proses. Die vermoë van die stelsel was getoets met die uitdrukking van drie lignien waardetoevoegende ensieme, glucuronoyl esterase, sellobiose dehidrogenase en laccase. Ahoewel 'n identiese molekulêre en bioproses strategie gebruik was, was daar beduidende verskille in proteïen afskeiding van elke ensiem. Hierdie waarneming lê klem op die invloed van die geselekteerde gene op afskeiding vlakke in *P. pastoris*. Die proteïen opbrengste verkry in die huidige studie kan verbeter word op mikrobiologiese vlak deur afskeiding te verbeter met behulp van alternatiewe afskeiding faktore of gene manipulasie. Die bioproses kan verder verbeter word deur optimalisering van die gliserolvoerfase, en so die groeitempo nader aan die maksimum groeitempo van elke stam te hou. Voorlopige data dui ook daarop aan dat fermentasie temperatuur verlaging dalk voordelig vir laccase produksie sal wees.

Acknowledgements

I would like to thank the following people and institutions for their significant roles in ensuring the completion of this study:

- My supervisor, Prof Johann Görgens, for granting me the opportunity to do research at Process Engineering, all the academic support, and his lightning-fast email replies.
- My co-supervisor, Dr María García-Aparicio, for her contributions to the molecular work of this study, her constant academic support, cheerleading, and hugs.
- Prof Emile van Zyl, Prof Marinda Viljoen-Bloom, and all the members of the 'fun' Zyl lab at Microbiology for being extremely supportive and helpful colleagues.
- My colleagues and friends at Process Engineering, and the members of the Bio-resources research team, for being there through all the victories and failures of my study.
- My parents: my father, for encouraging me to keep working hard; and my mother, for reminding me to take it easy sometimes. Thank you for all the financial and emotional support, and for providing a welcoming home that I could always escape to. The rest of my family (Björn, Angelique, Peter, Kandas, Erica, Omi) for their unconditional support, encouragement, and for being consistently hilarious.
- Du Preez, for making the heaviest days much lighter.
- Mr Gerhardt Coetzee and Mr Arrie Arends for all the training and technical assistance with the bioreactors. Dr Rose Cripwell for her help with HPLC samples.
- The National Research Foundation (NRF), and Renewable and Sustainable Energy Scholarships (RSES) for their financial support.

Table of Contents

Summary iii
Opsomming v
Acknowledgements vii
List of Figures xi
List of Tables xiv
Nomenclature xv
Chapter 1: Introduction and Thesis Outline 2
Chapter 2: Literature Review 8
2.1 Outline 9
2.2 Lignocellulose 9
2.2.1 Hemicellulose 10
2.2.2 Lignin 10
2.2.3 Lignin-carbohydrate complexes 12
2.2.4 Technical Lignins 12
2.3 Biodegradation of Lignocellulose 15
2.3.1 Lignocellulose degrading enzymes 16
2.3.1.1 Laccase 17
2.3.1.2 Cellobiose Dehydrogenase 19
2.3.1.3 Glucuronoyl Esterase 21
2.4 Industrial Enzyme Production 23
2.4.1 Introduction 23
2.4.2 Recombinant enzyme production 24
2.4.3 The expression system 26
2.4.4 Bioprocessing aspects of recombinant protein production in *Pichia pastoris* 34
2.4.5 Recombinant laccase production in *P. pastoris* 37
2.4.6 Recombinant cellobiose dehydrogenase production in *P. pastoris* 38
2.4.7 Recombinant Glucuronoyl esterase production in *P. pastoris* 39
2.5 Molecular Genetics and Recombinant Enzyme Production: How Inherent Properties of Proteins affect their Production in Recombinant Systems 39
2.5.1 Gene Expression 40
2.5.2 Gene Translation 40
2.5.3 The Secretory System 41
2.5.4 Inherent Protein Properties affect Protein Yields 43
Chapter 3: Aims and Objectives 45
Chapter 4: Materials and Methods 49

4.1 Development of constitutive Pichia pastoris expression strains 51
4.1.1 Strains and plasmids 51
4.1.2 Plasmid construction 52
4.1.3 Transformation of Pichia pastoris 54
4.2 Screening of recombinant P. pastoris strains 55
4.2.1 Agar plate-based screening 55
4.2.3 Test-tube screening 55
4.2.4 Shake-flask screening 55
4.3 Bioreactor Production of Lignin-Modifying Enzymes 56
4.3.1 Inoculum preparation 56
4.3.2 Fermentation protocol 57
4.3.3 Sampling 58
4.3.4 Preliminary improvement of laccase production 58
4.3.6 Product Harvesting 59
4.3.7 Tangential flow filtration 59
4.3.8 Evaluation of freezing as a storage strategy 60
4.4 Analytical Techniques 61
4.4.1 Biomass concentration 61
4.4.2 Glycerol concentration 61
4.4.3 Enzyme Activity Assays 61
4.4.4 SDS-PAGE analysis (for bioreactor samples) 63
4.4.5 Protein Concentration 63
4.4.6 Statistical Analyses 63
Chapter 5: Results and Discussion 64
5.1 Screening of recombinant P. pastoris transformants 65
5.1.1 Cellobiose Dehydrogenase 65
5.1.2 Laccase 69
5.1.3 Glucuronoyl Esterase 70
5.2 Bioreactor Fermentations 71
5.2.1 Cellobiose Dehydrogenase Production 74
5.2.2 Laccase Production 75
5.2.3 Glucuronoyl Esterase Production 80
5.2.4 Comparison of protein concentrations between different expressed enzymes 81
5.2.5 Enzyme production using constant glycerol feed 83
5.3 Downstream Processing 87

5.3.1 Concentration of cell-free extracts using two-step tangential flow filtration.....87
5.3.2 Freezing as a storage strategy.....90
Chapter 6: Conclusions & Recommendations.....93
References.....97
Addendum A.....117
Plasmid maps and sequences of strains created in this study.....117
Addendum B.....138
Tabulated data of graphs reported in this study.....138
Addendum C.....142
Sample Calculations:.....142

List of Figures

FIGURE 1.1 POTENTIAL REVENUE STREAMS FOR THE MOST COMMON LIGNIN-CONTAINING WASTES (FROM GOSSELINK, 2015, PERMISSION FOR RE-USE GRANTED). 4

FIGURE 2.1 LIGNIN MONOMERS: MONOLIGNOLS AND THEIR CORRESPONDING PHENYLPROPANOID UNITS (ADAPTED FROM POLLEGIONI *ET AL*. 2015. COPYRIGHT LICENSE FOR RE-USE GRANTED). 11

FIGURE 2.2 SCHEMATIC REPRESENTATION OF METHYL GLUCURONOXYLAN SHOWING THE ESTER LINKAGE BETWEEN THE METHYL GLUCURONIC ACID (MEGLCA) MOIETIES AND AROMATIC ALCOHOLS OF LIGNIN. THE SITE OF ENZYMATIC ACTION OF GE IS MARKED WITH AN ARROW (FROM ŠPÁNIKOVÁ AND BIELY, 2006. COPYRIGHT LICENSE FOR RE-USE GRANTED). 12

FIGURE 2.3. POTENTIAL APPLICATIONS OF LIGNIN MODIFICATION VIA BIOLOGICAL PROCESSES (REDRAWN FROM LAURICHESSE AND AVÉROUS, 2014). 14

FIGURE 2.4. SUMMARY OF LIGNIN BIODEGRADATION PROCESSES (REDRAWN FROM DASHTBAN *ET AL.* 2010). LMS: LACCASE-MEDIATOR SYSTEM, LP: LIGNIN PEROXIDASE, MNP: MANGANESE PEROXIDASE, VP: VERSATILE PEROXIDASE, AAO: ARYL ALCOHOL OXIDASE, GLOX: GLYOXAL OXIDASE, QR: QUINONE REDUCTASES, AAD: ARYL-ALCOHOL DEHYDROGENASES. 16

FIGURE 2.5. 3D STRUCTURES OF TRAMETES VERSICOLOR LACCASE (RODGERS *ET AL.* 2009, PIONTEK *ET AL*. 2002. COPYRIGHT LICENCES FOR RE-USE GRANTED). 17

FIGURE 2.6. 3D STRUCTURE OF N. CRASSA CDH (TAN *ET AL.* 2015. MADEJ *ET AL.* 2014. COPYRIGHT LICENSE FOR RE-USE GRANTED). 19

FIGURE 2.7. SUGGESTED INTERACTION OF CDH IN LIGNOCELLULOSES BREAKDOWN BY: DIMINISHING SUBSTRATE INHIBITION OF CELLULASES (A), ACIDIFICATION AND SOLUBILISATION OF METAL IONS (B), REDUCTION OF TOXIC QUINONES (C), INTERACTION WITH LIGNIN PEROXIDASE OR LACCASE (D) AND MANGANESE PEROXIDASE (E), PRODUCTION OF HYDROXYL RADICALS (OH●) VIA THE FENTON REACTION (F) AND DIRECT PRODUCTION OF REACTIVE OXYGEN SPECIES (G) (LUDWIG *ET AL*. 2010. COPYRIGHT LICENSE FOR RE-USE GRANTED). 20

FIGURE 2.8. 3D STRUCTURE OF THE CATALYTIC DOMAIN OF *T. REESEI* GLUCURONOYL ESTERASE. THE PROTEIN CHAIN IS SPECTRUM-COLOURED FROM BLUE (N-TERMINUS) TO RED (C-TERMINUS). THE CATALYTIC TRIAD RESIDUES (SER278–HIS411–GLU301) ARE SHOWN IN STICK DRAWING IN ATOM TYPE COLOURS. THE TRIAD IS ON THE SURFACE OF THE MOLECULE FACING THE VIEWER IN THIS ORIENTATION (POKKULURI *ET AL.* 2011. COPYRIGHT LICENSE FOR RE-USE GRANTED). 21

FIGURE 4.1. SUMMARY FLOW DIAGRAM OF METHODOLOGY FOLLOWED FOR EACH TARGET ENZYME. EACH PHASE IS DELINEATED BY COLOUR AND THE MAIN DELIVERABLES OF EACH PHASE ARE HIGHLIGHTED IN BOLD TEXT. STRAIN DEVELOPMENT WAS COMPLETED IN COLLABORATION WITH THE DEPARTMENT OF MICROBIOLOGY, STELLENBOSCH UNIVERSITY. 50

FIGURE 4.2. PLASMID MAPS OF THE PJ905 VECTOR (A) AND PJ905_XN_FOPA CONSTRUCT (B) USED TO GENERATE THE BACKBONE FOR THE GENES EVALUATED. 51

FIGURE 5.1. TEST-TUBE SCREENING OF *P. PASTORIS* TRANSFORMANTS, GROWN IN BMGY (INVITROGEN) MEDIA FOR 24 HOURS, EXPRESSING A: *N. CRASSA* CDH, B: *M. THERMOPHILUM* CDH. 66

FIGURE 5.2. SHAKE-FLASK SCREENING RESULTS OF VOLUMETRIC ACTIVITY OF RECOMBINANT *N. CRASSA* AND *M. THERMOPHILUM* CDH AFTER GROWTH IN BMGY FOR 72 HOURS. C1, C2: NEGATIVE CONTROL STRAINS. M1.1-M5.3: TRANSFORMANTS EXPRESSING M. THERMOPHILUM CDH. N2.3-N5.7: TRANSFORMANTS EXPRESSING N. CRASSA CDH. 67

FIGURE 5.3. SDS-PAGE ANALYSIS USED FOR DENSITOMETRIC PROTEIN CONCENTRATION DETERMINATION BETWEEN *M. THERMOPHILUM* AND *N. CRASSA* CDH SECRETION. LANES 1-5: BSA PROTEIN STANDARDS, 4 MG/L, 8 MG/L, 12 MG/L, 16 MG/L, 20 MG/L RESPECTIVELY. LANES 8: 20 ML SAMPLES OF CELL-FREE EXTRACT OF *M. THERMOPHILUM* TRANSFORMANT. LANE 9-10: 20 ML SAMPLES OF CELL-FREE EXTRACT OF *N. CRASSA* CDH TRANSFORMANTS (N2.6, N5.3). 68

FIGURE 5.4. SHAKE-FLASK SCREENING RESULTS FOR *P. PASTORIS* GROWN IN BMGY FOR 72 HOURS, EXPRESSING TWO LACCASE ISOFORMS. C1, C2: NEGATIVE CONTROL STRAINS. LCC1.1-1.3: *P. PASTORIS* TRANSFORMANTS EXPRESSING LACCASE ISOFORM LCC1. LCC2.1-2.3: *P. PASTORIS* TRANSFORMANTS EXPRESSING LACCASE ISOFORM LCC2. 70

FIGURE 5.5. SDS-PAGE ANALYSIS OF 20 ML CELL-FREE EXTRACT OF RECOMBINANT *P. PASTORIS* STRAINS GROWN IN SHAKE FLASKS CONTAINING BMGY MEDIUM. LANE 1: WILD-TYPE *P. PASTORIS* DSMZ 70382 (CBS704); LANE 2-4: PJ905_CG_CIP2.1-3; LANE 5: PAGERULER PROTEIN MOLECULAR WEIGHT MARKER; LANE 6: PJ905_CG_CIP2.5; LANE 7: PJ905_CG_CIP2.6; LANE 8-10: PJ905_HJ_CIP2.1-3 71

FIGURE 5.6. VOLUMETRIC ACTIVITY (GREY LINE) AND BIOMASS YIELDS (DRY CELL WEIGHT PER LITRE) (ORANGE LINE) DURING BIOREACTOR CULTIVATIONS OF EACH TARGET PROTEIN. RED ARROWS INDICATE THE INITIATION OF THE GLYCEROL FED-BATCH PHASE (20 – 22H), WHERE A 50 % (W/V) GLYCEROL FEED SUPPLEMENTED WITH TRACE SALTS WAS ADDED AT A RATE OF 72.6 ML/HR. A: CELLOBIOSE DEHYDROGENASE. B: LACCASE. C: GLUCURONOYL ESTERASE. ERROR BARS INDICATE STANDARD DEVIATION FROM MEAN OF THREE FERMENTATION REPLICATES. 73

FIGURE 5.7. EFFECT OF FIVE CULTIVATION TEMPERATURES (12 °C, 15 °C, 23 °C, 30 °C, 33 °C) ON VOLUMETRIC ACTIVITY OF SECRETED LACCASE, WHEN GROWN IN BMGY IN SHAKE FLASKS FOR 120 HOURS. 78

FIGURE 5.8. EFFECT OF COPPER SUPPLEMENTATION ON VOLUMETRIC ACTIVITY OF LACCASE AFTER 72 HOURS OF GROWTH IN BMGY. 79

FIGURE 5.9. SDS-PAGE ANALYSIS OF FINAL FERMENTATION SAMPLES (EACH LANE REPRESENTING ONE REPLICATE OF A BIOREACTOR FERMENTATION) OF 20ML SUPERNATANT CONTAINING THE TARGET PROTEINS. LANE 1: PROTEIN MOLECULAR WEIGHT MARKER. LANE 2-4: CDH-CONTAINING SUPERNATANT (RCDH). LANES 5-7: GLUCURONOYL ESTERASE-CONTAINING SUPERNATANT (RGE). LANES 8-10: LACCASE-CONTAINING SUPERNATANTS (RLCC). 83

FIGURE 5.10. EXPONENTIAL DECLINE IN SPECIFIC GROWTH RATE DURING FED-BATCH STAGE OF THE FERMENTATION, WITH A CONSTANT GLYCEROL FEED OF 72.6 ML/HR. OF A: GLUCURONOYL ESTERASE; B: LACCASE; C: CELLOBIOSE DEHYDROGENASE. 86

FIGURE 5.11. SDS-PAGE ANALYSIS OF CONCENTRATED ENZYME EXTRACTS. 20ML OF SAMPLE WAS LOADED IN EACH LANE. LANE 1: PROTEIN MOLECULAR WEIGHT MARKER. LANE 2-4: CONCENTRATED CDH-CONTAINING EXTRACT (RCDH), AT NO DILUTION, 5X DILUTION AND 10X DILUTION. LANES 5-7: CONCENTRATED GLUCURONOYL ESTERASE-CONTAINING SUPERNATANT (RGE) AT NO DILUTION, 5X DILUTION AND 10X DILUTION. LANES 8-10: CONCENTRATED LACCASE-CONTAINING EXTRACT (RLCC) AT NO DILUTION, 5X DILUTION AND 10X DILUTION. 88

FIGURE 5.12. EFFECT OF GLYCEROL AS A CRYOPROTECTANT FOR A: LACCASE, AND B: GLUCURONOYL ESTERASE. ALL STANDARD DEVIATIONS BETWEEN TRIPLICATES WERE LESS THAN 5 % FROM THE MEAN. 92

List of Tables

TABLE 2.1 TARGET ENZYMES, BIOCATALYTIC MECHANISMS AND POTENTIAL APPLICATIONS IN LIGNIN VALORISATION. 16

TABLE 2.2. RECOMBINANT PROTEIN PRODUCTION IN *P. PASTORIS*-P_{GAP} EXPRESSION SYSTEM (ADAPTED FROM COS *ET AL.* 2006). 30

TABLE 2.3. SUMMARY OF PREVIOUS STUDIES THAT PRODUCED RECOMBINANT LIGNINOLYTIC ENZYMES OF INTEREST 31

TABLE 2.4. PROTEIN PROPERTIES OF LIGNINOLYTIC ENZYMES OF INTEREST 44

TABLE 4.1. PRIMERS USED FOR INTRODUCTION OF RESTRICTION ENZYMES AND SEQUENCING OF THE SELECTED CDS OF LACCASE. 52

TABLE 4.2. PRIMERS USED FOR INTRODUCTION OF RESTRICTION ENZYMES AND SEQUENCING OF THE SELECTED CDS OF GLUCURONOYL ESTERASE. 53

TABLE 4.3. PRIMERS USED FOR SEQUENCING OF THE SELECTED CDS OF CELLOBIOSE DEHYDROGENASE. 53

TABLE 5.1. SUMMARY OF PRODUCTIVITY AND ENZYMATIC YIELDS FOR BIOREACTOR CULTIVATIONS OF RECOMBINANT *P. PASTORIS* AT THE END OF THE GLYCEROL FED-BATCH STAGE. 72

TABLE 5.2 SUMMARY OF TANGENTIAL FLOW FILTRATION CONCENTRATION OF CELL-FREE EXTRACTS OBTAINED FROM BIOREACTOR FERMENTATIONS. 88

Nomenclature

CDH:	Cellobiose dehydrogenase
DO:	Dissolved oxygen
DCW:	Dry cell weight
GE:	Glucuronoyl esterase
LCC:	Laccase
P_{AOX1}:	Alcohol oxidase 1 promoter
P_{GAP}:	Glyceraldehyde-3-phosphate dehydrogenase promoter
$q_{p.max}$:	Maximum specific productivity (mg recombinant protein.biomass^{-1}.h^{-1})
$q_{p.mean}$:	Mean specific productivity (mg recombinant protein.biomass^{-1}.h^{-1})
SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFF:	Tangential flow filtration
U.L^{-1}:	Units of enzymatic activity per litre
Vvm:	Volume per volume per minute
μ:	Specific growth rate
μ_{max}:	Maximum specific growth rate

Chapter 1: Introduction and Thesis Outline

Lignin is a heterogeneous, aromatic polymer that provides strength and rigidity to land plants and acts as a recalcitrant barrier to pathogenic attack (Beckham *et al.* 2016). Lignin is the second most abundant material in nature, after cellulose, and is the most prevalent renewable resource of aromatic polymers (Pollegioni *et al.* 2015). Due to lignin's inherent recalcitrance and high calorific value, the vast majority of lignin-enriched waste in industry is burned as an energy feedstock (Ragauskas *et al.* 2014). Ironically, it is the industrially challenging complexity and variety of chemical functional groups found in lignin that offers significant opportunity for conversion to a number of valuable products such as biofuels, chemicals, polymers, aromatics and other bio-based materials (Strassberger *et al.* 2014).

A **biorefinery** is a processing plant that converts biomass feedstocks into a variety of valuable products such as fuels, energy, materials, and chemicals (Maity, 2015). In an ideal biorefinery, the production of waste- material is minimised, and a key objective is to up-cycle previously considered waste-streams to valuable products or energy (FitzPatrick *et al.* 2010). The underutilization of lignin-enriched wastes is a critical factor for modern biorefineries to improve, if they wish to reach their full economic potential. The need for lignin valorisation has become more pronounced as availability of **lignin-enriched wastes increases**. An established contributor to lignin-enriched wastes is the paper and pulp industry that produces several lignin-enriched wastes (i.e. technical lignins), such as black liquor (35-45% lignin), an effluent of chemical pulping processes. It is estimated that of the fifty million tonnes of lignin extracted by the pulp and paper industry in 2010, only 2% was used to produce commercially viable products such as surfactants, adhesives and dispersants (Laurichesse and Avérous, 2014). Currently, black liquor is largely dealt with in one of three ways; it is disposed of as a waste, it is combusted resulting in minimal energy recovery, or it is combusted resulting in energy and inorganic compound recovery. In the case of combustion, the products are recycled back into the pulping process.

In all three of these processes, black liquor offers an opportunity for production of valuable products, thereby enhancing the economic viability of pulp and paper mills. However, this is subject to the condition that the use of lignin-enriched wastes for manufacture of valuable products is more beneficial than the current use for the lignin feedstock. For instance, in terms of energy production from lignin feedstocks, the cost of alternative energy feedstocks should be offset by income streams created by lignin-valorisation. There are a number of potential income streams for lignin-enriched wastes, dependent on the purity and source of the lignin, as

illustrated by Figure 1.1. Examples for applications include production of phenolics, cement additives as well as production of biofuels.

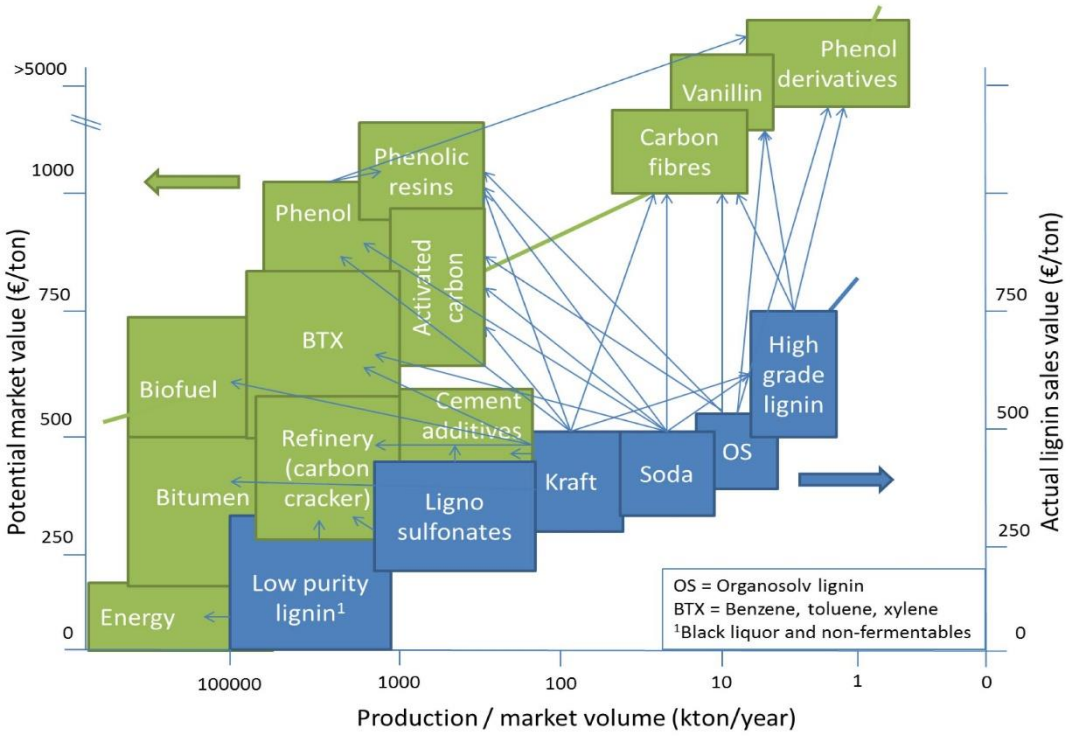


Figure 1.1 Potential revenue streams for the most common lignin-containing wastes (from Gosselink, 2015, permission for re-use granted).

Several routes of lignin valorisation have been investigated, including chemical, thermal and biological approaches. The enzymatic modification of technical lignin is a considerable challenge because of inert linkages (C-O ether or C-C bonds), the inherent heterogeneous structure of lignins and technical lignins, as well as differences in composition and chemical properties of industrial lignin-enriched wastes (Ragauskas *et al.* 2014). Native lignin modification and conversion have been successfully tackled in natural ligninolytic ecosystems. Several eukaryotic and prokaryotic microorganisms make use of **enzymatic systems** for lignin biodegradation. These enzymatic systems could potentially be harnessed and applied in lignin valorisation in an integrated biorefinery context. These **synergistic** lignocellulose-degrading systems can be divided into two general groups, namely (1) enzymes responsible for carbohydrate break down and (2) oxidative and auxiliary enzymes involved in lignin degradation (Martínez *et al.* 2005). Examples of carbohydrate degrading enzymes are the carbohydrate esterases (EC 3.1) and glycosylases (EC 3.2). While the carbohydrate-degrading enzymes are generally specific in terms of biocatalytic action, lignin degrading enzymes produce free radicals that are very reactive and not specific, resulting in the cleavage of the

otherwise inert carbon–carbon and ether inter-unit bonds (Bugg *et al.* 2011). Enzymes of importance in these ligninolytic systems are laccases, peroxidases, and a number accessory enzymes and radical compounds that aid in lignin degradation (Pollegioni *et al.* 2015). These oxidation reactions, however, are not limited to the lignin fraction, as carbohydrates are also predisposed to it. In fact, several carbohydrate oxidases are known to be part of the lignocellulose biodegradation process, while also producing highly reactive molecules that are required for other ligninolytic enzymes to function. An example of this is the mechanism of cellobiose dehydrogenase, that produces peroxide – a necessary compound for lignin peroxidase activity (Bey *et al.* 2011).

Lignin-degrading fungi and their associated enzymes have potential for application in several biotechnological processes, such as bleaching in paper and pulp industries, consolidated bioprocessing for ethanol production, biosensors, bioremediation (treatment of xenobiotic compounds) and textile decolourisation (Zhang *et al.* 2011. Fan *et al.* 2012). Three **enzymes of interest** for lignocellulose degradation and modification were selected in this study for recombinant enzyme production: glucuronoyl esterase (GE), cellobiose dehydrogenase (CDH) and laccase (LCC). These enzymes were selected based on novelty and their broad range of functions in the lignocellulose biodegradation process. These enzymes are representative of carbohydrate degradation (GE, CDH: Fenton reaction) and lignin degradation and/or modification (LCC). Carbohydrate degradation, such as that performed by GE, can be exploited in the enzymatic hydrolysis of pre-treated lignocellulose to improve fermentable sugar availability, and it can be applied to yield purer, higher quality technical lignin residues (D'Errico *et al.* 2016. Lyczakowski *et al.* 2017). Laccases are oxidative enzymes that catalyse repolymerisation or depolymerisation of lignin sub-units; this quality has resulted in the application of laccase in a number of lignin-valorisation strategies (Giardina *et al.* 2010). Cellobiose dehydrogenase acts synergistically in the degradation of lignin, and may be applied in the hydrolysis of carbohydrates from technical lignins (Huang *et al.* 2001). Although there are enzyme preparations of laccases commercially available, these contain undesired side activities and additives that may not be suitable for specific applications. Pure enzyme preparations of laccase and cellobiose dehydrogenase are provided in small amounts by different suppliers (i.e. Sigma, Megazyme, Creative-Enzymes, Jena Bioscience) at prohibitive costs for industrial application. Moreover, preparations of pure glucuronoyl esterase are not commercially available.

Pichia pastoris has been shown to be an excellent host for heterologous protein production, reaching extremely high cell densities (40-50 % [v/v]) and often reaching gram per litre quantities of expressed recombinant proteins (Daly and Hearn, 2005). Of utmost importance in the current study, is the ability of *P. pastoris* to perform a number of post-translational modification processes, such as correct protein folding, glycosylation, and proteolytic processing, which are essential in the secretion and eventual catalytic functioning of the three target enzymes presented here (Zhang *et al.* 2009). Further, the more extensively studied yeast host, *Saccharomyces cerevisiae*, has proved problematic by producing comparably low amounts of secreted heterologous proteins, performing incorrect or over-glycosylation of proteins, as well as the fact that it is a Crab-tree positive organism (Demain and Vaishnav, 2009).

However, despite the potential for high levels of recombinant protein secretion in *P. pastoris*, secretion levels are highly dependent on the protein and gene itself. In addition, the transcriptional regulation of the recombinant protein is an important factor to consider for industrial enzyme production (Cereghino *et al.* 2002). However, a major bioprocessing concern is the routine use of the methanol-induced AOX1 promoter. Methanol is undesirable in an industrial context due to the increased fire or explosion risks, complicated process monitoring requirements, as well as limitations in application due to the petro-chemical origin of methanol (Macauley-Patrick *et al.* 2005). The main alternative to this inducible promoter is the constitutive P_{GAP} promoter. The constitutive promoter expresses the recombinant gene throughout the growth cycle without the need for methanol induction.

Despite the promise of the alternative P_{GAP} promoter, there are significant shortcomings in literature on evaluating the feasibility of constitutive expression systems for heterologous production of the selected proteins. Considering this gap in literature and methanol-associated disadvantages, this study evaluated the production and secretion levels of the *P. pastoris* strain DSMZ-70382 for three selected lignocellulose-degrading enzymes (GE, CDH, LCC) when expressed under the control of the constitutive GAP promoter (P_{GAP}).

This study aims to assess the efficacy of a constitutive, patent-free, P_{GAP}-*P. pastoris* expression system, for the production of three enzymes associated with lignin valorisation, namely laccase, cellobiose dehydrogenase and glucuronoyl esterase, using a constant glycerol feeding strategy in a bioreactor cultivation. Both the enzyme production itself as well as downstream processing of the enzyme into a stable product for subsequent use, were considered.

This thesis is a multi-disciplinary study, that combines aspects of molecular biology and bioprocess engineering. The thesis is structured into 7 chapters: Chapter 2 consists of the literature review, which highlights the potential of technical lignins for production of biochemical and biomaterials with special emphasis on enzyme modification and recombinant enzyme production technology. The aims and objectives of the study are outlined in Chapter 3. The materials and methods are then reported in Chapter 4. Chapter 5 presents the results and discussion of the study. Conclusions and future recommendations are presented in Chapter 6. The final chapter contains the list of all literature cited.

Chapter 2: Literature Review

2.1 Outline

The following review introduces the structure of lignocellulose constituents, their biodegradation and discusses how these biodegradative enzyme systems can be harnessed by applying modern biotechnological techniques. Three lignocellulose-degrading enzymes of interest, namely cellobiose dehydrogenase, laccase, and glucuronoyl esterase were evaluated. Recombinant enzyme production and yeast expression hosts, particularly *Pichia pastoris*, were assessed in the context of the target recombinant enzymes. Lastly the molecular genetics of recombinant enzyme production was briefly elaborated on in the context of how inherent properties of proteins may affect their production in recombinant systems.

2.2 Lignocellulose

Lignocellulosic biomass comprises approximately half of the phytomass in the biosphere and is an abundant renewable resource (Bugg *et al.* 2011). Lignocellulose is a complex macromolecule that forms the structural material in plant cell walls, and is the main constituent of plant biomass. Lignocellulose is comprised of three major structural components, namely, cellulose, hemicellulose, and lignin, in addition to extractives such as pectin and nitrogenous compounds (Laurichesse and Avérous, 2014). The three major components are generally found in plant cell walls in a 4:3:3 ratio of cellulose: hemicellulose: lignin (Chen, 2014). Cellulose is a linear polysaccharide that consists of β-1,4-linked D-glucose monomers and is found in crystalline arrangements and in amorphous cellulose chains (Pérez *et al.* 2002). In the secondary cell wall of plants, cellulose, hemicellulose and lignin are chemically intertwined (Laurichesse and Avérous, 2014). Cellulose molecules are bonded together in a parallel fashion by hydrogen bonds that form between adjacent molecules. These bonded cellulose molecules form elementary fibrils, which in turn form fibrils which consist of 60-80 cellulose molecules. Four fibrils then constitute a thicker microfibril, and more than one microfibril forms a macrofilament (cellulose molecule < elementary fibril < fibril < microfibril < macrofilament) (Chen, 2014. Pérez *et al.* 2002). Hemicellulose is found between the elementary fibrils, in close association with the cellulose, via hydrogen bonding (Bugg *et al.* 2011). Microfibrils are enveloped in both hemicellulose and lignin, the latter of which contributes to the hardness, and mechanical and compressional strength of the plant cell wall. Industrially, the lignin and hemicellulose fractions of lignocellulose present a processing challenge, due to the inherent recalcitrance of lignin (Section 2.2.2) and the formation of lignin-carbohydrate complexes (Section 2.2.3) (Zeng *et al.* 2014). Specifically, in biofuel production as well as the paper and

pulp industries, cellulose is the target biomass fraction. However, cellulose is intertwined in a network containing lignin, hemicellulose and lignin-carbohydrate complexes (Guerriero *et al.* 2016). Therefore, the efficient separation of these biomass components is a key step, and remains an industrial challenge due to the inherent properties of lignocellulosic biomass. Additionally, in an integrated biorefinery, produced lignin-rich waste streams must be efficiently utilised and valorised.

2.2.1. Hemicellulose

Hemicelluloses are polysaccharides with structure that varies widely between different plant groups. Hemicelluloses are crudely defined as polysaccharides that are neither cellulose nor pectin and consist of varying amounts and combinations of D-glucose, D-mannose, D-galactose, L-arabinose, D-galacturonic acid, 4-O-methyl-glucuronic, L-glucoronic or D-xylose (Scheller and Ulvskov, 2010. Pérez *et al.* 2002). These sugar monomers are chiefly connected by β-1,4-linkages but β-1,3-linkages have also been found (Sanchéz, 2009). The main difference between cellulose and hemicellulose is that hemicellulose is branched and contains short lateral chains that consist of varying sugars, while cellulose consists of linear chains of glucose molecules only, resulting in some crystalline regions (Pérez *et al.* 2002). Additionally, hemicellulose does not appear in crystalline form, making it easier to hydrolyse than cellulose (Pérez *et al.* 2002. Sanchéz, 2009). The most common hemicelluloses found in terrestrial plants are xyloglucans, mannans, xylans and glucomannans (Scheller and Ulvskov, 2010). Xylan is the most predominant carbohydrate in grass- and hardwood-originating hemicellulose and is a linear polymer consisting of D-xylosyls chains that may be substituted with varying combinations of glucuronic acid, 4-O-methylglucuronic acid, acetyl groups, and arabinose (Guerriero *et al.* 2016. Pérez *et al.* 2002).

2.2.2 Lignin

The chemical structure of lignin has been a topic of study for approximately 170 years after Anselme Payen first discovered it in wood, calling it an ‘incrusting material’ (Adler, 1977). Lignin is a term used to describe a heterogenous group of complex aromatic heteropolymers that consist of phenylpropanoid units bound together by various irregular ether and carbon-carbon bonds in a three-dimensional network. Lignin consists of three dominant hydroxycinnamyl alcohols (monolignols), namely *p*-coumaryl alcohol, sinapyl alcohol and coniferal alcohol (Fig. 2.1). The monolignols are differentiated by their varying degrees of methoxylation. These aromatic alcohol precursor molecules undergo enzyme-mediated dehydrogenation, resulting in the formation of radical monolignol units. The monolignol units

polymerise via radical-radical coupling to construct lignin (Chen, 2014. Laurichesse and Avérous, 2014. Bugg *et al.* 2011. Sanchéz, 2009). The composition of lignin in terms of the phenylpropanoid units varies among different plant taxa and species and is also affected by environmental conditions during plant growth. Different forms of lignin are described using the presence and ratio of each of the phenylpropanoid units found in the particular lignin compound (Bugg *et al.* 2011. Wong, 2009).

The most prominent linkage found in natural lignins is the β-aryl ether (β-O-4) linkage, although five other bonds are also found. Chemically, the β-aryl ether bond is most easily cleaved, and is largely removed during the delignification process in paper pulping. The wide variation in chemical bonds found in lignin is as a result of resonance delocalisation in the three dominant radical monolignols. This allows for coupling at different sites between the monolignols (Wong, 2009). The five less common linkages are as follows: biphenyl (5-5) aryl-aryl/carbon-carbon bond, diaryl propane (β-1) carbon-carbon bond, diaryl ether (5-O-4) bond, phenylcoumarane [β-5 (α-O-4)] heterocyclic bond, and resinol (β-β) heterocyclic bond (Boerjan *et al.* 2003. Bugg *et al.* 2011. Chen, 2014. Wong, 2009). Once the three dominant hydroxycinnamyl alcohols are incorporated into the lignin polymer, they are known as phenylpropanoid units. The three phenylpropanoid units are *p*-hydroxyphenyl (H unit: from *p*-coumaryl alcohol), syringyl (S unit: from sinapyl alcohol) and guaiacyl (G unit: from coniferal alcohol) (Fig. 2.1) (Boerjan *et al.* 2003. Wong, 2009. Chen, 2014).

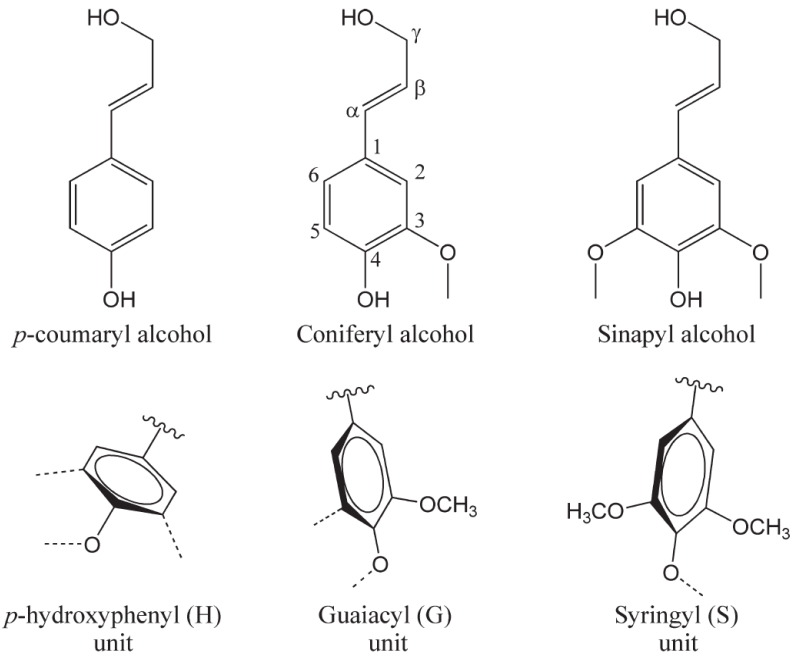


Figure 2.1 Lignin monomers: monolignols and their corresponding phenylpropanoid units (adapted from Pollegioni *et al.* 2015. Copyright license for re-use granted).

2.2.3. Lignin-carbohydrate complexes

LCCs are defined as macromolecular structures that consist of isolated lignin molecules that are covalently bound with hemicellulose (d'Errico *et al.* 2015). Covalent bonds are formed between hemicellulose side-chains and lignin, forming lignin-carbohydrate complexes (LCCs), where the polysaccharide components are cemented together by lignin. This creates a protective barrier against chemical or physical damage (Howard *et al.* 2003). Therefore, lignin isolated from natural lignocellulosic plant biomass contains small amounts of carbohydrate in the form of LCCs. The bonds between lignin and hemicellulose are challenging to fully characterise due to the difficulty in isolating pure, well-defined representative LCC samples. In spite of this, investigations have theorised that the dominant LCC linkages in wood are benzyl ether, esters and phenyl glycoside bonds (Balakshin *et al.* 2011) (Fig. 2.2).

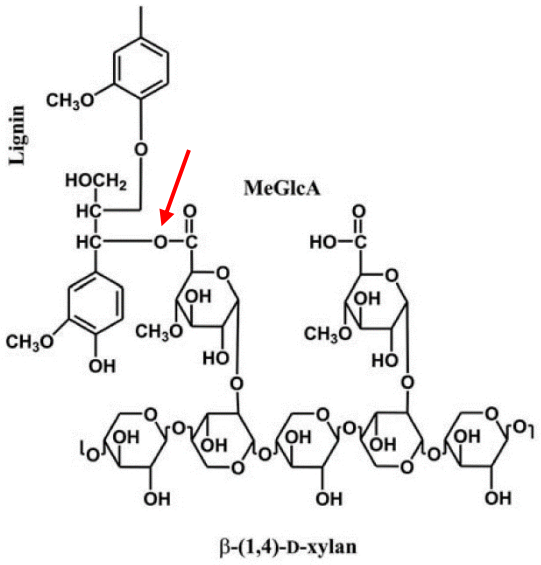


Figure 2.2 Schematic representation of methyl glucuronoxylan showing the ester linkage between the methyl glucuronic acid (MeGlcA) moieties and aromatic alcohols of lignin. The site of enzymatic action of GE is marked with an arrow (from Špániková and Biely, 2006. Copyright license for re-use granted).

2.2.4. Technical Lignins

Technical lignins, also known as spent lignins, are the fraction of lignin that is left over after the cellulosic portion of the biomass has been isolated for an industrial use such as paper manufacture or bioethanol production. In addition to the natural biomass source, technical lignins are affected by industrial delignification processes as well as the isolation methods used (Laurichesse and Avérous. 2014). There are two main types of technical lignins, namely sulfur lignins and sulfur-free lignins.

Sulfur lignins are produced by lignin extraction processes that use sulfur-containing chemicals. Industrial chemical pulping processes mainly produce sulfur lignins, e.g. Kraft lignin and lignosulfonates. Kraft lignin, produced by the Kraft lignin isolation process, has low sulfur content that is present in the form of sulphide bonds formed during the cooking stage of Kraft extraction (Vishtal and Kraslawski, 2011). Kraft lignin contains high levels of condensed moieties and phenolic hydroxyl groups (Laurichesse and Avérous. 2014). This altered chemical structure can be explained by the fact that a large proportion of β-aryl bonds in the native lignin are cleaved during the cooking process of Kraft lignin extraction forming hydroxyl groups (Monsouri and Salvado, 2006. Gordobil *et al.* 2016). Further, the condensed nature of Kraft lignin is attributed to the extreme conditions present during the cooking process (Vishtal and Kraslawaski, 2011). Lignosulfonates, which are produced by the sulfite lignin extraction process, contain a high proportion of sulfur as sulfonate groups in aliphatic side chains (Laurichesse and Avérous, 2014). Lignosulfonates are water-soluble, unlike Kraft lignins that are only soluble in organic solvents and alkaline solutions. Lignosulfonates have a higher mean molecular mass than Kraft lignin, with a broad dispersity (Đ) index, i.e. a wide range of possible molecular weights in a given sample (Laurichesse and Avérous, 2014. Mansouri and Salvado, 2006).

Sulfur-free lignins are much less predominant in industry, but are increasing in prevalence as a result of their vast potential and unique properties that make them alluring. Sulfur-free technical lignins, being free of sulfur with moderate molecular mass, resemble native lignins more than sulfur lignins (Lora and Glasser, 2002). Sulfur-free lignins include soda lignin, organosolv lignin and more recently, hydrolysis lignin and ionic liquid lignin (Mahmood *et al.* 2016. Vishtal and Kraslawski, 2014). Soda lignin is produced by alkaline pulping processes, while organosolv lignin is produced by solvent pulping. Hydrolysis lignin is the residue after pre-treatment and hydrolysis processing of lignocellulosic biomass in cellulosic ethanol plants, which is typically an acidic process (e.g. steam explosion, dilute acid or liquid hot water pre-treatments). This type of lignin is expected to become a significant contributor of technical lignin as cellulose-derived bioethanol and chemical 'bio'-industries grow (Mahmood *et al.* 2016). Similarly, ionic liquid lignins are an emerging group of technical lignins. Ionic liquids are used as a 'green' or environmentally friendly solvent in the extraction of lignin from various biomass sources (Pu *et al.* 2007). Ionic liquid lignins are similar to organosolv lignins and can be used in the same applications currently employed using organosolv lignins (Vishtal and Kraslawski, 2011).

Industrially, the valorisation of cellulose and hemicelluloses has been favoured due to their lucrative carbohydrate-based products such as paper, and second-generation biofuels. As a result, the valorisation of cellulose and hemicellulose has been relatively well optimised, while native and technical lignin feedstocks have largely been neglected because of their heterogeneous and recalcitrant nature (Strassberger *et al.* 2014. Linger *et al.* 2014). Lignin has mainly been used as an energy feedstock by being burned, however recent advances in biorefinery concepts has begun to explore lignin as a possible precursor to various value-added products (Linger *et al.* 2014. Ragauskas *et al.* 2014. Strassberger *et al.* 2014). Lignin is an abundant source of renewable aromatic materials. Aromatic materials are of importance as they form the basis of the petrochemical industry that is currently reliant on the use of fossil fuels. Therefore, the search for better, or equivalent products to the petrochemical industry from renewable resources (such as plant biomass), is of global interest. However, because of its structure, lignin remains a challenge to efficiently valorise in an industrial context, presenting an opportunity for the development of efficient, lucrative bioprocesses utilising enzymes (Fig. 2.3).

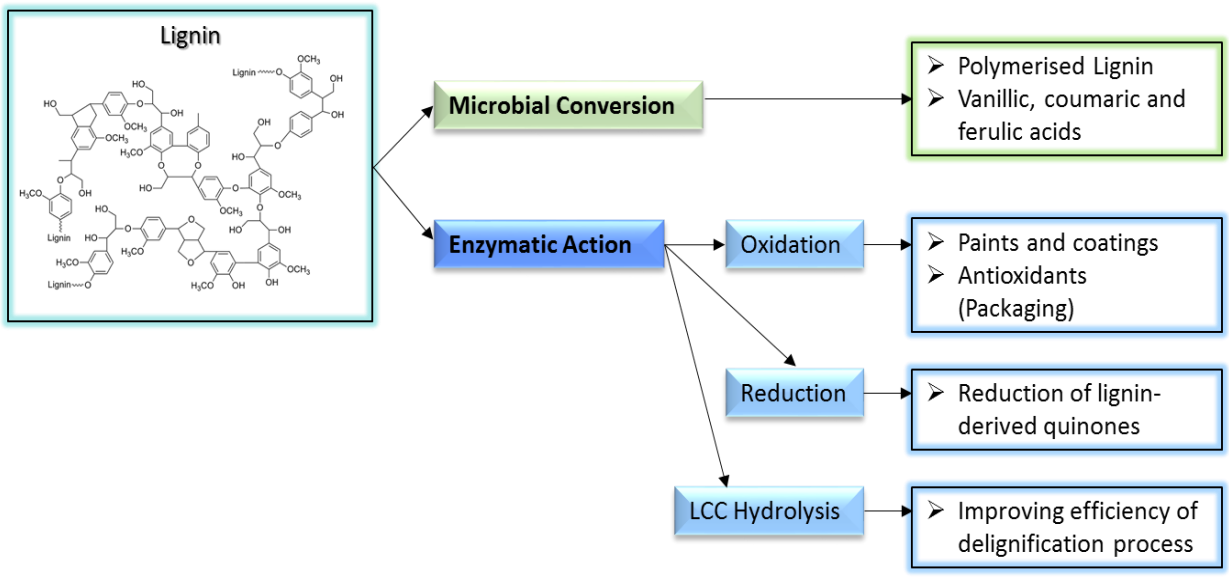


Figure 2.3. Potential applications of lignin modification via biological processes (redrawn from Laurichesse and Avérous, 2014).

2.3 Biodegradation of Lignocellulose

The recalcitrance of lignocellulosic biomass and lignin in particular poses a challenge to both anthropogenic industry and natural ecosystems. This robust feedstock offered an evolutionary niche for the development of organisms capable of degrading it for nutrients (Barr and Aust, 1994). These organisms were previously thought to be predominantly eukaryotic fungi; however, prokaryotic bacteria are now beginning to emerge as degraders of lignocellulose (Brown and Chang, 2014).

White-rot fungi have been extensively studied over the last century because of their lignin-degrading abilities. These fungi secrete a cocktail of low-specificity lignin-degrading enzymes. The low-specificity of the enzymes evolved as a result of the heterogeneous nature of natural lignin in plant biomass (Barr and Aust, 1994). The most commonly secreted lignin degrading enzymes are laccases, in combination with one or more peroxidases such as lignin peroxidase, manganese peroxidase or versatile peroxidase, and accessory enzymes such as quinone reductase and aryl alcohol oxidase (Fig. 2.4). (Pollegioni *et al.* 2015). In addition to direct enzymatic attack, these enzymes produce low molecular weight oxidants that also degrade lignin extracellularly. Examples of oxidants are manganese cations (Mn^{3+}), free radicals produced during enzymatic lignin degradation and reactive oxygen species, mainly the hydroxyl radical as well as the superoxide anion and hydrogen peroxide (Guillén *et al.* 1997). Hydroxyl radicals are among the strongest oxidants in the extracellular ligninolytic system. Enzymes that directly or indirectly contribute to hydroxyl radical production include cellobiose dehydrogenase (via Fenton's reagent), lignin peroxidase and laccase (Gómez-Toribio *et al.* 2009). Redox-cycling in a cell-bound quinone reductase system has also been shown to produce extracellular hydroxyl radicals for lignin degradation and an extracellular quinone reductase has also been isolated from *P. chrysosporium* (Guillén *et al.* 1997. Westermark and Eriksson, 1974).

Briefly, the mechanism of lignin degradation generally occurs by the oxidation of lignin, catalysed by laccases or ligninolytic peroxidases, to produce aromatic radicals. These aromatic radicals are then modified in a number of chemical processes, including aromatic ring cleavage, C4-ether and Cα-Cβ breakdown, and demethoxylation (Martinez *et al.* 2005). These processes effectively degrade lignin into aromatic derivatives, which in a biorefinery context may have potential for use in manufacture of value-added products.

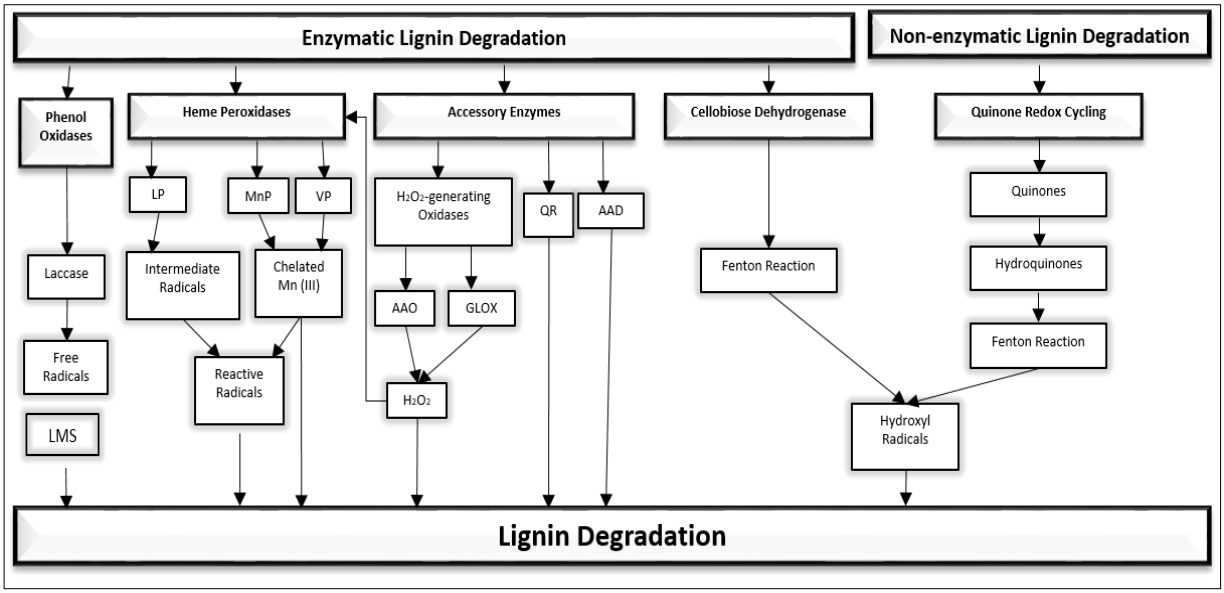


Figure 2.4. Summary of lignin biodegradation processes (redrawn from Dashtban *et al.* 2010). LMS: Laccase-mediator system, LP: Lignin peroxidase, MnP: Manganese peroxidase, VP: Versatile peroxidase, AAO: Aryl alcohol oxidase, GLOX: Glyoxal oxidase, QR: Quinone reductases, AAD: Aryl-alcohol dehydrogenases.

2.3.1 Lignocellulose degrading enzymes

Considering the multi-faceted nature of native lignin-degrading systems, a number of potential targets for biotechnological applications exist. Laccases have been extensively studied and utilized for industrial application, including the delignification and bleaching of paper and in biosensors (Bugg and Rahmanpour, 2015. Roth and Spiess, 2015). This study has focus on three ligninolytic enzymes, namely glucuronoyl esterase, cellobiose dehydrogenase, and laccase. These enzymes were selected based on their broad range of lignin-modifying biotechnological application streams, varying enzymatic mechanisms and protein structure, as well as the novelty of their production in the selected *P. pastoris* expression system (Table 2.1).

Table 2.1 Target enzymes, biocatalytic mechanisms and potential applications in lignin valorisation.

Enzyme	Biocatalytic Mechanism of Lignin Modification	Potential Applications	References
Laccase	Polymerisation and depolymerisation of lignin derivatives	Lignin-based (resin-free) particle boards, antimicrobial adhesives, biodegradable plastics.	Cannatelli and Ragauskas, 2016
Cellobiose dehydrogenase	Reduction of lignin derivatives	Homogenisation of lignin-rich effluents for use in biopolymers	Laurichesse and Avérous, 2014
Glucuronoyl esterase	Hydrolysis of lignin-carbohydrate complexes	Biofuels: Improved fractionation of plant biomass components – lignin and carbohydrates	d'Errico *et al.* 2016

2.3.1.1 Laccase

Laccase is a multi-copper oxidase that was first discovered in the sap of *Rhus vernicifera* trees (Gianfreda *et al.* 1999). Laccases have since predominantly been isolated from fungal sources, and more recently, from bacteria (Chandra and Chowdhary, 2015). White-rot basidiomycete fungi are the most prevalent source of laccase, which is secreted as part of the ligninolytic enzyme consortium during mineralisation of lignocellulose (Gianfreda *et al.* 1999; Yang *et al.* 2017). *Trametes versicolor* and *Pleurotus ostreatus* laccases are the most extensively studied in both fundamental and applied contexts (Yang *et al.* 2017).

Most fungal laccases are monomeric spheroproteins with glycosylation, ranging from 50 – 100 kDa in size, with a mode value of approximately 70 kDa (Cassland and Jönsson 1999; Shleev *et al.* 2004; Claus 2004; Bohlin *et al.* 2006; Giardina *et al.* 2010). Laccases contain at least four copper atoms that act as the catalytic core; each copper atom is further classified into three different types, namely, Type I, II, and III (Claus, 2004). Type I copper atoms are paramagnetic, with absorbance at 610 nm and lend the characteristic blue colour to laccase. Type II copper atoms are paramagnetic, with no absorption in the visible spectrum, while Type III copper atoms are diamagnetic, binuclear centres with electron adsorption at 330 nm (Claus 2004. Shleev *et al.* 2004. Giardina *et al.* 2010). Typically, the catalytic core will contain single Type I and Type II copper atoms, and two Type III atoms (Antošová and Sychrová, 2016). The Type II and Type III copper atoms are arranged in a tri-nuclear cluster (Fig. 2.5) (Claus, 2004). Structurally, laccases are composed of three sequential domains. These domains have greek key β-barrel topology, a distinctive characteristic of multi-copper oxidases (Giardina *et al.* 2010). The Type I copper atom is in domain 1, while the tri-nuclear cluster is located between domains 2 and 3 (Claus, 2004). Two disulfide bridges stabilize the laccase structure between domains 1 and 3, and domains 1 and 2 (Giardina *et al.* 2010).

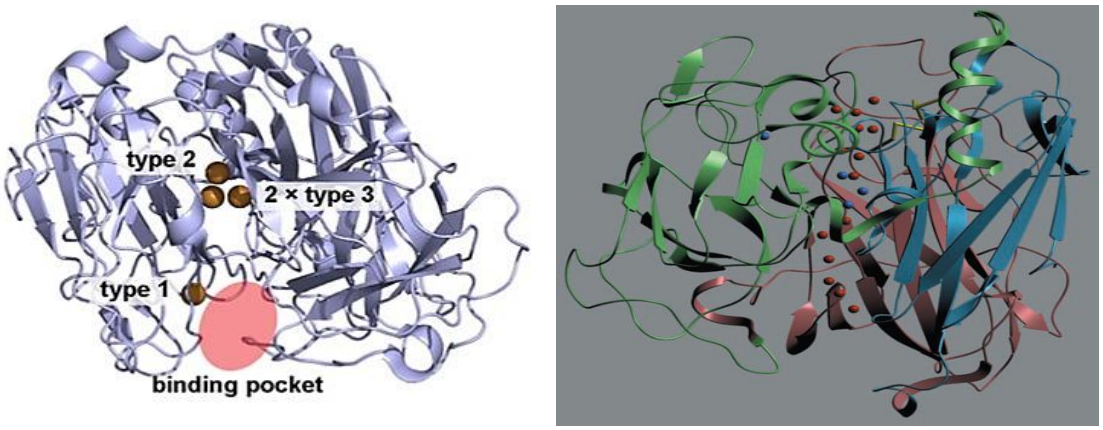


Figure 2.5. 3D Structures of *Trametes versicolor* laccase (Rodgers *et al.* 2009, Piontek *et al.* 2002. Copyright licences for re-use granted).

Laccases are oxidases that catalyse the one-electron oxidation of a wide range of substrates, reducing molecular oxygen to water. Laccase substrates comprise several aromatic compounds, including phenols, hydroxyindoles, amines, carbohydrates, small organic compounds, and benzenethiols (Giardina *et al.* 2010; Yang *et al.* 2017). Native laccases may be secreted or intracellular, where the enzyme catalyses polymerisation and depolymerisation reactions, as well as acting as a virulence factor in some laccase-producing fungi (Mayer and Staples, 2002; Riva, 2006). An example of polymerisation by laccases are cell-wall formation and lignification in plants, while depolymerisation is performed by fungal laccases which act during biodegradation of lignin (Antošová and Sychrová, 2016).

During a typical catalytic cycle, the laccase catalytic core oxidises the substrate via four one-electron oxidations with four-electron reduction of molecular oxygen to water (Antošová and Sychrová, 2016). The oxidised substrates form radicals, which may participate in various enzymatic and non-enzymatic reactions, to form dimers, oligomers, or polymers (Riva, 2006). Oxidation of the substrate occurs at the Type I copper site, because of its high redox potential. Further, the substrate oxidation is the rate limiting step of laccase catalysis, thus the redox potential of the Type I copper sites is of importance when screening laccases (Shleev *et al.* 2004). Reduction of molecular oxygen to water takes place at the trinuclear cluster of Type II and Type III copper atoms (Claus, 2004).

The non-specific nature of laccase oxidation has made it an attractive target for biotechnological applications in the biofuel, textile, food, bioremediation, pharmaceutical, cosmetic, and paper and pulp industries (Mayer and Staples, 2002). Currently, the most common industrial applications of laccases are related to dye decolourization in the textile and dye industries, or delignification in pulp and paper industries (Riva, 2006). Of interest for the current study is the potential for laccase-catalyzed conversion of lignin-containing waste to valuable products.

In a lignocellulosic biorefinery, there are three main avenues for laccase application, namely, during biomass pre-treatment, for lignin depolymerisation and modification, and for removal of microbial-inhibitory phenolic compounds from cellulose hydrolysate (Roth and Spiess, 2015). Laccase can catalyse lignin depolymerization and polymerization reactions, for instance, in the oxidative cross-coupling of lignin monomers (Riva, 2006). This property can be exploited to develop biopolymers (Laurichesse and Avérous, 2014). Laccases can also be

used as an active ingredient in packaging as they catalyse oxygen scavenging, increasing the shelf-life of oxidation-sensitive products (Johansson *et al.* 2014).

Laccase is unique from other lignin-degrading peroxidases because it does not require the use of environmentally harmful co-substrates, such as peroxide. Additionally, molecular techniques for recombinant production are considerably more developed, and the use of laccase at pilot scale for pulp delignification has already been demonstrated in the Lignozym® process, further cementing its feasibility in a biorefinery (Call and Mücke 1997). While commercial preparations of laccase do exist, the enzyme remains expensive and often contains a number of impurities and side-activities that are undesirable. To overcome this challenge, this study will evaluate the production potential of recombinant laccase production in a well-known expression system, *P. pastoris*.

2.3.1.2 Cellobiose Dehydrogenase

Cellobiose dehydrogenase (CDH) is an extracellular flavocytochrome protein first isolated in the white-rot basidiomycetous fungus, *P. chrysosporium* (Westermark and Eriksson, 1974). Cellobiose dehydrogenase has since been isolated from a number of wood-degrading, saprophytic and phytopathogenic fungi, eluding to its potential biotechnological application in lignocellulosic biomass degradation (Zamocky *et al.* 2006).

Figure 2.6. 3D Structure of *N. crassa* CDH (Tan *et al.* 2015. Madej *et al.* 2014. Copyright license for re-use granted).

It is a monomeric enzyme consisting of two distinct domains that are attached via a fifteen amino acid linker (Fig. 2.6). The first domain is a FAD-containing flavin unit and the second is a heme domain that contains a cytochrome *b* type heme. Typically, CDH is a glycosylated monomeric protein, 80-115 kDa in size, and is relatively thermo- and pH-stable. The temperature optimum is approximately 60 °C, with activity being reported up to 90 °C and optimal pH is 3-5, with activity being reported between pH 2-10 (Zamocky *et al.* 2006. Cameron and Aust, 2001).

The precise biological function of CDH is still unknown, however current literature speculates that it plays a role in degradation and modification of lignocellulose biopolymers (cellulose, hemicelluloses and lignin) by producing hydroxyl radicals via the Fenton reaction (Fig. 2.7) (Cameron and Aust, 2001. Harreither *et al.* 2011. Zamocky *et al.* 2006). CDH catalysis involves the oxidation of saccharides to the corresponding lactone, which spontaneously converts to a lactobionic acid with concomitant oxidation and reduction of the flavin prosthetic group (Sulej *et al.* 2015. Henriksson *et al.* 2000). Simply put, *in vivo*, CDH binds to cellobiose and dehydrogenates the compound to produce cellobiolactone (Fan *et al.* 2012). An oxidative system has also been proposed where CDHs cooperate with lytic polysaccharide monoxygenases (LPMOs) to catalyse oxidative glycosidic bond hydrolysis in starch, crystalline cellulose and hemicelluloses (Tan *et al.* 2015).

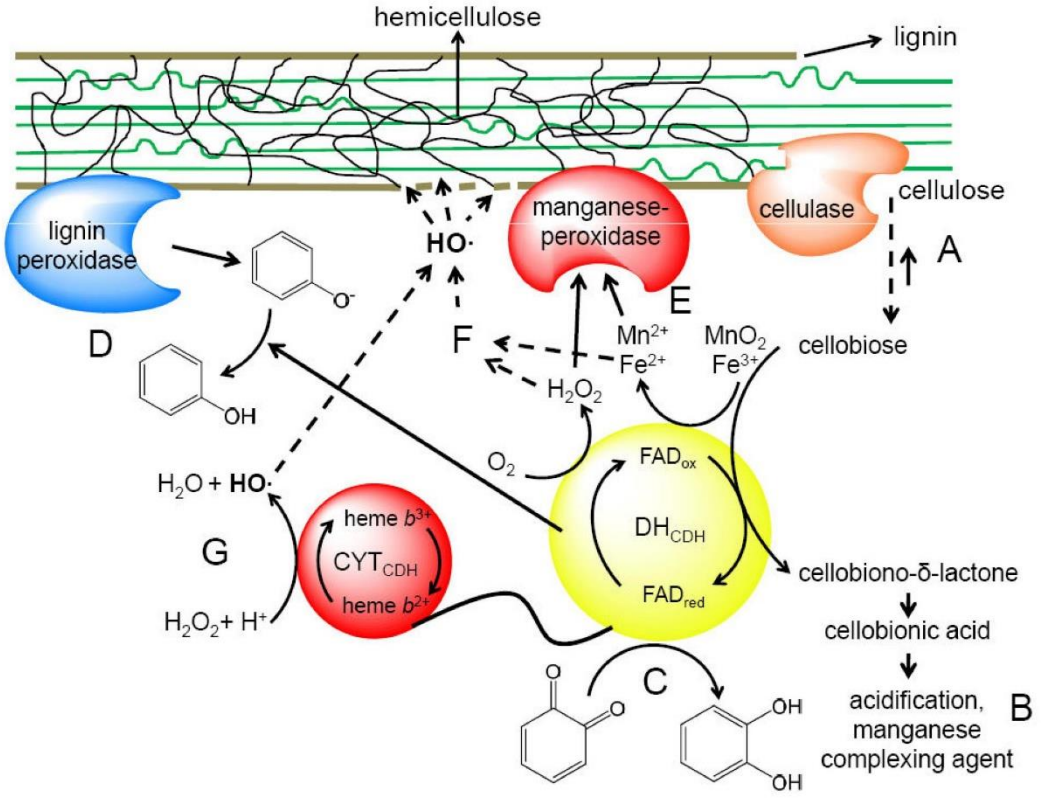


Figure 2.7. Suggested interaction of CDH in lignocelluloses breakdown by: diminishing substrate inhibition of cellulases (A), acidification and solubilisation of metal ions (B), reduction of toxic quinones (C), interaction with lignin peroxidase or laccase (D) and manganese peroxidase (E), production of hydroxyl radicals (OH●) via the Fenton reaction (F) and direct production of reactive oxygen species (G) (Ludwig *et al.* 2010. Copyright license for re-use granted).

CDH has been evaluated for potential use in a number of biotechnological applications including bleaching in paper and pulp industry, consolidated bioprocessing for ethanol production, biosensors, bioremediation and textile decolourisation (Zhang *et al.* 2011. Fan *et al.* 2012). Additionally, studies have shown CDH to enhance ligninolytic activity of peroxidase enzymes, thus showing potential for their use in lignin valorisation in a biorefinery context (Fang *et al.* 1999). Unfortunately, commercial preparations of CDH are costly and this hampers research and development in biocatalysis using CDH at an industrial scale. For CDH biocatalysis in biorefineries to be a realistic choice, cost-effective production of bulk amounts of enzyme must be achieved. To overcome this challenge, this study will evaluate the production potential of recombinant CDH production in a well-known expression system, *P. pastoris*.

2.3.1.3. Glucuronoyl Esterase

Glucuronoyl esterase is a recently defined carbohydrate esterase of family 15 (CE-15) that was first discovered by Špániková and Biely (2006) in the wood rotting fungus *Schizophyllum commune*. Since 2006 glucuronoyl esterase has been isolated from a number of different microorganisms including *Trichoderma reesei* (synonym: *Hypocrea jecorina*), *Myceliophthora thermophila* (synonyms: *Sporotrichum thermophile, Chrysosporium thermophilum*), *Phanerochaete chrysosporium*, and *Cerrena unicolor* (Li *et al.* 2007a. Vafiadi *et al.* 2008. Duranová *et al.* 2009a. Topakas *et al.* 2010. d'Errico *et al.* 2015).

Characterisation of these glucuronoyl esterases revealed that two structural types exist; one containing a catalytic domain linked to a carbohydrate-binding domain (CBM) and another with only a catalytic domain without the CBM (Fig. 2.8). Glucuronoyl esterase is reported to be *N*- and *O*-glycosylated when expressed in eukaryotic hosts (d'Errico *et al.* 2016. Duranová *et al.* 2009b). Glucuronoyl esterases have been found to be 42-60 kDa in

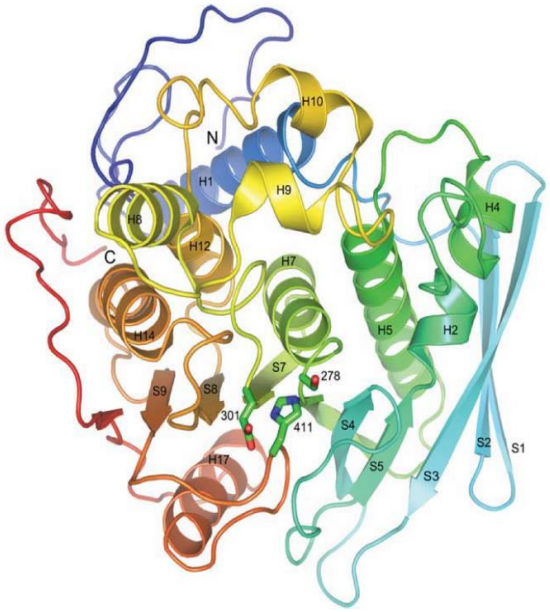


Figure 2.8. 3D structure of the catalytic domain of *T. reesei* glucuronoyl esterase. The protein chain is spectrum-coloured from blue (N-terminus) to red (C-terminus). The catalytic triad residues (Ser278–His411–Glu301) are shown in stick drawing in atom type colours. The triad is on the surface of the molecule facing the viewer in this orientation (Pokkuluri *et al.* 2011. Copyright license for re-use granted).

size (depending on the presence of the CBM and glycosylation patterns), with temperature and pH activity optima of 40-60 °C and 5-7, respectively (Li *et al.* 2007a. Vafiadi *et al.* 2008. Duranová *et al.* 2009a. d'Errico *et al.* 2015. Topakas *et al.* 2010).

The mechanism of action of glucuronoyl esterase has not been fully elucidated, because the chemistry of lignin-carbohydrate complexes remains a research challenge. Špániková and Biely (2006) initially described the functioning of glucuronoyl esterase as the cleavage of methyl esters from free or linked 4-O-methyl-D-glucuronic acid in lignocellulosic substrates. One of the covalent bonds connecting lignin and hemicellulose in plant biomass is an ester linkage between glucuronoxylans and the hydroxyl groups of lignin primary alcohols. Specifically, it is the ester bond between either the 4-O-methyl-D-glucuronic acid or D-glucuronic acid moieties of the glucuronoxylans and hydroxyl groups of lignin alcohols (Watanabe and Koshijima, 1988). Thus, glucuronoyl esterase was first hypothesised to play a role in the separation of hemicellulose and lignin by hydrolysing one of the main bonds that interlink these two plant biomass components. This hypothesis has since been validated in a number of studies that tested the activity of various glucuronoyl esterases on synthetic lignin-carbohydrate substrates (Špániková *et al.* 2007. Li *et al.* 2007a. Vafiadi *et al.* 2008. Duranová *et al.* 2009b).

The use of lignocellulosic biomass as an energy resource has been extensively investigated for decades in a bid to reduce dependence on fossil fuels. Bioethanol is the dominant biofuels for transportation on a global scale, based on current production volumes, resulting in a formidable biofuel industry. During pre-treatment and enzymatic hydrolysis of lignocellulosic feedstocks for second generation bioethanol production, a key objective is the separation of the three major biomass components – cellulose, hemicellulose and lignin – in order to access the fermentable carbohydrate monomers of the plant biomass (Lee, 1997). However, these pre-treatments are often energy-intensive and do not comprehensively fractionate the biomass components. Chemical bonds, including esters, linking carbohydrates to lignin are hypothesised to complicate the separation of lignin from cellulose and hemicellulose (Balakshin *et al.* 2014). These lignin-carbohydrate complexes that largely consist of lignin covalently bonded to hemicelluloses therefore contribute to the recalcitrance of lignocellulosic biomass. The use of glucuronoyl esterases may improve the degradability of lignocellulosic biomass by hydrolysing the ester bonds between hemicellulose and lignin. d'Errico *et al.* (2016) explored the potential of glucuronoyl esterases in the hydrolysis of lignin-carbohydrate complexes that impede bioethanol production processes. It was found that, in the presence of the glucuronoyl esterase-

producing *Aspergillus* spp., heat pre-treated corn fibre released improved amounts of glucose, glucuronic acid and C5 sugars – a promising result that shows the potential application of glucuronoyl esterases in improving yields of fermentable sugars from lignocellulose for biofuel production (d'Errico *et al.* 2016). Further, it was recently reported that the removal of glucuronic acid branches from the hemicellulose, xylan, significantly improved release of fermentable sugars for biofuels production (Lyczakowski *et al.* 2017). In addition to bioethanol production, glucuronoyl esterase has potential for application in other biorefineries, with the exception of alkali-based lignin extraction, where lignin-carbohydrate complexes hamper separation of lignin and polysaccharides required for valuable products (Balakshin *et al.* 2011). Glucuronoyl esterase is an exciting production target since, despite its industrial potential, very limited recombinant production has been reported, and no attempts at large scale production (besides that reported in this study) have been attempted to date.

2.4 Industrial Enzyme Production

2.4.1 Introduction

Enzymes are catalysts of biological reactions. Also known as biocatalysts, enzymes are favoured over traditional chemical catalysts for industrial applications because of their 1) high specificity, which reduces production of undesirable by-products and thus negates expensive downstream purification processes, 2) high efficiency, 3) lower associated costs, 4) relatively mild reaction conditions in terms of pH, temperature and pressure which uses less water and energy, while producing less toxic wastes, thus resulting in 5) a more positive environmental impact (Waites *et al.* 2001, Kirk *et al.* 2002).

Enzymes have inadvertently been used for centuries in the making of food products, such as beer, and in textiles, such as leather and linen. These ancient processes made use of spontaneous microbial growth of enzyme-producing organisms or the application of natural enzyme sources like the rumen of calves (Kirk *et al.* 2002). More recently, the power of these enzymes has been harnessed with the application of controlled, scientific principles. The development of controlled fermentation processes allowed for the mass production of pure native enzymes, which could then be used in large scale manufacturing processes, such as in the paper, detergent and textile industries (Aehle, 2007). With the advent of recombinant DNA technology, the efficiency of these fermentation processes was significantly improved and the production of enzymes previously thought to be unfeasible was made possible. Additionally, advances in

modern biotechnological techniques, including protein engineering and '-omics' based systems have further revolutionised the industrial enzyme production process (Kirk *et al.* 2002).

The production and application of enzymes in various industries is of enormous economical and scientific significance. Of particular interest for the current study are the pulp and paper industry, which make use of a number of enzymes including xylanases in bleaching processes, as well as biofuel (bioethanol) industries where enzymes are utilised both in pre-treatment processes and saccharification (Kirk *et al.* 2002).

2.4.2 Recombinant enzyme production

Every year, thousands of tonnes of commercial enzymes are produced, with a total value in excess of US $1.5bn. The majority of these enzymes are produced by microorganisms as a result of numerous advantages over alternative hosts such as plants, mammalian cells and insects (Waites *et al.* 2001). These advantages include (1) high surface area to volume ratio, which allows for rapid assimilation of nutrients resulting in high metabolic and biosynthesis rates, (2) the wide diversity of microbial catabolic and anabolic metabolic reactions available for exploitation, (3) adaptability and robustness of microbial growth, (4) inexpensive growth substrates, even use of waste materials, and (5) availability of established recombinant DNA techniques, allowing for relatively effective genetic engineering, which facilitates increased production of recombinant products and modification of the gene product to desirable specifications (Demain, 2000).

More than 60% of the enzymes used in food, detergent and starch processing industries are recombinant gene products (Adrio and Demain, 2010. Waites *et al.* 2001). Recombinant gene products are produced when a native gene from an organism that naturally produces a target gene product (enzyme) is excised from the native organism's genome and inserted into a vector, creating a recombinant DNA (rDNA) molecule. This rDNA is subsequently transformed into an appropriate host, creating a genetically modified microorganism capable of target gene expression and thus recombinant protein production (Klug and Cummings, 2011). The use of recombinant DNA techniques has resolved a number of enzyme production issues. For example, in cases where the native organism was problematic to grow or genetically manipulate, a simpler host may be genetically modified with the target gene. Additionally, in cases where the native organism was pathogenic or produced toxins, the recombinant protein could be produced in a safer, alternative host. In terms of productivity and efficiency, genetic engineering allows for increased production with the use of strong promotor sequences,

multiple gene copies as well as improvements in secretory signals. Further, protein engineering, or modification of the target genetic open reading frame, has facilitated improved enzyme stability, specificity and activity (Adrio and Demain, 2010). In addition to advantages provided by genetic manipulation, the replacement of the natural microorganism with recombinant microorganisms such as yeast, as in the case of the current study, results in high concentrations of the target protein that are relatively pure. This contrasts with protein or enzyme cocktails produced by most native microorganisms, especially ligninolytic fungi, which complicates downstream processing (Dashtban *et al.* 2010).

According to Waites *et al.* (2001), the process of industrial enzyme production can be divided into two main stages, namely upstream processing and downstream processing. Upstream processing deals with all processes leading up to and including the fermentation itself. Upstream processing can be further divided into three main categories, the first involves all aspects of the enzyme-producing microorganism including obtaining the target organism, procedures for improving the microorganism for use in an industrial environment, preparation of an inoculum and long-term storage and improvement of the microorganism. The second upstream processing category deals with the fermentation media, which includes the selection of efficient and cost-effective carbon, energy and essential nutrient sources. This step is often crucial to ensure maximum yield for the lowest cost. The last upstream processing step is the fermentation itself, where a number of variables are optimised under controlled conditions to enhance the growth of the host microorganism to ensure maximal recombinant product yield (Waites *et al.* 2001). The current study will focus on a number of upstream processing aspects, namely development of production strains, use of a defined fermentation culture medium and fermentation of the developed recombinant organisms for enzyme production.

Downstream processing involves all the processes succeeding the fermentation. The critical outcome of downstream processing is to reliably, safely and efficiently recover the maximum amount of target recombinant enzyme, while maintaining optimal specifications (enzyme purity, activity and stability), at minimal cost (Dutta, 2008). The stages in downstream processing are inherently reliant on the upstream processes. In general, the enzyme will be separated from either the fermentation medium in the case of extracellularly produced enzymes or from the biomass for intracellularly produced enzymes in a process known as primary recovery. This is followed by product purification to obtain a high purity product from a crude supernatant or extract. Lastly, the recombinant enzyme undergoes finishing processes, which may include crystallisation and packaging to prepare the product for its ultimate use (Waites *et*

al. 2001. Dutta *et al.* 2008). The enzymes produced in the current study are extracellularly secreted, through the use of native secretion signals, thus downstream processing will be simplified and involve separation of biomass from the enzyme-containing fermentation medium and enzyme-concentration steps using tangential flow filtration. This simplified downstream process will allow the enzyme products to be used directly in application studies, reducing over-all costs and equipment requirements, while increasing the time-efficiency of the production process. Additionally, the storage of the target enzymes by freezing are evaluated in the current study.

2.4.3 The expression system

A crucial initial step in developing an industrial fermentation process is the selection of a suitable expression system and microbial host. An expression system is comprised of an expression vector, into which the open reading frame and upstream and downstream genetic regulators known as promotors and terminators of the target gene, are cloned (Palomares *et al.* 2004). In some cases, an affinity tag or signal sequence may be present to allow for effective protein transport and separation. Eukaryotic protein expression systems are favoured over their more simplistic prokaryotic counterparts (e.g. *Escherichia coli*) in cases where the target recombinant protein requires post-translational modification and secretion processes to be biologically active or functional (Waites *et al.* 2001). These post-translational modifications only naturally occur in eukaryotes and include glycosylation, phosphorylation, methylation, disulphide linkage formation, proteolytic cleavage or subunit binding in multi-unit proteins (Mann and Jenson, 2003).

A number of factors are considered for optimal recombinant protein production, namely vector maintenance and copy number, promoters, transcription terminators, and selectable markers. Each factor may have a positive or negative effect on the resultant recombinant protein yield and it is an important step to evaluate each of these factors in the host microorganism (Strausberg and Strausberg, 1995).

2.4.3.1 Pichia pastoris

P. pastoris is a methylotrophic yeast that first gained attention as a potential source of high protein animal feed. The Phillips Petroleum Company was the first to research and develop growth media and protocols for growing *P. pastoris* at high cell densities using methanol in a continuous culture. Unfortunately, during this time it became economically unfeasible to continue with developing *P. pastoris*-based animal feed because the price of soybeans, an

alternative protein source, decreased while methanol prices increased (Higgins and Cregg, 1998). Later, the Phillips Petroleum Company teamed with the Salk Institute Biotechnology/Industrial Associates, Inc (SIBIA) to research and develop *P. pastoris* as a recombinant protein expression system. This undertaking resulted in the isolation of the *AOX1* gene (and promoter) as well as the extensive development of molecular techniques by SIBIA, which when coupled with the earlier work of Phillips Petroleum Company, created a successful recombinant protein expression system. The patent for this expression system was sold to Research Corporation Technologies (RCP) in 1993 and has since expired, allowing for companies such as DNA 2.0 to create and sell *P. pastoris* expression vectors which may be commercially used without payment of licensing fees to patent holders. Nevertheless, the *P. pastoris* expression system is also available from Invitrogen Corporation in kit form. In addition to this expression system, *P. pastoris* has been developed as a model eukaryotic organism in a number of cell biology research avenues (Higgins and Cregg, 1998. Macauley-Patrick *et al.* 2005).

P. pastoris has been utilised in the production of over 500 recombinant proteins, the majority being biopharmaceuticals and industrial enzymes, with some expression levels reaching 80% of the total secreted protein (Potvin *et al.* 2012. Ahmad *et al.* 2014). *P. pastoris* has rapidly been accepted and utilized as a recombinant protein expression system as a result of a number of host-specific advantages, such as the availability of the tightly controlled, highly expressed *AOX1* promoter. *P. pastoris* (1) maintains a rapid growth rate on inexpensive basal growth media, (2) is easily maintained at high cell-densities during fermentation, (3) produces high concentrations of recombinant proteins and does not secrete significant amounts of endogenous proteins, thereby decreasing complexities in downstream processing and purification, (4) allows for relatively simple genetic manipulation because well-characterized yeast expression vectors (some derived from the extensively studied *S. cerevisiae*) are available and commercial expression kits are available, (5) does not present any known human pathogenicity, (6) produces structurally and functionally correct recombinant eukaryotic proteins as a result of its capability to perform a variety of post-translational modifications including glycosylation, polypeptide folding, acylation, proteolytic processing, methylation, and targeting to subcellular compartments, and (7) offers secretion of extracellular proteins as well as the option to genetically engineer secretion signals into expression vectors, reducing downstream purification requirements (Li *et al.* 2007b. Macauley-Patrick *et al.* 2005. Calik *et al.* 2015).

In order to develop a successful *P. pastoris* recombinant protein expression system as part of the upstream processing of an industrial production system, there a few crucial steps. Evaluating the choice of expression vector and host strain are both integral in the development of a recombinant protein expression system. Further, the selection of promoter-terminator systems, selection markers, and consideration of secretion signals for extracellular secretion all affect recombinant protein production (Ahmad *et al.* 2014).

2.4.3.2.1 *P. pastoris* promoter systems

The oldest and most widely used promoter in *P. pastoris* expression systems is P_{AOX1} of the alcohol oxidase gene. P_{AOX1} was first discovered and applied by SIBIA during the 1970s and is a strong, tightly regulated inducible promoter. The use of this promoter is based on methylotrophic metabolism in *P. pastoris*, where methanol is utilised as a carbon and energy source. The basis for these *P. pastoris* expression systems is the fact that enzymes required for the metabolism of methanol are only expressed when yeasts are grown on methanol substrates (Macauley-Patrick *et al.* 2005). Methanol is metabolised by first being oxidised to formaldehyde via the alcohol oxidase enzyme. Thus, expression of the *AOX1* gene is initiated by P_{AOX1}, which is induced in the presence of methanol (Cregg and Higgins, 1998). The main advantages of P_{AOX1} are the strength and extent of regulation, the fact that it can be used to produce recombinant products toxic to the expression host and the added control of inducible expression (Vogl and Glieder, 2013). While P_{AOX1} has been successfully applied in a number of recombinant protein production systems, there are some disadvantages that have warranted the development and use of alternative promoters. Methanol is a potential fire hazard and storing large quantities in industrial environments is a risk. It is also of petro-chemical origin, which makes methanol unsuitable in the production of certain food additives or products. In addition, two carbon sources are required as methanol can only be added at the induction stage of fermentation and lastly, methanol is toxic to cells over certain concentrations and must be closely monitored throughout the fermentation process, adding many complexities to process design optimisation (Macauley-Patrick *et al.* 2005). Thus, in cases where the recombinant product is not toxic to the expression host, a strong constitutive promoter system can be applied, which allows for a reduction in culture optimisation strategies and high recombinant protein production in a shorter amount of time (Vogl and Glieder, 2013).

Constitutive promoter systems have become a popular option in recombinant protein expression, due to the ease of process control and omission of potentially hazardous inducers

such as methanol (Ahmad *et al.* 2014). The most commonly used constitutive promoter is P_{GAP} of the glyceraldehyde-3-phosphate gene. P_{GAP} was first isolated in 1997 by Waterham and colleagues, whom concluded that P_{GAP} had similar expression levels to P_{AOX1} when grown on glucose. P_{GAP} is a strong, constitutive promoter system that does not require optimisation of the methanol-induction step, is more suited to industrial scale operations as it negates the potential risk and expense in storing and transporting large amounts of methanol, and does not require strict optimisation and monitoring of culture conditions. Additionally, methylotrophic cell metabolism produces detrimental by-products such as formaldehyde and hydrogen peroxide (Calik *et al.* 2015). These advantages all contribute to the cost-effectiveness of industrial production of non-toxic recombinant proteins using the *GAP* promoter system (Cos *et al.* 2006).

Empirical data suggests that P_{AOX1} expression systems often produce higher protein concentrations; however, this is only true under tightly regulated, extensively optimised process conditions. Additionally, some contrasting results have been reported when comparing recombinant protein concentrations of P_{GAP} and P_{AOX1} expression systems. In the production of human chitinase using fed-batch fermentation, P_{GAP} was found to be superior to P_{AOX1} (Goodrick *et al.* 2001). Similarly, P_{GAP} produced yields of 1.3-fold higher than P_{AOX1} of fructose-releasing exo-levanase (LsdB). Conversely, P_{AOX1} has been found to be superior in a number of proteins including cellobiohydrolase, Hepatitis B virus surface antigen (HBsAG), and β-glucuronidase (Boer *et al.* 2000. Vassileva *et al.* 2001. Sears *et al.* 1998). Other studies have found similar protein production between the two promoter systems, such as in the production of the endoglucanases, where Varnai *et al.* (2014) reported that P_{GAP} performed similarly or better than P_{AOX1}.

2.4.3.2.2. Recombinant protein production in *P. pastoris*-P_{GAP} expression systems

The *P. pastoris*-P_{GAP} expression system has been used to successfully produce a wide variety of eukaryotic recombinant proteins (Table 2.2). *P. pastoris* produces high titres of recombinant proteins, with some reaching gram per litre levels. An example of this is the optimised production of S-Adenosyl-L-methionine (SAM) synthetase 2 under P_{GAP} regulation in *P. pastoris* that reached 2.49 g/L after 3 days of fermentation (Cos *et al.* 2006).

Table 2.2. Recombinant protein production in *P. pastoris*-P_{GAP} expression system (adapted from Cos *et al.* 2006).

Protein	Substrate	Protein concentration (mg/L)	Scale	Reference
Insect esterase	Glucose	80	Shake flasks	Delroisse *et al.* 2005
Fructose exo-levanase	Glycerol	26.6	Fed-Batch, 7.5 L Bioreactor	Menendez *et al.* 2003
Human granulocyte-macrophage colony-stimulating factor	Glucose	90	Shake flasks	Wu *et al.* 2003.
h-Chitinase	Glucose	450	Fed-Batch, 3 L Bioreactor	Goodrick *et al.* 2001

In terms of the three ligninolytic enzymes of interest, limited to no literature is available on recombinant enzyme production using the proposed *P. pastoris*-P_{GAP} expression system for the specific genes selected for this study, emphasising the novelty in producing the enzymes using this expression system. However, previous research has been done on the target enzymes using alternative expression systems and recombinant genes, which will be discussed in the following paragraphs (Table 2.3).

Table 2.3. Summary of previous studies that produced recombinant ligninolytic enzymes of interest

Enzyme	Promoter	Enzyme Activity	Enzyme Assay	Protein concentration (mg/L)	Mode of Operation	Reference
Laccase	P_{AOX1}	23.9 U/L	Spectrophotometric: ABTS oxidation	-	Shake Flasks	O'Callaghan *et al.* 2002
	P_{AOX1}	140000 U/L, 1380 U/mg	Spectrophotometric: ABTS oxidation	130	Fed-Batch, 2.5 L Bioreactor	Hong *et al.* 2002
	P_{AOX1}	2520 U/L, 2.6 U/mg	Spectrophotometric: ABTS oxidation	120	Fed-Batch, 2 L Bioreactor	Colao *et al.* 2006
	P_{AOX1}	83000 U/L	Spectrophotometric: ABTS oxidation	-	Shake flasks	Hong *et al.* 2002
	P_{AOX1}	34231 U/L	Spectrophotometric: ABTS oxidation	-	Shake flasks	Li *et al.* 2014a
	P_{GAP}	9030 U/L	Spectrophotometric: ABTS oxidation	-	Shake flasks	Liu *et al.* 2003

P_{GAP}	2.8 U/L	Spectrophotometric: ABTS oxidation	100	Fed-Batch, 500 ml Bioreactor	Bohlin *et al.* 2006
P_{GAP}	53300 U/L, 40 U/mg	Spectrophotometric: ABTS oxidation	517	Fed-Batch, 5 L Bioreactor	Kittl *et al.* 2012
P_{GAP}	451.08 U/L	Spectrophotometric: ABTS oxidation	416.40	Shake flasks	Rivera-Hoyos *et al.* 2015
P_{GAP}	3000 U/L	Spectrophotometric: N,N-dimethyl-p-phenylenediamine (DMPPDA) oxidation	-	Shake flasks	Balakumaran *et al.* 2016
P_{GAP}	60000 U/L	Spectrophotometric: ABTS oxidation	-	Fed-Batch, 2.5 L Bioreactor	Pezzella *et al.* 2017
P_{AOX1}	1800 U/L	Spectrophotometric: Reduction of *cytochrome* c in presence of cellobiose	79	Shake flasks	Yoshida *et al.* 2001

Cellobiose dehydrogenase	P_{AOX1}	7955 U/L, 1040 U/mg	Spectrophotometric: Reduction of *cytochrome* c in presence of cellobiose	7.68	Shake flasks	Stapleton *et al.* 2004
	P_{AOX1}	2150 U/L, 3.4 U/mg	Spectrophotometric: DCPIP reduction	-	Fed-batch, 4 L Bioreactor	Zamocky *et al.* 2008.
	P_{AOX1}	652 IU/L,	Spectrophotometric: DCPIP reduction	306.6	Shake flasks	Zhang *et al.* 2011
	P_{AOX1}	7800 U/L, 22.2 U/mg	Spectrophotometric: DCPIP reduction	-	Fed-batch, 1 L Bioreactor	Bey *et al.* 2011
	P_{AOX1}	376 U/L, 9.4 U/mg	Spectrophotometric: DCPIP reduction	633	Fed-batch, 7 L Bioreactor	Ma *et al.* 2017
Glucuronoyl esterase	P_{AOX1}	5.2 U/L	HPLC: measuring decrease of MeGlcA2	-	Shake flasks	Topakas *et al.* 2010

ABTS: 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), substrate sensitive to laccase oxidation

DCPIP: 2,6-Dichlorophenolindophenol, substrate that decolourizes when reduced by CDH

2.4.4 Bioprocessing aspects of recombinant protein production in *Pichia pastoris*

2.4.4.1 *Effects of cultivation conditions on recombinant protein production in* Pichia pastoris

Recombinant protein production levels are affected by strain-specific factors as well as cultivation-dependent factors. Temperature, pH, and dissolved oxygen (DO) concentrations have been shown to be notable cultivation-dependent factors in the production of recombinant proteins. These factors influence recombinant protein production at the molecular level or through affecting cell growth (Potvin *et al.* 2012).

The optimal temperature for *P. pastoris* growth is 30 °C (Cos *et al.*2006). However, the effects of temperature on protein secretion and protein stability must also be considered during recombinant protein production. Lower cultivation temperatures have been shown to improve recombinant protein secretion in some cases. This may be attributed to the fact that at higher temperatures, the stability of the recombinant protein may be decreased, proteases are released from dead cells, and protein folding errors occur (Macauley-Patrick *et al.* 2005). Lowering of cultivation temperature has improved protein secretion in *P. pastoris* for recombinant herring antifreeze protein, galactose oxidase, human m-opioid receptor fusion protein, endoglucanases, and single chain antibodies (Daly and Hearn, 2005). Of note in the current study are the results of Hong *et al.* (2002) and Bohlin *et al.* (2006), where higher laccase production was achieved by decreasing cultivation temperature. However, lower temperatures may also affect the growth rate of recombinant *P. pastoris*, and therefore the time and productivity of the bioprocess may be affected. In the context of the current study, where baseline levels of recombinant protein were determined, the recommended temperature of 30 °C ensured optimal growth of *P. pastoris*. In addition, the target enzymes are stable at this temperature, and would not be degraded.

Recombinant enzymes are sensitive to degradation when they are exposed to pH values above or below their optimal pH range. In addition, the activity of certain extracellular proteases is affected by pH (Potvin *et al.* 2012). Since *P. pastoris* is capable of growth in a wide pH range, the pH setpoint of a particular cultivation process should be dependent on the requirements of the recombinant protein itself, as well as its effect on protease activity (Macauley-Patrick *et al.* 2005). Therefore, the most commonly used pH values in *P. pastoris* cultivation are between

pH 5 and pH 6, as this reduces protease activity, while still maintaining protein stability (Cos *et al.* 2006).

The most widely used dissolved oxygen (DO) setpoints used in *P. pastoris* cultivation range between 20 – 30 %. DO concentration may be controlled by agitation rate as well as air or oxygen flow rate (Li *et al.* 2007). It is generally accepted that *P. pastoris* requires fully aerobic conditions for optimal growth at high cell densities. *P. pastoris* is a 'Crabtree' negative microorganism, and is therefore more sensitive to oxygen availability. Decreased oxygen availability causes a shift in core metabolism from respiratory to fermentative growth. This shift in metabolism affects both biomass growth, protein production and the protein secretion pathway (Baumann *et al.* 2010).

Considering this, it is surprising that conflicting results have been reported in the effect of DO concentration on recombinant protein production. Hypoxic conditions have been found to improve recombinant protein production in a number of cases (Hu *et al.* 2008. Baumann *et al.* 2008). Conversely, increasing DO tension has also improved the production of a recombinant peptide (Lee *et al.* 2003. Jin *et al.* 2010). The mechanisms by which DO concentration affect recombinant protein production are complex and difficult to singularly identify. Baumann *et al.* (2010) found hypoxia to influence transcriptomic, proteomic and metabolic fluxes in *P. pastoris*. Further, it was found that adaptation to lowered oxygen availability affected stress response as well as protein folding and secretion. In order to keep the fermentative conditions consistent throughout the current study, an aerobic fermentation strategy was selected, at a level of 30 %.

2.4.4.1 Modes of operation for constitutive recombinant protein production in Pichia pastoris

In addition to determining cultivation conditions, the selection and development of the operational mode of a bioprocess is essential for producing high concentrations of recombinant protein. The most widely used operational modes in recombinant protein production are batch or fed-batch fermentations (Potvin *et al*. 2012). Fed batch fermentations involve feeding of a selected substrate continuously or intermittently, as determined by monitoring substrate demand of the culture. Fed-batch fermentations are widely implemented as they increase biomass growth and increase yields of recombinant protein products (Lee *et al*. 1999).

While a wealth of literature exists for the optimisation of P_{AOX1} operational modes, much less information exists for the use of P_{GAP}. Since P_{GAP} expression has been shown to be largely growth associated, bioprocesses have been focussed on biomass cultivation. In general, the production process consists of two stages or operational modes. The first stage involves a batch phase, and the second involves a fed-batch stage (Calik *et al.* 2015).

During the batch phase, the main objective is biomass proliferation. A carbon source is supplied at concentration of 10 – 50 $g.L^{-1}$, and the culture is inoculated into the fermentation vessel. The culture then undergoes a typical growth curve, consisting of a lag phase, followed by an exponential growth phase, and finally a stationary phase. The batch phase is concluded, usually after 24 hours, when the carbon source has been depleted, and a certain biomass concentration is reached. The depletion of the carbon source is accompanied by a decrease in agitation rate, increase in DO concentration, and a slight increase in pH (Calik *et al*. 2015). Once this occurs, batch-operation is shifted to fed-batch operation.

During the fed-batch stage, the substrate (supplemented with additional trace salts) is fed into the bioreactor. The rate at which the substrate is fed is determined using several strategies. The most widely used strategies are feeding at a constant rate, using a DO-stat control, or the use of μ-stat control (Lee *et al*. 1999). Feeding at a constant rate requires the least complex monitoring, and can easily be implemented. DO-stat control involves an indirect feedback control system, where feeding is initiated or concluded (at a pre-determined rate), based on the DO concentration during the cultivation (Lee *et al*. 2003). Lastly, μ-stat control involves adjusting the feeding rate exponentially throughout the cultivation to maintain a certain specific growth rate (Potvin *et al.* 2012). Generally, the fed-batch stage is concluded once the volumetric limit of the bioreactor vessel is reached.

Unfortunately, the performance of each of these feeding strategies in a constitutive *P. pastoris* cultivation has not been systematically researched. Therefore, it is difficult to assume which strategy would be optimal for recombinant protein production. Considering this, it is important to investigate the effects of each of these strategies when optimising the bioprocess for recombinant protein production. However, to determine a baseline level of recombinant protein production, as was done in this study, a constant feeding strategy involves the least risk and the simplest execution of the three strategies.

2.4.5 Recombinant laccase production in *P. pastoris*

Laccase is arguably the most extensively studied lignin-degrading enzyme. Fungal laccases have been expressed in a wide variety of host organisms, the majority being yeasts and filamentous fungi, although there are examples of expression in bacteria (Antošová and Sychrová, 2016. Bohlin *et al.* 2006. Nicolini *et al.* 2013). Due to the ubiquity of the laccase enzyme, there are several source organisms from which laccase genes are isolated for heterologous expression (Upadhyay *et al.* 2016). The most common in published literature being *Trametes versicolor* laccase genes. Additionally, laccases are secreted by these source organisms in a number of isoforms with varying activities and biochemical characteristics (Jönsson *et al.* 1997. Colao *et al.* 2006). Most studies have employed the inducible P_{AOX1} promoter systems, however constitutive expression in laccase has been comparatively well-researched in terms of the three target enzymes of this study. Volumetric activity yields differ significantly (23.9 – 140000 U/L) between publications, owing to the varied recombinant genes, different laccase isoforms, differing cultivation strategies used, as well as differences in the assays that measure volumetric activities. The following paragraph will focus on the most relevant examples of fungal laccase expression in *P. pastoris* only.

The first example of expression in *Pichia pastoris* was reported by Jönsson *et al.* (1997), where *Trametes versicolor* laccase isoenzyme *lcc1* was expressed under P_{AOX1} regulation. The same gene used by Jönsson *et al.* was subsequently cloned by Hong *et al.* (2002) under P_{AOX1} regulation and expressed in *P. pastoris* in a bioreactor cultivation. Hong *et al.* (2002) reported a maximum activity of 140000 U/L under optimised cultivation conditions. Studies that followed have failed to improve on this volumetric activity value (Table 3). The second and third highest attained volumetric activity values, 83000 U/L and 34231 U/L, as reported by Hong *et al.* (2007) and Li *et al.* (2014), were both attained using isoforms of *T. versicolor* laccase, expressed under P_{AOX1} control, at shake flask-scale. Significantly lower volumetric activities were found for *Trametes trogii* laccase (*lcc1*) inducibly expressed in *P. pastoris* by Colao *et al.* (2006). These authors reported a maximum volumetric activity of 2520 U/L after cultivation in a 2.5 L bioreactor.

Constitutive expression of laccase in *P. pastoris* has shown promising results, although values remain below the benchmark value set by Hong *et al.* (2002). Liu *et al.* (2002) constitutively expressed *Fome lignosus* laccase (unspecified isoform) in *P. pastoris*, and reported a maximum activity of 9030 U/L. These authors showed that use of the native secretion signal was more

effective than the α-factor secretion signal, and that growth temperature and supplementation with copper were important process parameters. Bohlin *et al.* (2006) subsequently compared the expression of *T. versicolor* laccase (isoforms *lcc1* and *lcc2*) in *P. pastoris* and *Aspergillus niger*. Native secretion factors were used. The maximum activity of *lcc2* laccase in *P. pastoris* in a 500 ml fed-batch bioreactor cultivation was 2.8 U/L. This value is of importance in the current study as the *lcc2* isoform is constitutively expressed in *P. pastoris*. Following this work, the volumetric activity yields were vastly improved by researchers. Kittl *et al.* (2012), expressed *Botrytis aclada* laccase (isoform *Ba*Lac) in a 5 L bioreactor cultivation and reported a maximum activity of 53300 U/L, while Pezzella *et al.* (2017) recently reported a maximum activity of 60000 U/L of *Pleurotus ostreatus* laccase (isoform POXA1b) in a 2.5 L bioreactor cultivation. Both studies show that constitutive expression of laccase can viably compete with expression levels found in methanol-induced expression studies.

2.4.6 Recombinant cellobiose dehydrogenase production in *P. pastoris*

Cellobiose dehydrogenase (CDH) is an extracellular hemoflavoenzyme secreted by a number of white-rot and some brown-rot fungi (Section 3.1.2). Yoshida *et al.* (2001) published some of the earliest research in recombinant CDH production. These authors expressed a *Phanerochaete chrysosporium* CDH in a P_{AOX1} expression system and reported enzyme activity of 1800 U/L and protein concentration of 79 mg/L after four days of methanol-induction. This enzyme activity was significantly higher than that produced by the native strain and showed future potential for recombinant CDH production (Yoshida *et al.* 2001). Following Yoshida and colleagues, Stapleton *et al.* (2004) cloned and expressed a CDH of *Trametes versicolor* in *P. pastoris* under P_{AOX1} regulation. It was reported that a CDH activity of 1040 U/mg total soluble protein was found in the culture supernatant after 12 days in a small-scale baffled flask fermentation process. In 2008, Zamocky *et al.* cloned and expressed a thermostable CDH originally isolated from *Myriococcum thermophilum* in *P. pastoris* using the pPICZαB expression vector that contains the alcohol oxidase (*AOX1*) gene promoter and terminator. These authors obtained a maximum volumetric activity of 2150 U/L of the crude concentrated enzyme that was produced in a fed-batch system using glycerol for initial biomass growth. This was followed by Zhang *et al.* (2011), whom expressed a CDH of *Neurospora crassa* in *P. pastoris* using the *AOX1* inducible promoter. Zhang and colleagues performed the study at a small scale using 200 ml cultures and obtained 652 IU/L enzyme activity and total yield of 76.65 mg/L. Another study successfully expressed a CDH of *Pycnoporus*

cinnabarinus in *P. pastoris* under P_{AOX1} regulation, using the yeast α-factor secretion signal and a histidine tag for ease of purification. These authors report a maximum enzyme activity of 7800 U/L after optimisation of a 1 L fed-batch bioreactor fermentation process (Bey *et al.* 2011). This enzyme activity was much higher than previously found by other researchers, however direct comparisons of enzyme activity must be performed with caution as there may be differences in either the protein structures, thereby affecting correct folding and transport, or enzyme assay protocols that determined these activities.

2.4.7 Recombinant Glucuronoyl esterase production in *P. pastoris*

Glucuronoyl esterase is a newly identified enzyme, making it a novel target for production, and most literature has focussed on fundamental research aspects (Section 4.1.1). Recently, as the industrial significance of lignin-carbohydrate complexes has become clear, a small number of publications has explored their applications. Topakas *et al.* (2010) functionally expressed a *Sporotrichum thermophile* glucuronoyl esterase in *P. pastoris* under P_{AOX1} regulation and found an enzymatic activity of 5.2 U/L using 4-nitrophenyl 2-O-(methyl-4-O-methyl-α-D-glucopyrano- syluronate)-β-D-xylopyranoside (MeGlcA2) substrate. d'Errico *et al.* (2015) reported the recombinant expression of functionally active glucuronoyl esterases of both *Cerrena unicolor* and *Scizophyllum commune* in the industrial filamentous fungus *Aspergillus oryzae*.

2.5 Molecular Genetics and Recombinant Enzyme Production: How Inherent Properties of Proteins affect their Production in Recombinant Systems

The current study aims to constitutively express ligninolytic enzymes of fungal origin in *P. pastoris*. Yoshizumi and Ashikari (1987) reviewed potential reasons for inefficient fungal enzyme production in yeasts. Impacts of differences in promoters, introns, codon usage, secretion and glycosylation in filamentous fungi and yeasts were considered. The transcription of fungal genes in yeasts may be affected by differences in conserved intron sequences. Inaccurate excision of fungal introns in yeasts has previously been found to negatively affect recombinant fungal hydrolase production in yeasts. In addition, differences in molecular weights of native and recombinant enzymes may be present as a result of varying extents of glycosylation, although this was not found to affect the activity or physical properties of expressed enzymes.

Key molecular bottlenecks in the production of recombinant proteins are incorrect mRNA structure and gene copy number, limited transcription, translation and protein translocation into the endoplasmic reticulum (ER), partial protein folding and inefficient protein targeting to the extracellular environment (Ahmad *et al.* 2014).

2.5.1 Gene Expression

While recombinant gene expression is an important consideration in protein yields, it has become apparent that the efficacy of extracellular recombinant protein production is governed to a higher degree by post-translational processing of the protein. The processing capacity of the secretory pathway is finite, and therefore increased intracellular protein production may not reflect in the final protein yield. Over-expression may even be detrimental to recombinant protein production in some cases (Inan *et al.* 2005). Thus, optimal gene expression does not refer to the maximum level of gene expression, but rather to the ideal level of gene expression where enough of the protein is produced to be functionally secreted at an optimal rate without saturating the posttranscriptional modification- and secretion-molecular machinery (Wittrup *et al.* 1994. Inan *et al.* 2005).

2.5.2 Gene Translation

Translation is the ribosome-mediated process of translating mRNA to amino acids during protein synthesis. The efficiency of translation can affect optimal recombinant protein production (Kane, 1995). The most widely investigated effect in terms of translation is known as the codon bias. Codon bias refers to variation in the frequency of occurrence of synonymous codons in different organisms. Simply put, the DNA sequence used to encode a protein in one organism differs from the DNA sequence used to encode the same protein in a different organism. The frequencies of synonymous codons vary between organisms, high and low expression levels of the target gene in the organism and within operons. Available tRNAs for translation are ‘biased’ to more frequent codons and less prevalent codons have proportionally less available tRNAs, which serves to optimize the natural translation system. The difference in tRNA availability affects the rate and efficiency of translation, and therefore secretion and yield of heterologous proteins. A number of strategies to alleviate codon bias have been used, including the construction of synthetic recombinant genes using preferred codons of the host organism (codon optimisation), and over-expression of rare tRNA-encoding genes (Gustafsson *et al.* 2004).

The efficiency of translation initiation (and therefore elongation) is influenced by the arrangement of synonymous codons in the mRNA strand. In a number of genes, the first 30-50 codons are recognised by lower frequency tRNAs, effectively slowing the rate of translation initiation, before maintaining an optimal speed of translation during elongation. Slowing translation initiation is hypothesized to increase protein synthesis efficiency during heavy ribosomal traffic in highly expressed genes by preventing stalling during elongation and premature termination. Additionally, slow translation initiation may be beneficial for protein folding by facilitating interactions of the synthesised polypeptide with folding chaperone proteins, thus improving the proportion of correctly folded proteins (Fredrick and Ibba, 2010). Compounding to this is the concept of auto- and anti-correlated codons, which influences the speed of translation elongation. This refers to the particular arrangement of codons within an open reading frame. Cannarozzi *et al.* (2010) explored this concept in *S. cerevisiae* and found that translation efficiency increased in genes where the same codon was repeated and tRNAs could be 'recycled'. This was in comparison to genes composed of non-consecutive, varying codons that necessitated different tRNAs, where lower translation efficiency was found.

Lastly, the 5' upstream untranslated region (UTR) has also been targeted for improved recombinant protein production. The length and nucleotide composition of the UTR affects translation initiation and termination efficiency, as well as mRNA stability (Fleer, 1992). One strategy for improving recombinant protein yields is to optimise the 5' UTR by replacing the native 5' UTR with that of a highly expressed gene. An example of this is the use of an alcohol oxidase 5' UTR for expression of human serum albumin, where a 50-fold increase in protein production was attained. In terms of nucleotide sequence, a composition of more than 63% total adenine and uracil is generally preferred and the length of the 5' UTR should not exceed 126 nucleotides (Sreekrishna, 1993). In *P. pastoris* P_{AOX1} expression systems it has been found that the 5' UTR should be identical or as similar as possible to that of the *AOX1*-mRNA for optimal recombinant protein yields (Sreekrishna *et al.* 1997).

2.5.3 The Secretory System

Protein secretion in yeast is governed by an amino-terminal signal sequence that directs co- or post-translational translocation into the lumen of the endoplasmic reticulum (ER), after which the signal peptide is cleaved by a signal peptidase (Romanos *et al.* 1992). This intracellular protein 'trafficking' process to the ER is affected by protein properties. Two main translocation routes are used, namely co-translational translocation, where proteins are transported during

translation, or post-translational translocation where proteins are transported after translation. During early protein co-translocation, the protein targeting specificity is largely dependent on the hydrophobic core of the signal peptide sequence and its interaction with signal recognition particles in the cell cytoplasm. Thus, proteins with high hydrophobicity in this area are more likely to undergo co-translational translocation, while proteins with lower hydrophobicity are more likely to undergo post-translational translocation (Idiris *et al.* 2010. Johnson *et al.* 2013). The protein then undergoes one or many post-translational modifications depending on the functional structure of the target protein. These include folding into the functional state, disulphide-bond formation, and glycosylation (Ahmad *et al.* 2014). During high levels of expression, proteins may be retained in the ER, adding to inefficient protein production. Studies suggest that the limiting factor of secretion may be the preservation of the correct tertiary structure of recombinant proteins or inadequate levels of protein folding chaperones in the ER (Robinson *et al.* 1996. Rakestraw and Wittrup, 2005). Thus, the quality control system of the ER has been a target for improving recombinant protein yields (Idiris *et al.* 2010). The unfolded protein response (UPR) pathway is activated under ER-stress conditions and initiates transcription of ER chaperone proteins (Ma and Hendershot, 2001). Over-expression of ER-located folding helper proteins and a transcriptional regulator of the UPR, *HAC1*, have both been successful in overcoming the limitation of correct protein folding in the ER (Ahmad *et al.* 2014). Over-expression of the protein folding chaperones has shown increased protein yields, suggesting that improving protein folding capacity in the ER may improve recombinant protein yields (Damasceno *et al.* 2012). The modified proteins are then transported in vesicles to the Golgi apparatus, where further modifications to the attached glycosides are made. The proteins are then packaged into secretory vesicles that move to the cell surface and release the proteins extracellularly (Romanos *et al.* 1992).

Previous studies comparing production of different proteins in the same expression system have highlighted the importance of secretory system stresses in differing product yields. Rakestraw and Wittrup (2005) evaluated the impacts of secretory system stresses in two proteins with differing size and complexity, namely bovine pancreatic trypsin inhibitor (BPTI), a single domain protein containing 58 residues and three disulphide bonds, and lysozyme-binding single-chain antibody (scFv), a two-domain protein with two disulphide bonds. BPTI was produced in high concentrations while scFv was produced at much lower efficiency. It was concluded that the differing production yields were as a result of secretory stress mechanisms

between the two proteins and the fact that the proteins were affected differently by increased secretory load.

One of the major advantages of using *P. pastoris* in recombinant protein production is its extensive post-translational modification system. *P. pastoris* is capable of a number of post-translational modification processes, including processing of signal sequences, protein folding, disulphide bridge formation, selected addition of lipids, and *N*- and *O*-linked glycosylation (Cereghino and Cregg, 2000. White *et al.* 1994). Strategies to improve protein secretion in *P. pastoris* include the use of a recombinant signal peptide, mainly *S. cerevisiae* α-Mating Factor (MF) prepro signal sequence. Processing (cleavage) of the α-MF has been shown to be affected by both the surrounding amino acids and the tertiary structure of the recombinant protein (Section 6.4). In addition, the acid phosphatase (*PHO1*) signal peptide, native to *P. pastoris* has also been used with positive results (Cereghino and Cregg, 2000).

2.5.4 Inherent Protein Properties affect Protein Yields

From the above (Section 2.5) it is clear that different proteins will be produced in varying yields by the same expression system as a result of differences in protein folding requirements, sequences, and amino acid demands. These differences all contribute to varying amounts of cellular stress, which ultimately affects the final protein yield (Liu *et al.* 2012). This is illustrated in proteins of different sizes and complexity. Small, simple proteins that require less complex post-translational modification will be produced more efficiently with minimal reliance on cellular resources, while large, complex multi-domain proteins require more cellular resources in the form of folding chaperones of the ER and cellular secretory processes (Tutar and Tutar, 2010). Further, successful strategies for production of one protein will not necessarily work when implemented in a different protein (Liu *et al.* 2012). Possible factors that may affect secretion and therefore yield of the target proteins are summarised in Table 4.

Table 1.4. Protein Properties of Ligninolytic Enzymes of Interest

Enzyme	Source Organism	Molecular Weight (kDa)	Glycosylation	Structural Complexity	Post-translational Processing	Reference
Cellobiose Dehydrogenase	*Neurospora crassa*	80-115	Yes Mainly *N*-glycosylation ± 8 %	Two domains: Heme and FAD, linked via flexible polypeptide	FAD Domain: β-sandwich fold Heme Domain: β structure fold	Hallberg *et al.* 2002 Hallberg *et al.* 2000
Glucuronoyl Esterase	*Hypocrea jecorina*	43	Yes *N*-linked glycosylation site	Two domains: Catalytic domain and carbohydrate binding domain (CBM) linked via polypetide	3 Disulphide bonds, important for activity of catalytic domain Three-layer αβα-sandwich hydrolase fold	Charavgi *et al.* 2013 Pokkuluri *et al.* 2011
Laccase (isoform *lcc2*)	*Trametes versicolor*	74	Yes *N*-glycosylation sites	Three domains, with β-barrel architecture	2 Disulphide bonds, forming the tertiary protein structure	Piontek *et al.* 2002

Chapter 3: Aims and Objectives

Lignin valorisation is a key breakthrough in developing efficient integrated biorefineries. Unfortunately, this aspect has been largely neglected until recently, due to the recalcitrance and complexity of lignin-enriched feedstocks. Enzymatic modification or upgrading of technical lignin has garnered attention as a potential environmentally benign avenue for lignin valorisation.

A number of eukaryotic and prokaryotic microorganisms utilise enzymatic systems for lignin biodegradation. These enzymatic systems could potentially be harnessed and applied in an integrated biorefinery context for valorisation of lignin. Enzymes of importance in these enzyme systems are laccases, peroxidases, and a number accessory enzymes as well as radical compounds that aid in lignin degradation (Pollegioni *et al.* 2015). Considering the multi-faceted nature of native lignin-degrading systems, a number of potential targets for biotechnological applications exist.

For enzyme-mediated upgrading of technical lignins to be a feasible strategy for lignin valorisation, the enzymes must first be produced or sourced in large quantities. In terms of the current study, three ligninolytic enzymes, namely glucuronoyl esterase, cellobiose dehydrogenase and laccase have been selected for production for future use in lignin valorisation application studies. These enzymes are representative of carbohydrate degradation (GE, CDH: Fenton reaction) and lignin degradation and/or modification (LCC). Although there are enzyme preparations of laccases commercially available, these contain undesired side activities and additives that may not be suitable for specific applications. Pure enzyme preparations of laccase and cellobiose dehydrogenase are provided in small amounts by different suppliers (i.e. Sigma, Creative-Enzymes, Jena Bioscience) at prohibitive costs for industrial application. Moreover, preparations of pure glucuronoyl esterase are not commercially available.

While *P. pastoris* has significant potential for production of the selected target enzymes, in terms of functional secretion in appreciable amounts, some limitations exist for industrial application of the *P. pastoris* expression system. One these obstacles is the routine use of a methanol-induced promoter (P_{AOX1}) in the recombinant protein expression strategy. The use of methanol at a large scale poses a number of challenges in that it is toxic (both to human operators and to the yeast itself), flammable, more costly (as it requires an additional carbon source in the process), and restricts downstream applications as it is of petro-chemical origin

(Calik *et al.* 2015. Macauley-Patrick *et al.* 2005). The main alternative to P_{AOX1} is the constitutive P_{GAP} promoter that expresses the recombinant gene throughout the growth cycle. The use of P_{GAP} in non-toxic recombinant protein expression in *P. pastoris* has steadily increased due to the simplicity of the bioprocess, comparable protein production concentrations, and reduced over-all risk.

Still, there are significant shortcomings in literature on evaluating the feasibility of constitutive expression systems for heterologous production of the selected proteins. A single study exists for constitutive expression of *T. versicolor* laccase *lcc2* (Bohlin *et al.* 2006). *N. crassa* cellobiose dehydrogenase expression has only been investigated using inducible expression (Zhang *et al.* 2011). No studies have attempted any expression of *H. jecorina* glucuronoyl esterase.

Downstream processing of heterologously produced enzymes is an extremely important factor to consider for the economic feasibility of an enzyme production process (Dutta, 2008). Concentrated enzyme products are required for future lignin valorisation application studies. Tangential flow filtration coupled to an ultrafiltration membrane is a relatively simple method of concentration for extracellularly secreted proteins. Simplified downstream processing allows the concentrated enzyme product to be used directly in application studies, reducing over-all costs and equipment requirements, while increasing the time-efficiency of the production process.

In light of the above, this study **aims** to assess the efficacy of a constitutive, patent-free, P_{GAP}-*P. pastoris* expression system, for the production of three enzymes associated with lignin valorisation, namely laccase, cellobiose dehydrogenase and glucuronoyl esterase, using a constant glycerol feeding strategy in a bioreactor cultivation.

In order to achieve this aim, the following specific **objectives** were identified:

- Cloning of selected target genes into the pJexpress 905 vector, which contains the constitutive GAP promoter. This expression vector is free of intellectual property restrictions, and will facilitate industrial implementations of any created strains. This objective was completed in collaboration with Dr García Aparicio at the Department of Microbiology, Stellenbosch University.
- Selection of the best recombinant *P. pastoris* strains for each target enzyme by shake-flask screening and evaluation of enzyme activity or protein concentration. This

assesses clonal variation in terms of protein secretion and gives the highest probability of acceptable secretion levels at bioreactor scale.

- Since limited or no commercial preparations of the target enzymes are available, their production at large quantities will greatly facilitate their use in lignin valorisation research. Additionally, limited to no literature is available for bioreactor cultivations for constitutive expression of the target enzymes. Thus, the selected recombinant *P. pastoris* strains were applied in 14 L bioreactor fermentations using established fermentation conditions and defined glycerol growth media. The following sub-objectives were identified for this process:
    - Create enzyme production profiles during the fermentation process
    - Assess the efficacy of a constant glycerol feeding strategy in terms of biomass and growth kinetics
    - Compare production yields between the three target enzymes
    - Compare enzyme production yields to published data
- Since the three target enzymes were produced for their eventual application in lignin valorisation studies, the final product required concentration and processing for storage. Freezing as a storage strategy was evaluated.

Chapter 4: Materials and Methods

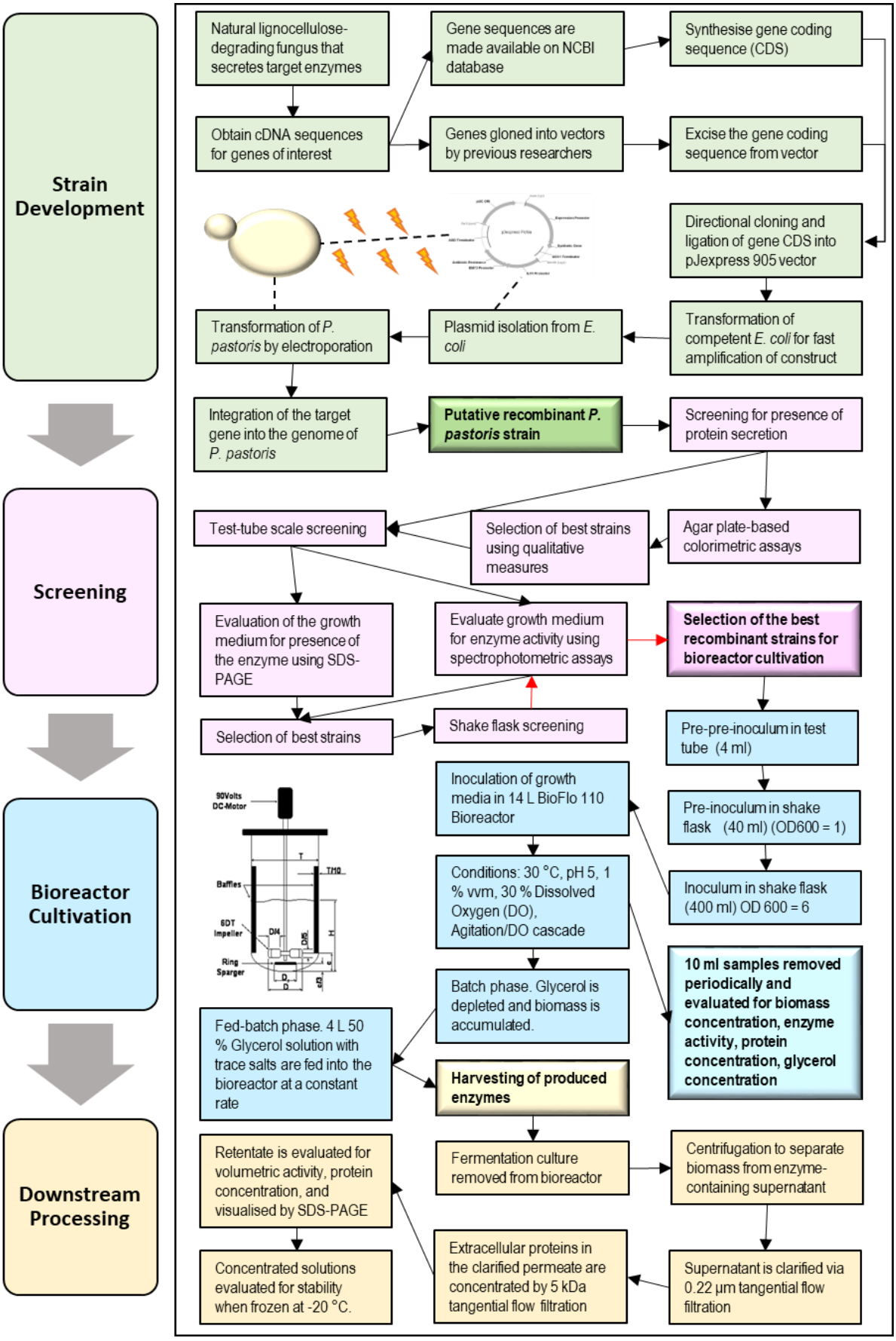


Figure 4.1. Summary flow diagram of methodology followed for each target enzyme. Each phase is delineated by colour and the main deliverables of each phase are highlighted in bold text. Strain development was completed in collaboration with the Department of Microbiology, Stellenbosch University.

4.1 Development of constitutive *Pichia pastoris* expression strains

The recombinant strain development was done in collaboration with Dr García-Aparacio at the Department of Microbiology, Stellenbosch University. *P. pastoris* transformants expressing cellobiose dehydrogenase and laccase were created by Dr García Aparicio. *P. pastoris* transformants expressing glucuronoyl esterase were created by Dr García Aparicio with assistance from Ms Conacher in work pertaining to glucuronoyl esterase described in Sections 4.1.1, 4.1.2 and 4.1.3.

4.1.1 Strains and plasmids

The shuttle expression vector, pJ-express pJ905, was used (DNA2.0, Inc., USA). Six constructs, two for each of the target enzymes, were generated. The commercial pJexpress 905 vector, named pJ905, (DNA 2.0, USA) was used. This expression construct is designed for use in *P. pastoris* and is preferable to other available systems as it avoids intellectual property restrictions. The expression vector includes a zeocin resistance marker, the GAP1 promoter, AOX1 terminator and no secretion signal sequences (Fig 4.2A).

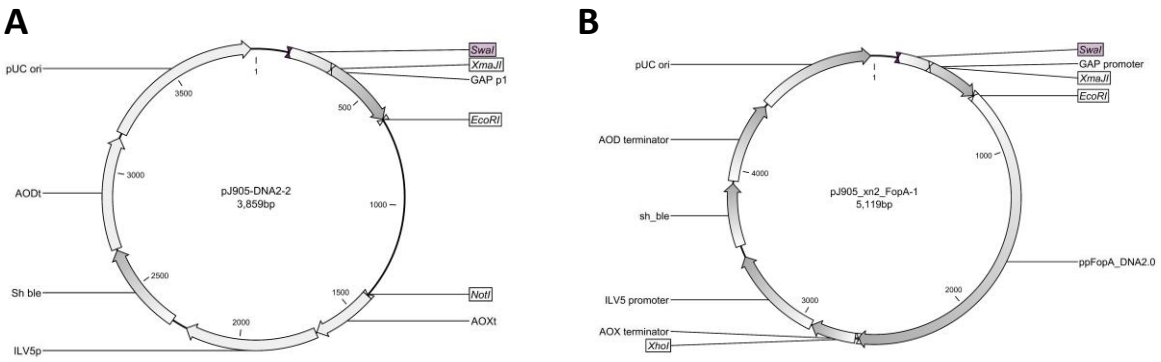


Figure 4.2. Plasmid maps of the Pj905 vector (A) and Pj905_xn_FopA construct (B) used to generate the backbone for the genes evaluated.

Escherichia coli DH5α (New England Biolabs, Midrand, South Africa) competent cells were prepared as per Ausubel *et al.* (1994). *E. coli* DH5α cells were grown in low salt or conventional Luria Bertani broth (Sigma) supplemented with 25 µg/ml zeocin, at 37 °C. The competent cells were used as the microbial host for amplification of all the constructs evaluated.

The *Pichia pastoris* type strain DSMZ 70382 (CBS704) was selected as the expression host of the three target genes. *P. pastoris* cells were grown in YPD/YD and YPDS, containing increasing concentrations of the selective agent, zeocin (100-1000 µg/ml). The zeocin and ampicillin were purchased from Melford Laboratories Ltd, UK or Invitrogen; and Roche, respectively.

4.1.2 Plasmid construction

The coding regions of the three target genes were obtained either by direct digestion for directional cloning with the vector of interest, or by PCR using high fidelity *taq* polymerase Phusion (NEB, England) when the introduction of restriction enzymes of interest was required.

The laccase coding sequence (CDS) for the LCC1 and LCC2 proteins of *Trametes versicolor*, with the native secretion signal, were isolated from a previously cloned pBluescript SK II vectors, kindly provided by Umeå University in Sweden. In order to directionally clone the genes into the pJexpress vector, it was necessary to build in the NotI and EcoRI restriction sites into the gene by PCR using the primers listed in Table 4.1 generating fragment sizes of 1596 and 1591 bp for LCC1 and LCC2, respectively.

Table 4.1. Primers used for introduction of restriction enzymes and sequencing of the selected CDS of laccase.

Genes for LCC	Purpose	Sequence primers (F, forward; R, reverse)	
LCC1, *T. versicolor*	Introduce EcoRI and NotI sites	F	5'- CGA TCC GAA TTC ATG GGC AGG TTC TCA TCT CTC -3'
		R	5'- GCA ATG CGG CCG CTT GAA CCG ATT AGA GGT C -3'
	Sequencing/PCR confirmation	F	5' ACGCATGTCATGAGATTATTGG -3' (GAPp)
		R1	5'- GCA AAT GGC ATT CTG ACA TCC-3' (AOXt)
		R2	5' GATACGAAGGAGACGTCGTT 3' (Internal primer)
LCC2, *T. versicolor*	Introduce EcoRI and NotI sites	F	5'- CGA TCC GAA TTC ATG TCG AGG TTT CAC TCT CTT -3'
		R	5'- GCA ATG CGG CCG CCA TTT ACT GGT CGC TCG G -3'
	Sequencing/PCR confirmation	F	5' ACGCATGTCATGAGATTATTGG -3' (GAPp)
		R1	5'- GCA AAT GGC ATT CTG ACA TCC-3' (AOXt)
		R2	5' GCCATCATAGCGGAGGAT 3' (Internal primer)

The glucuronoyl esterase CDS for the CIP2 protein of *Hypocrea jecorina*, with the native secretion signal was isolated from the previously cloned vector, pMU2097_CIP2, as a 1546 bp EcoRI-XhoI fragment by digesting it with EcoRI and XhoI (Fermentas/Roche). The glucuronoyl esterase CDS for the CIP2 protein of *Chaetomium globosum*, with the native secretion signal was isolated from the previously cloned vector, pMU2095_CIP2, and digested it with EcoRI and NotI (Fermentas/Roche).

Table 4.2. Primers used for introduction of restriction enzymes and sequencing of the selected CDS of glucuronoyl esterase.

Genes for GE	Purpose	Sequence primers (F, forward; R, reverse)	
CIP2 *H. jecorina*	Introduction of EcoRI and XhoI sites	F	5'- CGA TCC GAA TTC ATG GCC TCT AGA TTT TTT GCT -3'
		R	5'- GGA CTA CTC CAA CTT TGT CTT AAT GA C TCG AGG C -3'
Both	Sequencing confirmation (Inqaba)	F	5' ACGCATGTCATGAGATTATTGG -3'
		R	5'- GCA AAT GGC ATT CTG ACA TCC-3'
CIP2 *C. globosum*	Introduction of EcoRI and XhoI sites	F	5'- CGA TCC GAA TTC ATG TTG TCT CCA GCT TTG GCT TC -3'
		R	5'- TTG TCT TAA TGA GGC GCG CCC CTC GAG GC -3'

Two cellobiose dehydrogenase (CDH) CDS from *Neurospora crassa* (NC_CDH) and *Myriococcum thermophyllum* (MT_CDH) with the native secretion signal were evaluated. NC_CDH and MT_CDH were isolated as EcoRI-XhoI digest products from the constructs pMUC1531_NC_CDH and pMUC1531_MT_CDH.

Table 4.3. Primers used for sequencing of the selected CDS of cellobiose dehydrogenase.

Genes for CDH	Purpose	Sequence primers (F, forward; R, reverse)	
CDH, *Neurospora crassa*	Sequencing/PCR confirmation	F1	5' ACGCATGTCATGAGATTATTGG -3' GAPp
		F2	5' TCAATTAGGTTGGGTTCAAGCATTT 3'internal forward
		R1	5' CAATTGTCTTACAATACCATCTGCA 3'internal reverse
		R2	5'- GCA AAT GGC ATT CTG ACA TCC-3'AOXt
CDH, *Myriococcum thermohilum*	Sequencing/PCR confirmation	F1	5' ACGCATGTCATGAGATTATTGG -3' GAPp
		F2	5' TTCAGATGCAAGAACTGCTTGCAA 3'
		R1	5' CAGTCCATTGTAATTGTCTGACGAT 3'
		R2	5'- GCA AAT GGC ATT CTG ACA TCC-3' AOXt

The PCR fragments were ligated into the pJET1.2/blunt (Thermo Scientific CloneJET PCR Cloning Kit). These plasmids were digested with EcoRI and XhoI or EcoRI-NotI. The digested mixtures were run on an agarose gel (0.8% w/v) and DNA was purified using the Zymoclean™ Gel DNA Recovery kit. The purified inserts were then ligated into the same restriction sites of the backbone by carrying out a ligation using T4 DNA Ligase from New England Biolabs. The pJ905 was isolated from pJ905_FopA (Fig. 4.1.B) as a 3400 bp fragment previously digested with EcoRI and XhoI (Thermoscientific) for the CDH and GE. In the case of LCC, the empty plasmid was digested with EcoRI and NotI (fragment of 3086 bp). The recombinant plasmids were named pJ905_Hj_cip2, pJ905_Cg_cip2, pJ905_Tv_Lcc2, pJ905_Tv_Lcc1, pJ905_Mt_cdh and pJ905_Nc_cdh (See Addendum for plasmid maps and sequences).

The recombinant plasmids were then transformed into *E. coli* DH5α for amplification of the constructs and subsequent confirmation by restriction digest. The sequence of the constructs was further confirmed by SANGER sequencing using the primers specified in Table 4.1, Table

4.2 and Table 4.3 (Inqaba Biotechnical Industries (Pty) Ltd, South Africa; CAF Sequencing Facilities, Stellenbosch University).

4.1.3 Transformation of *Pichia pastoris*

Verified constructs were isolated from *E. coli* DH5α using the alkaline lysis method. Plasmids were linearised with *SwaI* digestion. A condensed protocol, which combines heat shock and electroporation, was followed for preparation of competent cells and transformation of *P. pastoris* with the linearized constructs (Lin-Cereghino *et al.* 2005). The electroporation was carried out in electroporation cuvettes (gap, 2.0 mm) in a Gene Pulser® II electroporator (Bio-Rad Laboratories, Hercules, CA, USA) with charging voltage of 1500 V, resistance of 200 Ω and capacitance of 25 μF, resulting in a time constant of about 4.5 ms. After electroporation, the cells were incubated in YPDS overnight. After recovery, transformed cells were spread out in YPDS media with different concentrations of zeocin (100, 250, 500, or 1000 μg/ml). Competent cells with no construct were plated as negative control. The zeocin-containing plates were incubated at 30 ˚C for up to 3 days until colonies grew. Zeocin plates were covered with foil to prevent light degradation.

All transformed colonies were inoculated into 5 ml YPD and grown overnight at 30 °C, with aeration. These cultures were then streaked on YPD agar plates and incubated for 24 hours at 30 °C. To confirm stable integration of the construct (containing the zeocin-resistance marker), single colonies were inoculated into 2 ml 96-well plates, containing 1 ml SC-media supplemented with zeocin (100 μg/ml). The 96-well plate was incubated at 30 °C, at 200 rpm shaking, for 24 hours, after which they were evaluated for growth by OD_{600} measurement. All cultures that did not grow were eliminated. The cultures that grew under zeocin selection pressure underwent total genomic DNA extraction. The genomic DNA was then used as a template for PCR amplification of the target gene, to confirm the integration of the gene into the *P. pastoris* genome, as described in the following paragraph.

Transformants were confirmed by sequencing and detection of the insert at the GAP1 locus in the genomic DNA of the host. The following sequencing primers were used as forward and reverse primers, respectively: 5’ ACGCATGTCATGAGATTATTGG -3’ and 5’- GCA AAT GGC ATT CTG ACA TCC- 3’ (Inqaba). 35 cycles of PCR as recommended by Kappa consisted of: 95˚C-3 min; 95˚C 1 min; 45˚C 15 sec; 72˚C 30 sec; 72˚C 1.21 min; 4˚C (band of 1350 pb).

4.2 Screening of recombinant *P. pastoris* strains[1]

4.2.1 Agar plate-based screening

For putative laccase *P. pastoris* transformants, double strength synthetic complete (SC) agar plates were prepared, supplemented with 0.2 mM ABTS (2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich, USA) and 0.1 mM $CuSO_4$. *P. pastoris* transformant colonies, as well as control strains, were point inoculated on the plates, incubated for 24 hours at 30 °C, and examined for a green halo, indicating ABTS oxidation.

For putative CDH *P. pastoris* transformants, a plate-based assay that contained DCPIP (2,6-Dichlorophenolindophenol) (Sigma-Aldrich, USA) as an electron acceptor was used. The agar media contained the following: 1 % glucose, 0.5 % peptone, 0.2 % yeast extract, 0.1% KH_2PO_4, 0.05 % $MgSO_4 \cdot 7H_2O$, 2 % agar, and 1mM DCPIP. The selected transformants, as well as negative control strains, were point-inoculated directly onto the DCPIP plates and incubated at room temperature for 24 h, followed by 4 °C for a further 24 h. The cool incubation temperature was used after it was observed that the DCPIP in the growth media became reduced when left at warm temperatures for an extended period. The plates were evaluated for clear halos around the inoculated colonies, showing reduction of the DCPIP.

No agar plate-based assays for glucuronoyl esterase are available at this time.

4.2.3 Test-tube screening

To confirm results of the plate-based assays, CDH (*N. crassa* and *M. thermophilum*) transformant colonies showing clear halos were further evaluated for enzyme activity. The active transformants were grown in 5 ml YPD in test tubes for 24 hours. Samples of the 24-hour growth medium were centrifuged at 14000 RPM for 5minutes to separate the yeast biomass from the supernatant. The supernatant was used to confirm enzyme activity.

4.2.4 Shake-flask screening

Growth conditions

Selected transformants were grown in triplicate in BMGY media (Invitrogen, 2002) (supplemented with 0.1 mM $CuSO_4$ for laccase expression), in baffled flasks, at 30 °C, shaken at 220 rpm for 96 hours. *Pichia pastoris* DSMZ 70382 (CBS704) and *Pichia pastoris* DSMZ

[1] Raw data for screening of *T. versicolor* laccase-expressing transformants only were provided by Dr García-Aparico. All other screening (for cellobiose dehydrogenase- and glucuronoyl esterase – expressing transformants) was performed by Ms Conacher.

70382 (CBS704) transformed with an empty PJ905 plasmid were used as negative controls. Samples were taken every 24 hours. Samples of the growth medium were centrifuged at 14000 RPM for 5 minutes to separate the yeast biomass from the supernatant. The supernatant was subsequently used for enzyme activity determination and/or SDS-PAGE. The volumetric activity at 72 hours was reported only.

The selected transformants were as follows: Five transformants expressing *N. crassa* CDH and five transformants expressing *M. thermophile* CDH, three transformants expressing *T. versicolor* laccase isoform *lcc1*, three transformants expressing *T. versicolor* laccase isoform *lcc2*, six transformants with confirmed integration of the *C. globosum* glucuronoyl esterase gene, and two transformants with confirmed integration of the *H. jecorina* glucuronoyl esterase gene.

SDS-PAGE analysis (for shake-flask samples)

To determine the concentration of recombinant *N. crassa* cellobiose dehydrogenase produced by *P. pastoris* transformants, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) densitometry analysis was done. SDS-PAGE was used to visualise the presence of the glucuronoyl esterase protein in the growth medium supernatant of the PCR-confirmed recombinant *P. pastoris* strains. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10 % polyacrylamide gel in the Bio-Rad Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, Inc., Hercules, California, USA). PageRuler Prestained Protein Ladder (Thermo Fisher Scientific Inc, Waltham, MA, USA) was used as a protein size marker and proteins were visualized by silver nitrate staining. BSA at varying known concentrations was used as a standard. Images of the stained polyacrylamide gels were analysed with Image J software.

4.3 Bioreactor Production of Lignin-Modifying Enzymes

4.3.1 Inoculum preparation

Cultures were prepared by inoculating single colonies from YPD agar plates into 5 ml of buffered glycerol complex medium (BMGY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34 % YNB, 4×10−5% biotin, 1 % glycerol) in test tubes. The culture was then used to inoculate 50 ml YNB in 250 ml baffled flasks, to a final OD_{600} of 0.1. The flasks were grown at 30 °C, shaken at 220 rpm, to an OD_{600} of 1. The cultures were then used to prepare the inoculum by inoculating BMGY to a final volume of 400 ml, and OD_{600} of 0.1, in

2 L Erlenmeyer flasks. The culture was grown at 30 °C, shaken at 220 rpm, until a final OD_{600} of 6. This culture was used to inoculate 0.36 L of fermentation basal salts media (per litre: 40 g glycerol, 26.7 ml phosphoric acid (85 %), 0.93 g calcium sulphate, 18.2 g potassium sulphate, 14.9 g magnesium sulphate heptahydrate, 4.13 g potassium hydroxide, and 4.35 ml PTM_1 trace salts [per litre: 6.0 g cupric sulphate pentahydrate, 0.08 g sodium iodide, 3.0 g magnesium sulphate monohydrate, 0.2 g sodium molybdate dehydrate, 0.02 g boric acid, 0.5 g cobalt chloride, 20.0 g zinc chloride, 65.0 g ferrous sulphate heptahydrate, 0.2 g biotin, 5.0 ml sulphuric acid], supplemented with 1 % (w/v) casein hydrolysate) in the bioreactor to obtain a starting OD_{600} of 0.6.

4.3.2 Fermentation protocol

Fermentations were conducted in triplicate 14 L New Brunswick BioFlo 110 bioreactors, with 9 L working volumes, under control of BioCommand® software. The Invitrogen Pichia Fermentation Process Guidelines was used as the general protocol. This protocol was selected as it has been shown to be a reliable heterologous protein production protocol. Each of the fermentation parameter setpoints (temperature, dissolved oxygen, pH, and agitation) are selected as they are optimal for *P. pastoris* growth. The bioreactors were equipped with temperature, dissolved oxygen (DO), and pH probes, with agitation control. The fermentation conditions were identical for all fermentations, conducted at 30.0 °C, pH 5.00 (maintained with 28 % ammonium hydroxide solution), DO maintained at 30.0 %, and an aeration rate of 5 vvm. The temperature was maintained within one decimal point of the setpoint temperature. The DO probe was calibrated using nitrogen for the zero-point (0.0 %) and compressed atmospheric air for the maximum point (100.0 %). The DO was measured within one decimal point of the setpoint. The DO and agitation rate were controlled in a cascade (maintained by the proportional (P) and integral (I) controllers) where, as the DO levels dropped below 30.0 %, the agitation rate increased (between 100 – 1000 RPM). The pH probe was calibrated using standard solutions of pH 4.00 and pH 7.00. The pH was measured within 2 decimal points of the set point. Pure oxygen was sparged into the bioreactor when DO levels dropped significantly below 30.0 % and agitation was at maximum. The end of the batch phase occurred once the glycerol was depleted from the media, as indicated by an increase in the DO levels, drop in agitation, and slight increase in pH.

The fed-batch stage was subsequently initiated, where 4 L of 50 % (w/v) glycerol feed, supplemented with 12 ml PTM_1 trace salts solution per litre, was fed at a constant rate of 72.6

ml/hr. The flow rate of 72.6 ml/h was maintained with the use of a calibrated peristaltic pump. Calibration was done with the use of a standard curve that related a range of peristaltic pump settings (5 %, 10 %, 25 %, 50 %, 100 %) to the flow rate (ml/h) at that setting. This rate is recommended in the Invitrogen Pichia Fermentation Process Guidelines. The recommended glycerol feeding rate should be optimal in terms of *P. pastoris* growth, while preventing accumulation of glycerol, that may lead to toxicity. The constant feeding strategy was selected to keep the fermentation conditions identical across the three target enzymes. The current study aimed to report baseline production levels of the target enzymes, and the constant feeding strategy was sufficient for this purpose. In addition, this strategy requires less optimisation and a lower level of process monitoring, which would be advantageous in the final production bioprocess. The fed-batch stage was concluded when the 4 L of feed had been added to the reactor (after approximately 48 hours).

4.3.3 Sampling

Samples of 10 ml were aseptically taken at intervals between 3-12 hours throughout the fermentation process. The samples were analysed for dry cell weight, wet cell weight (data not discussed), enzyme activity, and glycerol concentration (during fed-batch stage). The final samples were used to determine the total protein concentrations and specific activity conversion factors, via SDS-PAGE densitometry.

4.3.4 Preliminary improvement of laccase production

Since the productivity of the laccase production process reported in the current study was the lowest of the three target enzymes, two strategies for laccase productivity improvement were investigated.

Growth conditions

The strain selected for laccase production was grown in BMGY media (Invitrogen, 2002), in baffled flasks, shaken at 220 rpm for 96 hours. Samples were taken every 24 hours. Samples of the growth medium were analysed for biomass growth by OD_{600} measurement, and subsequently centrifuged at 14000 RPM for 5 minutes to separate the yeast biomass from the supernatant. The supernatant was subsequently used for enzyme activity determination.

Investigation of cultivation temperature

Production of heterologous laccase has been shown to be particularly sensitive to temperature. Specifically, lowering of the cultivation temperature has previously improved laccase production (Hong *et al.* 2002. Jönsson *et al.* 1997. Cassland and Jönsson, 1999).

The shake flasks were incubated at five different temperatures (12, 15, 23, 30, 33 °C). The temperature range was selected to accommodate *P. pastoris* growth. Temperatures within this range were determined using a central composite design (CCD) in Statistica. The resultant model was not significant, however differences in volumetric activity between the selected temperatures are discussed. Incubation at 12 and 15 °C was conducted in a water-bath shaking incubator, stored in an 8 °C refrigerator. No copper was supplemented into the growth medium.

Supplementation of copper

Two concentrations of copper, 1 mM and 2 mM, were assessed for their effect on laccase volumetric activity. Copper sulphate was supplemented into the BMGY medium to a final copper concentration of 1 mM and 2 mM. These concentrations were selected as they were found to be in an optimal range for laccase production (Balakumaran *et al.* 2016).

4.3.6 Product Harvesting

The culture was harvested immediately after the fed-batch phase was concluded to minimize degradation of the enzyme product. The resultant fermentation culture of approximately 9 L was pumped out of the reactor and into sterile containers. The total culture was transferred to sterile 500 ml centrifugation bottles and centrifuged at 8000 rpm for 10 minutes at 4 °C. The cell-free extracts were then transferred to sterile containers and stored at 4 °C until filtration.

4.3.7 Tangential flow filtration

The Pellicon 2.0 tangential flow filtration apparatus (Merck, South Africa) was used, as per the manual instructions. The supernatant was first filtered through a 0.22 µm filter cassette (Durapore® PVDF, Merck Millipore). The feed pressure was kept at a maximum of 10 bar, and the retentate pressure was kept at a maximum of 2 bar. The pressure was measured with gauges attached to the feed and retentate outlets of the TFF apparatus. The feed and retentate pressures were adjusted using a clamp on the retentate outlet. The transmembrane pressure (TMP) was 2.5 bar.

The crude enzyme extract was filtered through the 0.22 µm filter once, in a single step process, with no recycling of the permeate or retentate into the feeding reservoir. The resultant clarified permeate was then concentrated through ultrafiltration using a 5 kDa filter cassette (Biomax™ 5, Merck Millipore) with the retentate containing the protein of interest. The retentate was recycled into the feeding reservoir, while the permeate was collected in a separate, sterile vessel. The concentration process was concluded once the volume of the retentate/feeding reservoir was decreased by a concentration factor of 10. The schematic of the concentration process is given by Figure 4.3.

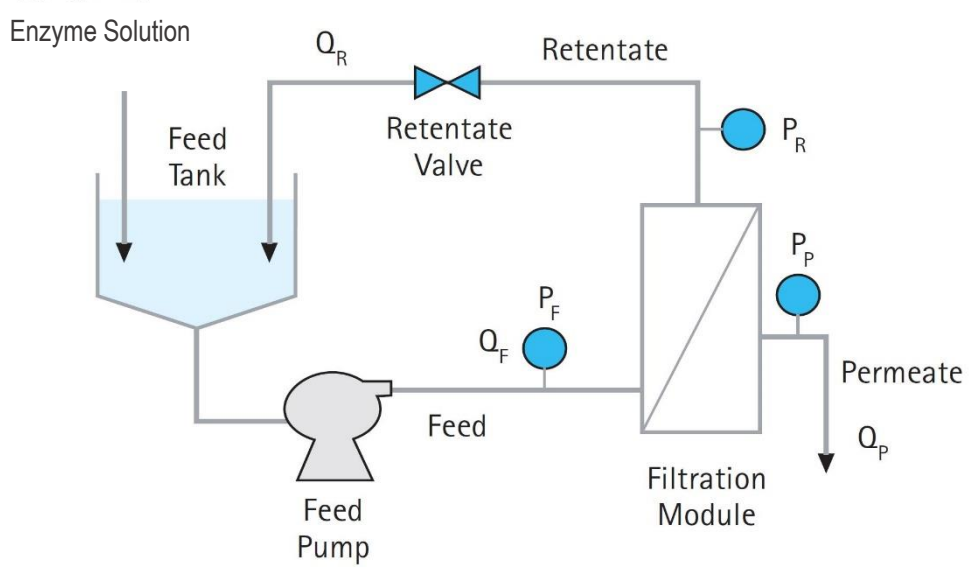


Figure 4.3. Schematic of enzyme concentration process set-up.

Samples were taken of each permeate and retentate and analysed for volumetric activity. Volumes of the feed, permeate and retentate of each filtration process was measured.

4.3.8 Evaluation of freezing as a storage strategy

The concentrated enzyme products were then evaluated for stability after freezing at -20 °C. This temperature was chosen as it is routinely used in commercial enzyme storage. Concentrated enzyme products were aliquoted into Eppendorf tubes and frozen at -20 °C overnight. Enzyme activity was evaluated before and after freezing for one freeze-thaw cycle. The frozen samples were rapidly defrosted at room temperature and left on ice during enzyme assay preparations. Finally, the effect of glycerol as a cryoprotectant was evaluated. 10 % and 25 % (v/v) final concentrations of glycerol were tested. Glycerol was added to the concentrated enzyme products, and the enzyme activity was evaluated before and after freezing.

4.4 Analytical Techniques

4.4.1 Biomass concentration

Biomass growth was measured by determining the dry cell weight per volume. Cell dry weight was measured in triplicate by adding 1 ml samples of fermentation culture into pre-weighed 1.5 ml Eppendorf tubes, followed by centrifugation at 14800 rpm for 5 minutes. The cell pellets were washed, centrifugation was repeated, and the resultant cell pellets were allowed to dry at 70 °C until no changes in weight were observed. The resultant cell-pellets were weighed and converted to g/L quantities.

Maximum growth rate (μ_{max}) was determined graphically by plotting the natural logarithm of the biomass concentration (g/L) during batch phase against fermentation time, and determining the gradient of the exponential growth phase, using at least four points. All trends were fitted to a linear regression with R^2 values of above 0.98.

4.4.2 Glycerol concentration

Glycerol concentration was analysed by HPLC using an Phenomenex RHM Monosaccharide column fitted with a guard column. A Refractive Index (RI) detector (Thermo-Scientific, Surveyor) was used to measure the glycerol. The Phenomenex RHM Monosaccharide column was maintained at 60 °C with 5 mM H_2SO_4 mobile phase at a flow rate of 0.6 mL/min for 25 minutes. Samples were acidified with H_2SO_4 to a final concentration of 0.5 % (v/v), and filtered through 0.22 μm nitrocellulose filters.

4.4.3 Enzyme Activity Assays

One enzymatic unit was defined as the amount of enzyme that releases 1 μmol of product per minute during the respective assays. All enzymatic assays were designed for use at a micro-scale, with a maximum final volume of 250 μl.

Activity of recombinant laccase

The time-dependent reduction of 0.5 mM 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (Sigma-Aldrich, USA) at a wavelength of 420 nm (ε_{420} =36000 M^{-1} cm^{-1}) in 100 mM sodium acetate buffer, pH 4.5. The reaction was done by adding 100 μL supernatant to 100 μL of the ABTS-based assay solution in the microtitre plate well and reading the absorbance in a microtitre plate reader (Bio-Rad) before and after incubation for 10 minutes at 30 °C.

Activity of recombinant cellobiose dehydrogenase

Two microtitre-plate spectrophotometric enzyme assays were evaluated. The first assay, reported by Brugger *et al.* (2014) used lactose as enzymatic substrate, while the second assay, reported by Sygmund *et al.* 2013, used cellobiose dehydrogenase as enzymatic substrate. The assay reported by Brugger *et al.* (2014) was found to be less effective than the second assay, reported by Sygmund *et al.* (2013), thus the second assay was used. The assay measured the time-dependent reduction of 300 µM 2,6-dichloroindophenol (DCPIP) at a wavelength of 520 nm (ε_{520} =6.8 mM^{-1} cm^{-1}) in 100 mM sodium acetate buffer, pH 5.5, into which 30 mM cellobiose was dissolved. The reaction was done by adding 50 µL supernatant to 150 µL of the DCPIP-based assay solution in the microtitre plate well and reading the absorbance in a microtitre plate reader before and after 5 minutes of incubation at 30 °C.

Activity of recombinant glucuronoyl esterase

Glucuronoyl esterase activity was measured spectrophotometrically by using a benzyl D-glucuronic acid (BnGlcA) uronate dehydrogenase-coupled screening assay developed by Sunner *et al.* (2015), with the exception of performing both the initial and detection reactions in a microplate. The initial glucuronoyl esterase-dependent reaction was done by incubating the crude enzyme extract with a commercial substrate, benzyl D-glucuronic acid (BnGlcA), which resembles postulated LCC bonds in plant biomass. The ester bond in the substrate is enzymatically cleaved by glucuronoyl esterase, producing D-glucuronic acid. The k-URONIC kit (Megazyme, Ireland) subsequently detects the concentration of released D-glucuronic acid by oxidation via uronate dehydrogenase. This oxidation releases NADH that is spectrophotometrically measured at 340 nm (Sunner *et al.* 2015). To complicate matters, the BnGlcA substrate spontaneously hydrolyses at the assay pH 6 and temperature, creating background activity in the assay. To account for this, a substrate control and incubated substrate control is included in the assay. The substrate control shows the amount of substrate hydrolyses before it is incubated with the enzyme extract, while the incubated substrate control shows the amount of substrate hydrolysed during incubation with the enzyme extract. The total concentration of D-glucuronic acid in each water-diluted crude enzyme extract was calculated by comparison to a standard curve and related in terms of total enzymatic activity. Total enzyme activity was expressed in volumetric activity units (U/L).

4.4.4 SDS-PAGE analysis (for bioreactor samples)

Total extracellular proteins in the fermentation samples were visualised by using tris-tricine SDS-PAGE, as described in Current Protocols for Immunology (Gallagher, 2012) for one dimensional gel electrophoresis of proteins. Briefly, the separating and stacking gels consisted of 10 % and 4 % acrylamide/bisacrylamide (Sigma-Aldrich, USA), respectively. The gels were run at 30 V until the loading dye-front had moved through the stacking gel, followed by 200 V until the loading dye-front ran off the gel. The gels were fixed in fixing solution (25 % isopropanol, 10 % acetic acid) for 25 minutes, stained overnight in Coomassie Brilliant Blue G250 (0.025 % [w/v] in 10 % acetic acid), and finally destained (10 % acetic acid). All preparations were made with MilliQ water. Note the more sensitive SDS-PAGE method implemented during shake flask screening.

4.4.5 Protein Concentration

Total Protein Concentration

The total protein concentration of the cell-free extracts was determined using the bicinchoninic acid (BCA^{TM}) microassay (Sigma-Aldrich, USA) with bovine serum albumin as the standard. Cell-free extracts were diluted in MilliQ water, mixed with BCA solution and 4 % $CuSO_4 \cdot 5H_2O$, as per the kit protocol, and incubated for 30 minutes at 37 °C. The absorbance was subsequently measured at 562 nm on a microtiter-plate spectrophotometer (Bio-Rad).

Densitometry

Protein concentration of the target proteins were determined by using densitometry. The cell-free extracts were separated via SDS-PAGE gels, which were subsequently stained (as described in Section 4.4.4). Photographs of the SDS-PAGE gels were analysed using ImageJ® software. The target protein was expressed as a percentage of the total extracellular protein, as determined by the BCA assay.

4.4.6 Statistical Analyses

Single-factor analysis of variance (ANOVA), at a significance level of 0.05, was used to determine significant differences between enzyme activity values.

Chapter 5: Results and Discussion

5.1 Screening of recombinant *P. pastoris* transformants

Following the molecular work of constructing recombinant *P. pastoris* strains constitutively expressing the target enzymes (Chapter 4, Section 4.1), a sufficient number of transformants of each strain were screened, to select the most promising candidates for subsequent bioreactor cultivations.

5.1.1 Cellobiose Dehydrogenase

Two cellobiose dehydrogenase genes from different source organisms, namely *Neurospora crassa* and *Myriococcum thermophilum* were expressed in the current study. These two genes were selected based on the novelty of their constitutive expression in *P. pastoris*, and the differences in their structure. *M. thermophlium* is a thermophilic organism, and it has been shown to be a good source of thermostable enzymes (Zamocky *et al.* 2008). Thermostability is an attractive property for industrial enzymes, as it increases their tolerance for the harshness of industrial processes.

Agar plate-based screening

Transformants of the two cellobiose dehydrogenase (CDH) genes, isolated from *N. crassa, M. thermophilum,* were qualitatively screened for cellobiose dehydrogenase activity by using a DCPIP-containing agar plate-based assay. Twenty active transformants were each selected for *N. crassa* and *M. thermophilum* CDH transformants, respectively.

Test-tube screening of cellobiose dehydrogenase transformants

Forty positive transformants, based on the plate assay, 20 expressing *N. crassa* CDH and 20 expressing *M. thermophilum* CDH, were assessed in test-tube cultures to confirm and quantify enzyme activity. The enzyme activity values obtained after 24 hours are represented in Figure 5.1. Except for transformant 2.5, all *N. crassa* transformants showed CDH activity above 40 U/L, with a mean value of 44.29 ± 9.48 U/L (Fig 5.1A). There were no significant differences in volumetric activity between the CDH-active transformants.

Seventeen of the *M. thermophilum* CDH transformants showed activity (Fig 5.1B). Significantly lower levels of volumetric activity were found in comparison to the *N. crassa* CDH, with a mean value of 3.70 ± 0.89 U/L. There were no significant differences in volumetric activity between the transformants.

Therefore, the validity of the plate assay showing putative CDH expression was confirmed. To scale up the screening process, 5 of each *M. thermophilum* and *N. crassa* CDH transformants were randomly selected for further screening in shake flasks.

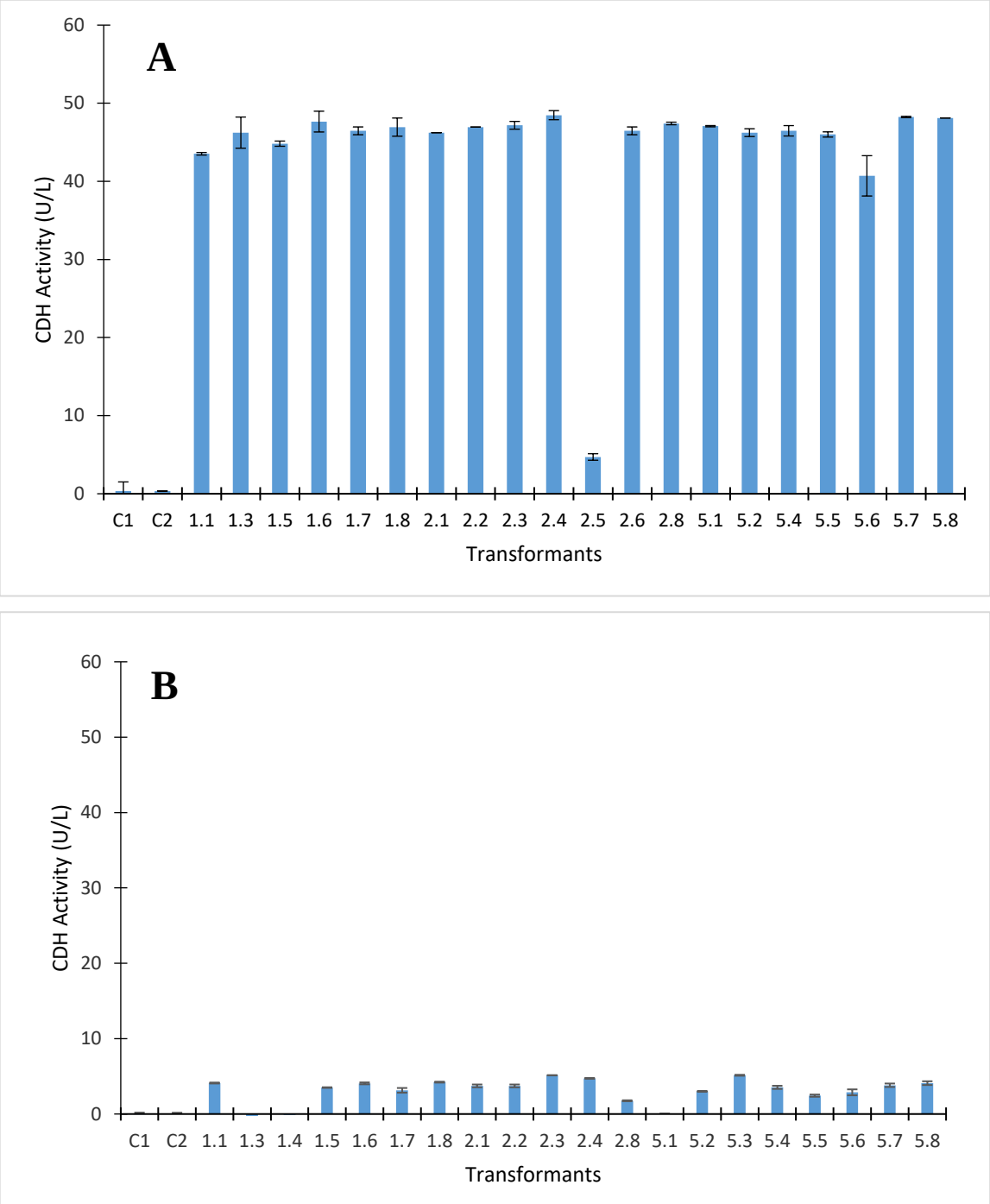


Figure 5.1. Test-tube screening of *P. pastoris* transformants, grown in BMGY (Invitrogen) media for 24 hours, expressing A: *N. crassa* CDH, B: *M. thermophilum* CDH.

Shake-flask screening

The ten selected transformants, as well as negative control strains, were grown in 250 ml baffled shake flasks for 72 hours and evaluated for maximum volumetric activity (U/L). The results mimicked that of the test-tube experiments. *M. thermophilum* CDH transformants exhibited significantly lower levels of volumetric activity when compared to the *N. crassa* CDH transformants. The best performing *M. thermophilum* CDH transformant, M2.4, yielded 12.69 U/L (Fig. 5.2). The *N. crassa* transformants showed significantly higher activity than the *M. thermophilum* transformants. A mean final activity value of 67.29 ± 2.08 U/L was found, 5-fold higher than the best performing *M. thermophilum* CDH transformant.

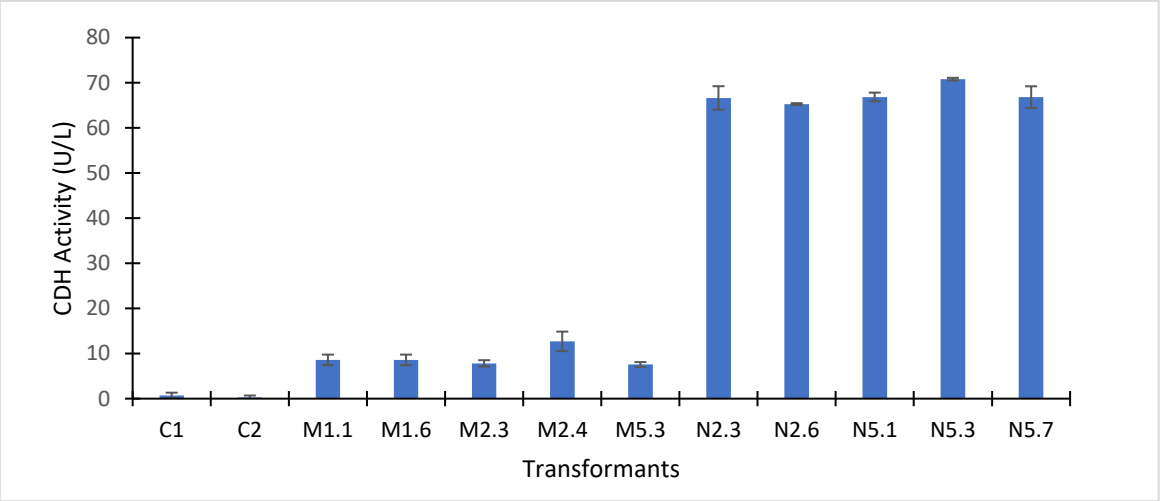


Figure 5.2. Shake-flask screening results of volumetric activity of recombinant *N. crassa* and *M. thermophilum* CDH after growth in BMGY for 72 hours. C1, C2: Negative control strains. M1.1-M5.3: transformants expressing *M. thermophilum* CDH. N2.3-N5.7: transformants expressing *N. crassa CDH*.

The low volumetric activity of the *M. thermophilum* CDH transformants may be as a result of low secretion of the recombinant protein, or the conditions of the enzymatic assay were not optimal for the enzyme. To investigate the possible reasons for the low activity, the protein was visualised by SDS-PAGE densitometry. It was found that the protein was indeed secreted successfully, but at lower relative concentrations than the *N. crassa*, CDH, as can be seen by the intensity of the protein bands (Fig. 5.3). The magnitude of differences in volumetric activity (5-fold) observed between the two source-organisms made it clear that the *N. crassa* CDH transformants were more successful secretors of functional, recombinant CDH. Thus, for the purposes of the current study, it was decided to move forward only with *N. crassa* CDH transformants. Since no significant differences in volumetric activity was found between the 5 screened transformants, nor in specific activity of the protein (Fig. 5.3), transformant N2.6 was randomly selected for bioreactor cultivation.

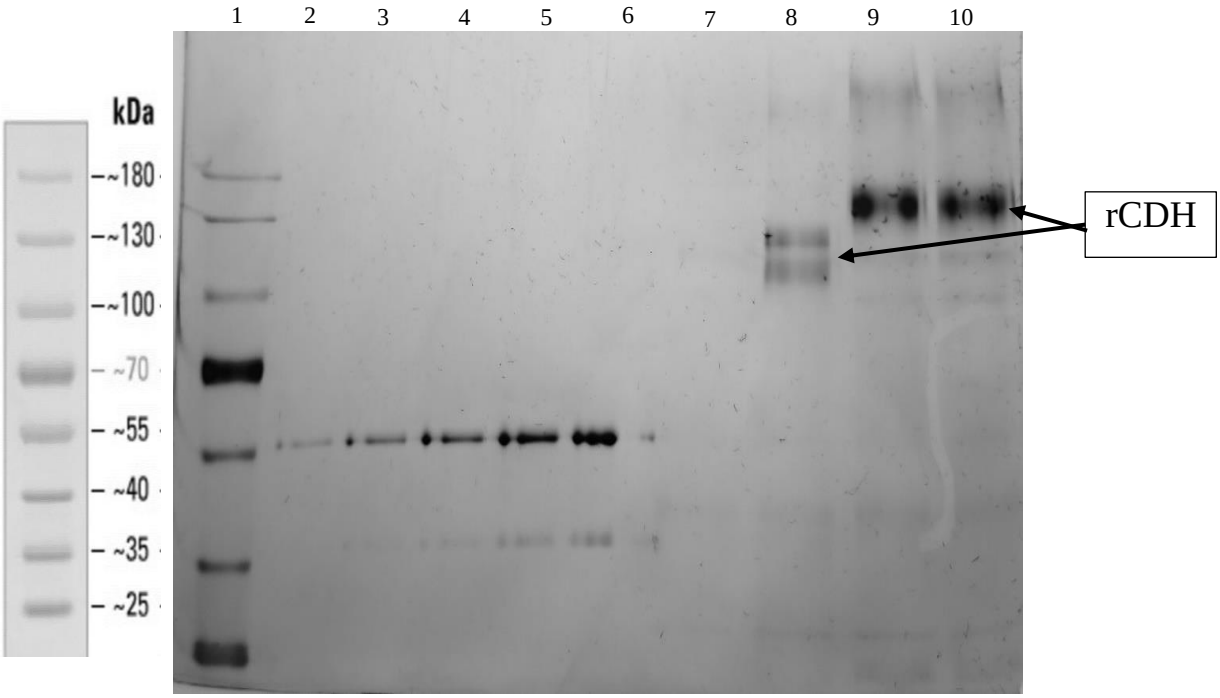


Figure 5.3. SDS-PAGE analysis used for densitometric protein concentration determination between *M. thermophilum* and *N. crassa* CDH secretion. Lanes 1-5: BSA protein standards, 4 mg/l, 8 mg/L, 12 mg/l, 16 mg/L, 20 mg/l respectively. Lanes 8: 20 µl samples of cell-free extract of *M. thermophilum* transformant. Lane 9-10: 20 µl samples of cell-free extract of *N. crassa* CDH transformants (N2.6, N5.3).

Interestingly, the clonal variation observed was minimal. Except for one transformant, no significant differences in volumetric activity were observed between transformants of the same gene. Generally, *P. pastoris* is reported to exhibit substantial clonal variation, and thus screening of several transformants is recommended in order to identify the best protein secretor (Looser *et al.* 2014). Clonal variation has been speculated to be due to differences in copy number, integration sites, and even the transformation process itself (Viader-Salvadó *et al.* 2006; Schwarzhans *et al.* 2016). However, despite empirical evidence for clonal variation and various correlations being made between the causes and effects for it, there is no definite conclusion on the mechanisms and workings of clonal variation in *P. pastoris* (Aw and Polizzi, 2016). The lack of clonal variation observed in the current study may be due to lowered stringency of the transformation process. After electroporation, the putative transformants were grown in growth media overnight. This may have caused single transformants to multiply, and increase the probability of selecting genetically identical colonies for screening. It may, therefore, be possible to observe higher levels of clonal variation if the incubation period is shortened.

5.1.2 Laccase[1]

Two isoforms of *Trametes versicolor* laccase, *lcc1* and *lcc2*, were expressed in *P. pastoris*. Native *T. versicolor* fungi are known to produce several isoforms of laccase proteins (Lorenzo *et al.* 2006). These isoforms usually have amino acid sequence homology of between 60 – 80 % and are expressed during different physiological conditions in nature (Li *et al.* 2014b). The majority of publications have expressed other isoforms, but examples of *lcc2* expression are popular for comparisons between laccase isoforms (Lorenzo *et al.* 2006. Li *et al.* 2014b).

Agar plate-based screening

Transformants were randomly selected and screened for laccase expression through activity on ABTS-containing agar plates. Six positive colonies, 3 expressing *lcc1* and 3 expressing *lcc2* were selected for further shake flask screening. The number of transformants selected for further shake flask screening was lowered, as after extensive screening of the CDH transformants, it was found that minimal clonal variation was observed with the transformation protocol applied in the current study.

Shake-flask screening

Strains showing expression of the target laccase genes were screened in shake flasks to select the most promising isoform and strain for production at bioreactor scale. It was found that isoenzyme *lcc2* performed significantly better than isoenzyme *lcc1*. The highest volumetric activity of *lcc1* transformants were 1.7 U/L, compared to 36.3 U/L of *lcc2* transformant lcc2.3 (Fig 5.4). This difference in recombinant expression between the *T. versicolor* laccase isoforms was also reported by Bohlin *et al.* (2006), where the *lcc2*-expressing *P. pastoris* strains showed approximately 4-fold higher volumetric activity than *lcc1*-expressing *P. pastoris* strains. The decreased activity of *lcc1*-expressing transformants can be explained by point mutations that were observed in the coding sequence of the lcc1 gene, causing improper transcription or translation of the protein.

Based on these results, it was decided to select a transformant expressing *lcc2* laccase. The differences in volumetric activity between the three *lcc2* transformants were not statistically significant, therefore, transformant lcc2.3 was randomly selected for bioreactor fermentation.

Raw data for screening of *T. versicolor* laccase-expressing transformants (Section 5.1.2 only) were provided by Dr García-Aparico. All interpretation and written discussion was completed by Ms Conacher. All other screening for cellobiose dehydrogenase and glucuronoyl esterase-expressing transformants (Section 5.1.1 and Section 5.1.3) was performed by Ms Conacher.

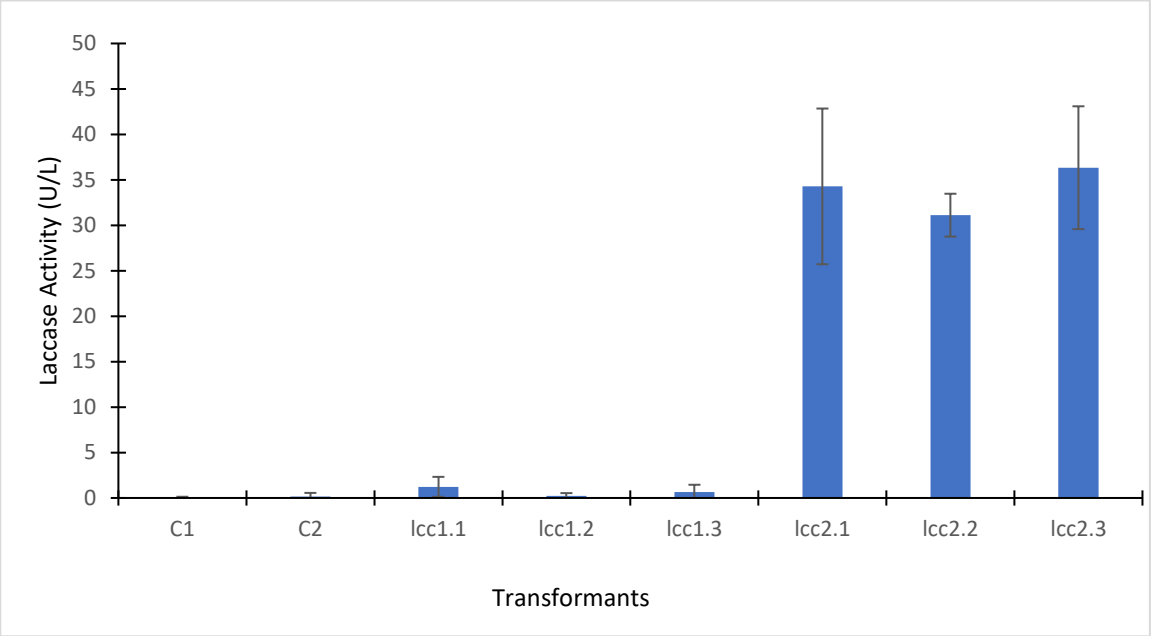


Figure 5.4. Shake-flask screening results for *P. pastoris* grown in BMGY for 72 hours, expressing two laccase isoforms. C1, C2: Negative control strains. Lcc1.1-1.3: *P. pastoris* transformants expressing laccase isoform *lcc1*. Lcc2.1-2.3: *P. pastoris* transformants expressing laccase isoform *lcc2*.

5.1.3 Glucuronoyl Esterase

Shake-flask screening

No agar-plate based assays or colorimetric spectrophotometric methods are available for glucuronoyl esterase (GE), thus, for screening, focus was placed firstly on molecular screening, followed by screening for secretion of the target protein. Forty strains were screened using PCR amplification to confirm integration of the target gene in the *P. pastoris* genome. Of these, 8 were positive for gene integration, showing an amplified band of approximately 1600-1700 bp in size. To confirm expression, these 8 strains with confirmed integration of the *H. jecorina* and *C. globosum* target genes, were screened for the presence of secreted glucuronoyl esterase using SDS-PAGE analysis. Of these, two transformants with the *H. jecorina* cip2 gene were found to express the target protein (Fig. 5.5). The calculated activity values were 6.00 U/L and 6.15 U/L, respectively. No significant difference in activity was found between the two transformants, thus pJ905_Hj_cip2.3 was chosen for bioreactor cultivation.

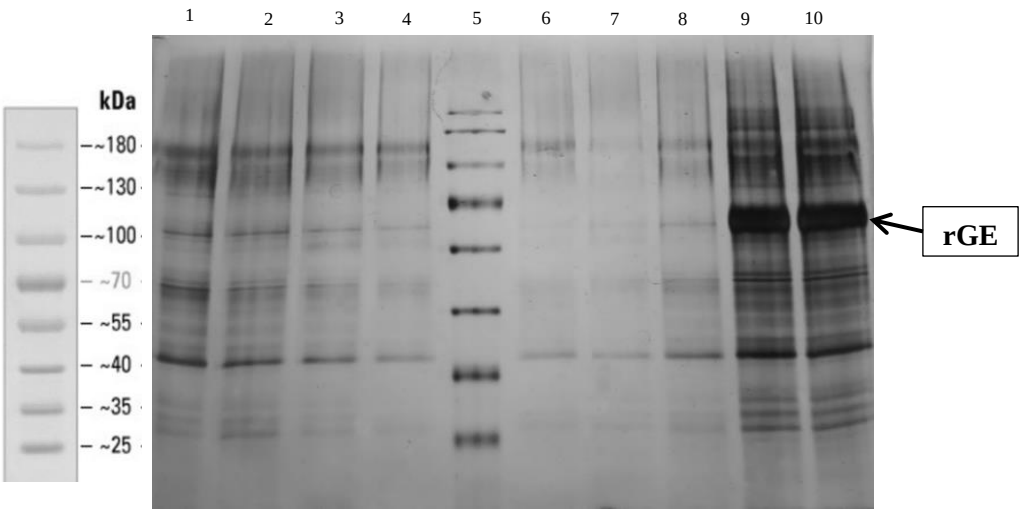


Figure 5.5. SDS-PAGE analysis of 20 µl cell-free extract of recombinant *P. pastoris* strains grown in shake flasks containing BMGY medium. Lane 1: Wild-type *P. pastoris* DSMZ 70382 (CBS704); lane 2-4: pJ905_Cg_cip2.1-3; lane 5: PageRuler protein molecular weight marker; lane 6: pJ905_Cg_cip2.5; lane 7:

5.2 Bioreactor Fermentation

The production of heterologous proteins at shake-flask level is limited by the small size, inadequate oxygen transfer capabilities, and a lack of substrate replenishment. Bioreactors are able to monitor and maintain optimal growth conditions for *P. pastoris*, by adjusting dissolved oxygen (DO) levels, pH, agitation, and temperature at predetermined optimal values. This controlled growth environment results in high cell densities, and therefore high recombinant protein concentrations (Macauley-Patrick *et al.* 2005). For the target enzymes of the current study to be applied in lignin valorisation research or application at industrial scale, large quantities of the enzymes are required. Currently, while cellobiose dehydrogenase (CDH) is available commercially, it is prohibitively expensive. Further, commercial preparations of laccase (LCC) are impure and often contain side activities, which complicate analytic methods used for research. Lastly, glucuronoyl esterase (GE) is not available commercially at all. The limited availability of pure extracts of these enzymes is a significant barrier to further research. Thus, an objective of this study was to establish baseline values of 14 L bioreactor fermentations of the constitutively expressed enzymes in *P. pastoris.*

To evaluate the feasibility of a constitutive *P. pastoris*-P_{GAP} expression system for production of lignocellulose degrading enzymes, the production of three enzymes were evaluated in a 14 L glycerol fed-batch bioreactor cultivation with constant feed strategy.

The general trends observed for all three fermentations are as follows (Fig. 5.6). During the batch phase, an initial lag phase of less than 6 hours was obtained, corresponding to minimal volumetric activity, followed by an exponential growth phase, where a linear increase in volumetric activity was observed until the end of the batch phase, between 20-22 hours. During the 48-hour glycerol fed-batch stage, there was a brief exponential growth phase of approximately 6 hours, followed by a stationary growth phase, where the agitation of the bioreactor remained at the maximum 1000 RPM, and oxygen sparging was required to maintain sufficient availability of dissolved oxygen in the culture. The maximum protein titre and volumetric activity of each fermentation was obtained at the end of the fed-batch stage. Peak specific productivity of protein was reached during the batch phase. During the fed-batch stage, specific productivity remained constant at a value close to the mean specific productivity value. The maximum growth rate of each strain was similar (0.15 – 0.17 h^{-1}), corresponding to expected values reported for recombinant *P. pastoris* strains growing on glycerol (0.15 – 0.20 h^{-1}) (Buchetics *et al.* 2011, Looser *et al.* 2014). Similarly, the biomass yields obtained (119.54 – 136.47 g_{DCW}/L) were satisfactory and comparable to previous bioreactor cultivations of recombinant *P. pastoris* (Cos *et al.* 2006, Hong *et al.* 2002).

A summary of the results of the bioreactor cultivations is given in Table 5.1. The following sub-sections will discuss Table 5.1, and the production of each enzyme in detail, followed by an assessment of the glycerol feed strategy, and finally a comparison in yields between the three target enzymes.

Table 5.1. Summary of productivity and enzymatic yields for bioreactor cultivations of recombinant *P. pastoris* at the end of the glycerol fed-batch stage.

Parameter	CDH	LCC	GE
Highest Volumetric Activity (U/L)	329.49	81.76	238.17
Highest Recombinant Protein Titre (mg/L)	1489.30	778.54	2778.01
% recombinant protein vs total protein in supernatant	50.89 %	37.65 %	93.61 %
Biomass Conc. (g_{DCW}/L)	136.47	119.54	120.94
μ_{max} (h^{-1})	0.16	0.17	0.15
$q_{p.max}$ (mg recombinant protein/g biomass·h)	0.76	0.13	1.22
$q_{p.mean}$ (mg recombinant protein/g biomass·h)	0.43	0.11	0.52

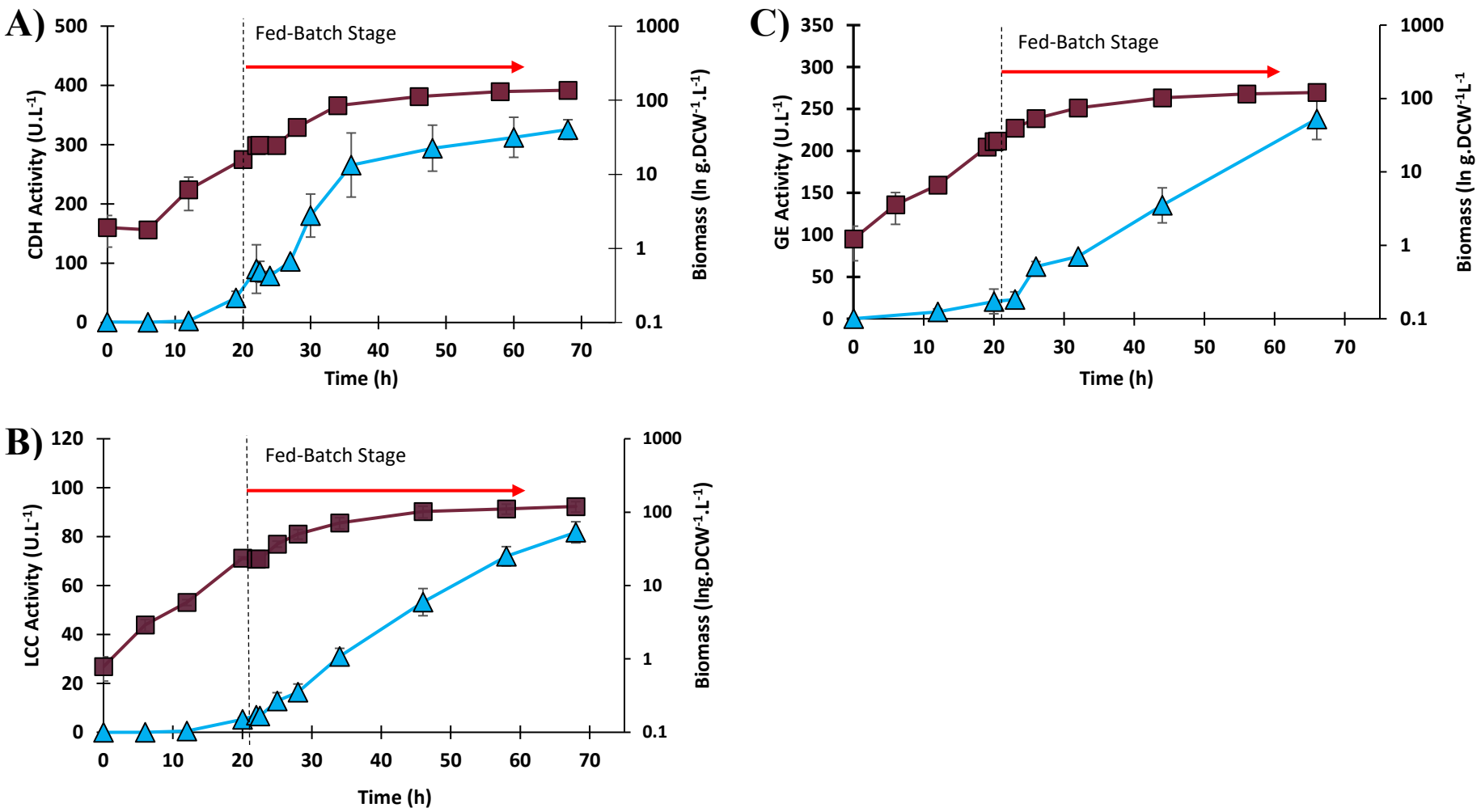


Figure 5.6. Volumetric activity (U.L^{-1}) (blue triangles) and biomass yields (dry cell weight per litre) (maroon squares) during bioreactor cultivations of each target protein. Red arrows indicate the initiation of the glycerol fed-batch phase (20 – 22h), where a 50 % (w/v) glycerol feed supplemented with trace salts was added at a rate of 72.6 ml/hr. A: Cellobiose Dehydrogenase. B: Laccase. C: Glucuronoyl esterase. Error bars indicate standard deviation from mean of three fermentation replicates.

5.2.1 Cellobiose Dehydrogenase Production

Bioreactor cultivation of *P. pastoris* constitutively expressing *N. crassa* CDH returned a final mean volumetric activity of 329.49 U/L and a final mean recombinant protein concentration of 1489.30 mg/L (Fig. 5.6A). This volumetric activity was 5-fold more than that found in shake-flask experiments (Section 5.1.1). The volumetric activity was detectable after 12 hours of batch cultivation, where a maximum volumetric activity of 90 U/L was reached at 22 hours. The glycerol fed-batch stage was subsequently initiated, where, following a brief lag phase, a steep linear increase in volumetric activity was observed between hours 24-36. Peak specific productivity of protein secretion ($q_{p.max}$) of 0.76 mg/g_{DCW}·h was reached at the end of the batch phase, corresponding to the end of the exponential growth phase at 22 hours. The mean specific productivity of protein was 0.43 mg/g_{DCW}·h.

The only other publication which has expressed *N. crassa* CDH in *P. pastoris*, to the best of the author's knowledge, returned higher volumetric activity yields of 652 U/L but lower protein concentrations of 306.6 mg/L total protein, at shake flask scale (Zhang *et al.* 2011). This difference in volumetric activity observed may be as a result of differing enzyme assay conditions. Further, these authors used a different expression vector, namely the Easy Select Pichia Expression System from Invitrogen, under regulation of the inducible P_{AOX1} promotor, and the α-secretion factor signal peptide. This is in contrast to the pJ-Express vector, P_{GAP} promotor, and native secretion signal used in the current study. Total protein in the cell-free extract reported by Zhang *et al.* (2011), 306.6 mg/L, was considerably less than the final CDH protein titre found in the current study (1489.3 mg/L) (Table 5.1). The final biomass yield obtained in the current study is 136.47 g_{DCW}/L.

Several studies have expressed CDH genes under P_{AOX1} regulation from alternative source organisms in *P. pastoris*. The highest reported volumetric activity (7955 U/L) was found with *T. versicolor* CDH inducibly expressed and grown at shake flask scale (Stapleton *et al.* 2004). However, these authors report considerably less secreted protein than the current study, at 7.68 mg/L, compared to 1489.30 mg/L reported here. The second highest volumetric activity yield was obtained with *Pycnoporus cinnabarinus* CDH inducibly expressed and grown in 1 L bioreactor, at 7800 U/L, however the authors do not report protein concentrations (Bey *et al.* 2011). Harreither *et al.* (2012) produced recombinant *Corynascus thermophilus* CDH and reported a total protein concentration of 633 mg/L. Sygmund *et al.* (2013) expressed a mutated

M. thermophilum CDH in a 7 L bioreactor cultivation and reported a CDH protein concentration of 950 mg/L after purification with hydrophobic interaction chromatography.

The current study reports the only constitutively expressed *N. crassa* CDH in *P. pastoris*, to the best of the author's knowledge. The volumetric activity yield of *N. crassa* CDH obtained in the current study is approximately 2-fold less than what was obtained using the same gene under regulation of the inducible P_{AOX1} promoter, and is considerably less than that found with alternative CDH genes expressed with P_{AOX1} regulation. While volumetric activity is a useful indicator of the functionality of the target enzymes, direct comparisons between publications in terms of volumetric activity yields should be approached with caution. Volumetric activity is extremely specific to the exact conditions of the enzyme assay used, and the calculation method. In contrast, when comparing the secreted protein concentrations of the current study, the results are significantly more promising. In terms of bioreactor cultivations of recombinant CDH, the current study reports higher protein titres (1489. 30 mg/L) to previously published data on CDH expression under $_{PAOX1}$ regulation (633 – 950 mg/L) (Harreither *et al.* 2012. Sygmund *et al.* 2013). Due to the fact that the biomass yields and protein titre was satisfactory, the low volumetric activity is most likely an issue of differing enzyme activity assay protocols and not the heterologous protein expression itself.

5.2.2 Laccase Production

The bioreactor cultivation for the constitutive expression of *T. versicolor* laccase *lcc2* returned a final volumetric activity yield of 81.76 U/L, corresponding to 778.54 mg/L of recombinant protein (Table 5.1). The volumetric activity was detectable after 12 hours of batch cultivation, where a maximum volumetric activity of 6.69 U/L was reached at 22 hours (Fig. 5.6B). The glycerol fed-batch stage was subsequently initiated, and a steep linear increase in volumetric activity was seen throughout the fed-batch phase. The maximum volumetric activity yield, 81.76 U/L, was reached at the end of the fed-batch process. The volumetric activity did not reach a plateau, however the specific protein productivity was constant during the fed-batch phase. Peak specific productivity ($q_{p.max}$) of 0.13 U/g_{DCW}·h was reached at the end of the batch phase, corresponding to the end of the exponential growth phase at 22 hours. The mean specific productivity of protein was 0.11 mg/g_{DCW}·h, the lowest of the three enzyme production bioprocesses.

The only other publication to constitutively express *T. versicolor* laccase *lcc2*, to the best of the author's knowledge, reported a final yield of 2.8 U/L after fed-batch cultivation with glucose in a 500 ml bioreactor (Bohlin *et al.* 2006). These authors reported a final protein concentration of 100 mg/L and a final biomass of 130 g_{DCW}/L. In comparison to this publication, the volumetric production of laccase *lcc2* under P_{GAP} regulation in the current study (81.76 U/L) is a significant improvement. The protein concentration of 778.54 mg/L is more than 7-fold higher. The final biomass yields are similar, with the current study reaching 119.54 g_{DCW}/L (versus 130 g_{DCW}/L). This means that the specific productivity (mg/g biomass·h) of the current bioprocess was higher than for Bohlin and colleagues. Therefore, the current study has shown the highest constitutive expression of *T. versicolor* laccase *lcc2* in *P. pastoris*.

Constitutive expression of alternative laccase genes in *P. pastoris* has been well studied due to the industrial importance of laccase. When compared to alternative source genes and expression strategies for laccase, the volumetric activity yields of the current study are significantly lower. The benchmark for constitutive laccase expression in *P. pastoris* is that of the *Pleurotus ostreatus* laccase isoform POXA1b, with a final volumetric activity of 60000 U/L and specific activity of 230 U/mg (protein concentrations not reported) after cultivation in a 2 L bioreactor cultivation (Pezzella *et al.* 2017). Kittl *et al.* (2012) constitutively expressed *Botrytis aclada* laccase in a 5 L bioreactor cultivation and reported 517 mg/L production, and a volumetric activity yield of 53300 U/L. Despite the low productivity of the laccase production process in the current study, the protein concentration of laccase, 778.54 mg/L, is still comparable and even a slight improvement on protein secretion yields reported in constitutive laccase expression studies.

In general, reported laccase volumetric activities are similar in P_{AOX1} and P_{GAP} expression systems, however there are more examples of extremely high yields for P_{AOX1} expression (Table 2.3). Kittl *et al.* (2012) found comparable protein concentrations between laccase expressed constitutively and inducibly. Pezella *et al.* (2017) compared the expression of *P. ostreatus* laccase under inducible and constitutive expression and found the P_{GAP} expression system to perform better under bioreactor cultivation (42000 U/L for P_{AOX1} and 60000 U/L for P_{GAP}). These authors motivate the use of the P_{GAP} expression system in terms of economic feasibility and showed by techno-economic analysis that the P_{GAP} expression system is more

economic than the P_{AOX1} expression system since it has shorter cultivation time and, in their case, higher volumetric activity.

Preliminary improvement of laccase production

The mean specific productivity of the laccase production bioprocess was the lowest of the three enzymes, and the final protein titre was the lowest (Table 5.1; Fig. 5.9). There are two widely-studied strategies that can be explored to improve the productivity value of laccase production reported in the current study. These are the investigation of fermentation temperature, as well as the addition of copper ions to the fermentation media (Balakumaran *et al.* 2016. Bohlin *et al.* 2006. Hong *et al.* 2002) Both of these aspects were evaluated in preliminary studies at shake flask scale.

Investigation of cultivation temperature

Five cultivation temperatures for recombinant laccase were assessed in shake flasks, ranging from 12 – 33 °C, as these temperatures still allow for appreciable *P. pastoris* growth. Biomass was monitored by measurement of OD_{600}, however no significant differences in final biomass was found. It was found that the best cultivation temperature in terms of volumetric activity of laccase was 23 °C, reaching 5.38 U/L after 120 hours, 3.4-fold higher than the second-best final volumetric activity yield of 1.59 U/L observed at 15 °C (Fig. 5.7). Further, no decreases in volumetric activity were observed at 23 °C, indicating the stability of laccase at 23 °C. The general trends observed show that the laccase *lcc2* isoform is degraded at temperatures of 30 °C and higher. This degradation may be due to sensitivity of the enzyme to temperature itself, increased protease activity at higher temperatures, or acceleration of the death phase (Jahic *et al.* 2003). At 30 °C, the volumetric activity decreased linearly after 24 hours, until 96 hours, where no volumetric activity was observed. This finding is of importance since the bioreactor fermentations were conducted at 30 °C. This indicates that the accumulated laccase in the fermentation medium would undergo degradation at the fermentation temperature. These effects were more severe at 33 °C, where the maximum volumetric activity observed at 24 hours was 1.88 U/L, almost half of that observed at 30 °C, and all activity ceased after 72 hours of growth. The lower spectrum of cultivation temperatures (12, 15 °C) showed the opposite trend, with volumetric activity showing a slight linear increase in volumetric activity throughout the cultivation period. Although the volumetric activity did not decrease at these

low cultivation temperatures, the final volumetric activity observed was significantly lower than that observed for the optimal cultivation at 23 °C.

It has been found that lower fermentation temperatures may be beneficial for laccase production. Hong *et al.* (2002) improved the volumetric activity yield of recombinant laccase expressed in *P. pastoris* by 5-fold by decreasing the fermentation temperature from 30 °C to 20 °C. Similarly, when *T. versicolor lcc1* was expressed in *P. pastoris*, there was a 5-fold improvement in activity when cultures were grown at 16-19 °C as opposed to 28 °C (Jönsson *et al.*1997). Comparable results have been reported in different yeast expression hosts, such as *S. cerevisiae*, expressing the same *T. versicolor lcc2* isoform used in the current study, where a 16-fold increase was observed in cultures grown at 19 °C as opposed to 28 °C (Cassland and Jönsson, 1999). This dependence on temperature is not seen in all expressed proteins, and seems to be gene specific (Cassland and Jönsson, 1999). The improved volumetric activity at lower temperatures may be attributed to reduced extracellular proteolysis and improved

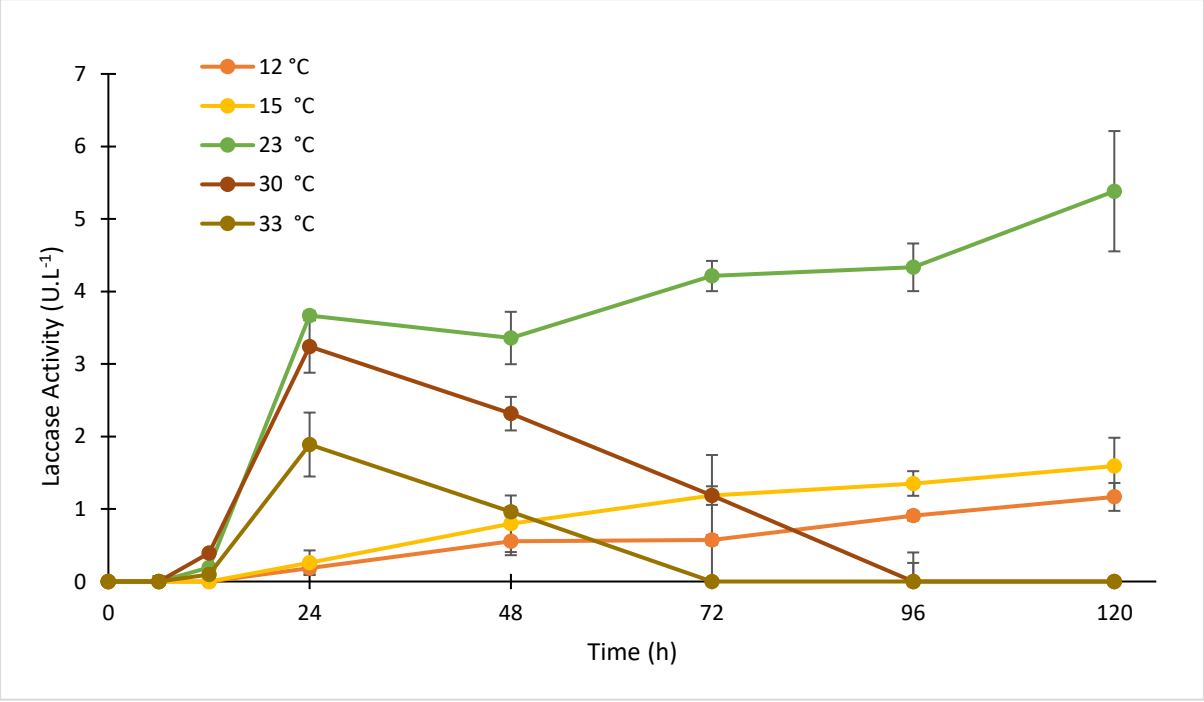


Figure 5.7. Effect of five cultivation temperatures (12 °C, 15 °C, 23 °C, 30 °C, 33 °C) on volumetric activity of secreted laccase, when grown in BMGY in shake flasks for 120 hours.

secretion dynamics in the yeast cell. Secretion comprises heterologous protein production and folding, as well as secretion trafficking. These processes are reported to be improved at lower induction temperatures compared to higher induction temperatures. Huang *et al.* (2008) studied these effects in *S. cerevisae* and found that the unfolded protein response (UPR) (See

Chapter 2, Section 6.3) was delayed when heterologous proteins were secreted at 20 °C as opposed to 30/37 °C.

Supplementation of copper

Two concentrations of copper supplementation (1 mM and 2 mM) were studied at shake flask scale. The growth of the recombinant *P. pastoris* was unaffected by the added copper, as seen in near identical growth in terms of OD_{600} for all cultures. Surprisingly, no improvements in volumetric activity were observed when copper was supplemented (Fig. 5.8).

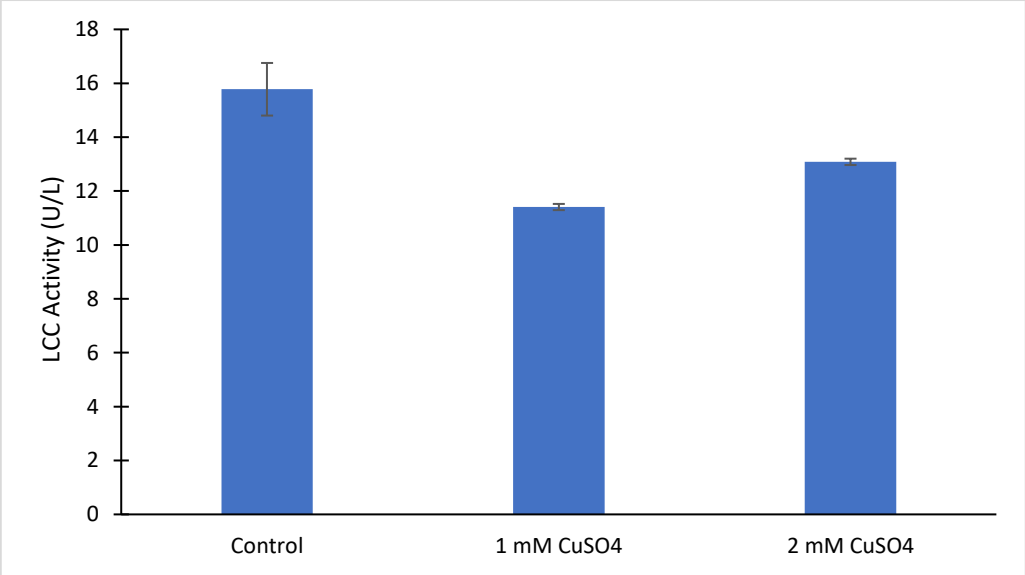


Figure 5.8. Effect of copper supplementation on volumetric activity of laccase after 72 hours of growth in BMGY.

The use of copper as a fermentation growth medium additive has been evaluated for recombinant laccase expression. The laccase active site is comprised of four copper atoms, thus during over-expression of the enzyme, the addition of copper has been shown to increase levels of the secreted enzyme (Neifar *et al.* 2009. Nishibori *et al.* 2012. Balakuruman *et al.* 2016). This is speculated to be as a result of replenishing the intracellular copper reservoir required for the production of copper-containing enzymes (Balakumaran *et al.* 2016). The importance of copper in the protein synthesis process for laccase has been proven, and it is even speculated that copper acts as a transcriptional inducer for laccase gene expression (Galhaup and Haltrich, 2001). In contrast, preliminary results presented here show no benefit to supplementation of copper. However, the scale of the experiment, and nutrient availability, should be considered. During high cell density cultivation in a bioreactor, the trace amounts of copper in the growth medium may be removed by biomass to a much larger extent than that experienced in a shake

flask. The trace metal solution used in the glycerol feed solution is designed to maintain optimal copper concentrations for *P. pastoris* growth only, as copper is essential in a number of metabolic processes (Balakumuran *et al.* 2016). It is therefore recommended that these experiments be repeated at bioreactor scale.

5.2.3 Glucuronoyl Esterase Production

The bioreactor cultivation for the expression of *H. jecorina* glucuronoyl esterase returned a final volumetric activity yield of 238.17 U/L, and a final recombinant protein titre of 2778.01 mg/L (Table 5.1). This volumetric activity was 39.6-fold higher than that found in shake-flask experiments (6 U/L, Section 5.1.3). The volumetric activity was detectable after 12 hours of batch cultivation, where a maximum volumetric activity of 20.69 U/L was reached at 20 hours (Fig. 5.6C). The glycerol fed-batch stage was subsequently initiated, where there was a three-hour lag phase, followed by a linear increase in volumetric activity, which was seen throughout the fed-batch phase. The volumetric activity did not reach a plateau; however, the specific protein productivity was constant at 0.34 mg/ g_{DCW}·h. during the fed-batch phase. Peak specific productivity ($q_{p.max}$) of 1.21 mg/g_{DCW}·h was reached at hour 12 of the batch phase, corresponding to mid-exponential growth phase. The mean specific productivity of protein was 0.52 mg/g_{DCW}·h, the highest of the three enzyme production bioprocesses. The biomass yield of 120.94 g_{DCW}/L was satisfactory and corresponded well to the biomass yields observed in the production of CDH and laccase in the current study as well as to published high cell density cultivations of recombinant *P. pastoris* (Bohlin *et al.* 2006. Maurer *et al.* 2006)

Very little published literature on recombinant expression of glucuronoyl esterase is available, with most studies focussing on native protein production in wild-type *H. jecorina* (Li *et al.* 2007a. Duranová *et al.* 2009. Sunner *et al.* 2015). The only published example, to the best of the author's knowledge, of quantitative heterologous glucuronoyl esterase expression in *P. pastoris* was reported by Topakas *et al.* (2010). These authors expressed *S. thermophile* glucuronoyl esterase in *P. pastoris* under P_{AOX1} regulation and the α-factor secretion signal. They reported a maximum volumetric activity of 5.2 U/L after 7 days of cultivation at shake-flask scale. Thus, the current study reports the first incidence of recombinant *H. jecorina* glucuronoyl esterase expression, and the highest volumetric activity (238.17 U/L) of constitutively expressed glucuronoyl esterase to date.

Other examples of glucuronoyl esterase expression in *P. pastoris* have been published, however the authors did not quantify enzyme activity, but rather made use of qualitative methods to confirm expression (Huynh and Arioka 2016. Sasagawa *et al.* 2011. d'Errico *et al.* 2015). While comparisons of enzyme production cannot be made with other examples of recombinantly produced glucuronoyl esterase, it is possible to compare levels of native expression in white-rot fungi. Native glucuronoyl esterase production in genetically modified *H. jecorina* grown on cellulose reached a final protein titre of 80 mg/L after 7 days of growth in shake flasks (Li *et al.* 2007a). The current study reports a final protein titre of 2778.01 mg/L, much higher than the 80 mg/L reported by Li *et al.* (2007b), further confirming the success and validity of the bioprocess reported here.

5.2.4 Comparison of protein concentrations between different expressed enzymes

The argument of whether protein properties affect their secretion in *P. pastoris* is an interesting and controversial one. It is obvious that different proteins are secreted in different amounts in *P. pastoris*. However, the exact reasons why, and how to predict these differences, are still elusive. Intuitively, the more complex a protein is (in terms of number of amino acids, post-translational modifications, and tertiary structure), the more taxing it is on a cell to perform transcription and translation during protein synthesis. However, these translational bottlenecks have been shown to be much more prominent in prokaryotic hosts, such as *Escherichia coli* than in eukaryotic hosts such as *P. pastoris*, due to the lower specific productivity values of the latter host (Hohenblum *et al.* 2004). Comparisons between publications are extremely difficult to make since the expression and secretion of proteins are affected by an enormous number of genetic and process-specific factors. Very limited research exists in which researchers have evaluated differences in protein yields when using identical expression strategies and fermentation conditions. One such example was conducted by Love *et al.* (2012), where the expression of three genes of varying complexity was evaluated during constitutive expression in *P. pastoris.* It was found that the rate limiting step in heterologous protein production, was the secretion process, regardless of protein complexity. This result was mirrored in a study by Pfeffer *et al.* (2012), which highlighted the importance of the endoplasmic reticulum associated degradation (ERAD) pathway during constitutive protein expression in *P. pastoris*. Based on these results, it seems that intracellular processes associated with secretion may be the most important in determining recombinant protein yields. However, this still does not explain the link between differing secretion rates in different genes. Rakestraw and Whittrup (2005)

studied the effects of protein complexity in inducing secretory system stress in *S. cerevisiae* and found that the more complex post-translational processing a protein requires, the higher the likelihood of misfolding, and being recognized by the quality control system of the endoplasmic reticulum, ultimately resulting in degradation of the misfolded proteins.

The current study employed the use of identical molecular techniques and process conditions to produce three different heterologous proteins. Indeed, there were significant differences in target protein titres. The most efficiently secreted protein was glucuronoyl esterase, while the least efficient was laccase (Fig. 5.9). Thus, the size of the protein did not give an indication of secretion efficiency, since the largest protein, CDH (127 kDa) was secreted more efficiently than the smaller laccase protein (104 kDa), and the smallest protein, glucuronoyl esterase (78 kDa) was secreted at the highest protein concentrations. Interestingly, the protein with the highest number of disulphide bonds, glucuronoyl esterase, was secreted most efficiently. Disulphide bonds are post-translational modifications that are formed during secretion. In contrast to what was observed in the current study, disulphide bonds have previously been identified as rate-limiting in the secretion of recombinant proteins in *P. pastoris* and *S. cerevisiae* (Macauley-Patrick *et al.* 2005. Rakestraw and Whittrup, 2005). However, *P. pastoris* has been shown to be capable of secreting proteins with highly complex disulphide bonds, such as invertase and a fragment of thrombomodulin (White *et al.* 1994). The main structural difference between poorly secreted laccase and the other two enzymes are the number of domains that make up the protein. Laccase is comprised of three domains, while both cellobiose dehydrogenase and glucuronoyl esterase are comprised of two domains. Based on the literature discussed above, and the fact that identical genetic modification procedures and bioprocess conditions were used in the current study, it can be speculated that these differences in protein production were as a result of bottlenecks in the secretory pathway of *P. pastoris*, which are dependent on specific protein properties. However, the scope and objective of the current study was limited to establishing baseline titres and observing differences in production yields. Therefore, a promising future study would be to evaluate the mechanisms behind the differences in protein production observed in the current study.

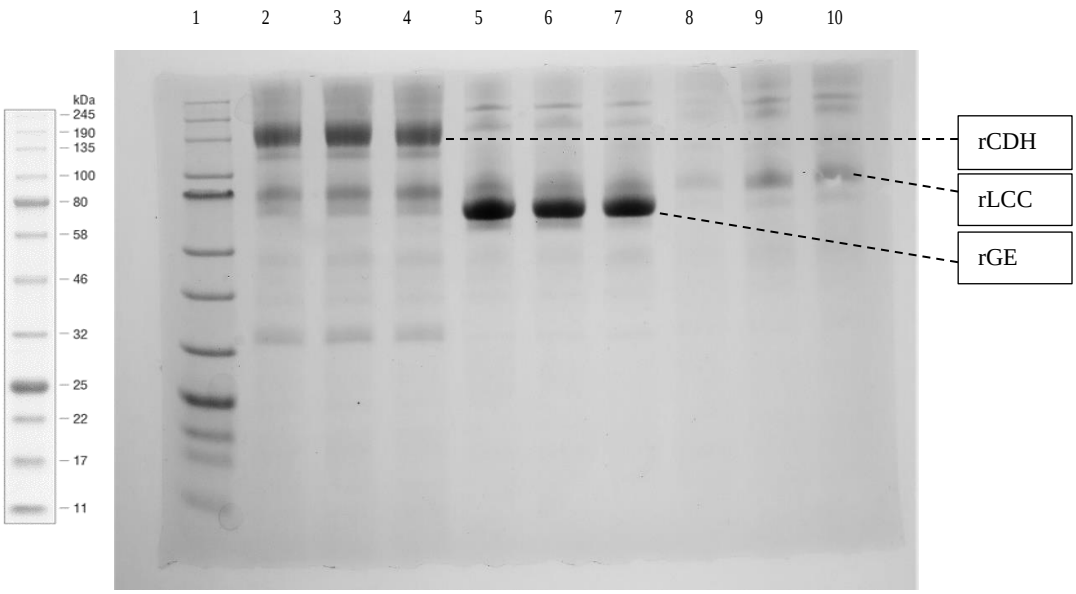


Figure 5.9. SDS-PAGE analysis of final fermentation samples (each lane representing one replicate of a bioreactor fermentation) of 20µl supernatant containing the target proteins. Lane 1: Protein molecular weight marker. Lane 2-4: CDH-containing supernatant (rCDH). Lanes 5-7: Glucuronoyl esterase-containing supernatant (rGE). Lanes 8-10: Laccase-containing supernatants (rLCC).

5.2.5 Enzyme production using constant glycerol feed

To establish baseline secretion levels through constitutive expression of the three target enzymes, a constant glycerol fed-batch bioreactor process was evaluated. The constant feed strategy was selected to avoid possible glycerol accumulation in the fermentation media, which has been shown to be detrimental at concentrations over 4 % (w/v) (Cos *et al.* 2006). The most popular choices of carbon source for constitutive recombinant protein production in *P. pastoris* are glucose and glycerol. The advantages of glycerol are the cost effectiveness, little to no accumulation of fermentative by-products such as acetate and ethanol, as well as satisfactory productivity levels in comparison to glucose (Calik *et al.* 2015). In addition, the scope of the current study is for the incorporation of lignin-modifying enzymes into biorefinery concepts. Crude glycerol-containing wastes, including those from biodiesel production, may be used to produce recombinant proteins, making glycerol the more industrially efficient carbon source (Tang *et al.* 2009, dos Santos *et al.* 2012). Taking into consideration the industrial potential, economic benefits, and novelty of the method, it was decided to test the production levels and growth rate of recombinant *P. pastoris* in a constant glycerol feed fed-batch process.

In the current study, three different lignin-modifying enzymes, isolated from three different source organisms were expressed using identical molecular strategies as well as identical growth conditions. The maximum specific growth rate was determined during the batch phase under identical growth conditions. Cellobiose dehydrogenase-, laccase-, and glucuronoyl esterase-expressing *P. pastoris* had similar μ_{max} values of 0.16 h^{-1}, 0.17 h^{-1}, and 0.15 respectively h^{-1} (Table 5.1). Specific growth rate is an important parameter to evaluate during recombinant protein production as it directly affects the productivity of the process (Looser *et al.* 2015). Of note in the current study is the exponential decrease in specific growth rate observed during the final stages of the fed-batch process (Fig 5.10). According to Maurer *et al.* (2006), ideally, the maximum specific growth rate (obtained during the batch phase) should be maintained for the first few hours, followed by a steady decrease in specific growth rate. In the current study, it was observed that after initiation of the glycerol fed-batch phase, there was a spike in specific growth rate (after 3-6 hours of glycerol feed), followed by a steady decline in specific growth rate, according to a decreasing exponential trend (Fig 5.10). This mimics the optimal specific growth rate model of Maurer *et al.* (2006), who used an exponential glucose feed strategy. Further, the results correlate well with models of what is expected in a constant feed fed-batch process, showing that the process conditions were correctly executed (Looser *et al.* 2015). Specifically, the amount of biomass in the fermentation vessel increased with time, but the amount of glycerol fed into the reactor remained constant, effectively decreasing the ratio of available carbon source per gram of biomass, resulting in a decrease in growth rate with time. The majority of kinetic studies in recombinant *P. pastoris* have shown that in a constitutive system, heterologous protein production is proportional to the specific growth rate. Thus, the specific growth rate should ideally be maintained at a value nearer to μ_{max} of a particular strain, however, this is not the case for all proteins and this factor should be evaluated on a case-by-case basis (Looser *et al.* 2015. Garcia-Ortega *et al.* 2013).

Limited research has been done on the evaluation of constant glycerol feeds as a carbon source in constitutive recombinant protein production in *P. pastoris*. Hu *et al.* (2008) investigated the effects of limited versus unlimited glycerol feeding in fed-batch fermentations constitutively expressing a model protein. These authors found that unlimited feeding of glycerol yielded the highest recombinant protein secretion, owing to improved oxygen transfer rates. A handful of studies have evaluated feeding strategies for P_{GAP}-*P. pastoris* bioprocesses for other carbon sources, such as glucose. These studies have reported conflicting results, showing that the

optimum production process is significantly influenced by the properties of the heterologous protein being produced. Maurer *et al.* (2006) modelled an optimised glucose feeding profile, based on the specific growth rate. These authors found that the optimum feed phase consisted of an exponential feed, corresponding to the maintenance of the maximum specific growth rate for a predetermined time period, followed by a phase with linearly increasing feed rate, corresponding to a decrease in specific growth rate. Similarly, a number of authors investigated the use of a μ-stat strategy, which modifies the feed rate throughout the fermentation based on a fixed specific growth rate (Zhao *et al.* 2008, Garcia-Ortega *et al.* 2013). Zhao *et al.* (2008) and Garcia-Ortega *et al.* (2013) reported contrasting conclusions. Zhao *et al.* tested four specific growth rates ranging between 0.1 – 0.25 h^{-1} and reported an optimum specific growth rate equal to 0.15 h^{-1} in the production of lipase, while Garcia-Ortega *et al.* found no significant differences between 0.1 h^{-1} and 0.15 h^{-1} for the production of human antigen binding fragment. A number of studies have investigated the glycerol feeding stage, prior to methanol induction, in P_{AOX1}-*P. pastoris* bioprocesses, however, application of these strategies in a constitutive system would yield different results as the mode of expression affects the growth characteristics of a particular strain (Potvin *et al.* 2012).

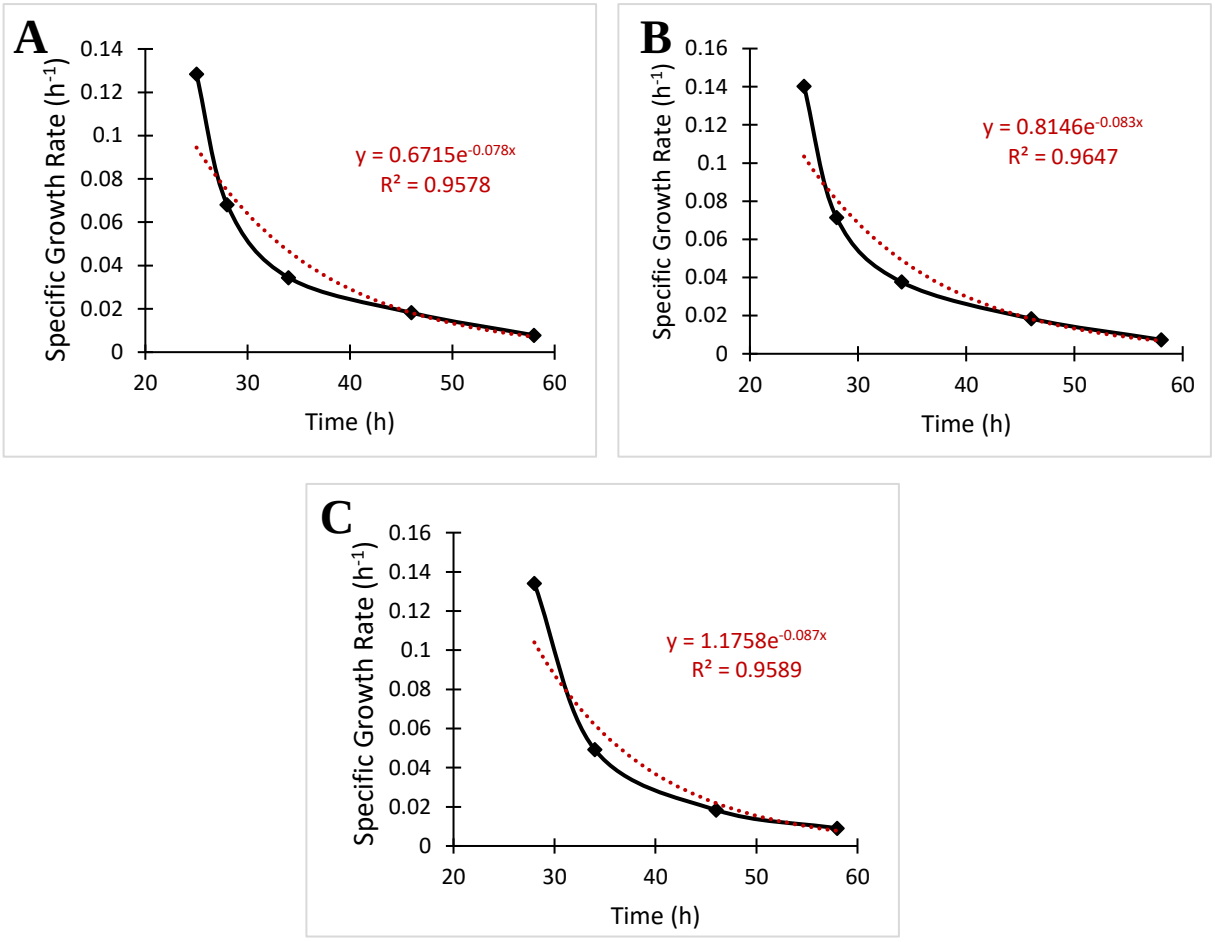


Figure 5.10. Exponential decline in specific growth rate during fed-batch stage of the fermentation, with a constant glycerol feed of 72.6 ml/hr. of A: Glucuronoyl esterase; B: Laccase; C: Cellobiose dehydrogenase.

Over-all, the productivity of the fermentation bioprocesses reported here, in terms of recombinant protein concentrations, were satisfactory (Table 5.1). The protein titres ranged between 778.54 – 2778.01 mg/L, which are exemplary values for recombinant protein production in *P. pastoris*. In terms of biomass, the glycerol feeding strategy returned satisfactory results, with yields ranging between 119.54 - 136.47 g_{DCW}/L. It can be argued that the yields of these fermentations would be improved by using an exponential feeding strategy to maintain the specific growth rate near to μ_{max}. However, this correlation may not be maintained in some heterologous protein expression systems due to the secretion bottlenecks experienced during high cell density fermentations (Puxbaum *et al.* 2015). Glycerol was measured throughout the fed-batch process and it was found to be at concentrations below the detectable limit of the HPLC analysis. This shows that the glycerol was efficiently assimilated by the biomass, and when considering the decrease in specific growth rate, this is likely to be an indication of glycerol limitation in the bioprocess. It follows that the glycerol constant-feed

strategy applied in the current study may be improved by either increasing the constant glycerol feed rate or by replacing the constant feed strategy with an exponential feeding strategy.

5.3 Downstream Processing

The successful production of the target enzymes was the first step in bridging knowledge gaps in literature, and overcoming limited availability of the enzymes. The next step is the evaluation of the ability of these enzymes to act on lignins or lignin-carbohydrate complexes in technical lignins. In order for the proposed biocatalysis in lignin valorisation to be evaluated in future studies, the cell-free extracts were filtered and concentrated. In addition, the concentrated extracts require storage until future use, therefore the feasibility of freezing the concentrated enzyme solutions at -20 °C was evaluated.

5.3.1 Concentration of cell-free extracts using two-step tangential flow filtration

Two-step tangential flow filtration (TFF) was employed to concentrate the cell-free extracts of each enzyme-expressing strain. Ultrafiltration is a common industrial method used to concentrate protein solutions, with human antibodies being the most prominent example (Van Reis and Zydney, 2001). Firstly, a 0.22 µm filtration cassette was used for microfiltration, which removed any cell debris and clarified the enzyme solution. The permeate from this microfiltration process was then used in a subsequent ultrafiltration process where a 5 kDa filtration cassette concentrated the enzyme solution. While this is an effective method to concentrate the protein of interest in terms of volumetric activity, it must be noted that all proteins above the size of 5 kDa are also concentrated. Thus, while the process significantly improves the volumetric activity of the cell-free extract, the specific activity of the solution (activity units per gram total protein) will only be slightly improved or unchanged, due to the majority of native secreted proteins being larger than 5 kDa (Fig. 4.11) (Pezzell*a et al.* 2017).

The results of the concentration process are summarised in Table 5.2 and represented in Figure 5.11. The concentration process was successful in terms of volumetric activity and protein concentration. The final volume was concentrated approximately 10-fold for all cell-free extracts. In terms of volumetric activity, the CDH, LCC, and GE were concentrated 5.20-, 6.01-, and 4.95-fold, respectively. The final volumetric activity yield, calculated as a percentage of the total number of enzymatic units in solution before and after each filtration step, of CDH, LCC, and GE were 50.00, 60.04, and 49.57 % respectively. The total protein concentration in

Table 5.2 Summary of tangential flow filtration concentration of cell-free extracts obtained from bioreactor fermentations.

	Filtration Step	Volumetric Activity (U/L)	Total Activity (U)	Yield (%, volumetric activity)	Total Protein Concentration (g/L)
Cellobiose Dehydrogenase	**Cell-free extract**	203.92	407.84	100.00	2.93
	0.22 µm permeate	196.07	352.93	86.54	-
	5 kDa retentate (concentrate)	1019.61	203.92	50.00	8.31
	5 kDa permeate	2.11	2.95	-	-
Laccase	**Cell-free extract**	75.56	151.12	100.00	2.07
	0.22 µm permeate	75.37	135.67	89.78	-
	5 kDa retentate (concentrate)	453.70	90.74	60.04	4.99
	5 kDa permeate	0.00	0.00	-	-
Glucuronoyl esterase	**Cell-free extract**	40.77	81.54	100.00	2.97
	0.22 µm permeate	39.34	70.81	86.84	-
	5 kDa retentate (concentrate)	202.08	40.42	49.57	10.16
	5 kDa permeate	0.03	0.04	-	-

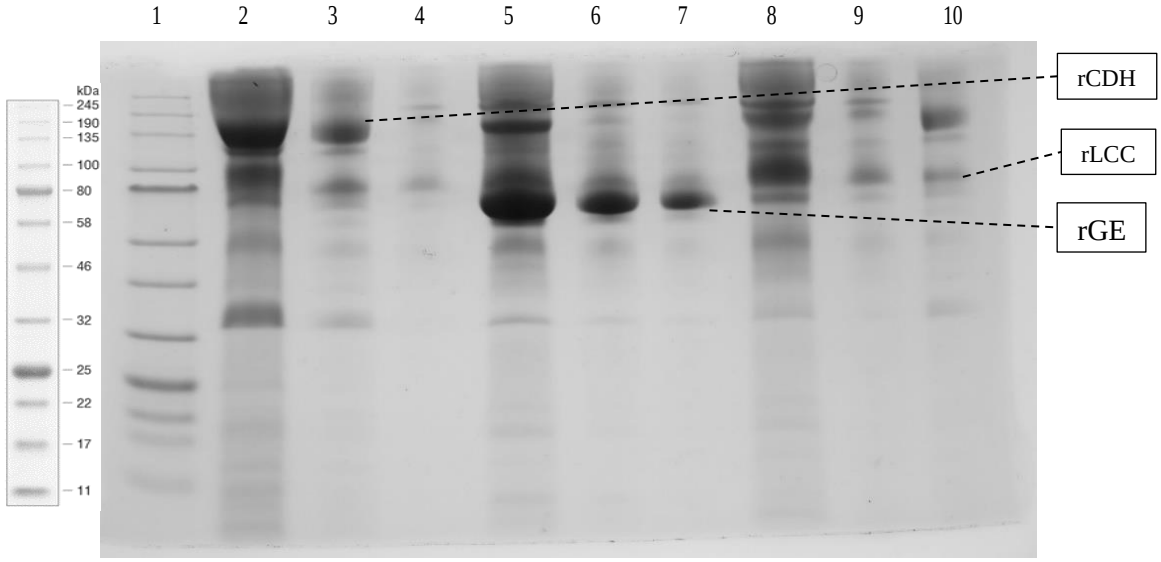


Figure 5.11. SDS-PAGE analysis of concentrated enzyme extracts. 20µl of sample was loaded in each lane. Lane 1: Protein molecular weight marker. Lane 2-4: Concentrated CDH-containing extract (rCDH), at no dilution, 5X dilution and 10X dilution. Lanes 5-7: Concentrated glucuronoyl esterase-containing supernatant (rGE) at no dilution, 5X dilution and 10X dilution. Lanes 8-10: Concentrated laccase-containing extract (rLCC) at no dilution, 5X dilution and 10X dilution.

the enzyme extracts was increased 2.84-, 2.41-, 3.69-fold for CDH, LCC, and GE respectively (Fig. 5.11).

The microfiltration process returned satisfactory volumetric activity yields (86.54-89.77 %), and the loss in activity units can be explained by the inherent loss of sample volume in the filtration apparatus itself. The ultrafiltration process returned volumetric activity yields that were acceptable, but still lower than expected (49.57-60.04 %). Previously reported volumetric activity yields for ultrafiltration-based concentration of cell-free extracts containing *P. pastoris*-secreted recombinant proteins, range between 42.9 – 96.2 %, with the modal value of reported yields in the range of 90 % (Zhong *et al.* 2004. Sulej *et al.* 2015. Stapleton *et al.* 2004. Eggert *et al.* 1996. Tien and Kirk, 1988. Li *et al.* 2014a. Colao *et al.* 2006). The permeate of the ultrafiltration process contained minimal total volumetric activity (0.00 – 2.95 U_{Total}/L), showing acceptable integrity of the membrane, and ruling this out as a possible reason for the observed losses in volumetric activity (Table 5.2). It is therefore hypothesised that the recombinant protein is being retained or degraded in the filtration apparatus itself.

The ultrafiltration membrane used in the current study is a polyethersulphone (PES) membrane. PES membranes are hydrophilic, and should have low, non-specific protein binding properties. Despite this, membrane fouling and retention is a common issue experienced in the ultrafiltration of protein solutions (Shi *et al.* 2014. Jones and O'Melia, 2001). Proteins are known to adsorb to membranes as a result of their affinity to the membrane surface, often causing permanent fouling (Jones and O'Melia, 2001). Proteins are complex macromolecules with a number of functional groups – some of these functional groups (e.g. hydrophobic acids) confer lipophilicity to the protein. This property allows the protein to interact and bind with the membrane surface, causing membrane fouling (Shi *et al.* 2014). This results in blockages of the membrane pores, and the formation of a protein mono-layer on the surface of the membrane, known as a fouling cake (Kim *et al.* 1992). Possible strategies to improve future enzymatic activity yields by preventing membrane fouling include i) modification of the membrane surface through presorption with a surface-active agent, such as ionic or anionic surfactants and polymers, or ii) optimization of the operating parameters, e.g. transmembrane pressure, cross-flow velocity and permeate flux (Hilal *et al.* 2005. Brink and Romijn, 1990).

5.3.2 Freezing as a storage strategy

Enzymes are sensitive to conformational and chemical degradation in aqueous environments due to changes in pH, temperature fluctuations, proteases, and a host of other protein-denaturing factors (Iyer and Ananthanarayan, 2008). Of particular interest for *P. pastoris*, are the action of native secreted proteases that cleave peptide bonds in secreted proteins, causing proteolytic degradation and rendering them inactive (Macauley-Patrick *et al.* 2005). It was observed that during refrigeration of the cell extracts, activity decreased with time, as illustrated by the differences in volumetric activity values directly after fermentation (Fig 5.6) and before filtration (Table 5.2). Thus, it was necessary to devise a long-term plan for storage of the concentrated enzyme products. Freezing at -20 °C is a commonly used strategy for long term storage of protein products, and negates the protein degradation risks of the freeze-drying process (Manning *et al.* 2010). It was therefore decided to test the efficiency of freezing at -20 °C as a storage strategy.

Of the three enzymes, cellobiose dehydrogenase showed no change in activity before and after freezing. However, laccase and glucuronoyl esterase showed activity losses of 5.84 % and 10.71 %, respectively, after freezing. Proteins are subject to degradation at freezing temperatures, and during thawing. This occurs due to temperature decrease, ice formation at the ice-water interface, concentration of solutes due to the crystallization of water, and pH changes that occur (Cao *et al.* 2003). Interestingly, glucuronoyl esterase had the highest protein concentration and experienced the highest sensitivity to freezing. This may be explained by the formation of protein aggregates at high protein concentrations (Shire *et al.* 2004). Protein aggregation has been shown to reduce activity of enzymes, and is aggravated during the freezing process (Wang, 1999).

A significant amount of research has been done on using additives, known as cryoprotectants, to reduce the degradation experienced during freezing and increase stability of proteins during the freezing process (Iyer and Ananthanarayan, 2008). Polyols, glycerol in particular, have been extensively studied for the use as stabilizers of protein solutions (Gekko, 1981). Glycerol is a hydrophilic solvent that reduces protein flexibility, and interacts with hydrophobic protein surfaces, thereby inhibiting protein unfolding and stabilizing the protein (Vagenende *et al.* 2009). In light of these findings, the addition of glycerol at two different percentages that are routinely used in enzyme preparations, 10 % (v/v) and 25 % (v/v), as a cryoprotectant for laccase and glucuronoyl esterase was assessed (Fig. 5.12).

The addition of 10 % (v/v) and 25 % (v/v) glycerol reduced the laccase activity loss to 1.3 % and 1.20 %, respectively. This shows that glycerol is a suitable cryoprotectant for concentrated laccase solutions. Therefore, since the different concentrations did not yield significantly different losses in enzyme activity, it was decided to select 10 % (v/v) glycerol as the most economical glycerol concentration.

In contrast, unexpected results were obtained when glucuronoyl esterase was frozen with glycerol. It was observed that the addition of glycerol increased the loss of enzyme activity to approximately 23 %, at both concentrations. This increase cannot be explained by interference of glycerol with the enzymatic assay, as glycerol is not detected by the D-Glucuronic Acid Kit (Megazyme, USA). The glucuronoyl esterase protein is hydrophobic in nature (Li *et al.* 2007a). Vaganende *et al.* (2009) studied the mechanism of glycerol binding preferentially to hydrophobic surfaces of proteins. It was found that the properties of the specific protein interface alters the way in which glycerol binds to the protein surface. Further, these authors show that glycerol inhibits hydrophobic interactions in proteins. When considering the three-dimensional conformation of glucuronoyl esterase, and the fact that the catalytic triad is located on the surface of the protein (as opposed to being buried within the three-dimensional structure, as is seen in laccase and cellobiose dehydrogenase), it can be hypothesised that the glycerol-protein interaction created steric hindrance for interaction between the substrate and the catalytic site. However, further study is required to examine these unexpected results. In addition, more cryoprotectants, such as sucrose or bovine serum albumin (BSA) should be investigated to reduce the apparent effects of freezing on glucuronoyl esterase activity (Brougham and Johnson, 1981). Nonetheless, for the purposes of the current study, it is more efficient to freeze the concentrated enzyme solutions (without glycerol) and sacrifice the 10 % volumetric activity, than to store the enzyme product at 4 °C where more extensive degradation occurs.

It is therefore recommended that the cellobiose dehydrogenase and glucuronoyl esterase concentrates be frozen without glycerol, and the laccase should contain 10 % (v/v) glycerol as a cryoprotectant.

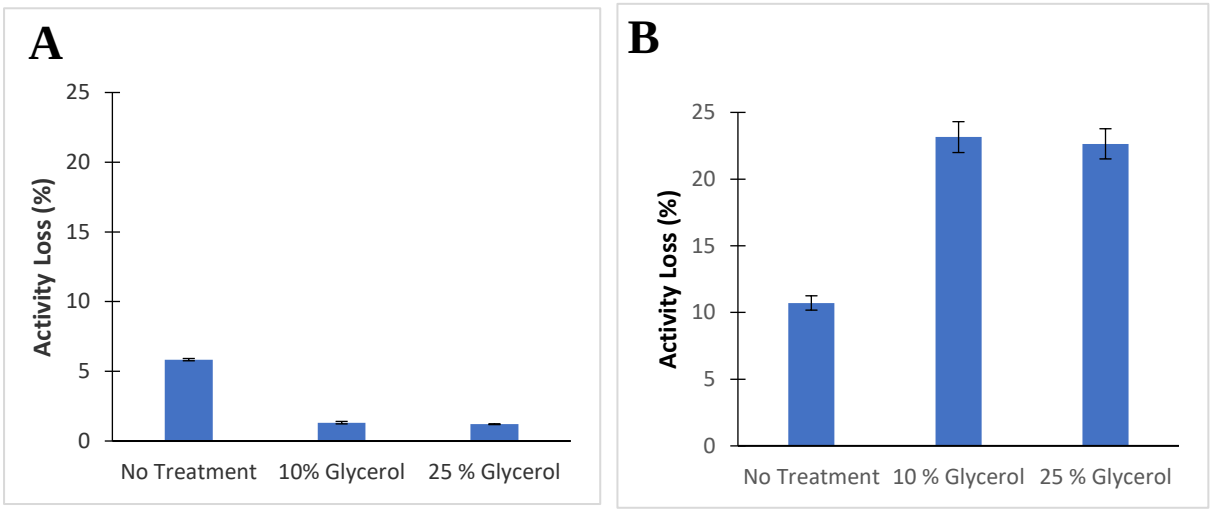


Figure 5.12. Effect of glycerol as a cryoprotectant for A: Laccase, and B: Glucuronoyl esterase.

Chapter 6: Conclusions & Recommendations

6.1 Conclusions

The present study aimed to provide fundamental data of the capabilities of a constitutive *P. pastoris* expression system to produce three enzymes, namely cellobiose dehydrogenase, laccase, and glucuronoyl esterase; all associated with lignin valorisation. A two-stage fermentation strategy, consisting of a batch phase, followed by a constant glycerol fed-batch stage was implemented in a 14 L bioreactor.

Three P. *pastoris* strains expressing the three target enzymes, namely *N. crassa* cellobiose dehydrogenase, *T. versicolor* laccase isoform *lcc2*, and *H. jecorina* glucuronoyl esterase were successfully created by making use of the DNA 2.0 (USA) pJexpress 905 plasmid, in collaboration with the Department of Microbiology, University of Stellenbosch. For the range of screened transformants, similar levels of secretion were observed among transformants of the same gene, implying low levels of clonal variation for the transformation procedure used.

The enzyme production process was scaled up in a 14 L bioreactor. The growth patterns of each strain were similar throughout the bioprocess. The time-profile of enzyme production during the batch phase was consistent for all strains. During the fed-batch stage, protein secretion plateaued at different stages for each of the strains, with cellobiose dehydrogenase reaching plateau sooner than laccase and glucuronoyl esterase. Large quantities of the target proteins were successfully produced.

A constant glycerol feed supplemented with trace salts was used in the fed-batch stage of all fermentations. No accumulation of glycerol was observed in the growth medium. Exponential growth rate decline was observed during the fed-batch stage, corresponding to a decreasing ratio of glycerol feed per unit of biomass. Improvements in productivity may be observed if an increased glycerol feeding rate or exponential feeding strategy is used, to maintain the maximum growth rate of each strain obtained in the current study.

Identical fermentation procedures were used to produce all three enzymes. This allowed for comparison in production kinetics and trends in protein secretion between the three strains. It was found that glucuronoyl esterase yielded the highest titre of protein, followed by cellobiose dehydrogenase and laccase, respectively. It is hypothesised that secretory processes are the main bottleneck in protein production and secretion between the three strains, however, further study on expression levels is required to make definitive conclusions. Preliminary assessments

indicate that the low productivity of the laccase fermentation may be improved by lowering the fermentation temperature.

A simple two-step tangential flow filtration strategy was successfully used for concentration of the harvested cell-free enzyme extracts, with final volumetric activity yields of 49.57, 50.00, and 60.04 % for cellobiose dehydrogenase, laccase, and glucuronoyl esterase, respectively. The decreased volumetric activity yields may be due to membrane fouling caused by the proteins. Cellobiose dehydrogenase was found to be the most stable after freezing at -20 °C, laccase was stabilized during freezing by the addition of 10 % (v/v) glycerol, and glucuronoyl esterase was the most sensitive to loss of activity during freezing and refrigeration.

This study has successfully expressed and produced three enzymes that may be applied in lignin valorisation in a constitutive *P. pastoris* expression system at 14 L bioreactor scale. Fermentation profiles of biomass and volumetric activity yields have provided essential fundamental data for future optimisation studies. The production of these enzymes will contribute to research on the enzymatic valorisation of lignin-enriched residues.

6.2 Recommendations

Recombinant protein production yields should be optimised in future studies by using the baseline data generated in the current study. Optimisation of the expression system itself could include the use of alternative secretion factors, such as the α-secretion factor, as well as alternative source genes from organisms that have shown benchmark levels of volumetric activity yields.

Improvement of the bioreactor process could be achieved by increasing the glycerol feed rate, since the cultures were glycerol limited, and recombinant protein production is usually growth-associated under regulation of the pGAP promoter. Further, an exponential feed could be attempted, however, caution should be taken to keep the glycerol concentrations below inhibitory levels.

Laccase fermentation productivity may be improved by lowering the fermentation temperature and by the addition of copper supplements. In addition, the economic trade-off between decreasing growth temperature and effects on productivity may be investigated.

Limitations in the secretion capacities of the current strains could be investigated further by evaluating gene dosage and expression levels using quantitative PCR of cDNA during the

enzyme production process. Studies on the secretory pathway of the strains generated in this study, will guide the development of tailor-made expression host for optimized expression of these recombinant enzymes.

Regarding the downstream processing, volumetric activity yields can be improved by investigating ultrafiltration process parameters, and attempting alternative membrane polymers or pore sizes. A mass-balance should be performed to identify the amount of enzyme product that is retained in the TFF membrane. A diafiltration strategy should be investigated whereby the addition of a buffer to the final product may prevent degradation of the protein product. In terms of the long-term storage of the target enzymes, the storage of glucuronoyl esterase should be further optimised by testing different cryoprotectants, such as sucrose or BSA. In addition, lyophilisation may be an alternative storage strategy, however this strategy would also require optimisation of additives (sugars, proteins) to prevent losses of enzyme activity.

When holistically considering the importance of optimising either the upstream or downstream processes it is difficult to speculate which of these stages would offer the best prospects for ultimately improving the resultant protein yields. Clearly, optimisation of upstream processes has been shown to improve recombinant protein yields dramatically. However, if the produced recombinant proteins are lost during downstream processing, is it worth the time and monetary cost of optimising the upstream process? Due to the fact that the current study returned relatively high recombinant protein concentrations after fermentation (and the fact that protein secretion levels are finite in *P. pastoris*), it can be argued that there would be more scope in optimisation of the downstream process to maximise the preservation of the recombinant proteins that were produced. In addition, ultrafiltration of concentrated protein solutions has been shown to be problematic, therefore, having a lower initial protein concentration may be beneficial (Shire *et al.* 2004). However, it may well be that a higher initial protein concentration, or a more stable recombinant protein (as a result of optimisation of upstream parameters) will still return higher protein yields after ultrafiltration, regardless of the downstream process. In addition, should the recombinant protein be produced in high enough concentrations, concentration of the protein solution through ultrafiltration would be unnecessary. Further, optimisation of both upstream and downstream processes will be specific to each enzyme, and the optimal process for one of the enzymes will not necessarily translate to an optimised process for a different enzyme. Therefore, it is recommended that optimisation of both upstream and downstream processes be considered with equal importance.

References

Adler E. 1977. Lignin chemistry: past, present and futures. *Wood Science and Technology*. 11: 169- 218.

Adrio JL and Demain AL. 2010. Recombinant organisms for production of industrial products. *Bioengineered Bugs*. 2: 116-131.

Aehle W. 2007. *Enzymes in industry*. 3rd Ed. Weinheim: Wiley-VCH.

Ahmad M, Hirz M, Pichler H, and Schwab H. 2014. Protein expression in *Pichia pastoris*: Recent achievements and perspectives for heterologous protein production. *Applied Microbiology and Biotechnology*. 98: 5301-5317.

Antošová Z and Sychrová H. 2016. Yeast Hosts for the Production of Recombinant Laccases: A Review. *Molecular Biotechnology*. 58: 93–116.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 1994. Current protocols in molecular biology. John Wiley and Sons, Inc., New York, NY

Aw R and Polizzi KM 2016. Liquid PTVA: a faster and cheaper alternative for generating multi - copy clones in *Pichia pastoris*. *Microbial Cell Factories*. 15: 29.

Balakshin M, Capanema E, and Berlin A. 2014. Isolation and analysis of lignin-carbohydrate complex preparations with traditional and advanced methods: A review. In: *Studies in Natural Products Chemistry Vol 42*. Ed: Atta-ur-Rahman, FRS. Elsevier, United Kingdom.

Balakshin M, Capanema E, Gracz H, Chang, H and Jameel H. 2011. Quantification of lignin-carbohydrate linkages with high-resolution NMR spectroscopy. *Planta*. 6: 1097-1110.

Balakumaran PA, Förster J, Zimmermann M, Charumathi J, Schmitz A, Czarnotta E, Lehnen M, Sudarsan S, Ebert BE, Blank LM and Meenakshisundaram S. 2016. The trade-off of availability and growth inhibition through copper for the production of copper-dependent enzymes by *Pichia pastoris*. *BMC biotechnology*. 16: 20.

Barr DP and Aust SD. 1994. Conversion of lignin peroxidase compound III to active enzyme by cation radicals. *Archives of Biochemistry and Biophysics*. 312: 511–515.

Baumann K, Carnicer M, Dragosits M, Graf AB, Stadlmann J, Jouhten P, Maaheimo H., Gasser, B., Albiol, J., Mattanovich, D., Ferrer, P., 2010. A multi-level study of recombinant Pichia pastoris in different oxygen conditions. BMC Syst. Biol. 4.

Baumann, K., Maurer, M., Dragosits, M., Cos, O., Ferrer, P., Mattanovich, D., 2008. Hypoxic fed-batch cultivation of Pichia pastoris increases specific and volumetric productivity of recombinant proteins. Biotechnol. Bioeng. 100, 177–183

Beckham GT, Johnson CW, Karp EM, Salvachua D, and Vardon DS. 2016. Opportunities and challenges in biological lignin valorisation. *Current Opinion in Biotechnology*. 42: 40-53.

Bey M, Berrin JG, Poidevin L, and Sigoillot JC. 2011. Heterologous expression of *Pycnoporus cinnabarinus* cellobiose dehydrogenase in *Pichia pastoris* and involvement in saccharification processes. *Microbial Cell Factories.* 10: 113.

Boer H, Teeri TT, and Koivula A. 2000. Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters. *Biotechnology and Bioengineering.* 69: 486-494.

Boerjan W, Ralph J, and Baucher M. 2003. Lignin biosynthesis. *Annual Reviews in Plant Biology.* 54: 519–546.

Bohlin C, Jönsson LJ, Roth R and van Zyl WH. 2006. Heterologous expression of *Trametes versicolor* laccase in *Pichia pastoris* and *Aspergillus niger*. *Applied biochemistry and biotechnology*, 129–132: 195–214.

Brink LES and Romijn DJ. 1990. Reducing the protein fouling of polysulfone surfaces and polysulfone ultrafiltration membranes: Optimization of the type of presorbed layer. *Desalination*. 78:209–233.

Brougham MJ and Johnson DB. 1981. Glycerol, α-glycerophosphate and other compounds as stabilizers of alcohol dehydrogenase from yeast. *Enzyme and Microbial Technology*. 3: 225–228.

Brown ME and Chang MCY. 2014. Exploring bacterial lignin degradation. *Current Opinion in Chemical Biology*. 19: 1–7.

Brugger D, Krondorfer I, Zahma K, Stoisser T, Bolivar JM, Nidetzky B, Peterbauer CK, and Haltrich D. 2014. Convenient microtiter plate-based, oxygen-independent activity assays for flavin-dependent oxidoreductases based on different redox dyes. *Biotechnology Journal*. 9:474–82.

Buchetics M, Dragosits M, Maurer M, Rebnegger C, Porro D, Sauer M, Gasser B and Mattanovich D. 2011. Reverse engineering of protein secretion by uncoupling of cell cycle phases from growth. *Biotechnology and Bioengineering*, 108: 2403–2412.

Bugg TDH and Rahmanpour R. 2015. Enzymatic conversion of lignin into renewable chemicals. *Current Opinion in Chemical Biology*. 29: 10-17.

Bugg TDH, Ahmad M, Hardiman EM and Rahmanpour R. 2011. Pathways for degradation of lignin in bacteria and fungi. *Natural Product Reports*, 28: 1883.

Calik P, Ata O, Gunes H, Massahi A, Boy E, Keskin A, Ozturk S, Zerze GH, and Ozdamar TH. 2015. Recombinant protein production in *Pichia pastoris* under glyceraldehyde-3-phosphate dehydrogenase promoter: from carbon source metabolism to bioreactor operation parameters. *Biochemical Engineering Journal.* 95: 20–36.

Call HP and Mücke I. 1997. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems Lignozym®-process., *Journal of Biotechnology* 53: 163–202.

Cameron MD and Aust SD. 2001. Cellobiose dehydrogenase-an extracellular fungal flavocytochrome. *Enzyme and Microbial Technology*. 28: 129–138.

Cannarrozzi G, Schraudolph NN, Faty M, von Rohr P, Friberg MT, Roth AC, Gonnet P, Gonnet G, and Barral Y. 2010. A role for codon order in translation dynamics. *Cell.* 141: 355-367.

Cannatelli MD and Ragauskas AJ. 2017. Two Decades of Laccases: Advancing Sustainability in the Chemical Industry. *Chemical Record*. 17:122–140.

Cao E, Chen Y, Cui Z and Foster PR. 2003. Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. *Biotechnology and Bioengineering*, 82: 684–690.

Cassland P and Jönsson LJ. 1999. Characterization of a gene encoding *Trametes versicolor* laccase A and improved heterologous expression in *Saccharomyces cerevisiae* by decreased cultivation temperature. *Applied Microbiology and Biotechnology*, 52: 393–400.

Cereghino GPL, Cereghino JL, Ilgen C and Cregg JM. 2002. Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. *Current Opinion in Biotechnology.* 13: 329–332.

Cereghino JL and Cregg JM. 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiology Reviews.* 24: 45–66.

Chandra R and Chowdhary P. 2015. Properties of bacterial laccases and their application in bioremediation of industrial wastes. *Environmental Science: Processes Impacts*. 17: 326–342.

Charavgi MD, Dimarogona M, Topakas E, Christakopoulos P, and Chrysina ED. 2013. The structure of a novel glucuronoyl esterase from *Myceliophthora thermophila* gives new insights into its role as a potential biocatalyst. *Acta Crystallographica Biological Crystallography*. 69: 63–73.

Chen H. 2014. Chemical composition and structure of natural lignocellulose, In: *Biotechnology of Lignocellulose: Theory and Practice*. Chemical Industry Press, Beijing.

Claus H. 2004. Laccases: Structure, reactions, distribution. *Micron*, 35: 93–96.

Colao MC, Lupino S, Garzillo AM, Buonocore V and Ruzzi M. 2006. Heterologous expression of lcc1 gene from *Trametes trogii* in *Pichia pastoris* and characterization of the recombinant enzyme. *Microbial Cell Factories*. 5:31.

Cos O, Ramon R, Montesinos JL, and Valero F. 2006. Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review. *Microbial Cell Factories.* 5: 17.

Daly R and Hearn MTW. 2005. Expression of heterologous proteins in *Pichia pastoris*: A useful experimental tool in protein engineering and production. *Journal of Molecular Recognition*, 18: 119–138.

Damasceno LM, Huang CJ, and Batt CA. 2012. Protein secretion in *Pichia pastoris* and advances in protein production. *Applied Microbiology and Biotechnology*. 93:31–39.

Dashtban M, Schraft H, Syed TA and Qin W. 2010. Fungal biodegradation and enzymatic modification of lignin. *International Journal of Biochemical Molecular Biology.* 1: 36–50.

Delroisse JM, Dannau M, Gilsoul JJ, El Mejdoub T, Destain J, Portetelle D, Thonart P, Haubruge E, and Vandenbol M. 2005. Expression of a synthetic gene encoding a tribolium castaneum carboxylesterase in *Pichia pastoris*. *Protein Expression and Purification*. 42: 286-294.

Demain AL and Vaishnav P. 2009. Production of recombinant proteins by microbes and higher organisms. *Biotechnology Advances*. 27: 297–306.

Demain AL. 2000. Small bugs, big business: the economic power of the microbe. *Biotechnology Advances.* 18: 499-514.

dErrico C, Börjesson J, Din, H, Krogh,KBRM, Spodsberg N, Madsen R, and Monrad RN. 2016. Improved biomass degradation using fungal glucuronoyl-esterases-hydrolysis of natural corn fiber substrate. *Journal of Biotechnology.* 219: 117-123.

dErrico C, Jørgensen JO, Krogh KBRM, Spodsberg N, Madsen R, Monrad RN. 2015. Enzymatic degradation of lignin-carbohydrate complexes LCCs.: Model studies using a fungal glucuronoyl esterase from *Cerrena unicolor*. Biotechnology and Bioengineering. 112: 914–922.

dos Santos EO, Michelon M, Furlong E, Fernandes J, Kalil S and Veiga C. 2012. Evaluation of the Composition of Culture Medium for Yeast Biomass Production Using. *Brazilian Journal of Microbiology*. 43: 432–440.

Ďuranová M, Hirsch J, Kolenová K, and Biely P. 2009b. Fungal glucuronoyl esterases and substrate uronic acid recognition. *Bioscience, Biotechnology and Biochemistry*. 73: 2483–2487.

Ďuranová M, Špániková, S Wösten HAB, Biely P, and De Vries RP. 2009a. Two glucuronoyl esterases of *Phanerochaete chrysosporium*. 191: 133-140.

Dutta R. 2008. Fundamentals of Biochemical Engineering. Ane Books India.

Eggert C, Temp U, Eriksson KE, Eggert C and Temp U. 1996. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Applied and Environmental Microbiology*. 62:1151–1158.

Fan Z, Wu W, Hildebrand A, Kasuga T, Zhang R, and Xiong X. 2012. A novel biochemical route for fuels and chemicals production from cellulosic biomass. *PLoS ONE.* 2: e31693.

Fang J, Huang F, and Gao PJ. 1999. Optimization of cellobiose dehydrogenase production by *Schizophyllum commune* and effect of the enzyme on kraft pulp bleaching by ligninases. *Process Biochemistry*. 34: 957–961.

FitzPatrick M, Champagne P, Cunningham MF and Whitney RA. 2010. A biorefinery processing perspective: treatment of lignocellulosic materials for the production of value-added products. *Bioresource Technology*. 101: 8915–8922.

Fleer R. 1992. Engineering yeast for high level expression. *Current Opinion in Biotechnology*. 3: 486- 496.

Fredrick K and Ibba M. 2010. How the sequence of a gene can tune its translation. *Cell*. 14: 227–229.

Galhaup C and Haltrich D. 2001. Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. *Applied Microbiology and Biotechnology*. 56: 225–232.

Gallagher SR. 2012. One-Dimensional SDS Gel Electrophoresis of Proteins. *Current Protocols in Molecular Biology*. 97:II:10.2A:10.2A.1–10.2A.44

Garcia-Ortega X, Ferrer P, Montesinos JL and Valero F. 2013. Fed-batch operational strategies for recombinant Fab production with *Pichia pastoris* using the constitutive GAP promoter. *Biochemical Engineering Journal*. 79: 172–181.

Gekko K. 1981. Mechanism of polyol-induced protein stabilization: solubility of amino acids and diglycine in aqueous polyol solutions. *Journal of biochemistry*. 90: 1633–1641.

Gianfreda L, Xu F and Bollag JM. 1999. Laccases: A Useful Group of Oxidoreductive Enzymes. *Bioremediation Journal*. 3: 1–26.

Giardina P, Faraco V, Pezzella C, Piscitelli A, Vanhulle S and Sannia G. 2010. Laccases: A never-ending story. *Cellular and Molecular Life Sciences*. 67: 369–385.

Gómez-Toribio V, Garcia-Martin AB, Martinez MJ, Martinez AT, and Guillén F. 2009. Induction of extracellular hydroxyl radical production by white-rot fungi through quinone redox cycling. *Applied and Environmental Microbiology.* 75: 3944-3953.

Goodrick JC, Xu M, Finnegan R, Schilling BM, Schiavi S, Hoppe H, and Wan NC. 2001. High-level expression and stabilization of recombinant human chitinase produced in a continuous constitutive *Pichia pastoris* expression system. *Biotechnology and Bioengineering.* 74:492-497

Gordobil O, Moriana R, Zhang L, Labidi J, and Sevastyanova O. 2016. Assesment of technical lignins for uses in biofuels and biomaterials: Structure-related properties, proximate analysis and chemical modification. *Industrial Crops and Products.* 83: 155-165.

Gosselink, R. 2015. Lignin valorisation towards materials, chemicals and energy. 2nd Lund symposium on lignin and hemicellulose valorisation, November 3-4, 2015, Lund.

Guerriero G, Hausman JF, Strauss J, Ertan H, and Siddiqui KS. 2016. Lignocellulosic biomass: biosynthesis, degradation, and industrial utilization. *Engineering in Life Sciences.* 16: 1-16.

Guillén F, Martínez MJ, Muñoz C, and Martínez AT. 1997. Quinone redox cycling in the ligninolytic fungus *Pleurotus eryngii* leading to extracellular production of superoxide anion radical. *Archives of Biochemistry and Biophysics.* 339: 190-199.

Gustafsson C, Govindarajan S, and Minshull J. 2004. Codon bias and heterologous protein expression. *Trends Biotechnology*. 22: 346–353.

Hallberg BM, Bergfors T, Baeckbro K, Pettersson G, Henriksson G, and Divne C. 2000. A new scaffold for binding haem in the cytochrome domain of the extracellular flavocytochrome cellobiose dehydrogenase. *Structure*. 8:79–88.

Hallberg MB, Henriksson G, Pettersson G, and Divne C. 2002. Crystal structure of the flavoprotein domain of the extracellular flavocytochrome cellobiose dehydrogenase. *Journal of Molecular Biology*. 315: 421–434.

Harreither W, Felice AKG, Paukner R, Gorton L, Ludwig R, and Sygmund C. 2012. Recombinantly produced cellobiose dehydrogenase from Corynascus thermophilus for glucose biosensors and biofuel cells. *Biotechnology Journal*. 71: 1359–1366.

Harreither W, Sygmund C, Augustin M, Narciso M, Rabinovich ML, Gorton L, Haltrich D, and Ludwig R. 2011. Catalytic properties and classification of cellobiose dehydrogenases from ascomycetes. *Applied and Environmental Microbiology.* 77: 1804–1815.

Henriksson G, Johansson G, and Pettersson G. 2000. A critical review of cellobiose dehydrogenases. *Journal of Biotechnology.* 78: 93-113.

Higgins DR and Cregg JM. 1998. Introduction to *Pichia pastoris.* In: *Pichia* Protocols, Methods in Molecular Biology Vol. 103. Totowa NJ: Humana Press Inc.

Hilal N, Ogunbiyi OO, Miles NJ and Nigmatullin R. 2005. Methods Employed for Control of Fouling in MF and UF Membranes: A Comprehensive Review. *Separation Science and Technology*. 40:1957–2005.

Hohenblum H, Gasser B, Maurer M, Borth N, and Mattanovich D. 2004. Effects of Gene Dosage, Promoters, and Substrates on Unfolded Protein Stress of Recombinant Pichia pastoris. *Biotechnology and Bioengineering*. 85: 367–375.

Hong F, Meinander NQ, and Jönsson LJ. 2002. Fermentation strategies for improved heterologous expression of laccase in Pichia pastoris. *Biotechnology and Bioengineering*, 79: 438–449.

Howard RL, Abotsi E, Jansen van Rensburg EL, and Howard S. 2003. Lignocellulose biotechnology: issues of bioconversion and enzyme production. *African Journal of Biotechnology*. 12: 602–619.

Hu XQ, Chu J, Zhang Z, Zhang SL, Zhuang YP, Wang YH, Guo MJ, Chen HX, Yuan ZY. 2008. Effects of different glycerol feeding strategies on S-adenosyl-l-methionine biosynthesis by PGAP-driven Pichia pastoris overexpressing methionine adenosyltransferase. *Journal of Biotechnology*. 137: 44–49

Huang D, Gore PR, Shusta EV. 2008. Increasing yeast secretion of heterologous proteins by regulating expression rates and post-secretory loss. *Biotechnology and Bioengineering*. 101: 1264–1275.

Huynh HH and Arioka M. 2016. Functional expression and characterization of a glucuronoyl esterase from the fungus *Neurospora crassa:* Identification of novel consensus sequences containing the catalytic triad. *The Journal of General and Applied Microbiology*. 224: 1–8.

Idiris A, Tohda H, Kumagai H, and Takegawa K. 2010. Engineering of protein secretion in yeast: strategies and impact on protein production. *Applied Microbiology and Biotechnology*. 86: 403–417.

Inan M, Aryasomayajula D, Sinha J and Meagher MM. 2005. Enhancement of protein secretion in *Pichia pastoris* by overexpression of protein disulfide isomerase. *Biotechnology and Bioengineering*. 93:771–778.

Iyer PV and Ananthanarayan L. 2008. Enzyme stability and stabilization-Aqueous and non-aqueous environment. *Process Biochemistry*. 43: 1019–1032.

Jahic M, Wallberg F, Bollok, M, Garcia P and Enfors SO. 2003. Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. *Microbial cell factories*. 2:6.

Johansson K, Gillgren T, Winestrand S, Järnström L, Jönsson LJ. 2014. Comparison of lignin derivatives as substrates for laccase-catalyzed scavenging of oxygen in coatings and films. *Journal of Biological Engineering*, 8: 1.

Johnson N, Powis K, and High S. 2013. Post-translational translocation into the endoplasmic reticulum. *Biochimica et Biophysica Acta.* 1833: 2403-2409.

Jones KL and O'Melia CR. 2001. Ultrafiltration of protein and humic substances: Effect of solution chemistry on fouling and flux decline. *Journal of Membrane Science*. 193:163–173.

Jönsson LJ, Saloheimo M, Penttilä M. 1997. Laccase from the white-rot fungus *Trametes versicolor*: cDNA cloning of lcc1 and expression in *Pichia pastoris*. *Current genetics*. 32: 425–430.

Kane JF. 1995. Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Current Opinion in Biotechnology.* 6: 494–500.

Kim KJ, Fane AG, Fell CJD and Joy DC. 1992. Fouling mechanisms of membranes during protein ultrafiltration. *Journal of Membrane Science*. 68:79–91.

Kirk O, Borchert TV, and Fuglsang CC. 2002. Industrial enzyme applications. *Current Opinion in Biotechnology*. 4: 345-351.

Kittl R, Gonaus G, Pillei C, Haltrich D, Ludwid R. 2012. Constitutive expression of Botrytis aclada laccase in *Pichia pastoris*. *Bioengineered*. 19: 263–267.

Klug WS and Cummings MR. 2011. *Concepts of Genetics*. 10th Ed. U:er Saddle River, NJ: Prentice Hall.

Laurichesse S and Avérous L. 2014. Chemical modification of lignins: towards biobased polymers. *Progress in Polymer Science*. 39: 1266–1290.

Lee J. 1997. Biological conversion of lignocellulosic biomass to ethanol. *Journal of Biotechnology*. 56: 1–24.

Lee, C.Y., Nakano, A., Shiomi, N., Lee, E.K., Katoh, S., 2003. Effects of substrate feed rates on heterologous protein expression by Pichia pastoris in DO-stat fed-batch fermentation. Enzyme Microb. Technol. 33, 358–365.

Lee, J., Yup, S., Park, S., Middelberg, A.P.J., 1999. Control of fed-batch fermentations. Biotechnol. Adv. 17, 29–48.

Li P, Anumanthan A, Gao X, Ilangovan K, Suzara VV, Düzgüneş N, and Renugopalakrishnan, V. 2007. Expression of recombinant proteins in *Pichia pastoris*. *Applied Biochemistry and Biotechnology*. 142: 105-124.

Li Q, Ge L, Cai J, Pei J, Xie J and Zhao L. 2014b. Comparison of two laccases from *Trametes versicolor* for application in the decolorization of dyes. *Journal of Microbiology and Biotechnology*. 24:545–555.

Li Q, Pei J, Zhao L, Xie J, Cao F and Wang G. 2014. Overexpression and characterization of laccase from *Trametes versicolor* in *Pichia pastoris. Applied Biochemistry and Microbiology*, 50:140–147.

Li Q, Pei J, Zhao L, Xie J, Cao F, Wang G. 2014. Overexpression and characterization of laccase from *Trametes versicolor* in *Pichia pastoris*. *Applied Biochemistry and Microbiology*, 50: 140–147.

Li XL, Špániková S, de Vries RP and Biely P. 2007. Identification of genes encoding microbial glucuronoyl esterases. *FEBS Letters*. 581:4029–4035.

Lin-Cereghino J, Wong WW, Xiong S, Giang W, Luong LT, Vu J, Johnson SD and Lin-Cereghino GP. 2005. Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris. BioTechniques.* 38:44–48.

Linger JG, Vardon DR, Guarnieri MT, Karp EM, Hunsinger GB, Franden MA, Johnson CW, Chupka G, Strathmann TJ, Pienkos PT, and Beckham GT. 2014. Lignin valorisation through integrated biological funneling and chemical catalysis. *Proceedings of the Natural. Academy of Sciences*. 111: 12013–12018.

Liu W, Chao Y, Liu S, Bao H and Qian S. 2003. Molecular cloning and characterization of a

laccase gene from the basidiomycete *Fome lignosus* and expression in *Pichia pastoris*. *Applied Microbiology and Biotechnology*. 63:174–181.

Liu Z, Tyo KEJ, Martinez JL, Petranovic D, and Nielson J. 2012. Different expression systems for production of recombinant proteins in *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering.* 109: 1259-1268.

Looser V, Brühlmann B, Bumbak F, Stenger C, Costa M, Camattari A, Fotiadis D, Kovar K. 2014. Cultivation strategies to enhance productivity of *Pichia pastoris*: A review. *Biotechnology Advances*. 33: 1177–1193.

Lora JH and Glasser WG. 2002. Recent Industrial Applications of Lignin: a Sustainable Alternative to Nonrenewable Materials. *Journal of Polymers and the Environment.* 10: 39-48.

Lorenzo M, Moldes D, Sanromán MÁ. 2006. Effect of heavy metals on the production of several laccase isoenzymes by *Trametes versicolor* and on their ability to decolourise dyes. *Chemosphere*, 63: 912–917.

Love KR, Politano TJ, Panagiotou V, Jiang B, Stadheim TA, Love JC. 2012. Systematic single-cell analysis of *Pichia pastoris* reveals secretory capacity limits productivity. *PLoS ONE*. 76: 1–11.

Ludwig RL, Harreither W, Tasca F, and Gorton L. 2010. Cellobiose Dehydrogenase: A Versatile Catalyst for Electrochemical Applications. *Journal of Chemical Physics and Physical Chemistry.* 13:2674-2697.

Lyczakowski JJ, Wicher KB, Terrett OM, Faria-Blanc N, Yu X, Brown D, Krogh KBRM, Dupree P and Busse-Wicher M. 2017. Removal of glucuronic acid from xylan is a strategy to improve the conversion of plant biomass to sugars for bioenergy. *Biotechnology for Biofuels*. 10: 224.

Ma S, Preims M, Piumi F, Kappel L, Seiboth B, Record E, Kracher D and Ludwig R. 2017. Molecular and catalytic properties of fungal extracellular cellobiose dehydrogenase produced in prokaryotic and eukaryotic expression systems. *Microbial Cell Factories*. 16:37.

Ma Y and Hendershot LM. 2001. The unfolding tale of the unfolded protein response. *Cell*. 107: 827– 830.

Macauley-Patrick S, Fazenda ML, McNeil B, and Harvey LM. 2005. Heterologous protein production using the *Pichia pastoris* expression system. *Yeast.* 22: 249–270.

Madej T, Lanczycki CJ, Zhang D, Thiessen PA, Geer RC, Marchler-Bauer A, and Bryant SH. 2014. MMDB and VAST+: tracking structural similarities between macromolecular complexes. *Nucleic Acids Resonance.* 42: D297-303.

Mahmood N, Yuan Z, Schmidt J, and Xu C. 2016. Depolymerization of lignins and their applications for the preparation of polyols and rigid polyurethane foams: A review. *Renewable and Sustainable Energy Reviews.* 60: 317-329.

Maity SK. 2015. Opportunities, recent trends and challenges of integrated biorefinery: Part I. *Renewable and Sustainable Energy Reviews.* 43: 1427-1445.

Mann M and Jenson ON. 2003. Proteomic analysis of post-translational modifications. *Nature Biotechnology.* 21: 255-261.

Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. 2010. Stability of protein pharmaceuticals: An update. *Pharmaceutical Research*. 27: 544–575.

Mansouri NEE, and Salvado J. 2006. Analytical methods for determining functional groups in various technical lignins. *Industrial Crops and Products.* 26: 116–124.

Martínez ÁT, Speranza M, Ruiz-Dueñas FJ, Ferreira P, Camarero S, Guillén F, Martínez MJ, Gutiérrez A, Del Río JC. 2005. Biodegradation of lignocellulosics: Microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *International Microbiology*. 83: 195–204.

Maurer M, Kühleitner M, Gasser B, Mattanovich D. 2006. Versatile modeling and optimization of fed batch processes for the production of secreted heterologous proteins with *Pichia pastoris*. *Microbial cell factories*. 5: 37.

Mayer AM, Staples RC. 2002. Laccase: New functions for an old enzyme. *Phytochemistry*, 60: 551–565.

Menendez J, Valdes I, and Cabrera N. 2003. The ICL1 gene *of Pichia pastoris*, transcriptional regulation and use of its promoter. *Yeast.* 20: 1097-1108.

Neifar M, Jaouani A, Ellouze-Ghorbel R, Ellouze-Ghorbel S, and Penninck MJ. 2009. Effect of culturing processes and copper addition on laccase production by the white-rot fungus *Fomes fomentarius* MUCL 35117. *Letters in Applied Microbiology*. 49: 73-78.

Nicolini C, Bruzzese D, Cambria MT, Bragazzi NL and Pechkova E. 2013. Recombinant Laccase: I. Enzyme cloning and characterization. *Journal of Cellular Biochemistry*. 114:599–605.

Nishibori N, Masaki K, Tsuchioka H, Fujii T, and Lefuji H. 2013. Comparison of laccase production levels in *Pichia pastori*s and Cryptococcus sp. S-2. *Journal of Bioscience and Engineering.* 115: 394-399.

O 'Callaghan J, O 'Brien, M, Mcclean K and Dobson A. 2002. Optimisation of the expression of a *Trametes versicolor* laccase gene in *Pichia pastoris. Journal of Industrial Microbiology & Biotechnology*. 29:55–59.

Palomares LA, Estrada-Mondaca S, and Ramirez OT. 2004. Production of recombinant proteins: challenges and solutions. In: Recombinant gene expression. Eds: Balbas P, Lorence A. New York: Springer.

Pérez J, Muñoz-Dorado J, de la Rubia T, and Martínez J. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *International Microbiology*. 5: 53-63.

Pezzella C, Giacobelli VG, Lettera V, Olivieri G, Cicatiello P, Sannia G, Piscitelli A. 2017. A step forward in laccase exploitation: Recombinant production and evaluation of techno-economic feasibility of the process. *Journal of Biotechnology*. 259: 175–181.

Pfeffer M, Maurer M, Stadlmann J, Grass J, Delic M, Altmann F, Mattanovich D. 2012. Intracellular interactome of secreted antibody Fab fragment in *Pichia pastoris* reveals its routes of secretion and degradation. *Applied Microbiology and Biotechnology*, 93: 2503–2512.

Piontek K, Antorini M, Choinowski T. 2002. Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. *Journal of Biological Chemistry*. 277: 37663–37669.

Pokkuluri PR, Duke NEC, Wood SJ, Cotta MA, Li XL, Biely P. and Schiffer M. 2011. Structure of the catalytic domain of glucuronoyl esterase Cip2 from *Hypocrea jecorina. Proteins:*

Structure, Function and Bioinformatics. 79:2588–2592.

Pollegioni L, Tonin F, and Rosini E. 2015. Lignin-degrading enzymes. *FEBS Journal*. 282: 1190-1213

Potvin G, Ahmad A, and Zhang Z. 2012. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: a review. Biochemical Engineering Journal. 64: 91–105.

Pu Y, Jiang N, and Raguaskas AJ. 2007. Ionic liquid as a green solvent for lignin. *Journal of Wood Chemistry and Technology.* 27: 23-33.

Puxbaum V, Mattanovich D, Gasser B. 2015. Quo vadis? The challenges of recombinant protein folding and secretion in *Pichia pastoris*. *Applied Microbiology and Biotechnology*. 99: 2925–2938.

Ragauskas AJ, Beckham GT, Biddy MJ, Chandra R, Chen F, Davis MF, Davison BH, Dixon RA, Gilna P, Keller M, Langan P, Naskar AK, Saddler JN, Tschaplinksi TJ, Tukson G, and Wyman CE. 2014. Lignin valorisation: improving lignin processing in the biorefinery. *Science*. 344: 709-719.

Rakestraw A and Wittrup KD. 2005. Contrasting secretory processing of simultaneously expressed heterologous proteins in *Saccharomyces cerevisiae. Biotechnology and Bioengineering.* 93: 896-905

Riva S. 2006. Laccases: blue enzymes for green chemistry. *Trends in Biotechnology*. 24: 219–226.

Rivera-Hoyos CM, Morales-Álvarez ED, Poveda-Cuevas SA, Reyes-Guzmán EA, Poutou-Piñales, RA, Reyes-Montaño EA, Pedroza-Rodríguez AM, Rodríguez-Vázquez R and Cardozo-Bernal ÁM. 2015. Computational analysis and low-scale constitutive expression of laccases synthetic genes GLLCC1 from *Ganoderma lucidum* and POXA 1B from *Pleurotus ostreatus* in *Pichia pastoris. PLoS ONE*. 10: 1–21.

Robinson A, Bockhaus J, Voegler AC, and Wittrup K. 1996. Reduction of BiP levels decreases heterologous protein secretion in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry.* 271: 10017-10022.

Rodgers CJ, Blanford CF, Giddens SR, Skamnioti P, Armstrong FA and Gurr SJ. 2010. Designer laccases: a vogue for high-potential fungal enzymes? *Trends in Biotechnology*. 28:63–

72.

Romanos M, Scorer CA, and Clare JJ. 1992. Foreign gene expression in yeast: A review. *Yeast.* 8: 423-488.

Roth S and Spiess AC. 2015. Laccases for biorefinery applications: a critical review on challenges and perspectives. *Bioprocess and Biosystems Engineering.* 38: 2285-2313.

Sánchez C. 2009. Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnology Advances*. 27:185–194.

Sasagawa T, Matsui M, Kobayashi Y, Otagiri M, Moriya S, Sakamoto Y, Ito Y, Lee CC, Kitamoto K, Arioka M. 2011. High-throughput recombinant gene expression systems in *Pichia pastoris* using newly developed plasmid vectors. *Plasmid*. 65: 65–69.

Scheller HV and Ulvskov P. 2010. Hemicelluloses. *Annual Review of Plant Biology*. 61: 263–289.

Schwarzhans JP, Wibberg D, Winkler A, Luttermann, T, Kalinowski J, Friehs K. 2016. Integration event induced changes in recombinant protein productivity in *Pichia pastoris* discovered by whole genome sequencing and derived vector optimization. *Microbial Cell Factories*. 15: 84.

Sears IB, O'Connor J, Rossanese OW, and Glick BS. 1998. A versatile set of vectors for constitutive and regulated gene expression in *Pichia pastoris*. *Yeast*. 14: 783–90.

Shi X, Tal G, Hankins NP and Gitis V. 2014. Fouling and cleaning of ultrafiltration membranes: A review. *Journal of Water Process Engineering*. 1:121–138.

Shire SJ, Shahrokh Z, Liu JUN. 2004. Challenges in the Development of High Protein Concentration Formulations. *Journal of Pharmaceutical Sciences*, 93: 1390–1402.

Shleev SV, Morozova OV, Nikitina OV, Gorshina ES, Rusinova TV, Serezhenkov VA, Burbaev DS, Gazaryan IG, Yaropolov AI. 2004. Comparison of physico-chemical characteristics of four laccases from different basidiomycetes. *Biochimie*. 86: 693–703.

Špániková S, and Biely P. 2006. Glucuronoyl esterase—Novel carbohydrate esterase produced by *Schizophyllum commune*. *FEBS Letters*. 580: 4597–4601.

Špániková S, Poláková M, Joniak D, Hirsch J, and Biely P. 2007. Synthetic esters recognized by glucuronoyl esterase from *Schizophyllum commune*. *Archives of Microbiology.* 188: 185-189.

Sreekrishna K, Brankamp RG, Kropp KE, Blankenship D, Tsay JT, Smith PL, Wierschke JD, Subramaniam A, and Birkenberger LA. 1997. Strategies for optimal synthesis and secretion of heterologous proteins in methylotropic yeast *Pichia pastoris*. *Gene*. 190: 55-62.

Sreekrishna K. 1993. Strategies for optimising protein expression and secretion in the methylotrophic yeast *Pichia pastoris.* In: Industrial Microorganisms: Basic and Applied Molecular Genetics. Eds: Baltz RH, Hegemen GD and Skatrud PL. Washington DC: American Society for Microbiology.

Stapleton PC, O'Brien MM, O'Callaghan J and Dobson ADW. 2004. Molecular cloning of the cellobiose dehydrogenase gene from *Trametes versicolor* and expression in *Pichia pastoris. Enzyme and Microbial Technology.* 34:55–63.

Stapleton PC, OBrien MM, OCallaghan J, and Dobson ADW. 2004. Molecular cloning of the cellobiose dehydrogenase gene from *Trametes versicolor* and expression in *Pichia pastoris*. *Enzyme and Microbial Technology* 34: 55–63

Strassberger Z, Tanase S, and Rothenberg G. 2014. The pros and cons of lignin valorisation in an integrated biorefinery. *RSC Advances*. 4: 25310-25318.

Strausberg RL and Strausberg SL. 1995. Overview of protein expression in *Saccharomyces cerevisiae*. *Current Protocols in Protein Science.* 5.6.1-5.6.7.

Sulej J, Grzegorz J, Osinska-Jaroszuk M, Rachubik P, Mazur A, Komaniecka I, Choma A, and Rogalski J. 2015. Characterization of cellobiose dehydrogenase from a biotechnologically important *Cerrena unicolor* strain. *Applied Biochemistry and Biotechnology.* 6: 1638-1658.

Sulej J, Janusz G, Osinska-Jaroszuk M, Rachubik P, Mazur A, Komaniecka I, Choma A and Rogalski J. 2015. Characterization of Cellobiose Dehydrogenase from a Biotechnologically Important *Cerrena unicolor* Strain. *Applied Biochemistry and Biotechnology*. 176:1638–1658.

Sunner H, Charavgi MD, Olsson L, Topakas E and Christakopoulos P. 2015. Glucuronoyl Esterase Screening and Characterization Assays Utilizing Commercially Available Benzyl Glucuronic Acid Ester. *Molecules*. 20:17807–17817.

Sygmund C, Santner P, Krondorfer I, Peterbauer CK, Alcalde M, Nyanhongo GS, Guebitz GM, Ludwig R. 2013. Semi-rational engineering of cellobiose dehydrogenase for improved hydrogen peroxide production. *Microbial cell factories* 12: 38.

Tan TC, Kracher D, Gandini R, Sygmund C, Kittl R, Haltrich D, Hällberg BM, Ludwig R, and Divne C. 2015. Structural basis for cellobiose dehydrogenase action during oxidative cellulose degradation. *Nature Communications.* 6: 7542.

Tang S, Boehme L, Lam H, Zhang Z. 2009. *Pichia pastoris* fermentation for phytase production using crude glycerol from biodiesel production as the sole carbon source. *Biochemical Engineering Journal*, 43: 157–162.

Tien M and Kirk TK. 1988. Lignin peroxidase of *Phanerochaete chrysosporium. Methods in Enzymology*. 161:238–249.

Topakas E, Moukouli M, Dimarogona M, Vafiadi C, and Christakopoulos P. 2010. Functional expression of a thermophilic glucuronyl esterase from *Sporotrichum thermophile*: Identification of the nucleophilic serine. *Applied Microbiology and Biotechnology.* 87: 1765–1772.

Tutar L and Tutar Y. 2010. Heat shock proteins; an overview. *Current Pharmaceutical Biotechnology*. 11: 216–222.

Upadhyay P, Shrivastava R and Agrawal PK. 2016. Bioprospecting and biotechnological applications of fungal laccase. *3 Biotech*. 6:15.

Vafiadi C, Topakas E, Biely P, and Christakopoulos P. 2009. Purification, characterization and mass spectrometric sequencing of a thermophilic glucuronoyl esterase from *Sporotrichum thermophile*. *FEMS Microbiology Letters*. 296: 178–184.

Vagenende V, Yap MGS Trout BL. 2009. Mechanisms of protein stabilization and prevention of protein aggregation by glycerol. *Biochemistry*, 48: 11084–11096.

Van Reis R, Zydney A. 2001. Membrane separations in biotechnology. *Current Opinion in Biotechnology*, 12: 208–211.

Varnai A, Tang C, Bengtsson O, Atterton A, Mathiesen G, and Eijsink VGH. 2014. Expression of endoglucanases in *Pichia pastoris* under control of the GAP promoter. *Microbial Cell Factories.* 13: 57.

Vassileva A, Chugh DA, Swaminathan S, and Khanna N. 2001. Effect of copy number on the expression levels of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification.* 21: 71–80.

Viader-Salvadó, J. M, Cab-Barrera, E. L, Galán-Wong, L. J, Guerrero-Olazarán, M. 2006. Genotyping of recombinant *Pichia pastoris* strains. *Cellular & molecular biology letters*, 11: 348–359.

Vishtal A and Kraslawski A. 2011. Challenges in industrial applications of technical lignins. *Bioresources.* 6: 3547–3568.

Vogl T and Glieder A. 2013. Regulation of *Pichia pastoris* promoters and its consequences for protein production. *Nature Biotechnology*. 30: 385–404.

Waites MJ, Morgan NL, Rockey JS, and Higton G. 2001. *Industrial microbiology: an introduction*. United Kingdom: Blackwell Science.

Wang, W. 1999. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *International Journal of Pharmaceutics*. 185: 129-188.

Watanabe T and Koshijima T. 1988. Evidence for an ester linkage between lignin and glucuronic acid in lignin–carbohydrate complexes by DDQ-oxidation. *Agricultural and Biological Chemistry*. 52: 2953–2955

Waterham HR, Digan ME, Koutz PJ, Lair SV, and Cregg JM. 1997. Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene.* 186: 37–44.

Westermark U and Eriksson K. 1974. Cellobiose: quinone oxidoreductase, a new wood-degrading enzyme from white-rot fungi. *Acta Chemica Scandinvica B.* 28: 209–214.

White CE, Kempi NM, and Komives EA. 1994. Expression of highly disulphide-bonded proteins in *Pichia pastoris. Structure.* 2: 1003-1005.

Wittrup KD, Robinson AS, Parekh RN, and Forrester KJ. 1994. Existence of an optimum expression level for secretion of foreign proteins in yeast. *Annals of the New York Academy of Science.* 745: 321– 330.

Wong DWS. 2009. Structure and action mechanism of ligninolytic enzymes. *Applied Biochemistry and Biotechnology*. 157: 174–209.

Wu JM, Lin JC, Chieng LL, Lee CK, and Hsu TA. 2003. Combined used of GAP and AOX1 promoter to enhance the expression of human granulocyte-macrophage colony-stimulating factor in *Pichia pastoris*. *Enzyme and Microbial Technology*. 33: 453-459.

Yang J, Li W, Bun Ng T, Deng X, Lin J, Ye X. 2017. Laccases: Production, expression regulation, and applications in pharmaceutical biodegradation. *Frontiers in Microbiology*. 8: 832.

Yoshida M, Igarashi K, Kawai R, Aida K, and Samejima M. 2001. Differential transcription of beta-glucosidase and cellobiose dehydrogenase genes in cellulose degradation by the basidiomycete *Phanerochaete chrysosporium*. *FEMS Microbiology Letters.* 235: 177-182

Yoshizumi H and Ashikari T. 1987. Expression, glycosylation and secretion of fungal hydrolases in yeast. *Trends in Biotechnology.* 5: 277-281.

Zamocky M, Ludwig R, Peterbauer C, Hallberg BM, Divne C, Nicholls P, and Haltrich D. 2006. Cellobiose dehydrogenase—a flavocytochrome from wood-degrading, phytopathogenic and saprotropic fungi. *Current Protein and Peptide Science.* 7: 255–280.

Zamocky M, Schumann C, Sygmund C, OCallaghan J, and Dobson AD, Ludwig R, Haltrich D, and Peterbauer CK. 2008. Cloning, sequence analysis and heterologous expression in *Pichia pastoris* of a gene encoding a thermostable cellobiose dehydrogenase from *Myriococcum thermophilum*. *Protein Expression and Purification.* 59: 258-265.

Zeng Y, Zhao S, Yang S, and Ding SY. 2014. Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels. *Current Opinion in Biotechnology*. 27: 38-45

Zhang AL, Luo JX, Zhang TY, Pan YW, Tan YH, Fu CY, Tu FZ. 2009. Recent advances on the GAP promoter derived expression system of *Pichia pastoris*. *Molecular Biology Reports*, 36: 1611–1619.

Zhang R, Fan Z, and Kasuga T. 2011. Expression of cellobiose dehydrogenase from *Neurospora crassa* in *Pichia pastoris* and its purification and characterization. *Protein Expression and Purification*. 75: 63- 69.

Zhao W, Wang J, Deng R, Wang X. 2008. Scale-up fermentation of recombinant *Candida*

rugosa lipase expressed in *Pichia pastoris* using the GAP promoter. *Journal of Industrial Microbiology and Biotechnology*. 35: 189–195.

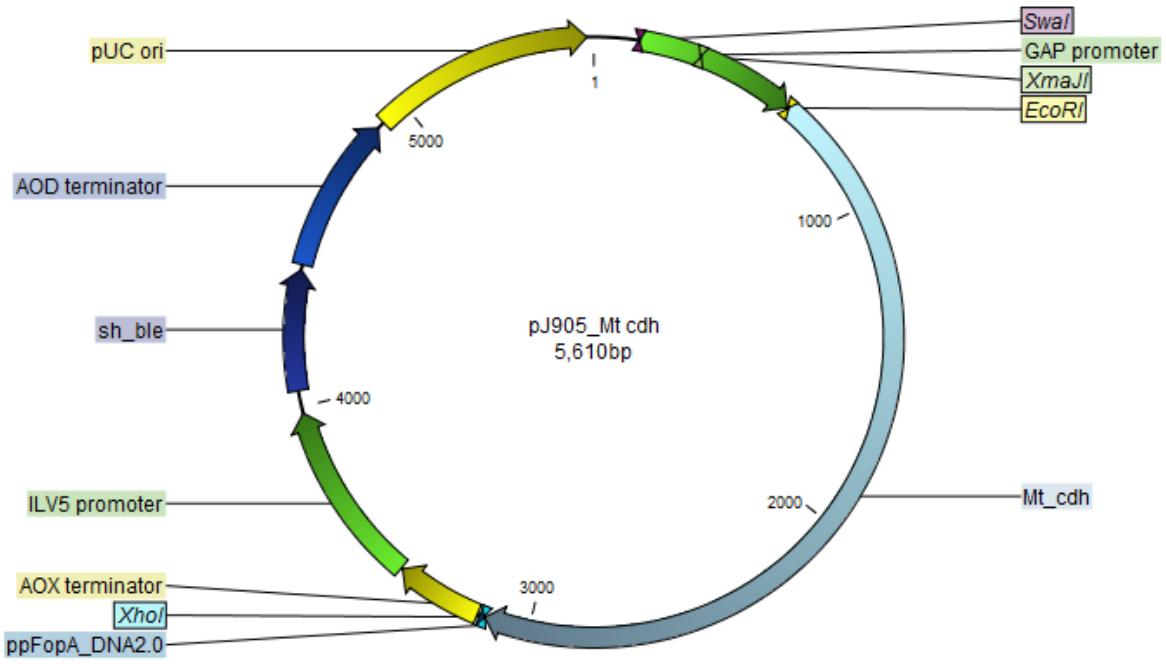
Zhong X, Peng L, Zheng S, Sun Z, Ren Y, Dong M and Xu A. 2004. Secretion, purification, and characterization of a recombinant *Aspergillus oryzae* tannase in *Pichia pastoris*. *Protein Expression and Purification*. 36:165–169.

Addendum A

Plasmid maps and sequences of strains created in this study

Cellobiose Dehydrogenase (CDH)

Myriococcum thermophilum CDH: pJ905-Mt-CDH



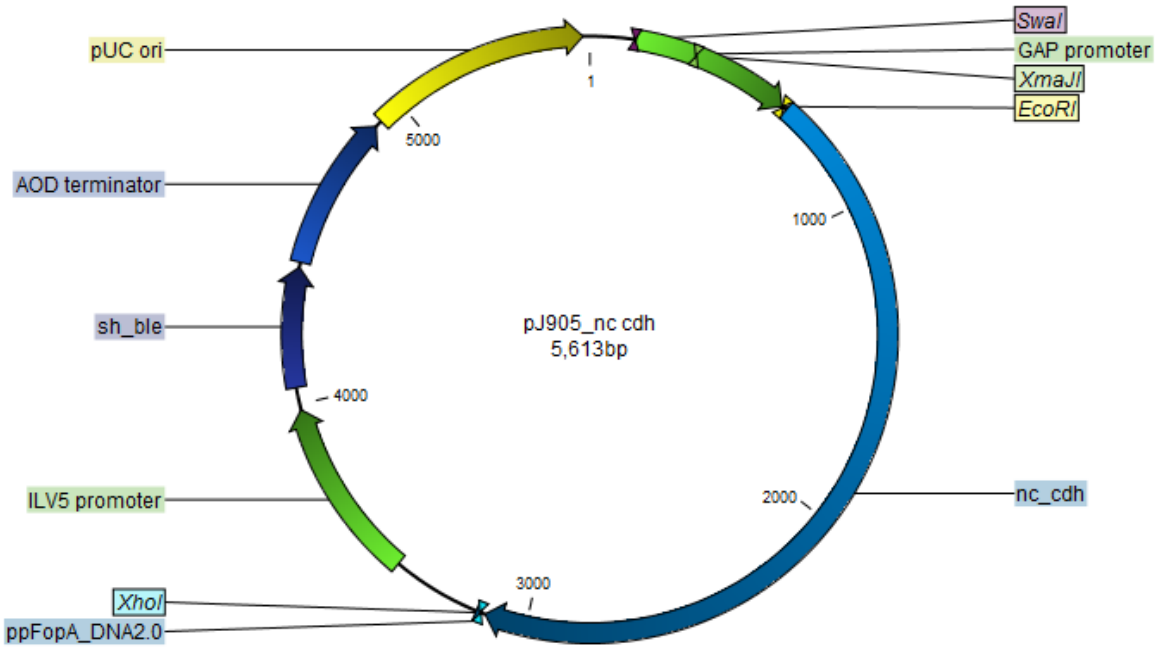
AGATCCAATTCCCGCTTTGACTGCCTGAAATCTCCATCGCCTACAATGATGACAT
TTGGATTTGGTTGACTCATGTTGGTATTGTGAAATAGACGCAGATCGGGAACACT
GAAAAATACACAGTTATTATTCATTTAAATTTTTTGTAGAAATGTCTTGGTGTCCT
CGTCCAATCAGGTAGCCATCTCTGAAATATCTGGCTCCGTTGCAACTCCGAACGA
CCTGCTGGCAACGTAAAATTCTCCGGGGTAAAACTTAAATGTGGAGTAATGGAA
CCAGAAACGTCTCTTCCCTTCTCTCTCCTTCCACCGCCCGTTACCGTCCCTAGGAA
ATTTTACTCTGCTGGAGAGCTTCTTCTACGGCCCCCTTGCAGCAATGCTCTTCCCA
GCATTACGTTGCGGGTAAAACGGAGGTCGTGTACCCGACCTAGCAGCCCAGGGA

TGGAAAAGTCCCGGCCGTCGCTGGCAATAATAGCGGGCGGACGCATGTCATGAG
ATTATTGGAAACCACCAGAATCGAATATAAAAGGCGAACACCTTTCCCAATTTTG
GTTTCTCCTGACCCAAAGACTTTAAATTTAATTTATTTGTCCCTATTTCAATCAAT
TGAACAACTATCAAAACACAGAATTcTTAATTAAAATGAGAACCTCTTCCAGATT
GATCGGTGCCTTAGCAGCCGCCTTGTTACCTTCAGCCTTAGCCCAAAATAATGTC
CCAAATACTTTCACTGATCCAGACAGTGGTATTACTTTTAATACCTGGGGTTTGG
ATGAAGACTCTCCTCAAACCCAAGGTGGTTTTACTTTCGGTGTTGCCTTGCCATCT
GATGCTTTAACTACAGACGCATCAGAGTTTATTGGTTACTTAAAGTGTGCAAGAA
ACGATGAATCCGGTTGGTGCGGTATAAGTTTGGGTGGTCCAATGACAAACTCTTT
GTTAATCACCGCTTGGCCTCATGAAGATACTGTTTATACATCTTTGAGATTCGCA
ACTGGTTATGCCATGCCAGATGTTTACGAAGGTGACGCTGAAATAACACAAGTA
TCTTCATCCGTTAATAGTACCCATTTCTCTTTGATATTCAGATGCAAGAACTGCTT
GCAATGGTCACACGGTGGTAGTTCTGGTGGTGCCTCAACATCCGGTGGTGTATTG
GTTTTAGGTTGGGTTCAAGCTTTCGATGACCCAGGTAATCCTACTTGTCCAGAAC
AAATCACATTACAACAACACGATAACGGTATGGGTATCTGGGGTGCACAATTGA
ACACCGATGCTGCAAGTCCTTCTTACACTGACTGGGCCGCTCAAGCTACTAAAAC
AGTTACCGGTGACTGCGAAGGTCCAACTGAAACAAGTGTTGTCGGTGTCCCAGT
ACCTACTGGTGTTTCTTTTGATTATATCGTAGTTGGTGGTGGTGCTGGTGGTATTC
CAGCAGCCGATAAATTGTCTGAAGCTGGTAAATCAGTTTTGTTGATAGAAAAGG
GTTTTGCATCTACCGCCAATACTGGTGGTACATTGGGTCCTGAATGGTTAGAAGG
TCATGATTTGACAAGATTCGACGTTCCAGGTTTATGTAACCAAATTTGGGTCGAT
TCAAAGGGTATAGCCTGTGAAGATACTGACCAAATGGCTGGTTGCGTATTAGGTG
GTGGTACAGCTGTTAATGCAGGTTTGTGGTTCAAGCCTTACTCTTTGGATTGGGA
CTACTTGTTCCCAGATGGTTGGAAGTACAATGACGTTCAACCTGCTATCAACAGA
GCATTGTCTAGAATACCAGGTACAGATGCTCCAAGTACCGACGGTAAAAGATAC
TACCAAGAAGGTTTCGAAGTATTGTCCAAGGGTTTAGCTGCTGGTGGTTGGACCA
GTGTTACTGCCAATAACGCTCCAGATAAAAAGAATAGAACTTTCGCCCATGCTCC
TTTTATGTTCGCAGGTGGTGAAAGAAACGGTCCATTAGGTACTTACTTCCAAACA
GCCAAAAAGAGAAACAACTTCGATGTTTGGTTGAATACCTCTGTCAAAAGAGTA
ATAAGAGAAGGTGGTCACATCACAGGTGTTGAAGTCGAACCTTTTAGAGATGGT
GGTTATGAAGGTATTGTACCAGTTACAAAAGTCACCGGTAGAGTAATATTATCTG
CTGGTACTTTCGGTTCAGCCAAGATTTTGTTAAGATCAGGTATAGGTCCAGAAGA
TCAATTGGAAGTCGTAGCCGCTTCCGAAAAAGACGGTCCTACAATGATCGGTAA

TTCATCCTGGATTAACTTACCAGTCGGTTACAATTTGGATGACCATTTGAACACC
GATACTGTAATTTCTCACCCTGATGTTGTCTTTTATGACTTCTACGAAGCTTGGGA
TGACCCAATAGAATCAGATAAAAATTCTTATTTGGAAAGTAGAACTGGTATTTTG
GCACAAGCAGCCCCTAACATAGGTCCAATGTTTTGGGAAGAAATAGTTGGTGCT
GATGGTATCGTCAGACAATTACAATGGACTGCAAGAGTTGAAGGTTCATTGGGT
GCCCCTAATGGTCATACAATGACCATGTCTCAATACTTAGGTAGAGGTGCTACAT
CAAGAGGTAGAATGACTATAACACCATCCTTAACCACTATCGTTAGTGATGTCCC
TTATTTGAAAGATCCAAATGACAAGGAAGCTGTTATTCAAGGTATTATAAATTTG
CAAAACGCCTTACAAAATGTCGCTAACTTGACATGGTTATTTCCTAACTCTACCA
TTACTCCAAGAGAATATGTTGAATCAATGGTAGTTTCACCATCCAATAGAAGATC
CAACCATTGGATGGGTACTAATAAGTTGGGTACAGATGACGGTAGAAAGGGTGG
TTCTGCTGTTGTTGATTTGGACACCAGAGTCTATGGTACTGATAATTTGTTCGTAA
TCGACGCTTCAATATTCCCTGGTGTTCCAACAACCAACCCTACTTCCTACATCGTT
GTCGCTGCTGAACACGCTAGTTCTAGAATTTTGGCATTACCAGATTTGGAACCAG
TTCCTAAATATGGTCAATGCGGTGGTAGAGAATGGACAGGTTCTTTCGTCTGTGC
CGATGGTTCTACTTGTGAATATCAAAATGAATGGTACTCCCAATGCTTGTAAGGC
GCGCCcTCGAGGCGCGCCCGGGGCGGCCGCTCAAGAGGATGTCAGAATGCCATT
TGCCTGAGAGATGCAGGCTTCATTTTTGATACTTTTTTATTTGTAACCTATATAGT
ATAGGATTTTTTTTGTCATTTTGTTTCTTCTCGTACGAGCTTGCTCCTGATCAGCCT
ATCTCGCAGCAGATGAATATCTTGTGGTAGGGGTTTGGGAAAATCATTCGAGTTT
GATGTTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAAAC
CTTCGTTTGTGCGGATCCTTCAGTAATGTCTTGTTTCTTTTGTTGCAGTGGTGAGC
CATTTTGACTTCGTGAAAGTTTCTTTAGAATAGTTGTTTCCAGAGGCCAAACATTC
CACCCGTAGTAAAGTGCAAGCGTAGGAAGACCAAGACTGGCATAAATCAGGTAT
AAGTGTCGAGCACTGGCAGGTGATCTTCTGAAAGTTTCTACTAGCAGATAAGATC
CAGTAGTCATGCATATGGCAACAATGTACCGTGTGGATCTAAGAACGCGTCCTAC
TAACCTTCGCATTCGTTGGTCCAGTTTGTTGTTATCGATCAACGTGACAAGGTTGT
CGATTCCGCGTAAGCATGCATACCCAAGGACGCCTGTTGCAATTCCAAGTGAGCC
AGTTCCAACAATCTTTGTAATATTAGAGCACTTCATTGTGTTGCGCTTGAAAGTA
AAATGCGAACAAATTAAGAGATAATCTCGAAACCGCGACTTCAAACGCCAATAT
GATGTGCGGCACACAATAAGCGTTCATATCCGCTGGGTGACTTTCTCGCTTTAAA
AAATTATCCGAAAAAATTTTCTAGAGTGTTGTTACTTTATACTTCCGGCTCGTATA
ATACGACAAGGTGTAAGGAGGACTAAACCATGGCTAAACTCACCTCTGCTGTTC

CAGTCCTGACTGCTCGTGATGTTGCTGGTGCTGTTGAGTTCTGGACTGATAGACT
CGGTTTCTCCCGTGACTTCGTAGAGGACGACTTTGCCGGTGTTGTACGTGACGAC
GTTACCCTGTTCATCTCCGCAGTTCAGGACCAGGTTGTGCCAGACAACACTCTGG
CATGGGTATGGGTTCGTGGTCTGGACGAACTGTACGCTGAGTGGTCTGAGGTCGT
GTCTACCAACTTCCGTGATGCATCTGGTCCAGCTATGACCGAGATCGGTGAACAG
CCCTGGGGTCGTGAGTTTGCACTGCGTGATCCAGCTGGTAACTGCGTGCATTTCG
TCGCAGAAGAGCAGGACTAACAATTGACACCTTACGATTATTTAGAGAGTATTTA
TTAGTTTTATTGTATGTATACGGATGTTTTATTATCTATTTATGCCCTTATATTCTG
TAACTATCCAAAAGTCCTATCTTATCAAGCCAGCAATCTATGTCCGCGAACGTCA
ACTAAAAATAAGCTTTTTATGCTCTTCTCTCTTTTTTTCCCTTCGGTATAATTATAC
CTTGCATCCACAGATTCTCCTGCCAAATTTTGCATAATCCTTTACAACATGGCTAT
ATGGGAGCACTTAGCGCCCTCCAAAACCCATATTGCCTACGCATGTATAGGTGTT
TTTTCCACAATATTTTCTCTGTGCTCTCTTTTTATTAAAGAGAAGCTCTATATCGG
AGAAGCTTCTGTGGCCGTTATATTCGGCCTTATCGTGGGACCACATTGCCTGAAT
TGGTTTGCCCCGGAAGATTGGGGAAACTTGGATCTGATTACCTTAGCTGCAGGTA
CCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTT
TTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGG
TTTGTTTGTCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAG
CAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCAC
TTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAG
TGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATA
GTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCC
CAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATG
AGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCG
GCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGG
TATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTG
ATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTT
ACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGGTACCC

Neurospora crassa CDH: pJ905-Nc-CDH



AGATCCAATTCCCGCTTTGACTGCCTGAAATCTCCATCGCCTACAATGATGACAT
TTGGATTTGGTTGACTCATGTTGGTATTGTGAAATAGACGCAGATCGGGAACACT
GAAAAATACACAGTTATTATTCATTTAAATTTTTTGTAGAAATGTCTTGGTGTCCT
CGTCCAATCAGGTAGCCATCTCTGAAATATCTGGCTCCGTTGCAACTCCGAACGA
CCTGCTGGCAACGTAAAATTCTCCGGGGTAAAACTTAAATGTGGAGTAATGGAA
CCAGAAACGTCTCTTCCCTTCTCTCTCCTTCCACCGCCCGTTACCGTCCCTAGGAA
ATTTTACTCTGCTGGAGAGCTTCTTCTACGGCCCCCTTGCAGCAATGCTCTTCCCA
GCATTACGTTGCGGGTAAAACGGAGGTCGTGTACCCGACCTAGCAGCCCAGGGA
TGGAAAAGTCCCGGCCGTCGCTGGCAATAATAGCGGGCGGACGCATGTCATGAG
ATTATTGGAAACCACCAGAATCGAATATAAAAGGCGAACACCTTTCCCAATTTTG
GTTTCTCCTGACCCAAAGACTTTAAATTTAATTTATTTGTCCCTATTTCAATCAAT
TGAACAACTATCAAAACACAGAATTcTTAATTAAAATGAGAACTACTTCCGCTTT
CTTGTCAGGTTTAGCCGCCGTCGCCTCCTTGTTGTCACCAGCCTTCGCCCAAACTG
CTCCAAAAACTTTTACTCATCCAGACACAGGTATTGTTTTCAATACTTGGTCCGCT
AGTGATTCTCAAACTAAAGGTGGTTTTACAGTTGGTATGGCTTTGCCTTCTAACG
CATTAACTACAGATGCCACAGAGTTTATTGGTTATTTGGAATGTTCTTCAGCTAA
GAATGGTGCAAACTCAGGTTGGTGCGGTGTTTCCTTGAGAGGTGCAATGACTAAT

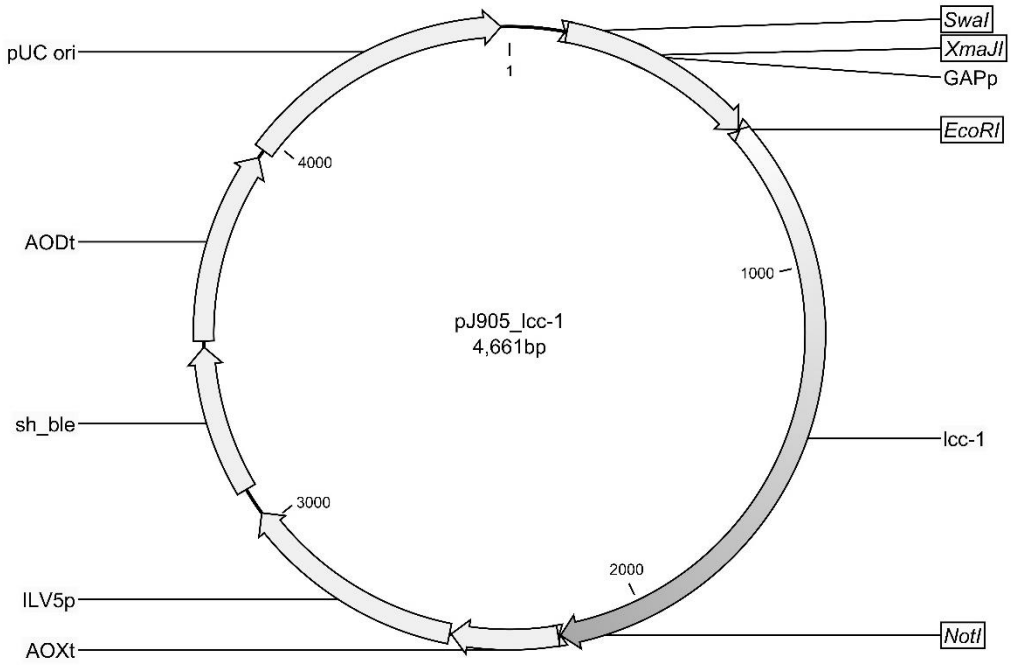
AACTTGTTAATAACAGCCTGGCCATCTGATGGTGAAGTTTACACCAATTTGATGT
TTGCCACTGGTTATGCTATGCCTAAAAACTACGCCGGTGACGCTAAGATCACTCA
AATTGCTTCCAGTGTCAACGCAACTCATTTCACATTGGTATTCAGATGTCAAAAC
TGCTTGTCTTGGGATCAAGACGGTGTCACCGGTGGTATTTCAACTTCCAATAAGG
GTGCTCAATTAGGTTGGGTTCAAGCATTTCCAAGTCCTGGTAATCCAACCTGTCC
TACTCAAATAACATTGTCTCAACACGACAACGGTATGGGTCAATGGGGTGCTGC
ATTCGATAGTAATATCGCTAACCCATCTTATACTGCCTGGGCCGCTAAAGCTACA
AAGACCGTTACTGGTACATGCTCAGGTCCTGTCACCACTTCCATCGCAGCCACCC
CAGTACCTACTGGTGTTTCATTTGACTACATCGTTGTCGGTGGTGGTGCTGGTGGT
ATTCCAGTTGCTGATAAGTTGAGTGAATCTGGTAAAAGTGTCTTGTTGATCGAAA
AGGGTTTCGCTTCTACAGGTGAACATGGTGGTACCTTGAAGCCAGAATGGTTAAA
TAACACCTCTTTGACTAGATTCGATGTTCCTGGTTTGTGTAACCAAATATGGAAG
GATAGTGACGGTATCGCCTGTTCTGATACTGACCAAATGGCTGGTTGCGTTTTAG
GTGGTGGTACAGCAATTAATGCCGGTTTGTGGTATAAACCATACACCAAGGATTG
GGACTATTTGTTTCCTTCAGGTTGGAAAGGTTCCGATATAGCCGGTGCTACTTCA
AGAGCTTTATCCAGAATCCCAGGTACAACCACTCCTTCACAAGATGGTAAAAGA
TACTTGCAACAAGGTTTTGAAGTATTGGCAAATGGTTTAAAAGCCTCCGGTTGGA
AGGAAGTTGATAGTTTGAAGGACTCTGAACAAAAGAATAGAACATTCTCACATA
CCTCCTATATGTACATTAACGGTGAAAGAGGTGGTCCATTGGCCACATATTTGGT
TTCTGCTAAGAAAAGATCAAACTTCAAGTTGTGGTTAAACACTGCTGTTAAGAGA
GTCATAAGAGAAGGTGGTCACATCACAGGTGTCGAAGTAGAAGCTTTTAGAAAT
GGTGGTTACTCAGGTATTATACCAGTTACTAACACAACCGGTAGAGTAGTTTTAT
CAGCAGGTACATTCGGTTCCGCCAAAATTTTGTTAAGATCCGGTATAGGTCCTAA
AGACCAATTGGAAGTCGTAAAGGCATCAGCCGATGGTCCAACTATGGTTTCCAA
TTCTTCATGGATTGATTTGCCTGTCGGTCATAATTTGGTAGATCACACCAACACTG
ACACAGTAATTCAACATAACAACGTTACATTCTATGATTTCTACAAAGCATGGGA
CAACCCAAACACTACAGATATGAATTTGTATTTGAACGGTAGATCAGGTATTTTT
GCTCAAGCTGCACCAAATATAGGTCCTTTATTCTGGGAAGAAATCACTGGTGCAG
ATGGTATTGTAAGACAATTGCACTGGACAGCTAGAGTTGAAGGTTCATTTGAAAC
CCCAGATGGTTATGCAATGACTATGTCCCAATACTTAGGTAGAGGTGCTACTAGT
AGAGGTAGAATGACATTGTCTCCTACATTAAATACCGTTGTCTCTGATTTGCCAT
ATTTGAAAGATCCTAACGACAAGGCCGCTGTAGTTCAAGGTATAGTCAATTTGCA
AAAGGCTTTGGCAAACGTAAAGGGTTTGACATGGGCATATCCATCAGCCAATCA

AACCGCAGCCGATTTTGTTGACAAACAACCTGTCACTTACCAATCTAGAAGATCA
AATCATTGGATGGGTACAAACAAGATGGGTACCGATGACGGTAGAAGTGGTGGT
ACTGCTGTCGTAGATACCAATACTAGAGTTTATGGTACAGACAACTTATACGTTG
TCGATGCATCTATTTTCCCAGGTGTTCCTACCACTAATCCAACAGCTTATATTGTA
GTTGCTGCTGAACACGCCGCTGCAAAAATATTGGCTCAACCAGCAAACGAAGCC
GTTCCTAAGTGGGGTTGGTGTGGTGGTCCAACATACACCGGTTCACAAACCTGTC
AAGCCCCATACAAATGTGAAAAGCAAAACGACTGGTACTGGCAATGCGTCTAAG
GCGCGCCcTCGAGGCGCGCCCGGGGCGGCCGCTCAAGAGGATGTCAGAATGCCA
TTTGCCTGAGAGATGCAGGCTTCATTTTTGATACTTTTTTATTTGTAACCTATATA
GTATAGGATTTTTTTTGTCATTTTGTTTCTTCTCGTACGAGCTTGCTCCTGATCAGC
CTATCTCGCAGCAGATGAATATCTTGTGGTAGGGGTTTGGGAAAATCATTCGAGT
TTGATGTTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAA
ACCTTCGTTTGTGCGGATCCTTCAGTAATGTCTTGTTTCTTTTGTTGCAGTGGTGA
GCCATTTTGACTTCGTGAAAGTTTCTTTAGAATAGTTGTTTCCAGAGGCCAAACA
TTCCACCCGTAGTAAAGTGCAAGCGTAGGAAGACCAAGACTGGCATAAATCAGG
TATAAGTGTCGAGCACTGGCAGGTGATCTTCTGAAAGTTTCTACTAGCAGATAAG
ATCCAGTAGTCATGCATATGGCAACAATGTACCGTGTGGATCTAAGAACGCGTCC
TACTAACCTTCGCATTCGTTGGTCCAGTTTGTTGTTATCGATCAACGTGACAAGGT
TGTCGATTCCGCGTAAGCATGCATACCCAAGGACGCCTGTTGCAATTCCAAGTGA
GCCAGTTCCAACAATCTTTGTAATATTAGAGCACTTCATTGTGTTGCGCTTGAAA
GTAAAATGCGAACAAATTAAGAGATAATCTCGAAACCGCGACTTCAAACGCCAA
TATGATGTGCGGCACACAATAAGCGTTCATATCCGCTGGGTGACTTTCTCGCTTT
AAAAAATTATCCGAAAAAATTTTCTAGAGTGTTGTTACTTTATACTTCCGGCTCGT
ATAATACGACAAGGTGTAAGGAGGACTAAACCATGGCTAAACTCACCTCTGCTG
TTCCAGTCCTGACTGCTCGTGATGTTGCTGGTGCTGTTGAGTTCTGGACTGATAGA
CTCGGTTTCTCCCGTGACTTCGTAGAGGACGACTTTGCCGGTGTTGTACGTGACG
ACGTTACCCTGTTCATCTCCGCAGTTCAGGACCAGGTTGTGCCAGACAACACTCT
GGCATGGGTATGGGTTCGTGGTCTGGACGAACTGTACGCTGAGTGGTCTGAGGTC
GTGTCTACCAACTTCCGTGATGCATCTGGTCCAGCTATGACCGAGATCGGTGAAC
AGCCCTGGGGTCGTGAGTTTGCACTGCGTGATCCAGCTGGTAACTGCGTGCATTT
CGTCGCAGAAGAGCAGGACTAACAATTGACACCTTACGATTATTTAGAGAGTAT
TTATTAGTTTTATTGTATGTATACGGATGTTTTATTATCTATTTATGCCCTTATATT
CTGTAACTATCCAAAAGTCCTATCTTATCAAGCCAGCAATCTATGTCCGCGAACG

TCAACTAAAAATAAGCTTTTTATGCTCTTCTCTCTTTTTTTCCCTTCGGTATAATTA
TACCTTGCATCCACAGATTCTCCTGCCAAATTTTGCATAATCCTTTACAACATGGC
TATATGGGAGCACTTAGCGCCCTCCAAAACCCATATTGCCTACGCATGTATAGGT
GTTTTTTCCACAATATTTTCTCTGTGCTCTCTTTTTATTAAAGAGAAGCTCTATATC
GGAGAAGCTTCTGTGGCCGTTATATTCGGCCTTATCGTGGGACCACATTGCCTGA
ATTGGTTTGCCCCGGAAGATTGGGGAAACTTGGATCTGATTACCTTAGCTGCAGG
TACCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTT
TTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGT
GGTTTGTTTGTCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTC
AGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACC
ACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACC
AGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACG
ATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACA
GCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCT
ATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAA
GCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCC
TGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTT
GTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTT
TTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGGTACC
C

Laccase

Trametes versicolor laccase isoform *lcc1*: pJ905-Tv-lcc1



Plasmid Sequence

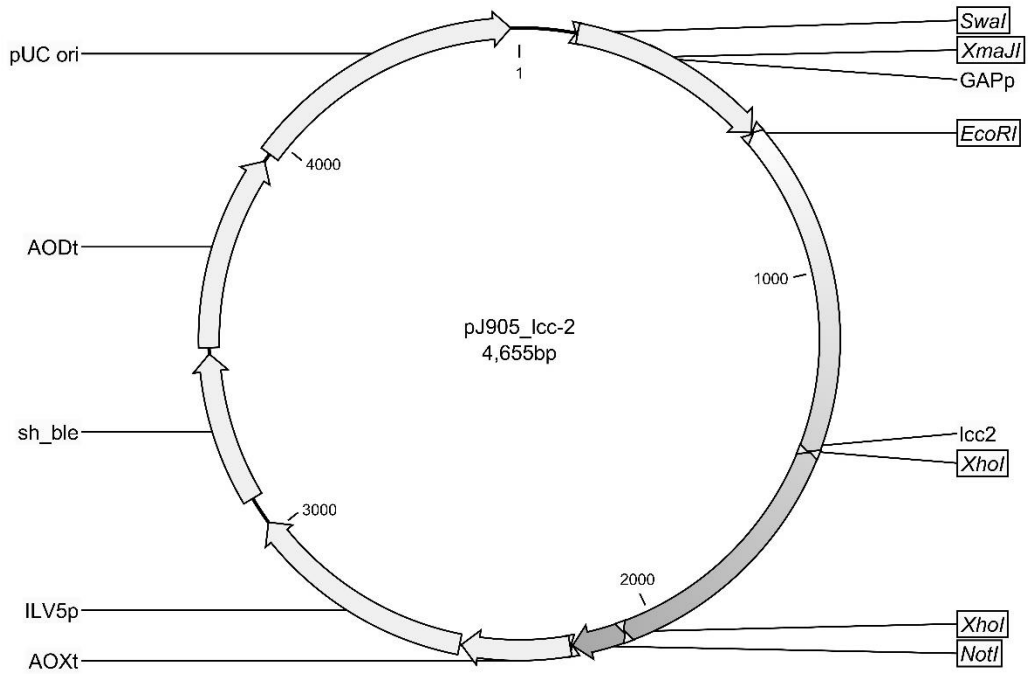
AGATCCAATTCCCGCTTTGACTGCCTGAAATCTCCATCGCCTACAATGATGACAT
TTGGATTTGGTTGACTCATGTTGGTATTGTGAAATAGACGCAGATCGGGAACACT
GAAAAATACACAGTTATTATTCATTTAAATTTTTTGTAGAAATGTCTTGGTGTCCT
CGTCCAATCAGGTAGCCATCTCTGAAATATCTGGCTCCGTTGCAACTCCGAACGA
CCTGCTGGCAACGTAAAATTCTCCGGGGTAAAACTTAAATGTGGAGTAATGGAA
CCAGAAACGTCTCTTCCCTTCTCTCTCCTTCCACCGCCCGTTACCGTCCCTAGGAA
ATTTTACTCTGCTGGAGAGCTTCTTCTACGGCCCCCTTGCAGCAATGCTCTTCCCA
GCATTACGTTGCGGGTAAAACGGAGGTCGTGTACCCGACCTAGCAGCCCAGGGA
TGGAAAAGTCCCGGCCGTCGCTGGCAATAATAGCGGGCGGACGCATGTCATGAG
ATTATTGGAAACCACCAGAATCGAATATAAAAGGCGAACACCTTTCCCAATTTTG
GTTTCTCCTGACCCAAAGACTTTAAATTTAATTTATTTGTCCCTATTTCAATCAAT
TGAACAACTATCAAAACACAGAATTCATGGGCAGGTTCTCATCTCTCTGCGCGCT
CACCGCCGTCATCCACTCTTTTGGTCGTGTCTCCGCCGCTATCGGGCCTGTGACCG
ACCTCACCATCTCCAATGCGGACGTTTCTCCCGACGGCTTCACTCGTGCCGCAGT
GCTTGCAAACGGCGTCTTCCCGGGTCCTCTTATCACGGGAAACAAGGGCGACAA

CTTCCAGATCAATGTTATCGACAACCTCTCTAACGAGACGATGTTGAAGTCGACC
TCCATCCATTGGCACGGCTTCTTCCAGAAGGGTACTAACTGGGCTGATGGAGCTG
CCTTCGTCAACCAGTGCCCTATCGCGACGGGGAACTCTTTCCTTTACGACTTCAC
CGCGACGGACCAAGCAGGCACCTTCTGGTACCACAGTCACTTGTCTACGCAGTAC
TGCGATGGTTTGCGGGGCCCGATGGTCGTATACGACCCGAGTGACCCGCATGCG
GACCTTTACGACGTCGACGACGAGACCACGATCATCACGCTCTCTGATTGGTATC
ACACCGCCGCTTCGCTCGGTGCTGCTTTCCCGATTGGCTCGGACTCTACCCTCATC
AACGGGTTGGGCCGTTTCGCCGGTGGCGACAGCACTGACCTTGCGGTCATCACG
GTTGAGCAGGGCAAGCGCTACCGTATGCGTCTTCTCTCGCTGTCTTGCGACCCCA
ACTATGTCTTCTCCATCGACGGTCACAACATGACCATCATCGAGGCCGACGCTGT
CAACCACGAGCCCCTCACGGTCGACTCCATCCAGATCTACGCCGGACAACGTTAC
TCCTTCGTCCTTACTGCTGACCAGGACATCGACAACTACTTCATCCGTGCCCTGCC
CAGCGCCGGTACCACCTCGTTCGACGGCGGCATCAACTCGGCTATCCTGCGCTAC
TCTGGTGCCTCCGAGGTTGACCCGACGACCACGGAGACCACGAGTGTCCTCCCCC
TCGACGAGGCGAACCTCGTGCCCCTTGACAGCCCCGCTGCTCCCGGTGACCCCAA
CATTGGCGGTGTCGACTACGCGCTGAACTTGGACTTCAACTTCGATGGCACCAAC
TTCTTCATTAACGACGTCTCCTTCGTATCCCCCACTGTCCCTGTCCTCCTCCAGAT
CCTTAGCGGCACCACCTCCGCGGCCGACCTCCTCCCCAGCGGCAGTCTCTTCGCG
CTCCCGTCCGACTCGACGATCGAGATCTCGTTCCCCATCACCGCGACGAACGCGC
CCGGCGCGCCGCATCCCTTCCACTTGCACGGTCACACCTTCTCTATCGTTCGTACC
GCCGGCAGCACGGATACGAACTTCGTCAACCCCGTCCGCCGCGACGTCGTGAAC
ACCGGTACCGCCGGCGACAACGTCACCATCCGCTTCACGACTGACAACCCCGGC
CCCTGGTTCCTCCACTGCCACATCGACTTCCACTTGGAGGCCGGTTTCGCCATCGT
CCTCAGCGAGGACACCGCCGACGTCTCGAACACGACCACGCCCTCGACTGCTTG
GGAAGATCTGTGCCCCACGTACAACGCTCTTGACTCATCCGACCTCTAATCGGTT
CAAGCGGCCGCTCAAGAGGATGTCAGAATGCCATTTGCCTGAGAGATGCAGGCT
TCATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATTTTTTTTGTCATT
TTGTTTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCAGATGAATA
TCTTGTGGTAGGGGTTTGGGAAAATCATTCGAGTTTGATGTTTTTCTTGGTATTTC
CCACTCCTCTTCAGAGTACAGAAGATTAAGTGAAACCTTCGTTTGTGCGGATCCT
TCAGTAATGTCTTGTTTCTTTTGTTGCAGTGGTGAGCCATTTTGACTTCGTGAAAG
TTTCTTTAGAATAGTTGTTTCCAGAGGCCAAACATTCCACCCGTAGTAAAGTGCA
AGCGTAGGAAGACCAAGACTGGCATAAATCAGGTATAAGTGTCGAGCACTGGCA

GGTGATCTTCTGAAAGTTTCTACTAGCAGATAAGATCCAGTAGTCATGCATATGG
CAACAATGTACCGTGTGGATCTAAGAACGCGTCCTACTAACCTTCGCATTCGTTG
GTCCAGTTTGTTGTTATCGATCAACGTGACAAGGTTGTCGATTCCGCGTAAGCAT
GCATACCCAAGGACGCCTGTTGCAATTCCAAGTGAGCCAGTTCCAACAATCTTTG
TAATATTAGAGCACTTCATTGTGTTGCGCTTGAAAGTAAAATGCGAACAAATTAA
GAGATAATCTCGAAACCGCGACTTCAAACGCCAATATGATGTGCGGCACACAAT
AAGCGTTCATATCCGCTGGGTGACTTTCTCGCTTTAAAAAATTATCCGAAAAAAT
TTTCTAGAGTGTTGTTACTTTATACTTCCGGCTCGTATAATACGACAAGGTGTAAG
GAGGACTAAACCATGGCTAAACTCACCTCTGCTGTTCCAGTCCTGACTGCTCGTG
ATGTTGCTGGTGCTGTTGAGTTCTGGACTGATAGACTCGGTTTCTCCCGTGACTTC
GTAGAGGACGACTTTGCCGGTGTTGTACGTGACGACGTTACCCTGTTCATCTCCG
CAGTTCAGGACCAGGTTGTGCCAGACAACACTCTGGCATGGGTATGGGTTCGTG
GTCTGGACGAACTGTACGCTGAGTGGTCTGAGGTCGTGTCTACCAACTTCCGTGA
TGCATCTGGTCCAGCTATGACCGAGATCGGTGAACAGCCCTGGGGTCGTGAGTTT
GCACTGCGTGATCCAGCTGGTAACTGCGTGCATTTCGTCGCAGAAGAGCAGGAC
TAACAATTGACACCTTACGATTATTTAGAGAGTATTTATTAGTTTTATTGTATGTA
TACGGATGTTTTATTATCTATTTATGCCCTTATATTCTGTAACTATCCAAAAGTCC
TATCTTATCAAGCCAGCAATCTATGTCCGCGAACGTCAACTAAAAATAAGCTTTT
TATGCTCTTCTCTCTTTTTTTCCCTTCGGTATAATTATACCTTGCATCCACAGATTC
TCCTGCCAAATTTTGCATAATCCTTTACAACATGGCTATATGGGAGCACTTAGCG
CCCTCCAAAACCCATATTGCCTACGCATGTATAGGTGTTTTTTCCACAATATTTTC
TCTGTGCTCTCTTTTTATTAAAGAGAAGCTCTATATCGGAGAAGCTTCTGTGGCCG
TTATATTCGGCCTTATCGTGGGACCACATTGCCTGAATTGGTTTGCCCCGGAAGA
TTGGGGAAACTTGGATCTGATTACCTTAGCTGCAGGTACCACTGAGCGTCAGACC
CCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTG
CTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGTCGGATCAA
GAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAA
ATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGC
ACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGC
GATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCG
CAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACG
ACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTT
CCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGG

AGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGT
CGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGG
CGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTT
GCTGGCCTTTTGCTCACATGTTCTTTCCTGCGGTACCC

Trametes versicolor laccase isoform *lcc2*: pJ905-Tv-lcc2



Plasmid Sequence

AGATCCAATTCCCGCTTTGACTGCCTGAAATCTCCATCGCCTACAATGATGACAT
TTGGATTTGGTTGACTCATGTTGGTATTGTGAAATAGACGCAGATCGGGAACACT
GAAAAATACACAGTTATTATTCATTTAAATTTTTTGTAGAAATGTCTTGGTGTCCT
CGTCCAATCAGGTAGCCATCTCTGAAATATCTGGCTCCGTTGCAACTCCGAACGA
CCTGCTGGCAACGTAAAATTCTCCGGGGTAAAACTTAAATGTGGAGTAATGGAA
CCAGAAACGTCTCTTCCCTTCTCTCTCCTTCCACCGCCCGTTACCGTCCCTAGGAA
ATTTTACTCTGCTGGAGAGCTTCTTCTACGGCCCCCTTGCAGCAATGCTCTTCCCA
GCATTACGTTGCGGGTAAAACGGAGGTCGTGTACCCGACCTAGCAGCCCAGGGA
TGGAAAAGTCCCGGCCGTCGCTGGCAATAATAGCGGGCGGACGCATGTCATGAG
ATTATTGGAAACCACCAGAATCGAATATAAAAGGCGAACACCTTTCCCAATTTTG
GTTTCTCCTGACCCAAAGACTTTAAATTTAATTTATTTGTCCCTATTTCAATCAAT
TGAACAACTATCAAAACACAGAATTCATGTCGAGGTTTCACTCTCTTCTCGCTTT

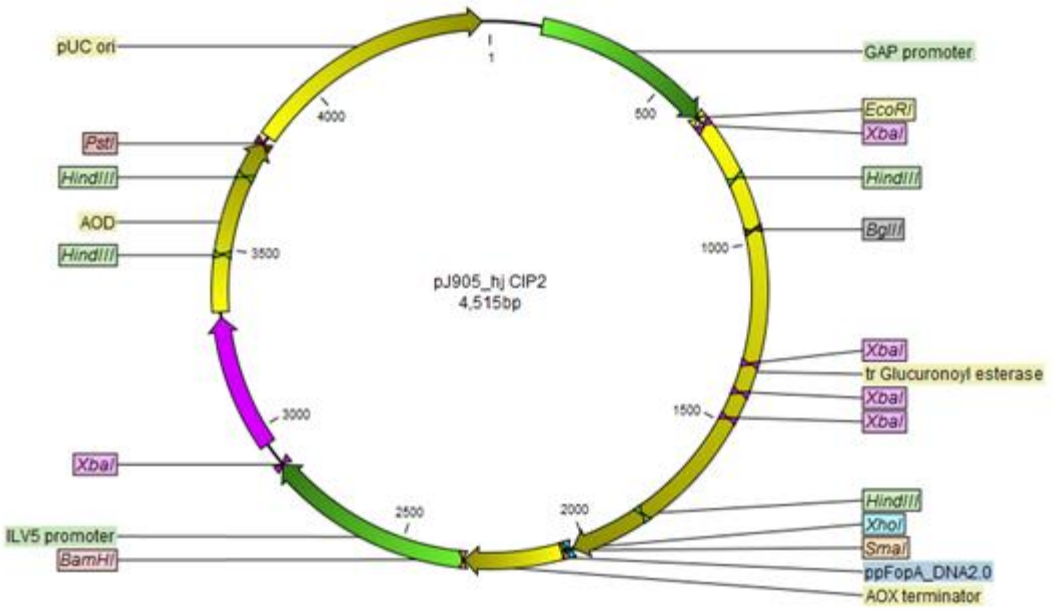
CGTCGTTGCTTCCCTTGCGGCTGTGGCCCACGCTGGTATCGGTCCTGTCGCCGAC
CTCACCATCACCAACGCAGCGGTCAGCCCCGACGGGTTTTCTCGCCAGGCCGTCG
TCGTGAACGGTGGCACCCCTGGCCCTCTCATCACCGGTAACATGGGGGATCGCTT
CCAGCTCAATGTCATCGACAACCTCACGGACCACACGATGCTGAAGAGCACCAG
TATTCACTGGCACGGTTTCTTCCAGAAGGGCACCAACTGGGCCGACGGTCCCGCC
TTCATCAACCAGTGCCCGATCTCATCTGGCCACTCGTTCCTGTATGACTTCCAGGT
TCCTGACCAGGCTGGCACCTTCTGGTACCACAGTCACTTGTCCACGCAGTACTGT
GATGGTCTGAGGGGTCCGTTCGTTGTTTACGACCCGAATGACCCGGCCGCCGACC
TGTACGACGTCGACAACGACGACACGGTCATTACCCTTGCGGATTGGTACCACGT
CGCCGCGAAGCTGGGCCCCGCATTCCCTCTCGGCGCCGACGCCACTCTCATCAAC
GGTAAGGGACGCTCCCCCAGCACGACCACCGCGGACCTCACTGTTATCAGCGTC
ACTCCGGGTAAACGTTACCGTTTCCGCCTGGTGTCCCTGTCGTGCGACCCCAACC
ACACCTTCAGCATCGATGGCCACAACATGACGATCATCGAGACCGACTCGATCA
ACACGGCGCCCCTCGTGGTCGACTCCATTCAGATCTTCGCTGCCCAGCGTTACTC
CTTCGTGCTCGAGGCCAACCAGGCCGTCGACAACTACTGGATTCGCGCCAACCCG
AGCTTCGGTAACGTCGGGTTCACCGGCGGGATCAACTCGGCTATCCTCCGCTATG
ATGGCGCCGCTGCCATCGAGCCCACCACCACGCAGACCACTTCGACCGAGCCGC
TCAACGAGGTCAACCTGCACCCGCTGGTTGCCACCGCTGTCCCTGGCTCTCCCGC
TGCGGGTGGTGTTGACCTGGCCATCAATATGGCGTTCAACTTCAATGGCACCAAC
TTCTTCATCAACGGCGCGTCTTTCACGCCCCCGACCGTGCCTGTCCTCCTCCAGAT
CATCAGCGGCGCGCAGAACGCGCAGGACCTCCTGCCCTCCGGCAGCGTATACTC
GCTCCCCTCGAACGCCGACATCGAGATCTCCTTCCCCGCCACCGCCGCTGCCCCC
GGTGCGCCCCACCCCTTCCACTTGCACGGGCACGCGTTCGCGGTCGTCCGCAGCG
CCGGCAGCACGGTCTACAACTACGACAACCCCATCTTCCGCGACGTCGTCAGCAC
GGGGACGCCTGCGGCCGGTGACAACGTCACCATCCGCTTCCGCACCGACAACCC
CGGCCCGTGGTTTCTCCACTGCCACATCGACTTCCACCTCGAGGCCGGCTTCGCC
GTCGTGTTTGCGGAGGACATCCCCGATGTCGCATCGGCGAACCCCGTCCCCCAGG
CGTGGTCCGACCTCTGCCCGACCTACGACGCGCTCGACCCGAGCGACCAGTAAA
TGGCGGCCGCTCAAGAGGATGTCAGAATGCCATTTGCCTGAGAGATGCAGGCTT
CATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATTTTTTTTGTCATTT
TGTTTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCAGATGAATAT
CTTGTGGTAGGGGTTTGGGAAAATCATTCGAGTTTGATGTTTTTCTTGGTATTTCC
CACTCCTCTTCAGAGTACAGAAGATTAAGTGAAACCTTCGTTTGTGCGGATCCTT

CAGTAATGTCTTGTTTCTTTTGTTGCAGTGGTGAGCCATTTTGACTTCGTGAAAGT
TTCTTTAGAATAGTTGTTTCCAGAGGCCAAACATTCCACCCGTAGTAAAGTGCAA
GCGTAGGAAGACCAAGACTGGCATAAATCAGGTATAAGTGTCGAGCACTGGCAG
GTGATCTTCTGAAAGTTTCTACTAGCAGATAAGATCCAGTAGTCATGCATATGGC
AACAATGTACCGTGTGGATCTAAGAACGCGTCCTACTAACCTTCGCATTCGTTGG
TCCAGTTTGTTGTTATCGATCAACGTGACAAGGTTGTCGATTCCGCGTAAGCATG
CATACCCAAGGACGCCTGTTGCAATTCCAAGTGAGCCAGTTCCAACAATCTTTGT
AATATTAGAGCACTTCATTGTGTTGCGCTTGAAAGTAAAATGCGAACAAATTAAG
AGATAATCTCGAAACCGCGACTTCAAACGCCAATATGATGTGCGGCACACAATA
AGCGTTCATATCCGCTGGGTGACTTTCTCGCTTTAAAAAATTATCCGAAAAAATT
TTCTAGAGTGTTGTTACTTTATACTTCCGGCTCGTATAATACGACAAGGTGTAAG
GAGGACTAAACCATGGCTAAACTCACCTCTGCTGTTCCAGTCCTGACTGCTCGTG
ATGTTGCTGGTGCTGTTGAGTTCTGGACTGATAGACTCGGTTTCTCCCGTGACTTC
GTAGAGGACGACTTTGCCGGTGTTGTACGTGACGACGTTACCCTGTTCATCTCCG
CAGTTCAGGACCAGGTTGTGCCAGACAACACTCTGGCATGGGTATGGGTTCGTG
GTCTGGACGAACTGTACGCTGAGTGGTCTGAGGTCGTGTCTACCAACTTCCGTGA
TGCATCTGGTCCAGCTATGACCGAGATCGGTGAACAGCCCTGGGGTCGTGAGTTT
GCACTGCGTGATCCAGCTGGTAACTGCGTGCATTTCGTCGCAGAAGAGCAGGAC
TAACAATTGACACCTTACGATTATTTAGAGAGTATTTATTAGTTTTATTGTATGTA
TACGGATGTTTTATTATCTATTTATGCCCTTATATTCTGTAACTATCCAAAAGTCC
TATCTTATCAAGCCAGCAATCTATGTCCGCGAACGTCAACTAAAAATAAGCTTTT
TATGCTCTTCTCTCTTTTTTTCCCTTCGGTATAATTATACCTTGCATCCACAGATTC
TCCTGCCAAATTTTGCATAATCCTTTACAACATGGCTATATGGGAGCACTTAGCG
CCCTCCAAAACCCATATTGCCTACGCATGTATAGGTGTTTTTTCCACAATATTTTC
TCTGTGCTCTCTTTTTATTAAAGAGAAGCTCTATATCGGAGAAGCTTCTGTGGCCG
TTATATTCGGCCTTATCGTGGGACCACATTGCCTGAATTGGTTTGCCCCGGAAGA
TTGGGGAAACTTGGATCTGATTACCTTAGCTGCAGGTACCACTGAGCGTCAGACC
CCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTG
CTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGTCGGATCAA
GAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAA
ATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGC
ACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGC
GATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCG

CAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACG
ACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTT
CCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGG
AGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGT
CGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGG
CGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTT
GCTGGCCTTTTGCTCACATGTTCTTTCCTGCGGTACCC

Glucuronoyl Esterase (GE)

Hypocrea jecorina GE: pJ905-Hj-ge



Plasmid Sequence

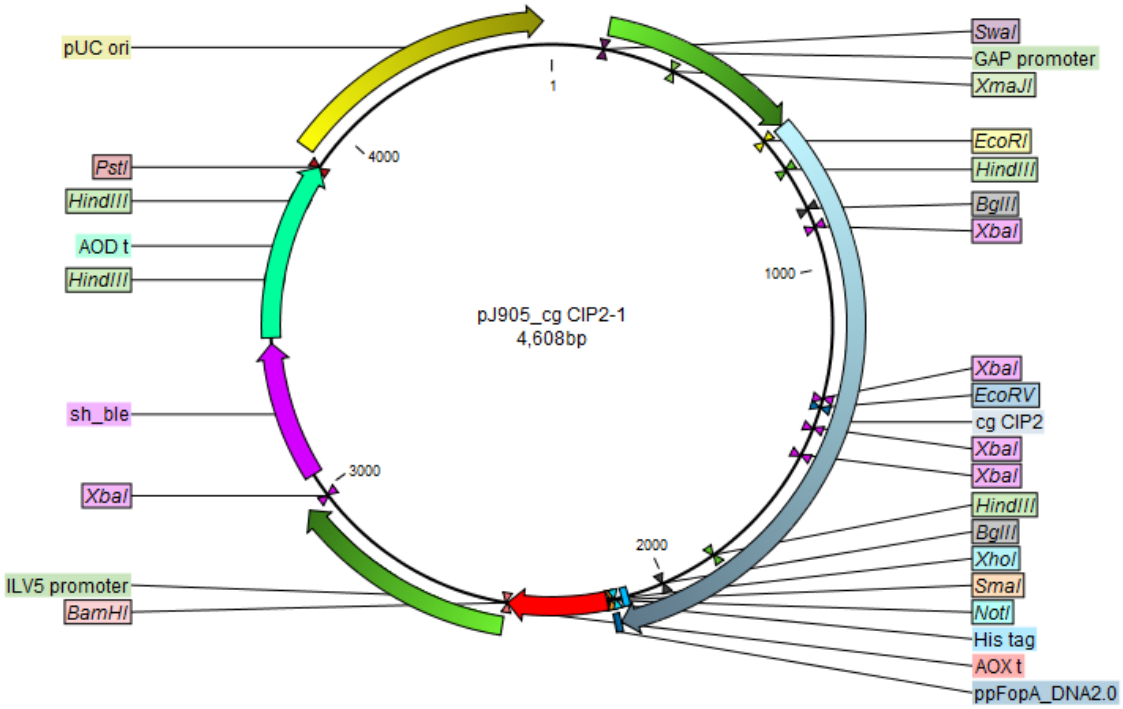
AGATCCAATTCCCGCTTTGACTGCCTGAAATCTCCATCGCCTACAATGATGACAT
TTGGATTTGGTTGACTCATGTTGGTATTGTGAAATAGACGCAGATCGGGAACACT
GAAAAATACACAGTTATTATTCATTTAAATTTTTTGTAGAAATGTCTTGGTGTCCT
CGTCCAATCAGGTAGCCATCTCTGAAATATCTGGCTCCGTTGCAACTCCGAACGA
CCTGCTGGCAACGTAAAATTCTCCGGGGTAAAACTTAAATGTGGAGTAATGGAA
CCAGAAACGTCTCTTCCCTTCTCTCTCCTTCCACCGCCCGTTACCGTCCCTAGGAA
ATTTTACTCTGCTGGAGAGCTTCTTCTACGGCCCCCTTGCAGCAATGCTCTTCCCA
GCATTACGTTGCGGGTAAAACGGAGGTCGTGTACCCGACCTAGCAGCCCAGGGA

TGGAAAAGTCCCGGCCGTCGCTGGCAATAATAGCGGGCGGACGCATGTCATGAG
ATTATTGGAAACCACCAGAATCGAATATAAAAGGCGAACACCTTTCCCAATTTTG
GTTTCTCCTGACCCAAAGACTTTAAATTTAATTTATTTGTCCCTATTTCAATCAAT
TGAACAACTATCAAAACACAGAATTCTTAATTAAAAACAAAATGGCCTCTAGAT
TTTTTGCTTTGTTGTTGTTGGCTATTCCAATTCAAGCTCAATCTCCAGTTTGGGGT
CAATGTGGTGGTATTGGTTGGTCTGGTCCAACTACTTGTGTTGGTGGTGCTACTTG
TGTTTCTTACAACCCATATTACTCTCAATGTATTCCATCTACTCAAGCTTCATCTT
CTATTGCTTCTACTACTTTGGTTACTTCTTTCACTACTACTACTGCTACTAGAACTT
CTGCTTCTACTCCACCAGCTTCATCTACTGGTGCTGGTGGTGCAACATGTTCTGCT
TTGCCAGGTTCTATTACTTTGAGATCTAACGCTAAGTTGAATGATTTGTTCACTAT
GTTTAACGGTGATAAGGTTACTACAAAGGATAAGTTCTCTTGTAGACAAGCTGAA
ATGTCTGAATTGATCCAAAGATATGAATTGGGTACTTTGCCAGGTAGACCATCTA
CTTTGACTGCTTCATTTTCTGGTAACACTTTGACTATTAACTGTGGTGAAGCTGGT
AAATCTATTTCTTTCACCGTTACTATTACTTATCCATCTTCTGGTACTGCTCCATAT
CCAGCTATTATTGGTTATGGTGGTGGTTCTTTGCCAGCTCCAGCTGGTGTTGCTAT
GATTAACTTCAACAACGATAATATTGCTGCTCAAGTTAATACTGGTTCTAGAGGT
CAAGGTAAATTTTATGACTTGTACGGTTCTTCTCATTCTGCTGGTGCTATGACTGC
TTGGGCTTGGGGTGTTTCTAGAGTTATTGATGCTTTGGAATTGGTTCCAGGTGCTA
GAATTGATACAACTAAGATTGGTGTTACTGGTTGTTCTAGAAATGGTAAAGGTGC
TATGGTTGCTGGTGCTTTTGAAAAAAGAATTGTCTTGACTTTGCCACAAGAATCT
GGTGCCGGTGGTTCTGCTTGTTGGAGAATTTCTGATTACTTGAAATCTCAAGGTG
CTAATATTCAAACTGCCTCCGAAATTATTGGTGAAGATCCATGGTTTTCTACTAC
CTTTAACTCCTACGTTAATCAAGTTCCAGTTTTGCCATTTGATCATCATTCTTTGG
CTGCTTTGATTGCTCCAAGAGGTTTGTTCGTCATCGATAACAACATTGATTGGTTG
GGTCCACAATCTTGTTTTGGTTGTATGACTGCTGCTCATATGGCTTGGCAAGCTTT
GGGTGTTTCTGATCATATGGGTTATTCTCAAATTGGTGCTCATGCTCATTGTGCTT
TTCCATCTAATCAACAATCTCAATTGACTGCTTTTGTTCAAAAATTCTTGTTGGGT
CAATCTACTAATACTGCTATTTTCCAATCTGATTTCTCTGCTAATCAATCTCAATG
GATTGATTGGACTACTCCAACTTTGTCTTAATGAGGCGCGCCCTCGAGGCGCGCC
CGGGGCGGCCGCTCAAGAGGATGTCAGAATGCCATTTGCCTGAGAGATGCAGGC
TTCATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATTTTTTTTGTCAT
TTTGTTTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCAGATGAAT
ATCTTGTGGTAGGGGTTTGGGAAAATCATTCGAGTTTGATGTTTTTCTTGGTATTT

CCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAAACCTTCGTTTGTGCGGATCC
TTCAGTAATGTCTTGTTTCTTTTGTTGCAGTGGTGAGCCATTTTGACTTCGTGAAA
GTTTCTTTAGAATAGTTGTTTCCAGAGGCCAAACATTCCACCCGTAGTAAAGTGC
AAGCGTAGGAAGACCAAGACTGGCATAAATCAGGTATAAGTGTCGAGCACTGGC
AGGTGATCTTCTGAAAGTTTCTACTAGCAGATAAGATCCAGTAGTCATGCATATG
GCAACAATGTACCGTGTGGATCTAAGAACGCGTCCTACTAACCTTCGCATTCGTT
GGTCCAGTTTGTTGTTATCGATCAACGTGACAAGGTTGTCGATTCCGCGTAAGCA
TGCATACCCAAGGACGCCTGTTGCAATTCCAAGTGAGCCAGTTCCAACAATCTTT
GTAATATTAGAGCACTTCATTGTGTTGCGCTTGAAAGTAAAATGCGAACAAATTA
AGAGATAATCTCGAAACCGCGACTTCAAACGCCAATATGATGTGCGGCACACAA
TAAGCGTTCATATCCGCTGGGTGACTTTCTCGCTTTAAAAAATTATCCGAAAAAA
TTTTCTAGAGTGTTGTTACTTTATACTTCCGGCTCGTATAATACGACAAGGTGTAA
GGAGGACTAAACCATGGCTAAACTCACCTCTGCTGTTCCAGTCCTGACTGCTCGT
GATGTTGCTGGTGCTGTTGAGTTCTGGACTGATAGACTCGGTTTCTCCCGTGACTT
CGTAGAGGACGACTTTGCCGGTGTTGTACGTGACGACGTTACCCTGTTCATCTCC
GCAGTTCAGGACCAGGTTGTGCCAGACAACACTCTGGCATGGGTATGGGTTCGT
GGTCTGGACGAACTGTACGCTGAGTGGTCTGAGGTCGTGTCTACCAACTTCCGTG
ATGCATCTGGTCCAGCTATGACCGAGATCGGTGAACAGCCCTGGGGTCGTGAGTT
TGCACTGCGTGATCCAGCTGGTAACTGCGTGCATTTCGTCGCAGAAGAGCAGGA
CTAACAATTGACACCTTACGATTATTTAGAGAGTATTTATTAGTTTTATTGTATGT
ATACGGATGTTTTATTATCTATTTATGCCCTTATATTCTGTAACTATCCAAAAGTC
CTATCTTATCAAGCCAGCAATCTATGTCCGCGAACGTCAACTAAAAATAAGCTTT
TTATGCTCTTCTCTCTTTTTTTCCCTTCGGTATAATTATACCTTGCATCCACAGATT
CTCCTGCCAAATTTTGCATAATCCTTTACAACATGGCTATATGGGAGCACTTAGC
GCCCTCCAAAACCCATATTGCCTACGCATGTATAGGTGTTTTTTCCACAATATTTT
CTCTGTGCTCTCTTTTTATTAAAGAGAAGCTCTATATCGGAGAAGCTTCTGTGGCC
GTTATATTCGGCCTTATCGTGGGACCACATTGCCTGAATTGGTTTGCCCCGGAAG
ATTGGGGAAACTTGGATCTGATTACCTTAGCTGCAGGTACCACTGAGCGTCAGAC
CCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCT
GCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGTCGGATCA
AGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCA
AATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAG
CACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGG

CGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGC
GCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAAC
GACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCT
TCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAG
GAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTG
TCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGG
GCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTT

Chaetomium globosum GE: pJ905-Cg-GE



Plasmid Sequence

AGATCCAATTCCCGCTTTGACTGCCTGAAATCTCCATCGCCTACAATGATGACAT
TTGGATTTGGTTGACTCATGTTGGTATTGTGAAATAGACGCAGATCGGGAACACT
GAAAAATACACAGTTATTATTCATTTAAATTTTTTGTAGAAATGTCTTGGTGTCCT
CGTCCAATCAGGTAGCCATCTCTGAAATATCTGGCTCCGTTGCAACTCCGAACGA
CCTGCTGGCAACGTAAAATTCTCCGGGGTAAAACTTAAATGTGGAGTAATGGAA
CCAGAAACGTCTCTTCCCTTCTCTCTCCTTCCACCGCCCGTTACCGTCCCTAGGAA
ATTTTACTCTGCTGGAGAGCTTCTTCTACGGCCCCCTTGCAGCAATGCTCTTCCCA
GCATTACGTTGCGGGTAAAACGGAGGTCGTGTACCCGACCTAGCAGCCCAGGGA

TGGAAAAGTCCCGGCCGTCGCTGGCAATAATAGCGGGCGGACGCATGTCATGAG
ATTATTGGAAACCACCAGAATCGAATATAAAAGGCGAACACCTTTCCCAATTTTG
GTTTCTCCTGACCCAAAGACTTTAAATTTAATTTATTTGTCCCTATTTCAATCAAT
TGAACAACTATCAAAACACAGAATTCTTAATTAAAAACAAAATGTTGTCTCCAGC
TTTGGCTTCTTCTTTGTTGGTTGTTTTGGGTGCTGCTGGTGCTCATGCTCAACAAA
GACAAGCTTTGTGGGGTCAATGTGGTGGTTCTGGTTGGAATGGTCCAACTGCTTG
TGTTGATGGTGCTTACTGTTTTCAACAAAATCAATGGTATCATCAATGTATTCCAG
GTTCTGATAGATCTCCATCTCCAACTAATCCACCACAACAATCTTCTACTTTGACT
ACTTCTAGAGTTACTGTTGCTCCACCAACTTCTACTTCTCCAGCTCCAGGTGGTGG
TGGTACTACTTGTCCAGCTACTCCATCTGGTCAAGGTTCTCCAGTTGCTAATGCTT
TGAATGATCCATTCACTTTTCATGGTGGTGGTAAAGTTTCTTCTAAAGCTGATTGG
ACTTGTAGACAAGCTGAAATTTCTAAGTTGTTGCAACAATATGAATTGGGTACTT
TGCCACCAAAACCATCTTCAGTTACTGCTTCATTTTCTGGTTCTACCTTGTCTATT
TCTGTTTCTGAAGGTGGTAAGTCTATTTCTTTCTCTGTTTCCATTAACAATAGACC
ATCTTCTTCAGGTCCACATCCAGCTATTATTAACTTTGGTACTTTTGGTGGTTTGC
CAGTTCCAGCTGGTGTTGCTACTATTAACTTCAACAACGACGAAATTGCTCAACA
ACAAGATCAATCTTCTAGAGGTAAAGGTAAATTCTATGATATCTACGGTTCTGGT
CATTCTGCTGGTGCTTTGACTGCTTGGGCTTGGGGTGTTTCTAGAATTATTGATGC
TTTGGAATTGACTAGAGCCCAAACTAATATTGATCCAGCTAGAATTGGTGTTACT
GGTTGTTCTAGAAATGGTAAAGGTGCTATGGTTGCTGGTGCATTGGAACCTAGAA
TTGCTTTGACTTTGCCAGAAGAATCTGGAGCTGGCGGTTCAGGTTGTTGGAGAAT
TGCTACTTGGCAAAAGAATAGAGGTGAAAACGTTCAAGAATCTAAGCAAATCGT
TACTGAAAATGTTTGGTTTTCTACCAATTTCAACTCTCAAGTTAACAATGTTAATG
CTTTGCCATTTGATCATCATATGTTGGCTGGTTTGATTGCTCCAAGAGCATTATAT
GTTATGGAAAACACTGATATGGAATGGTTGGGTAAAGTTTCAACTTATGGTTGTA
TGGGTATTGCTAGAAAACAATGGGAAGCTTTGGGTGCTTTGGATTCTTTTGGTTA
TTCTCAAGTTGGTGGTAATTCTCATTGCTCTTTTCCATCTTCTCAACAAGGTTCTG
AATTGAATGCTTTTATCGGTAAATACTTGTTGAACAATCCAAATGCTGGTAACAC
TAACATTTTTAGATCTACCGGTAATCAAAATTTCGATTTGAACTCTTGGTCACCAT
GGACTGTTCCAAAATTGTCTGATGATGATGACAAAGGTGGTTCTCCTCCTTCTCA
TCATCACCACCACCACTAATGAGGCGCGCCCTCGAGGCGCGCCCGGGGCGGCCG
CTCAAGAGGATGTCAGAATGCCATTTGCCTGAGAGATGCAGGCTTCATTTTTGAT
ACTTTTTTATTTGTAACCTATATAGTATAGGATTTTTTTTGTCATTTTGTTTCTTCT

CGTACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCAGATGAATATCTTGTGGTA
GGGGTTTGGGAAAATCATTCGAGTTTGATGTTTTTCTTGGTATTTCCCACTCCTCT
TCAGAGTACAGAAGATTAAGTGAAACCTTCGTTTGTGCGGATCCTTCAGTAATGT
CTTGTTTCTTTTGTTGCAGTGGTGAGCCATTTTGACTTCGTGAAAGTTTCTTTAGA
ATAGTTGTTTCCAGAGGCCAAACATTCCACCCGTAGTAAAGTGCAAGCGTAGGA
AGACCAAGACTGGCATAAATCAGGTATAAGTGTCGAGCACTGGCAGGTGATCTT
CTGAAAGTTTCTACTAGCAGATAAGATCCAGTAGTCATGCATATGGCAACAATGT
ACCGTGTGGATCTAAGAACGCGTCCTACTAACCTTCGCATTCGTTGGTCCAGTTT
GTTGTTATCGATCAACGTGACAAGGTTGTCGATTCCGCGTAAGCATGCATACCCA
AGGACGCCTGTTGCAATTCCAAGTGAGCCAGTTCCAACAATCTTTGTAATATTAG
AGCACTTCATTGTGTTGCGCTTGAAAGTAAAATGCGAACAAATTAAGAGATAATC
TCGAAACCGCGACTTCAAACGCCAATATGATGTGCGGCACACAATAAGCGTTCA
TATCCGCTGGGTGACTTTCTCGCTTTAAAAAATTATCCGAAAAAATTTTCTAGAG
TGTTGTTACTTTATACTTCCGGCTCGTATAATACGACAAGGTGTAAGGAGGACTA
AACCATGGCTAAACTCACCTCTGCTGTTCCAGTCCTGACTGCTCGTGATGTTGCT
GGTGCTGTTGAGTTCTGGACTGATAGACTCGGTTTCTCCCGTGACTTCGTAGAGG
ACGACTTTGCCGGTGTTGTACGTGACGACGTTACCCTGTTCATCTCCGCAGTTCA
GGACCAGGTTGTGCCAGACAACACTCTGGCATGGGTATGGGTTCGTGGTCTGGA
CGAACTGTACGCTGAGTGGTCTGAGGTCGTGTCTACCAACTTCCGTGATGCATCT
GGTCCAGCTATGACCGAGATCGGTGAACAGCCCTGGGGTCGTGAGTTTGCACTG
CGTGATCCAGCTGGTAACTGCGTGCATTTCGTCGCAGAAGAGCAGGACTAACAA
TTGACACCTTACGATTATTTAGAGAGTATTTATTAGTTTTATTGTATGTATACGGA
TGTTTTATTATCTATTTATGCCCTTATATTCTGTAACTATCCAAAAGTCCTATCTTA
TCAAGCCAGCAATCTATGTCCGCGAACGTCAACTAAAAATAAGCTTTTTATGCTC
TTCTCTCTTTTTTTCCCTTCGGTATAATTATACCTTGCATCCACAGATTCTCCTGCC
AAATTTTGCATAATCCTTTACAACATGGCTATATGGGAGCACTTAGCGCCCTCCA
AAACCCATATTGCCTACGCATGTATAGGTGTTTTTTCCACAATATTTTCTCTGTGC
TCTCTTTTTATTAAAGAGAAGCTCTATATCGGAGAAGCTTCTGTGGCCGTTATATT
CGGCCTTATCGTGGGACCACATTGCCTGAATTGGTTTGCCCCGGAAGATTGGGGA
AACTTGGATCTGATTACCTTAGCTGCAGGTACCACTGAGCGTCAGACCCCGTAGA
AAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGC
AAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGTCGGATCAAGAGCTAC
CAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGT

TCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCT
ACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGT
CGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGT
CGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACA
CCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAG
GGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGC
ACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTC
GCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCT
ATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCT
TTTGCTCACATGTTCTTTCCTGCGGTACCC

Addendum B

Tabulated data of graphs reported in this study

Screening data:

Cellobiose dehydrogenase:

Table B1. Test-tube screening of *P. pastoris* transformants, grown in BMGY (Invitrogen) media for 24 hours, expressing *N. crassa* CDH.

Strain Name	Volumetric Activity (U/L)	Standard Deviation (SD)
C1	0.352941176	1.164646463
C2	0.352941176	0
N1.1	43.52941176	0.166378066
N1.3	46.23529412	1.996536794
N1.5	44.82352941	0.332756132
N1.6	47.64705882	1.331024529
N1.7	46.47058824	0.499134198
N1.8	46.94117647	1.164646463
N2.1	46.23529412	0
N2.2	46.94117647	0
N2.3	47.17647059	0.499134198
N2.4	48.47058824	0.582323232
N2.5	4.705882353	0.415945165
N2.6	46.47058824	0.499134198
N2.8	47.41176471	0.166378066
N5.1	47.05882353	0.083189033
N5.2	46.23529412	0.499134198
N5.3	46.47058824	0.665512265
N5.5	46	0.332756132
N5.6	40.70588235	2.578860026
N5.7	48.23529412	0.083189033
N5.8	48.11764706	0

Table B2. Test-tube screening of *P. pastoris* transformants, grown in BMGY (Invitrogen) media for 24 hours, expressing *M. thermophilum* CDH.

Strain Name	Volumetric Activity (U/L)	Standard Deviation (SD)
C1	0	0.207972583
C2	0.022058824	0.145580808
M1.1	4.110294118	0.062391775
M1.3	-0.477941176	0.103986291
M1.4	-0.139705882	0.083189033
M1.5	3.507352941	0.041594517
M1.6	4.066176471	0.12478355
M1.7	3.139705882	0.311958874
M1.8	4.227941176	0.062391775
M2.1	3.727941176	0.187175324

M2.2	3.727941176	0.187175324
M2.3	5.139705882	0.020797258
M2.4	4.727941176	0.020797258
M2.8	1.757352941	0.062391775
M5.1	0.007352941	0.041594517
M5.2	3.007352941	0
M5.3	5.139705882	0.062391775
M5.4	3.536764706	0.207972583
M5.5	2.433823529	0.145580808
M5.6	2.875	0.395147907
M5.7	3.816176471	0.228769841
M5.8	4.095588235	0.249567099

Table B3. Shake-flask screening results of volumetric activity of recombinant *N. crassa* and *M. thermophilum* CDH after growth in BMGY for 72 hours. C1, C2: Negative control strains. M1.1-M5.3: transformants expressing *M. thermophilum* CDH. N2.3-N5.7: transformants expressing *N. crassa CDH*.

Strain Name	Volumetric Activity (U/L)	Standard Deviation (SD)
C1	0.699346405	0.615178176
C2	0.254901961	0.441503147
M1.1	8.588235294	1.169752058
M1.6	8.588235294	1.169752058
M2.3	7.830065359	0.689441804
M2.4	12.69281046	2.163655376
M5.3	7.568627451	0.537340769
N2.3	66.62309368	2.604745322
N2.6	65.27233115	0.199676501
N5.1	66.84095861	0.963544418
N5.3	70.81045752	0.272113203
N5.7	66.79738562	2.417443763

Laccase:

Table B4. Shake-flask screening results for *P. pastoris* grown in BMGY for 72 hours, expressing two laccase isoforms. C1, C2: Negative control strains. Lcc1.1-1.3: *P. pastoris* transformants expressing laccase isoform *lcc1*. Lcc2.1-2.3: *P. pastoris* transformants expressing laccase isoform *lcc2*.

Strain Name	Volumetric Activity (U/L)	Standard Deviation (SD)
C1	0.055555556	0.07856742
C2	0.222222222	0.314269681
lcc1.1	1.222222222	1.099943882
lcc1.2	0.222222222	0.314269681
lcc1.3	0.666666667	0.785674201
lcc2.1	34.27777778	8.563848794
lcc2.2	31.11111111	2.357022604
lcc2.3	36.33333333	6.756798131

Bioreactor Fermentation Data:

Cellobiose Dehydrogenase:

Table B5. Volumetric activity and biomass concentration during bioreactor cultivations of cellobiose dehydrogenase.

Time (h)	Dry Cell Weight (g/L)	SD	Volumetric Activity (U/L)	SD2
0	1.911111111	0.870079	0.980392157	0.54902
6	1.783333333	0.306413	0.431372549	0.610053
12	6.211111111	2.964294	2.784313725	1.637695
20	15.91666667	1.296362	41.56862745	11.09187
22	24.45555556	0.753019	90.19607843	40.94238
22.5	24.8	0.04714	85.49019608	17.74699
25	24.43333333	0.094281	78.43137255	7.76431
28	43.00	1.461354	102.9411765	1.386484
34	84.38888889	3.058019	265.6862745	54.07287
46	112.3	10.89806	294.1176471	38.82155
58	131.1666667	10.9284	312.4183007	33.8105
68	136.4666667	7.606138	325.4901961	16.63781

Laccase:

Table B6. Volumetric activity and biomass concentration during bioreactor cultivations of laccase.

Time (h)	Dry Cell Weight (g/L)	SD	Volumetric Activity (U/L)	SD2
0	0.783333333	0.2848	0.010493827	0.018176
6	2.908333333	0.530112	0.001234568	0.002138
12	5.883333333	0.542627	0.49691358	0.075412
20	23.68333333	0.924362	5.25308642	0.717777
22	22.81666667	0.614938	6.944444444	0.244977
22.5	23.15	0.964749	6.697530864	0.053458
25	36.80833333	3.421433	12.83950617	3.340185
28	50.40833333	7.543276	16.41975309	3.319588
34	71.44166667	12.66527	30.98765432	3.340185
46	101.6777778	18.36828	53.20987654	5.559669
58	111.0666667	17.23623	71.97530864	3.92546
68	119.5444444	19.43849	81.75925926	4.321208

Glucuronoyl Esterase:

Table B7. Volumetric activity and biomass concentration during bioreactor cultivations of glucuronoyl esterase.

Time (h)	Dry Cell Weight (g/L)	SD	Volumetric Activity (U/L)	SD2
0	1.222222222	0.604918	0.144345135	0.250013
6	3.588888889	1.655071	NM	NM
12	6.622222222	0.952968	8.299845262	3.57236
20	21.83333333	2.562117	20.68946935	14.72627
22	26.03333333	2.412698	NM	NM
22.5	26.35555556	1.787094	NM	NM
25	39.5	1.178983	23.0952216	9.014351
28	53.63333333	1.081665	62.06840805	6.124045
34	74.4	1.705221	74.33774452	1.020674
46	102.0777778	1.927674	135.2032764	20.88437
58	115.1	4.245128	NM	NM
68	120.9444444	5.707922	238.1694727	24.49618

Addendum C

Sample Calculations:

Maximum growth rate, μ_{max} (graphical method)

$$lnx = lnx_0 + \mu t$$

Where μ_{max} can be obtained from plotting $ln\ x$ vs. t, with μ_{max} represented by the maximum slope of the plot.

Productivity, (q_p), rate of recombinant protein production per gram of biomass (mg recombinant protein.g biomass^{-1}.h^{-1})

$$Productivity = \frac{p_i - p_0}{t_i - t_0}$$

The subscript "*0"* indicates conditions at inoculation or at time = 0 and subscript "*i*" refers to the conditions in the bioreactor at any sampling point thereafter.

Enzymatic Activity

$$Enzymatic\ Activity\ (U/L) = \frac{\Delta\text{Abs x F x Vt x } 10^3}{\varepsilon \text{ x d x Vs x t}}$$

ΔAbs= Abs in time final-Abs in time zero.

F = dilution factor

Vt = total volume of the assay (ie, 200 mcl)

10^3 = converts mM to μM

ε = molar attenuation coefficient

d = distance of cuvette (1 cm when you select "path-length correction" in the software)

Vs =volume of sample used in the assay

t = change in time (minutes)

Protein Concentration

$$Protein\ Concentration\ (\mathrm{mg.\,L^{-1}}) = \text{Conversion Factor}\ (\mathrm{U.\,mg^{-1}}) \times \text{Volumetric Activity}\ (\mathrm{U.\,L^{-1}})$$

The conversion factor was calculated as the number of units of enzyme activity per milligram of recombinant protein, as determined by SDS-PAGE densitometry.

Transmembrane Pressure (TMP)

$$TMP\ [bar] = [(P_{FEED} + P_{RETENTATE})/2] - P_{PERMEATE}$$