The Evaluation of Heterologous Eukaryotic Expression Systems for the Production of Biocatalytic Enzymes

by

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work with due recognition of the other contributors in Chapters 3, 5 and 6, and that I have not previously in its entirety or in part submitted it at any university for a degree.

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14 / 02 / 2008
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Abstract

Heterologous gene expression is of considerable interest for the production of proteins of therapeutic and industrial importance. As the nature of recombinant proteins has become more complex and as transformation systems have been established in more species, so the variety of hosts available for expression has increased. Every system available has both advantages and disadvantages. The research presented here highlights the advantages of selecting the most appropriate expression system for different recombinant proteins. Expression of different biocatalytically-relevant enzymes, epoxide hydrolases, halohydrin dehalogenase, laccase and mannanase, in different host systems is undertaken, and expression levels and activity are compared.

The development of *Yarrowia lipolytica* as a whole-cell biocatalyst is described. *Y. lipolytica* is used for the functional expression of epoxide hydrolases (EHs) and halohydrin dehalogenases. EHs are hydrolytic enzymes that convert epoxides to vicinal diols by ring-opening. Two new fungal EHs from *Rhodosporidium toruloides* NCYC 3181 and NCYC 3158 (a putative *Cryptococcus curvatus* strain) were identified and cloned. Additional EHs from different sources, including bacteria, yeasts, fungi and plants, were chosen for expression in *Y. lipolytica*, in order to determine its suitability as the expression system of choice for the production of EHs. Multi-copy integrants were developed, with the genes under control of the growth-phase dependent hp4d promoter. A *Saccharomyces cerevisiae* strain was developed, expressing the EH from *Rhodotorula araucariae*[1], to compare as a whole-cell biocatalyst with *Y. lipolytica*. This strain proved to be an exceptionally poor whole-cell biocatalyst. All the *Y. lipolytica* strains developed showed varying levels of activity towards different classes of epoxides. Some strains displayed opposite enantioselectivities, allowing for potential complete conversions of racemic epoxides to the desired enantiomeric product.

Halohydrins can be considered direct precursors of epoxides. Halohydrin dehalogenases catalyse the nucleophilic displacement of a halogen ion in halohydrins

[1] The construction of the *S. cerevisiae* epoxide hydrolase production strain was carried out by Dr Neeresh Rohitlall of CSIR Biosciences. The *Y. lipolytica* epoxide hydrolase strains were constructed by the author

by a vicinal hydroxyl group, yielding an epoxide, a proton and a halide ion. The *HheC* gene from *Agrobacterium radiobacter* AD1, codon-optimised to match the codon usage of *Y. lipolytica,* was over-expressed in *Y. lipolytica* by generation of multi-copy integrants, further expanding the use of this organism as a host strain for heterologous production of enzymes. Expression levels were maximised by creating tandem repeats of the introduced *HheC* gene. The ring-closure activity with 2-chloro-1-phenylethanol as substrate was demonstrated to be broadly dose-dependent.

The β-mannanase gene (*man1*) from *Aspergillus aculeatus* MRC11624 was expressed in *Y. lipolytica* with effective secretion in the presence of its native secretion signal, using the hp4d promoter. The same gene was expressed in *Aspergillus niger*[2] under control of the *A. niger* glyceraldehyde-3-phosphate dehydrogenase promoter (gpd_P) and the *Aspergillus awamori* glucoamylase terminator (gla_T). Following optimisation with copy numbers and culture conditions, maximal activity levels of 26,140 nkat.ml^{-1} for *Y. lipolytica*, and 16,596 nkat.ml^{-1} for *A. niger* were obtained.

Laccases are important enzymes for bioremediation, and the best characterised enzymes are from the fungus *Trametes versicolor.* The objective of this research was to optimise expression of *T. versicolor* laccases (*lcc1* and *lcc2*) in *A. niger* D15 and *Pichia pastoris*[3]. The Lcc1 enzyme was less active than Lcc2 in both hosts. *P. pastoris* secreted 0.4 U.L^{-1} Lcc1 and 2.8 U.L^{-1} Lcc2, compared to 2,700 U.L^{-1} produced by *A. niger*. The Lcc2 enzyme from recombinant *A. niger* was subsequently purified and characterised in terms of molecular weight and glycosylation, and compared to the wild-type enzyme purified from *T. versicolor*.

The work presented underscores the requirement for experimentation before finalising the choice of an expression system for the optimal production of the desired protein. Every system available has both advantages and disadvantages, and when considering which system to use for producing a recombinant protein, various factors must be taken into consideration. However, the choice is broad and each decision needs to be made empirically.

[2] The construction of Man1-producing *A. niger* strain was done by Dr Shaunita Rose of the University of Stellenbosch. The construction of *Y. lipolytica* Man1 production strains was done by the author.
[3] The expression of *T. versicolor* laccases in *P. pastoris* was done by Christina Bohlin of Karlstad University. *A niger* laccase production strains were created by the author.

Opsomming

Heteroloë geen uitdrukking is van groot belang vir die produksie van proteïene wat van terapeutiese en industriele belang is. Soos die aard van rekombinante proteïene meer ingewikkeld raak en getransformasie-sisteme vir verskeie spesies gevestig raak, is daar 'n groter verskeidenheid van gashere beskikbaar vir geenuitdrukking. Elke sisteem het beide sy voor- en nadele. Hierdie navorsing beklemtoon die voordele wanneer die mees gepaste uitdrukkingssiteem gekies word. Die uitdrukking van verskeie ensieme van biokatalities belang, epoksiedhidrolases, halohidrien dehalogenase, lakkase en mannanase in verskillende gasheersisteme is onderneem en die uitdrukkingsvlakke en aktiwiteite vergelyk.

Die ontwikkeling van *Yarrowia lipolytica* as 'n heelsel biokatalis word beskryf. *Y. lipolytica* word gebruik vir die funksionele uitdrukking van epoksiedhidrolases (EHs) en halohidrien dehalogenases. EHs is hidroliseringsensieme wat die epoksiede omskakel na aangrensende diole deur middel van ring-opening. Twee nuwe fungi EHs vanaf *Rhodosporidiom toruloides* NCYC 3181 en NCYC 3158 ('n moontlike *Cryptococcus curvatus*) is geïdentifiseer en gekloneer. Verdere EHs van verskillende bronne, insluitend bakterieë, giste, fungi en plante, is gekies vir uitdrukking in *Y. lipolytica* ten einde sy geskiktheid vir die produksie van EHs te bepaal. Multikopie integrante is ook ontwikkel met gene onder beheer van die groei-fase afhanklike hp4d promotor. 'n *Saccharomyces cerevisiae* ras is ook ontwikkel vir die uitdrukking van die EH van *Rhodotorula araucariae*[4] sodat dit met *Y. lipolytica* as 'n heelsel biokatalis vergelyk kan word. Hierdie ras was 'n buitengewone swak heelsel

[4] Die konstruksie van die *S. cerevisiae* epoksiedhidrolase-produserende ras is deur Dr Neeresh Rohitlall van CSIR Biosciences gedoen. Die *Y. lipolytica* epoxied hydrolase rasse is deur die outeur gemaak.

biokatalis. Al die *Y. lipolytica* rasse wat ontwikkel is het wisselende aktiwiteitsvlakke teenoor verskillende klasse van epoksiede getoon. Sommige rasse het teenoorgestelde enantio-selektiwiteit getoon en het die potensiaal om rasemiese epoksiede volledig na die gewensde enantiomeriese produk om te skakeling.

Halohidriene kan as direkte voorgangers van epoksiede beskou word. Halohidrien dehalogenases kataliseer die nukleofiliese vervanging van 'n halogeen-ioon in halohidriene deur 'n aangrensende hidroksiel groep, wat 'n epoksied, 'n proton en 'n halied-ioon lewer. Die *HheC* geen van *Agrobacterium radiobacter* AD1 is kodon–geöptimiseer om te pas by die kodon gebruik van *Y. lipolytica* en was uitgedruk in *Y. lipolytica* deur die skep van mulitkopie integrante, 'n verdere verbreeding van die toepaslikheid van die organisme as gasheerras vir die heteroloë produksie van ensieme. Maksimum uitdrukkingsvlakke is bereik deur die skep van opeenvolgende herhalings van die ingevoegde *HheC*-geen. Daar is ook gewys dat die ring-sluitings-aktiwiteit met 2-chloro-1-pheniel-etanol as substraat meestal dosis-afhanklik is.

Die β-mannanase geen (*man1)* van *Aspergillus aculeatus* MRC11624 is uitgedruk en effektief in *Y. lipolytica* mbv sy eie uitskeidings sein uitgeskei, met die gebruik van die groei-fase afhanklike hp4d promotor. Dieselfde geen is uitgedruk in *Aspergillus niger*[5] onder beheer van die *A. niger* gliseraldehied-3-fosfaat dehidrogenase promotor (gpd_p) en die *Aspergillus awamori* glikoamilase termineerder (gla_T). Verdere optimisering van kopiegetal en voedingskondisies het gelei tot maksimum aktiwiteitsvlakke van 26,140 nkat.ml^{-1} vir *Y. lipolytica* en 16,596 nkat.ml^{-1} vir *A. niger*.

[5] Die konstruksie van die Man1-produserende *A. niger* ras is deur Dr Shaunita Rose van die Universiteit van Stellenbosch gemaak. Die *Y. lipolytica* Man1 ras is gemaak deur die outeur.

Lakkases is belangrike ensieme vir bio-remediëring, en die ensieme van die fungus *Trametes versicolor* is die beste gekarateriseer. Die doelwit van hierdie navorsing was die optimisering van die uitdrukking van *T. versicolor* lakkases (*lcc1* en *lcc2*) in *A. niger* en *Pichia pastoris*[6]. Die Lcc1 ensiem was minder aktief as Lcc2 in altwee die gashere. *P. pastoris* het 0.4 $U.L^{-1}$ Lcc1 en 2.8 $U.L^{-1}$ Lcc2 onderskeidelik uitgeskei, in vergelyking met 2,700 $U.L^{-1}$ Lcc2 wat deur *A. niger* geproduseer is. Die Lcc2 ensiem afkomstig van die rekombinante *A. niger* is vervolgens gesuiwer en gekarakteriseer met betrekking tot molekulêre massa en glikosilering, en daarna vergelyk met die wilde-tipe ensiem wat deur *T. versicolor* geproduseer word.

Die werk wat hier aangebied word, beklemtoon die vereistes vir eksperimentering voor die finale keuse met betrekking tot 'n gepaste uitdrukkingsisteem gemaak kan word vir die optimale produksie van die gewensde proteïen. Elke sisteem het beide voordele en nadele, en wanneer 'n sisteem oorweeg word is daar verskeie faktore wat in ag geneem moet word. 'n wye verskeidenheid van keuses is beskikbaar en elke besluit moet empiries gemaak word.

[6] Die uitdrukking van *T. versicolor* lakkases in *P. pastoris* is gedoen deur Christina Bohlin van Karlstad University. Die *A niger* lakkase produksie ras is geskep deur die outeur.

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Biographical Sketch

Robyn Lindsay Roth was born in Johannesburg, South Africa, on 2 November 1972. She attended Waverley High School for Girls and matriculated in 1990.

Robyn enrolled at the University of Stellenbosch in 1991 and obtained a B.Sc. degree *cum laude* in Microbiology and Biochemistry in 1993. In 1994 she completed a B.Sc Honours degree *cum laude* at the same university. In 1997 she received her Masters degree in Microbiology. Her Masters thesis was entitled "Molecular cloning, manipulation and expression of the laccase gene (*lacA*) of *Pleurotus ostreatus* in *Saccharomyces cerevisiae*".

She enrolled at the University of Stellenbosch again at the end of 2002, to complete her PhD part-time, while working at the Council for Scientific and Industrial Research (CSIR).

Preface

This thesis is presented as a compilation of manuscripts. Each chapter is introduced separately and is written according to the style of the journal to which the manuscript was / will be submitted.

Chapter 3 Isolation of epoxide hydrolases, functional expression in *Yarrowia lipolytica*, evaluation of recombinant strains as whole-cell biocatalysts and comparison to recombinant *Saccharomyces cerevisiae* strains

Chapter 4 Functional expression of *Agrobacterium radiobacter* AD1 halohydrin dehalogenase in *Yarrowia lipolytica*

Chapter 5 Heterologous expression of *Aspergillus aculeatus* endo-1,4-β-mannanase in *Yarrowia lipolytica* and *Aspergillus niger*

Chapter 6 Heterologous expression of *Trametes versicolor* laccases in *Pichia pastoris* and *Aspergillus niger*

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Contents

Abbreviations and Definitions

ABTS	2,2'-azino-*bis*(3-ethylbenz-thiazoline-6-sulphonic acid)
ADH1	*Saccharomyces cerevisiae* alcohol dehydrogenase I gene, promoter used for heterologous expression
AEP	Alkaline extracellular protease of *Yarrowia lipolytica*, deleted in many strains, promoter used for heterologous expression
amdS	*Aspergillus niger* nutritive marker, a gene from *Aspergillus nidulans* that encodes acetamidase which hydrolyses acetamide to acetate and ammonium, allowing growth on acetamide as sole carbon source
AOX1	*Pichia pastoris* alcohol oxidase gene, promoter used for heterologous expression
ARS	Autonomously replicating sequence
ATP	Adenosine-5'-triphosphate
CEN	Centromere sequences
Ct	Cycle number where curve intersects threshold in RT-PCR
DCW	Dry cell weight
E	The enantioselectivity of a kinetic resolution can be described by the ratio of the individual reaction rates of the enantiomers, expressed as the 'enantiomeric ratio' (E).
ee	Enantiomeric excess, is a measure for how much of one enantiomer is present compared to the other.
Enantioselectivity	The degree to which one enantiomer is preferentially produced in a chemical reaction
echA	*Agrobacterium radiobacter* gene encoding sEH
eph	Gene encoding epoxide hydrolase
EHs	Epoxide hydrolases
GAL1	*S. cerevisiae* galactokinase gene, promoter inducible by galactose, used for heterologous expression
GAP	*P. pastoris* glyceraldehyde-3-phosphate, promoter used for heterologous expression
GC	Gas Chromatography
GFP	Green Fluorescent Protein
glaA	*A. niger* glucoamylase gene, promote used for heterologous expression
gpd	*S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase gene, promoter used for heterologous expression
GRAS	Generally Regarded As Safe
HhdH	Halohydrin dehalogenase
HheC	*A. radiobacter* gene encoding a halohydrin dehalogenase
HheC	Product of *HheC*
hp4d	Hybrid *Y. lipolytica* promoter composed of four tandem copies of the upstream activating sequence 1 of XPR2 promoter, inserted upstream of the minimal *LEU2* promoter
HPLC	High Pressure Liquid Chromatography
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
lcc1, *lcc2*	*Trametes versicolor* laccase-encoding genes
Lcc1, Lcc2	Products of *lcc1, lcc2*

LIP2	*Y. lipolytica* gene encoding extracellular lipase, secretion signal used for heterologous production
Man1	*Aspergillus aculeatus* β-mannanase-encoding gene
Man1	Product of *Man1*
mEHs	Microsomal EHs
min	Minute
PGK	*S. cerevisiae* phosphoglycerate kinase gene, promoter used for heterologous expression
pyrG	*A. niger* marker gene that encodes the enzyme orotidine-5'-phosphate decarboxylase, which catalyses the first enzymatic step in the *de novo* synthesis of uridine monophosphate.
Regioselectivity	The property of a chemical reaction of producing one structural isomer in preference to others that are theoretically possible
RT-PCR	Real-time polymerase chain reaction
SCP	Single cell protein
sEHs	Cytosolic EHs (soluble)
TLC	Thin Layer Chromatography
ura3d1	Non-defective marker for multi-copy transformant selection in *Y. lipolytica*
ura3d4	Defective marker for multi-copy transformant selection in *Y. lipolytica*
XPR2	*Y. lipolytica* gene encoding AEP
X-GAL	5-bromo-4-chloro-3-indolyl-β- D-galactopyranoside
YL-HmA	Recombinant *Y. lipolytica* strains without *LIP2* secretion signal
YL-HmL	Recombinant *Y. lipolytica* strains with *LIP2* secretion signal
zeta	Long terminal repeat of Ylt1 retrotransposon in *Y. lipolytica*, used for random integration in non-Ylt1-containing *Y. lipolytica* strains

Chapter 1

Introduction

Heterologous expression systems

Heterologous gene expression is of considerable interest for the production of proteins of therapeutic and industrial importance. Initially, commercial production was achieved using *Escherichia coli* as a host (Domínguez et al. 1998). The dominance of *E. coli* in this field is a reflection of the extent of information available on its genetic and biochemical systems, accumulated over many decades of research. However, as the nature of recombinant proteins has become more complex and as transformation systems have been established in more species, so the variety of hosts available for expression has increased. The expression systems currently most commonly used for academic or commercial purposes are bacteria, mammalian cell lines, insect cell lines, yeasts and fungi (Jana and Deb 2005; Rhie et al. 2005; Merten 2006; Ikonomou et al. 2003; Schuster et al. 2000; Nevalainen et al. 2005).

Every system available has both advantages and disadvantages, and when considering which system to use for producing a recombinant protein, the following factors should be considered: (1) origin of gene, *i.e.* prokaryotic or eukaryotic; (2) secretion versus intracellular expression; (3) post-translational modifications for optimal activity; (4) ease of use of expression system; (5) cost; (6) recovery of product; and (7) scalability of the process.

For prokaryotic gene products, prokaryotic expression systems are often suitable. However, recombinant proteins expressed in the cytoplasm of bacteria may be insoluble and therefore inactive (Geisse et al. 1996). Despite this potential difficulty, bacterial systems are simple and quick to use. *E. coli* facilitates protein expression by its relative simplicity, its inexpensive and fast high-density cultivation, the well-known genetics and the large number of compatible tools available (Sørensen and Mortensen 2005).

Eukaryotic gene expression is a more complicated process. Eukaryotic cells have the capacity to carry out post-translational modifications, such as glycosylation, phosphorylation on tyrosine, serine and threonine residues or the addition of fatty acid

chains (Geisse et al. 1996). Recombinant proteins requiring these modifications for activity or specificity will usually be more effectively produced in eukaryotic hosts.

This study explores some of the considerations to be taken into account when selecting the appropriate expression system. Expression of biocatalytically relevant enzymes in different host systems is undertaken, and expression levels and activity are compared. Four different enzymes have been selected: epoxide hydrolases, halohydrin dehalogenase, laccase and mannanase. These enzymes are variously expressed in *Yarrowia lipolytica* and *Aspergillus niger,* and compared with expression in more traditional hosts such as *Pichia pastoris* and *Saccharomyces cerevisiae*. The enzymes are described in more detail below.

Biocatalytic enzymes for heterologous expression

Epoxides (three-membered ring cyclic ethers that are also known as oxiranes or alkylene oxides, containing an oxygen atom bonded to two other atoms, usually of carbon) and their vicinal diols have value as synthetic intermediates of optically active drugs (Moussou et al. 1998). The need therefore exists to obtain these compounds in a high state of purity. In addition to traditional chemical methods, they can be obtained by using enzymes, i.e. with epoxide hydrolases, which catalyse the enantioselective hydrolysis of epoxides. Epoxide hydrolases (E.C. 3.3.2.3, EHs) belong to a sub-category of a broad group of hydrolytic enzymes that include esterases, proteases, dehalogenases and lipases (Fretland and Omiecinski 2000). They are cofactor-independent (Archelas and Furstoss 2001), making them, in theory, easy to use for organic synthesis. They are found widely in nature, from bacteria to humans, and microbial epoxide hydrolases exhibit high enantioselectivity as well as high activity. They therefore may enable the preparation of enantiopure epoxides in a very simple way starting from cheap and easily available racemic epoxides. Various epoxide hydrolases have been selected for expression in *Y. lipolytica* and *Saccharomyces cerevisiae*, and the levels of activity and selectivity obtained for the recombinant enzymes compared to those reported in literature for

recombinant epoxide hydrolases expressed in *A. niger* and other yeast-based expression systems of *S. cerevisiae* and *P. pastoris.*

Halohydrins can be considered direct precursors of epoxides. Halohydrin dehalogenases (HHdHs), also referred to as haloalcohol dehalogenases or halohydrin hydrogen-halide lyases, catalyse the nucleophilic displacement of the halogen ion in halohydrins by a vicinal hydroxyl group, yielding an epoxide, a proton, and a halide ion (Van Hylckama Vlieg et al. 2001). These enzymes also efficiently catalyse the reverse reaction which is the halogenation of epoxides, as well as the dehalogenation of vicinal chlorocarbonyls to hydroxycarbonyls. The interest in halohydrin dehalogenases increased when it was found that the dehalogenation of halohydrins may proceed with high enantioselectivity, making these enzymes useful catalysts for the production of optically pure epoxides and halohydrins.

The HHdH of the 1,3-dichloropropanol-utilising bacterium *Agrobacterium radiobacter* (also known as *Agrobacterium tumefaciens*) AD1 is encoded by the *HheC* gene (Lutje Spelberg et al 2001). HheC exhibits remarkable enantioselectivity with a broad range of aliphatic and aromatic halohydrins. Few reports of the heterologous expression of this enzyme are available, and its expression on *Y. lipolytica* is explored.

Endo-β-1,4-mannanase (β-mannanase, E.C. 3.2.1.78) belongs to the glycosyl hydrolase family 5 (Christgau et al. 1994) and has been cloned from bacterial and fungal origins. β-mannanases are useful in several industrial processes, such as extraction of vegetable oils from leguminous seeds, and the reduction of viscosity of coffee extracts during the manufacture of instant coffee (Wong and Saddler 1993). They can also be used for biobleaching of softwood Kraft pulps to enhance extractability of lignin (Montiel et al. 1999). In this study, β-Mannanase from *Aspergillus aculeatus* is expressed in *Y. lipolytica*, using its own secretion peptide, which is presumably recognized by the *Y. lipolytica* secretion machinery. This expression is compared to the activity of the same gene expressed in *A. niger*.

Laccases (E.C. 1.10.3.2) are part of a larger group of enzymes termed the multicopper enzymes, which include ascorbic acid oxidase and ceruloplasmin (Mayer and Staples 2002), and are found in higher plants and fungi. Laccases can degrade lignin in the absence of lignin peroxidase and manganese peroxidase, and therefore have the potential to be used in delignification in the pulp and paper industry. They also have potential uses in bleaching, and can be adsorbed onto solid surfaces, which has led to the development of an electrode for detecting azide (Leech and Daigle 1998). Laccases have also been used to reduce phenolic inhibitors in lignocellulose hydrolysates used for ethanol production in fermentation (Larsson et al. 2001). All these varied uses of laccases can be ascribed to one basic property of the enzyme: its ability to produce a free radical from a suitable substrate. The ensuing secondary reactions are responsible for the versatility of laccases in producing so many varied products. The use of laccases in bioremediation has also been proposed, again presumably due to this single basic reaction. In this study, laccase from the basidiomycete *Trametes versicolor* is expressed in *A. niger*, and compared to the recombinant enzyme in *P. pastoris*. The *A. niger*-expressed enzyme is purified to homogeneity and compared to the *T. versicolor*-produced enzyme.

Aims of this study

The aim of this study was to investigate various eukaryotic expression systems for the production of different classes of biocatalytically relevant enzymes. Two expression systems, *Aspergillus niger* and *Yarrowia lipolytica* were chosen, and different classes of enzymes were selected for comparison. The complex hydrolysing enzyme laccase, and the simple hydrolysing enzymes mannanase and epoxide hydrolases were chosen. Halohydrin dehalogenase from *A. radiobacter* AD1 was also expressed, using both its original sequence and a DNA sequence optimised for *Y. lipolytica* codon usage.

The specific aims of the present study are as follows:

1. Epoxide Hydrolases:
   a) Isolation of two novel fungal EH genes.
   b) Expression of various EHs in *Y. lipolytica* and *S. cerevisiae* for evaluation as whole-cell biocatalysts.

2. Halohydrin dehalogenase:
   a) Expression of codon-optimised *A. radiobacter* AD1 HHdH in *Y. lipolytica*.
   b) Scale-up of expression using tandem copies of expression cassette.

3. β-Mannanase:
   a) Expression of *A. aculeatus* endo-β-1,4-mannanase in *Y. lipolytica* in various constructs.
   b) Comparisons to *A. niger*-expressed endo-β-1,4-mannanase.
      [*In collaboration with the University of Stellenbosch*]

4. Laccase:
   a) Expression of *T. versicolor* laccase in *A. niger*.
   b) Comparison to *P. pastoris*-produced *T. versicolor* laccase.
      [*In collaboration with the University of Stellenbosch and Karlstad University, Sweden*]

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Chapter 2.

Literature Review:

The Evaluation of Host strains for Heterologous Expression of Biocatalytically Important Enzymes

Introduction

Recombinant DNA technology has provided several techniques for transferring and efficiently expressing desired genes in a foreign cell. This has provided the means not only to study gene function, but also to produce substantial amounts of protein (and non-protein) molecules for commercial and investigative use. It was thus thought that unlimited and inexpensive sources of required proteins would soon become available. It was, however, observed that the choice of host cell has a great influence on the quality and quantity of the produced recombinant protein. For example, mammalian cells can yield biologically active protein with all the required post-translational modifications, but the cultivation of mammalian cells is characterised by low yields, long cultivation times and a requirement for expensive bioreactors and medium components. All these have a great impact on the production costs. On the other hand, bacterial cultivation processes are based on inexpensive media in which fast growth and high cell concentrations can be obtained. These high cell concentrations combined with higher production rates of the bacterial expression system result in higher productivity. However, the production of recombinant proteins in bacteria such as *Escherichia coli* frequently yields an inactive protein, aggregated in the form of inclusion bodies.

The choice of expression system for the high-level production of recombinant proteins therefore depends on many factors, including growth characteristics, expression levels, intracellular versus extracellular expression, post-translational modifications required and the biological activity of the protein, as well as regulatory issues in the case of production of therapeutic proteins (Baneyx 1999). In addition, the selection of a particular expression system requires that cost, in terms of process, design and other economic considerations be taken into account. The quantity of protein desired is also relevant. There is no one system that is optimal for the expression and production of all recombinant proteins. Each situation must be evaluated independently.

Common problems encountered include loss of expression due to structural changes in the recombinant gene or the disappearance of the gene from the host cells, low expression

levels due to unstable mRNA, translational and post-translational difficulties such as incorrect codon usage, misfolding, aggregation and insolubility, as well as incorrect post-translational modifications (*e.g.* glycosylation).

The most commonly used expression systems are bacteria (*e.g.* gram negative *E. coli* and gram positive *Bacillus*), mammalian cell lines (*e.g.* Chinese hamster ovary and baby hamster kidney cells), insect cell lines (*e.g. Spodoptera frugiperda*), yeasts (*e.g. Saccharomyces cerevisiae* and *Pichia pastoris*) and fungi (*e.g. Aspergillus niger*). Transgenic plants have also gained increasing attention for their potential to produce pharmaceutical proteins and peptides.

As an illustration of the growing market for recombinant products, in 2004 it was estimated that 30 recombinant pharmaceutical compounds with a market volume of USD 50-60 million had been introduced as therapies, and about 300 compounds were estimated to be in development worldwide (Schmidt 2004). In 1997, the commercial enzyme market was worth USD 1 billion, and filamentous fungi were the sources for 40% of the available enzymes. This highlights the importance of systems capable of producing these enzymes in a reproducible, cost-effective, controllable and functional way.

This chapter summarises the different expression systems commonly available for the production of recombinant proteins and compounds, and highlights the two systems used in this research, the yeast *Yarrowia lipolytica* and the filamentous fungal species *Aspergillus niger*. Table 6 on page 44 provides a summary of the various systems discussed.

A. Expression Systems

A.1. Bacterial Expression Systems

The overwhelming genetic and physiological characterisation, the short generation time, ease of handling, established fermentation know-how and high capacity to accumulate foreign proteins to more than 20% of total cellular protein content have made the Gram negative bacterium *Escherichia coli* the most widely used of all prokaryotic organisms for recombinant protein production (Schmidt 2004). However, production of recombinant proteins in *E. coli* often results in the foreign protein being present in inclusion bodies, which results in insoluble protein aggregates that demand laborious and cost-intensive *in vitro* refolding and purification steps (Joosten et al. 2003). The final yield may therefore be only a small percentage of the protein that was initially present in the inclusion bodies. Production of proteins from eukaryotic sources in bacteria is particularly difficult. Difficulties in expression may be due to sequence features of the foreign gene, the stability and translational efficiency of the mRNA, the ease of protein folding, degradation of the protein by host cell proteases, differences in the codon usage between the host cell and the foreign gene, and the potential toxicity of the protein to the host (Jana and Deb 2005).

However, there are strategies that may assist in efficient production. These include the choice of host strain, changing the gene dosage through the use of plasmids with varying copy number, the choice of an inducible over a constitutive promoter, altering the codon usage to match *E. coli*'s preference, altering the growth temperature - which has an impact on protein folding and solubility, and optimising growth conditions (Jana and Deb 2005). Expression strains should be deficient in the most harmful native proteases and maintain stable plasmid expression (Sørensen and Mortensen 2005). mRNA is stabilised by efficient translation initiation and consequent immediate ribosomal protection from degradation and is achieved by selection of ribosomal binding sites lacking inhibitory secondary structures. Aggregation of recombinant protein in inclusion bodies can be minimised through the control of parameters such as temperature, expression rate, host

metabolism, target protein engineering including tag technology, and by the co-expression of plasmid-encoded chaperones (Jonasson et al. 2002). An advantage of producing proteins in inclusion bodies is that the protein is concentrated within one place in the cell, and is protected to a certain extent from cellular proteases (Marino 1989).

To achieve high gene dosage, foreign genes are cloned into plasmids that are present in 15 – 60 copies per cell (ColE1-derived plasmids) or a few hundred copies per cell (pMB1 derivatives) (Baneyx 1999). Many of the promoters used for heterologous protein production are derived from the *E. coli* lactose utilisation (*lac*) operon. The *lac* promoter and its close relative *lac*UV5 are rather weak and not normally used for the high-level production of recombinant polypeptides, though induction can be performed by the addition of the non-hydrolysable lactose analogue IPTG (isopropyl-β-D-1-thiogalactopyranoside). The synthetic *trc* and *tac* promoters consist of the -35 region of the *trp* promoter (tryptophan ulilisation) and the -10 region of the *lac* promoter. Both promoters are strong and can allow accumulation of polypeptides to about 15 – 30% of total cell protein. The pET vectors from Novagen (Madison, WI) use the bacteriophage T7 *lac* promoter. The arabinose promoter (*ara*BAD or P_{BAD}) uses the inexpensive sugar L-arabinose as an inducer and is somewhat weaker than the *tac* promoter, and have been commercialised by Invitrogen Corp (Carlsbad, CA).

Bacillus brevis, a Gram positive organism with low G+C content, also has an extremely high capacity for protein secretion and is used for the expression of prokaryotic and eukaryotic proteins on an industrial scale (Udaka and Yamagata 1993). As a "Generally Regarded As Safe" (GRAS) soil micro-organism, *Bacillus subtilis* has several advantages (Yin et al. 2007). Firstly, it does not produce any lipo-polysaccharide, a common by-product of *E. coli*, which can affect protein function Secondly, *B. subtilis* can be transformed readily with many bacteriophages and plasmids due to its genetic characteristics. Thirdly, the bacterium can secrete some well-processed proteins directly into the medium, facilitating further purification steps. However, some problems remain with the system, including degradation by proteases and instability of plasmids. Finally,

this cell type can grow to very high density in relatively simple and cheap media, and its growth and physiological properties have been well-studied.

Other bacteria used for the production of bacterial heterologous proteins include the Gram positive lactic acid bacterium *Lactococcus lactis* (Nouaille et al. 2003; Le Loir et al. 2005), and the Actinomycetes (Gram positive bacteria with high G+C content) *Streptomyces, Rhodococcus, Corynebacterium* and *Mycobacterium* (Nakashima et al. 2005). The internal environment of these actinomycetes is different to *E. coli*. The moderately halophilic bacteria of the family *Halomonadaceae* (*Halomonas*, *Chromohalobacter*, *Zymobacter*) also have promising applications in biotechnology, with their high salt tolerance (Vargas and Nieto 2004). This decreases the necessity for aseptic conditions, lowering the costs of initial sterilisation and aseptic maintenance.

A.2. Insect, Mammalian and Plant Expression Systems

Many recombinant therapeutic proteins are produced in mammalian expression systems (Giddings et al. 2000). A big advantage of these systems is that they can correctly synthesise and process mammalian proteins. Preferred cells in the pharmaceutical industry are Chinese hamster ovary (CHO) and baby hamster kidney (BHK) (Schmidt 2004), as they have the added advantage of being recognised as safe regarding infectious and pathogenic agents. However, in general in mammalian cells, product yields are low, and the requirement for foetal bovine serum in the growth medium makes production expensive. In addition, cultured mammalian cells are sensitive to shear forces that occur during industrial-scale culture, and to variations in temperature, pH, dissolved oxygen, and certain metabolites. This makes it necessary to control culture conditions carefully, because variation in cell growth can affect fermentation and product purity.

Insect cells transformed by baculovirus vectors have gained popularity, as they are considered more stress-resistant, easier to handle and more productive than mammalian expression systems (Schmidt 2004). However, insect cells also have their disadvantages. These include (1) inefficient processing and the impairment of folding and secretion

capacity due to baculovirus infection, (2) high protease activity, partly baculovirus-encoded, (3) insufficient expression levels, and (4) deviations in post-translational modification.

Producing therapeutic proteins in transgenic plants has many economic and qualitative benefits, including reduced health risks from pathogen contamination, comparatively high yields, and production in seeds or other storage organs (Giddings et al. 2000). The cultivation, harvesting, storage and processing of transgenic crops can also be done using existing infrastructure and would require relatively little capital investment. Plants are potentially a cheap source of recombinant products. Kusnadi et al. (1997) estimated that the cost of producing recombinant proteins in plants could be 10- to 50-fold lower than producing the same protein in *E. coli* by fermentation, depending on the crop.

Two transformation approaches are commonly used to produce recombinant protein in plants. In the first, transgenic plants are produced using *Agrobacterium*-mediated transformation or other standard transformation techniques such as microparticle bombardment (Bevan 1984; Lorence and Verpoorte 2004). *Nicotiana tabacum* is widely used as a model expression system, but other plants have also been used, including *Nicotiana benthamiana, Arabidopsis thaliana*, tomato, banana, turnip, black-eyed bean, canola, Ethiopian mustard, potato, rice, wheat and maize (Giddings et al. 2000). The second strategy is to infect non-transgenic plants with recombinant viruses that express recombinant proteins during their replication in the host (Mushegian and Shephard 1995). The two host-virus systems most commonly used are tobacco with tobacco mosaic virus (TMV) and cowpea with cowpea mosaic virus (CPMV).

Whereas tobacco production systems have been widely employed for research and proof-of-concept studies, they may not be ideal for commercial or other large-scale applications. Although such leafy crops are advantageous in terms of biomass yield, proteins that are expressed in leaves tend to be unstable, which means the harvested material has a limited shelf life and must be processed immediately after harvest. The removal and purification of recombinant products from plants such as tobacco is

inefficient and expensive, requiring the removal of a variety of metabolites, including nicotine. Seeds make up only a small percentage by weight of tobacco plants, and are therefore not ideal for large-scale production and storage of proteins. Grain and canola crops, such as maize, rice, wheat, soybeans and oilseed canola, would probably more suited to full-scale commercial production. Futhermore, proteins stored in seeds can be dried and could remain intact for long periods, making seeds a convenient method of storing, distributing and administering pharmaceuticals such as vaccines. Where extraction and purification is necessary, the current procedures of crushing and milling for the production of fractionated extracts such as meal and oil may be adapted for the extraction of recombinant products.

Plant cell cultures can be used for the production of small molecule drugs, but they are also advantageous for molecular farming because of the high level of containment that they offer and the possibility of producing proteins under current good manufacturing practice (cGMP) conditions (Fischer et al. 2004). Tobacco suspension cells are the most popular system at present, although pharmaceutical proteins have also been produced in soybean, tomato and rice cells and in tobacco hairy roots. More than 20 pharmaceutical proteins have been produced in plant cell-suspension cultures, including antibodies, interleukins, erythropoietin, human granulocyte-macrophage colony stimulating factor (hGM-CSF) and hepatitis B antigen. Unfortunately, few of these proteins have been expressed at yields sufficient for commercial production. The problem of poor yields could be addressed in part by the use of optimised regulatory elements. For example, the expression of hGM-CSF in rice suspensions using an inducible promoter produced far greater yields than was possible using tobacco cells and a constitutive promoter (Shin et al. 2003). Regulated promoters are increasingly used, particularly those that allow external regulation by physical or chemical stimuli. Several novel inducible promoters that may be useful in molecular farming applications have been described recently. For example, a peroxidase gene promoter isolated from sweet potato (*Ipomoea batatas*) was used to drive the *gusA* reporter gene in transgenic tobacco, induced in response to environmental stresses including hydrogen peroxide, wounding and UV treatment (Kim et al. 2003). A novel seed-specific promoter from the common bean (*Phaseolus vulgaris*)

has been used to express a single chain antibody in *Arabidopsis thaliana* (De Jaeger et al. 2002).

A.3. Yeast Expression Systems

Yeasts are very useful hosts in that they exhibit several advantages over other micro-organisms. Yeasts, mainly *Saccharomyces cerevisiae*, have been used for centuries for food production, and there is extensive information about their safety – many have GRAS status awarded by the American Food and Drug Administration (FDA) (Domínguez et al. 1998). By contrast, mammalian cells may contain oncogenic or viral DNA. The fermentation profiles of yeasts are well established and these organisms are able to grow rapidly in simple media up to high cell densities at a lower cost than any other fermentation systems. As eukaryotic micro-organisms, they have the ability to perform certain eukaryotic processing steps such as post-translational processing and modifications (disulphide bond formation, proteolytic maturation, glycosylation, etc), which are required for the functional synthesis of many proteins. Relative to more complex eukaryotic expression systems, such as CHO cells and baculovirus-infected plant cells, yeasts are economical, usually give higher yields and are less demanding in terms of time and effort (Cereghino and Cregg 1999). Although yeasts such as *S. cerevisiae* have a greater genetic complexity than bacteria, they share many of the technical advantages that permitted rapid progress in the molecular genetics of prokaryotes (Sherman 1998).

Many recombinant proteins expressed in yeasts have been expressed in *S. cerevisiae* as the host system owing to the ease with which it can be manipulated genetically and to the extraordinary amount of information accumulated about its molecular biology and physiology (Domínguez et al. 1998). Moreover, the sequence of its entire genome has been completed (Goffeau et al. 1996). Some of the non-conventional yeasts, such as *Kluyveromyces lactis* and the methylotrophs *Pichia pastoris* and *Hansenula polymorpha* (now known as *Pichia angusta*, Houard et al. 2002) have also been developed as expression systems. Like the bakers' yeast, these methylotrophs combine the ease of

genetic manipulations with characteristics favourable for a fermentation process (Hollenberg and Gellissen 1997).

Laboratory strains of *S. cerevisiae* are derived from industrial strains (Mortimer and Johnston 1986). These laboratory strains have several special features; they (1) are usually isogenic, (2) are haploid of either the a or α mating type, and (3) contain multiple auxotrophic mutations, such as *leu2, ura3, his3, trp1,* etc. These auxotrophies are indispensable for gene cloning purposes. Selection in *S. cerevisiae* occurs via complementation of the host's auxotrophy. The most widely-used marker in yeast vectors is the *URA3* gene, which encodes orotidine-5'-phosphate decarboxylase, an enzyme which is required for the biosynthesis of uracil (Sherman 1998), and complements *ura3* auxotrophy in the host. Another commonly used auxotrophic maker is *LEU2*. Many of the industrial strains used are polyploid, and dominant markers are therefore needed (Hansen et al. 2003). Resistance to the aminoglycoside antibiotic G418 is the system of choice, but others have been developed. Drug resistance can be conferred to chloramphenicol, methotrexate, glyophosate and phleomycin (Akada 2002). The genes encoding hygromycin B phosphotransferase (*hph*), nourseothricin *N*-acetyltransferase (*nat*) and a mutant inositol phosphoceramide synthase (*AUR1-C*) have all been used successfully as markers both in laboratory and industrial strains of *S. cerevisiae,* and in industrial strains of *Saccharomyces carlbergensis* and *Saccharomyces kluyveri* (Hansen et al. 2003).

There are numerous expression vectors available for producing heterologous proteins in *S. cerevisiae*. These are derivatives of YIp, YEp and YCp plasmids (Sherman 1998). Yeast integrative plasmids (YIps) do not replicate autonomously, but integrate into the genome at low frequencies by homologous recombination. They typically integrate as a single copy, but multiple integrations occur at low frequencies. Strains transformed with YIp plasmids are extremely stable, even in the absence of selective pressure. Yeast episomal plasmids (YEps) replicate autonomously because of the presence of a segment of the native yeast 2µm plasmid that serves as an origin of replication. The 2µm *ori* is responsible for the high copy number and high frequency of transformation of YEp

vectors. Most YEp plasmids are relatively unstable. Even under selective growth conditions, only 60-95% of the cells retain the YEp plasmid. The copy number of most YEp plasmids ranges from 10-40 per cell. However, the plasmids are not evenly distributed among the cells, and there is a high variance in the copy number per cell in populations. Yeast centromere plasmids (YCps) are autonomously replicating vectors containing centromere sequences (CEN) and autonomously replicating sequences (ARS). YCp vectors are typically present in very low numbers, from 1 to 3 per cell and are very stably inherited.

The native *S. cerevisiae* 2µm multicopy vector, upon which much of *S. cerevisiae* expression is based, can have inherent instability problems (Cereghino and Cregg 1999). There can also be wide variation in the productivity of different transformants when 2µm vectors are used (Romanos et al. 1992), which appears to be due to the unexplained variation in plasmid copy number between different transformants.

Numerous native and adapted yeast promoters are available for use in the expression of heterologous proteins. Some of these have been derived from genes encoding alcohol dehydrogenase I (*ADH1*), enolase I (*ENO1*), glyceraldehyde-3-phosphate dehydrogenase (*GPD*), phosphoglycerate kinase (*PGK*), triose phosphate isomerase (*TPI*), galactokinase (*GAL1*) and repressible acid phosphatase (*PHO*) (Sherman 1998). The twenty or so glycolytic genes of *S. cerevisiae* give rise to approximately 50% of the cell's total protein (Rallabhandi and Yu 1996) so it is not surprising that many of the commonly used promoters are derived from these genes. These genes are often in single copy, and the high-level of expression of these genes is presumed to be due to the specific strong promoters associated with them. They are not all constitutive, with some being induced or repressed depending upon the carbon sources available for growth.

The *PGK* gene is one of the most efficiently expressed genes in yeast, and the protein and mRNA comprise about 5% of the total cell protein and mRNA (Kingsman et al. 1990). The constitutive *PGK* promoter has been manipulated to produce a series of high-efficiency expression vectors which have been used to express a number of different

eukaryotic proteins. These include human interferons, calf chymosin, immunoglobulins, wheat α-amylase and HIV antigens. The *ADHI* promoter is also constitutive but not particularly efficient (Hitzeman et al. 1981). The *ADH2* promoter is both powerful and tightly regulated (Rallabhandi and Yu 1996). It is repressed 100-fold in the presence of glucose. The *GAL1* promoter is induced 2,000-fold in the presence of galactose (St John and Davis 1981).

Recently, the emergence of *S. cerevisiae* as a human pathogen has been reported (Murphy and Kavanagh 1999). Classically, *S. cerevisiae* was regarded as non-pathogenic, but there is some evidence that some isolates are virulent and have been implicated in the induction of disease, particularly in immuno-compromised individuals. In Europe, *S. cerevisiae* has been upgraded from GRAS to Biosafety Level 1, indicating the ability to cause superficial or mild systemic infections. Virulent isolates are defined as those capable of growth at 42°C.

P. pastoris is now the most frequently used yeast species for heterologous protein expression in general (Schmidt 2004). *P. pastoris* was initially developed by Phillips Petroleum Company for the production of single cell protein (SCP). Since *P. pastoris* has no native plasmids, expression of foreign genes was initially achieved by chromosomal integration of expression cassettes containing a promoter, the desired foreign gene, a terminator and marker genes (Domínguez et al. 1998). Such constructs are stable, and multicopy integrants can be obtained. Two autonomously replicating sequences (ARS) isolated from *P. pastoris* allow autonomous replication of plasmids containing these ARSs (Reiser et al. 1990). However, autonomous plasmids are of low copy number, unstable and a high frequency of plasmid integration is seen (Domínguez et al. 1998). As a methylotrophic yeast, *P. pastoris* can grow on methanol as its sole carbon and energy source. Almost all foreign genes are expressed under control of the *P. pastoris* alcohol oxidase 1 (*AOX1*) promoter. This promoter is tightly regulated by a carbon source-dependent repression/induction mechanism. Its expression is undetectable in cells grown on glucose or glycerol, but is maximally induced during growth on methanol. It can be induced >1,000-fold in cells shifted to methanol as a carbon source

(Ceregrinho and Cregg 1999). The promoter from the constitutive, highly-expressed glyceraldehyde-3-phosphate (*GAP*) gene is also available (Waterham et al. 1997). Because *P. pastoris* secretes only low levels of endogenous proteins, secreted heterologous proteins can constitute the vast majority of total protein in the medium (Ceregrinho and Cregg 2000). However, due to protein stability and folding requirements, secretion of foreign proteins is usually reserved for those proteins secreted in their native hosts.

H. polymorpha (*P. angusta*) is also a methylotrophic yeast able to grow on methanol as sole carbon source (Domínguez et al. 1998). The *MOX* methanol oxidase promoter is almost exclusively used for expression of heterologous proteins. As with *P. pastoris*' *AOX1* promoter, *MOX* is repressed by glucose and induced by methanol. However, in *H. polymorpha*, the inducible promoter elements from the methanol metabolism pathway can also be derepressed when grown on glycerol or xylitol as sole carbon source, leading to high yields in methanol-free fermentation. (Hollenberg and Gellissen 1997; van Dijk et al. 2000). *H. polymorpha* is used particularly for industrial applications because of its favourable fermentation characteristics, and is more thermo-tolerant than *P. pastoris* (30-43°C versus 30°C).

Some of the characteristics of these different yeasts are summarised in Table 1. These need to be taken into account when a decision is taken on which yeast expression system to use for the production of a heterologous protein.

Table 1. Comparison of some yeast characteristics (Schmidt 2004: modified from Swinkels et al. 1993)

Characteristic	***S. cerevisiae***	***P. pastoris***	***K. lactis***	***H. polymorpha***
Industrial application	+	+	+	+
Requirement for explosion-proof equipment	-	+	-	-
GRAS	+	-	+	-
Secretion efficiency	-	+	+	+
Hyper-glycosylation	+	-	-	-
Episomal vector stability	+	-	-	-

The most extensively used secretion signal for heterologous protein production in both conventional and methylotrophic yeasts is the *S. cerevisiae* mating alpha factor (*MFα1*), separated from the foreign protein by a Kex2 endopeptidase processing site (Brake et al. 1984; Hitzeman et al. 1990; Gellissen 2000; Ceregrinho and Cregg 2000). Producing heterologous proteins via the secretory pathway provides a system for modifying and processing the recombinant products. These can include disulphide bond formation, glycosylation, and sequence-specific endoproteolytic cleavage by Kex2-like proteins and sequence-specific exoproteolytic trimming by Kex1 proteins (Rallabhandi and Yu 1996).

Despite these many advantages, several limitations have been detected in the *S. cerevisiae* and other yeast systems. Examples of these limitations are possible low product yield and inefficient secretion. Many of the native *S. cerevisiae* proteins are not found free in the culture medium but rather are retained in the periplasmic space or associated within the cell wall (Buckholz and Gleeson 1991). This can also occur with heterologous proteins. Often the removal of the α-factor leader sequences by Kex2 is incomplete, resulting in hyper-glycosylated secreted material with amino-terminal extensions (Eckart and Bassineau 1996).

Conventional yeasts only synthesise oligosaccharide chains of the high mannose type and are unable to synthesise oligosaccharide chains of the mammalian complex type (Romanos et al. 1992). Eukaryotic cells assemble *O*-linked saccharides onto the hydroxyl groups of serine and threonine. In mammals, *O*-linked oligosaccharides are composed of a variety of sugars, including *N*-acetylglucosamine (GlcNAc), galactose, and sialic acid (Cereghino and Cregg 2000) (Figure 1; Marino 1989). In contrast, lower eukaryotes such as *P. pastoris* and *S. cerevisiae* add *O*-oligosaccharides composed solely of mannose (Man) residues. No consensus primary amino acid sequence for *O*-glycosylation appears to exist, meaning *O*-glycosylation cannot be completely predicted and that different hosts may add *O*-linked sugars to different residues in the same protein. Consequently, it should not be assumed that a heterologous protein will be expressed in a non-glycosylated state even if that protein is not glycosylated in its native host.

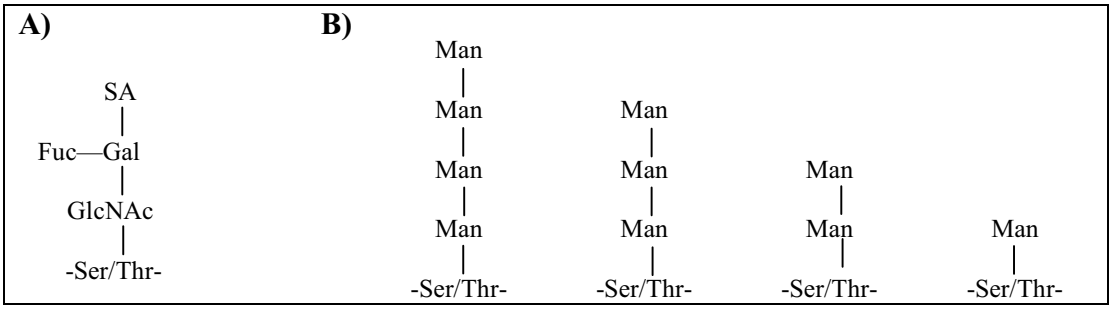

Figure 1. Structure of certain *O*-linked oligosaccharides (Marino 1989). Mammalian *O*-linked oligosaccharides are shown in (A) and *S. cerevisiae O*-linked oligosaccharides are shown in (B). SA = sialic acid, Fuc = fucose, Gal = galactose, GlcNAc = *N*-acetylglucosamine, Man = mannose.

In all eukaryotes, *N*-glycosylation begins in the ER with the transfer of a lipid-linked oligosaccharide unit, $Glc_3Man_9GlcNAc_2$ (Glc = glucose), to asparagine at the recognition sequence Asn-X-Ser/Thr, where X is any amino acid other than proline (Cereghino and Cregg 2000). Not all such sequences in a protein are necessarily glycosylated (Marino 1989). The oligosaccharide is then trimmed to $Man_8GlcNAc_2$ (Cereghino and Cregg 2000). At this point, glycosylation patterns of the lower and higher eukaryotes begin to differ. The mammalian Golgi performs a series of trimming and addition reactions that generate oligosaccharides composed of $Man_{5-6}GlcNAc_2$ (High mannose type), a mixture of several different sugars (complex type) or a combination of both (hybrid type) (Figure 2). In yeasts, *N*-linked core units are elongated in the Golgi through the addition of mannose outer chains. Since these chains vary in length, endogenous and heterologous secreted proteins from *S. cerevisiae* are heterogeneous in size. *S. cerevisiae* shows a tendency to hyper-glycosylate heterologous proteins (glycosylation by the addition of >40 mannose residues), while *P. pastoris* and *H. polymorpha* produce glycoproteins with an average mannose chain length of 8 – 14 (Buckholz and Gleeson 1991; Gellissen and Hollenberg 1997). *N*-linked high-mannose oligosaccharides added to proteins by yeast secretory systems represent a significant problem in the use of foreign-secreted proteins in the pharmaceutical industry. They can be exceedingly antigenic to animals. An additional problem caused by the differences between *N*-linked glycosylation patterns in most yeasts and mammals is that the longer outer chains can potentially interfere with the folding or function of a foreign protein. Glycosylation can also affect stability, thus influencing recovery, purification and *in vivo* half-life (Marino 1989).

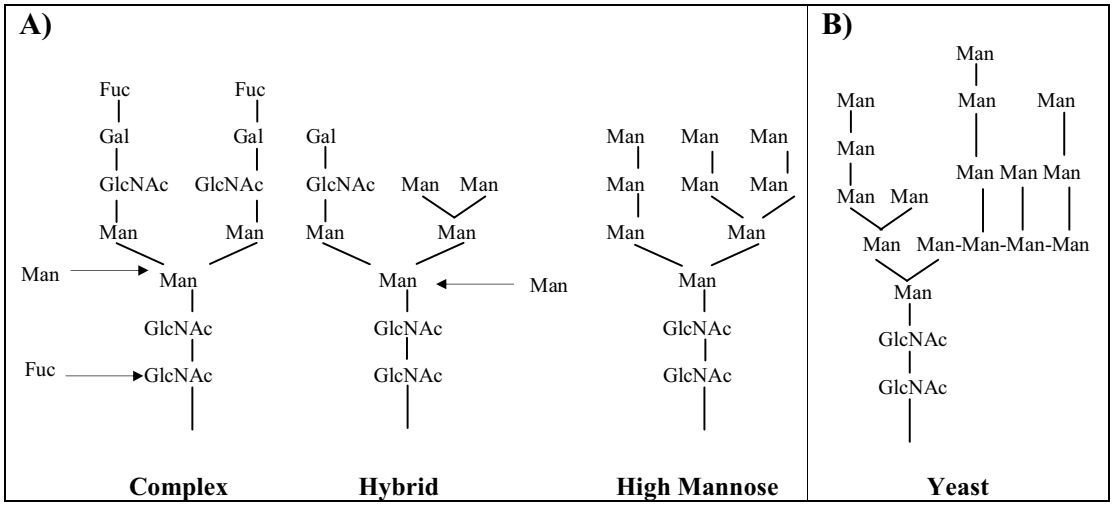

Figure 2. Structure of *N*-linked oligosaccharides. Mammalian *N*-linked oligosaccharides are shown in (A) and *S. cerevisiae N*-linked oligosaccharides are shown in (B). Fuc = fucose, Gal = galactose, GlcNAc = *N*-acetylglucosamine, Man = mannose.

One option to reduce the hyper-glycosylation of the high mannose type is to remove the *N*-glycosylation sites in the heterologously expressed protein (Ratner 1989). Engineering of the glycosylation pathways in yeast host strains has also attracted increasing attention. The pathway in P. pastoris was the first to be engineered, lacking Och1p (α-1, 6-mannosyltransferase) activity, and having added mannosidases (MnS) I and II, *N*-acetylglucosaminyl transferases (GnT) I and II and UDP- *N*-acetylglucosamine transporter in the ER and Golgi (Hamilton et al. 2003). This mutant was able to produce the complex human N-glycan GlcNac2Man3GlcNAc2.

Stress situations of host cells can influence the productivity of any expression system. Limitations in yeast systems that can reduce the final yield include potential bottlenecks because of the codon usage of the expressed gene, the gene copy number, efficient transcription by using strong promoters leading to the depletion of precursors and energy, translocation, processing and folding in the ER and Golgi, as well as protein turnover by proteolysis (Mattanovich et al. 2004). Cells which produce high levels of proteins can accumulate unfolded protein in the ER, which aggregates, overwhelms and eventually shuts down the secretory pathway (Cereghino and Cregg 1999). The yeast proteins that assist in folding and disulphide bond formation differ from their counterparts in higher eukaryotes, which may affect folding of foreign proteins (Romanos et al. 1992). In

certain cases, yeasts, like higher eukaryotes, perform the post-translational removal of the initiator methionine (Eckart and Bussineau 1996). In instances where this Met is retained, immunogenicity problems can arise.

Some recent examples of heterologous proteins expressed in *S. cerevisiae* and methylotrophic yeasts are shown in Table 2. Commercially produced therapeutic proteins are given in Table 3.

Table 2. Heterologous proteins expressed in *S. cerevisiae* and *P. pastoris*

Gene	Promoter	Intracellular (I) / Secretion signal	Production Levels	Reference
P. pastoris				
B. subtilis XZ2 protopectinase	*AOX1*	MFα1	4.14 mg/L	Liu et al. 2006
Co-produced *A. niger* endo-1,4-β-D-xylanases	*AOX1*	MFα1 Native leader peptides	140/180 mg/L 150/220 mg/L	Korona et al. 2006
A. awamori endo-1,4-β-xylanase, *XylA*	*AOX1*	*SUC2* signal peptide	60 mg/L	Berrin et al. 2000
Human single chain (scFv) antibodies	*AOX1*	MFα1		Miller et al. 2005
Aspergillus oryzae tannase	*AOX1*	MFα1	72 mg/L (7,000 IU/L)	Zhong et al. 2004
Candida parapsilosis lipase/acyltransferase	*AOX1*	MFα1	5.8 g/L	Brunel et al. 2004
Sphingomonas paucimobilis haloalkane dehalogenase	*AOX1*	MFα1	0.6 mg/L	Nakamura et al. 2006
A. oryzae acetyl xylan esterase	*AOX1*	MFα1	190 mg/L	Koseki et al. 2006
Rhozopus oryzae lipase	*AOX1* *FLD1* [1]	MFα1 MFα1	6,000 U/L 15,000 U/L	Cos et al. 2005
Tribolium castaneum carboxylesterase	*AOX1* *GAP* [2]	I I MFα1	40 mg/L 7 mg/L 80 mg/L	Delroisse et al. 2005
Rhodothermus marinus thermostable xylanase	*AOXI*	MFα1	3 g/L	Ramchuran et al. 2005
S. cerevisiae				
Antirrhinum majus (snapdragon) benzoic acid methyltransferase	*TPI* [3] and Cu^{2+}-inducible *CUP1*		1 mg methyl benzoate/L/day	Farhi et al. 2006
Hantavirus Puumala nucleocapsid (N) protein	Galactose-inducible *GAL10-PYK*	I	316 mg/L	Antoniukas et al. 2006
A. niger glucose oxidase	*PGK*	MFα1	100,000 U/L	Malherbe et al. 2003

[1] Formaldehyde dehydrogenase 1 [2] Glyceraldehyde-3-phosphate dehydrogenase [3] Constitutive Triosephosphate isomerise

Table 3. Therapeutic proteins produced in the yeasts *S. cerevisiae*, *P. pastoris* (Gerncross 2004), *H. polymorpha* and *K. lactis* (Schmidt 2004)

	Commercial name	Recombinant protein	Company
S. cerevisiae	Actrapid	Insulin	NovoNordisk
	Ambirix	Hepatitis B surface antigen	GlaxoSmithKline
	Comvax	Hepatitis B surface antigen	Merck
	Elitex	Urate oxidase	Sanofi-Synthelabo
	Glucagen	Glucagon	Novo Nordisk
	HBVAXPRO	Hepatitis B surface antigen	Aventis Pharma
	Hexavac	Hepatitis B surface antigen	Aventis Pasteur
	Infanrix-Penta	Hepatitis B surface antigen	GlaxoSmithKline
	Novolog	Insulin	Novo Nordisk
	Pediarix	Hepatitis B surface antigen	GlaxoSmithKline
	Procomvax	Hepatitis B surface antigen	Aventis Pasteur
	Refuldan	Hirudin/lepirudin	Hoechst
	Revasc	Hirudin/desirudin	Aventis
	Twinrix	Hepatitis B surface antigen	GlaxoSmithKline
P. pastoris	Angiostatin	Antiangiogenic factor	EntreMed
	Elastase inhibitor	Cystic fibrosis	Dyax
	Endostatin	Antiangiogenic factor	EntreMed
	Epidermal growth factor analogue	Diabetes	Transition Therapeutics
	Insulin-like growth factor-1	Insulin-like growth factor-1 deficiency	Cephalon
	Human serum albumin	Stabilizing blood volume in burns/shock	Mitsubishi Pharma (formerly Welfide)
	Kallikrein inhibitor	Hereditary angiodema	Dyax
H. polymorpha		Hepatitis B vaccine	Rhein-Biotech
K lactis		Trypsin	Roche

A.3.1. *Yarrowia lipolytica*

The Yeast *Y. lipolytica*

The ascomycetous yeast *Yarrowia lipolytica* (originally classified as *Candida lipolytica*) is one of the most intensely studied 'non-conventional' yeast species. The term 'non-conventional' was originally used to differentiate these yeasts from the commonly used 'conventional' and well-studied yeasts *S. cerevisiae* and *Schizosaccharomyces pombe.* There are now about ten non-conventional yeast species.

Y. lipolytica is a yeast species widely used in industrial applications such as citric acid production, peach flavour production and SCP production. With the emergence of SCP

production in the 1960's, a strong industrial interest arose from the fact that strains of this species were able to use cheap and abundant *n*-paraffins as sole carbon source (Barth and Gaillardin 1997). *Y. lipolytica* was also able to produce large amounts of organic acids (α-ketoglutaric acid and citric acid) when grown on these substrates. *Y. lipolytica* has been classified as having GRAS status by the American Food and Drug Administration (FDA) for citric acid production (Fickers et al. 2005a).

The species was originally classified as a *Candida* species since no sexual state had until then been described. The perfect form was identified in the late 1960's by Wickerham at the Northern Region Research Laboratory in Peoria (Wickerham et al. 1970). A culture isolated in 1945 from a corn processing plant was found to form asci attached to hyphal elements when put onto suitable media. One to four spores of various size and shape could be isolated from these asci, but the spore viability was found to be very low. Two mating types, called A and B, were identified among the progeny. Nearly all wild type isolates could mate to one of these two types, albeit at very low frequency, suggesting that most natural isolates are haploid (or near haploid). The perfect form was reclassified first as *Endomycopsis lipolytica* (Wickerham et al. 1970), then as *Saccharomycopsis lipolytica* (Yarrow 1972) and finally as *Yarrowia lipolytica*.

Wild type strains of *Y. lipolytica* exhibit various colony shapes, ranging from smooth and glistening to heavily convoluted and matt (Barth and Gaillardin 1997). The colony morphology is determined by both the growth conditions (aeration, carbon and nitrogen sources, pH, etc) and by the genetic background of the strain. Certain conditions are known to cause preferential formation of yeast cells or induce mycelial development (Ota et al. 1984). Mycelial development is inhibited by a deficiency of magnesium sulphate and ferric chloride, or by the addition of cysteine or reduced glutathione. Comparisons of the composition of yeast and hyphal cells have shown that hyphal walls exhibit a higher content of amino sugars and a reduced protein content (Vega and Dominguez 1986).

Most *Y. lipolytica* strains grow only up to 34°C, but there are some strains which are adapted to higher temperatures (Barth and Gaillardin 1996). The recommended

temperature for growth is 25 – 30°C. Most strains tolerate low pH levels well (down to pH 3), while growth is reduced above pH 7 and stops above pH 8. It is an obligate aerobe and cannot ferment without oxygen. Low O_2 pressure reduces the growth rate strongly. Because of its low growth temperature, it is not considered a possible human pathogen (Spencer et al. 2002).

Y. lipolytica diverges significantly from other ascomycetes. This is obvious in its high GC content (49.6-51.7%; Kurtzman and Phaff 1987), its unusual structure of rDNA genes coupled with a lack of the RNA Polymerase I consensus sequences found in other yeasts (van Heerikhuizen et al. 1985), snRNA of the size normally found in higher eukaryotes (Roiha et al. 1989) and the size of its 7S RNA (Poritz et al. 1988). Homologous proteins tend to display low levels of similarity (50-60% at amino acid level) to their counterparts in *S. cerevisiae, K. lactis* or *Candida albicans*. *Y. lipolytica* genes do not hybridise detectably to DNA from species of the genera *Saccharomycopsis, Endomyces* or *Endomycopsella*, some of which were formerly classified in the same genus as *Y. lipolytica* (Naumova et al. 1993). Data on 7S RNA genes, on intron structure, and on ARS (autonomously replicating sequence) function confirm that *Y. lipolytica* is quite peculiar when compared to most other yeasts. Evolutionary trees based on sequence comparison of genes encoding well-conserved functions (glycolytic genes, ribosomal RNA genes) locate *Y. lipolytica* on an isolated branch, clearly separated from *S. pombe* on the one hand and the majority of other ascomycetous yeasts on the other (Figure 3; Barnes et al. 1991). The codon bias of *Y. lipolytica* is more similar to *A. niger* than *S. cerevisiae* (Domínguez et al. 1998). The entire sequence *Y. lipolytica* has been determined (Sherman et al. 2004). *Y. lipolytica* can use *n*-alkanes and l-alkenes as carbon sources (Klug and Markowetz 1967). Although the phylogenetic position of *Y. lipolytica* is uncertain, it is clear that it is phylogenetically distant from other well-known and thoroughly studied alkane-degrading yeasts such as *Candida maltosa, C. tropicalis, Debaryomyces (Schwanniomyces) occidentalis, D. hansenii* and *Pichia guillermondi.*

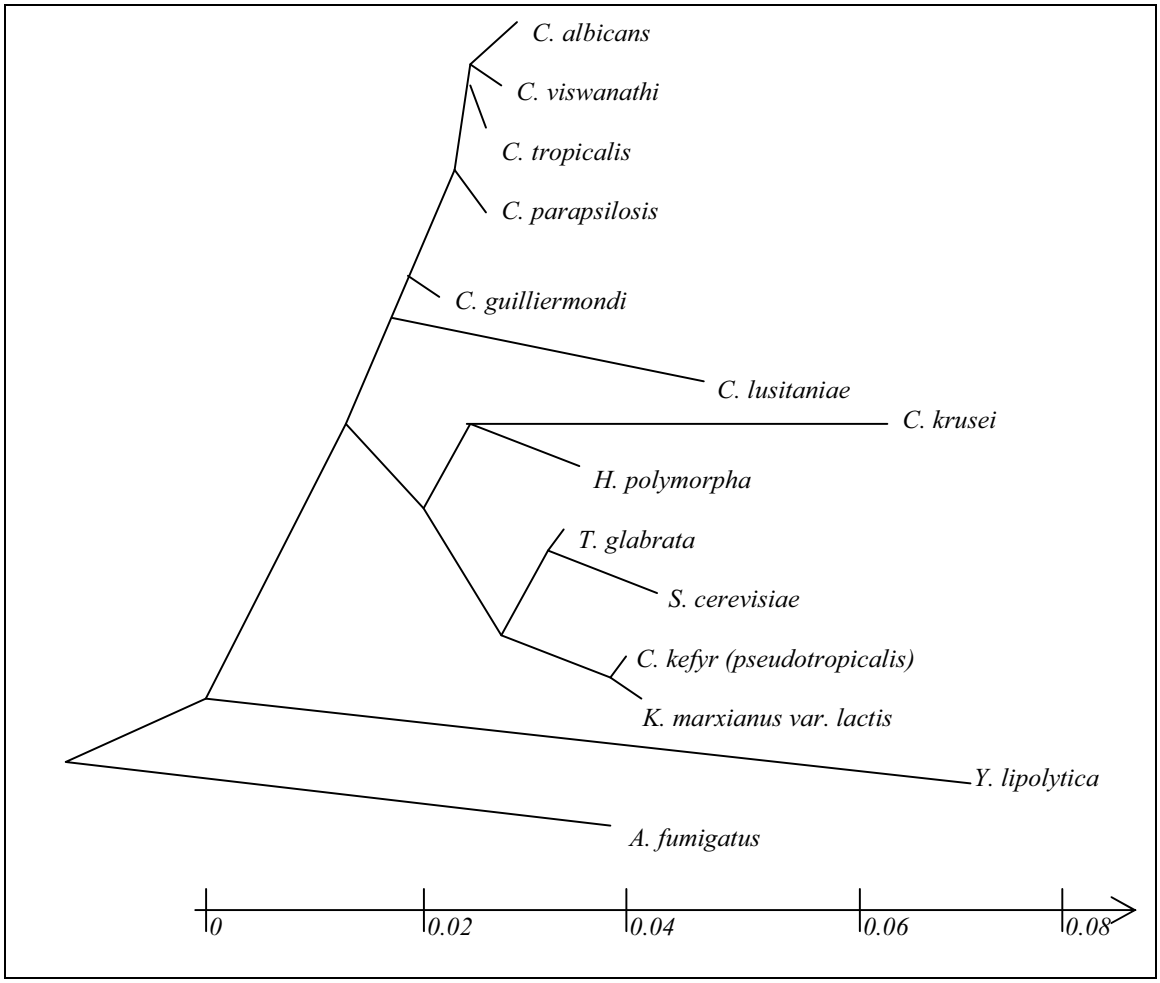

Figure 3. Evolutionary tree for members of the genus *Candida* and relatives (Barnes et al. 1991).

Although interest in bioconversion with *Y. lipolytica* initially focussed mainly on the production of SCP or citric acid from *n*-alkanes, attention then turned towards the production of primary oxidation products and other metabolites directly from alkanes, fatty acids or triglycerides. Several mono- and di-terminal oxidation products of long- and middle-chain alkanes and fatty acids, such as fatty alcohols, aldehydes and acids, ω-hydroxy fatty acids (HFA), wax esters, methyl citrate, and especially dicarboxylic acids (DCA), are of industrial importance in the production of detergents, surfactants, lubricants and cosmetics. Potential and actual utilisations of wild-type, mutant or recombinant strains in bioconversion, aroma production, single cell oil (SCO) production, intermediate-metabolite production, in bioremediation processes and in food technology are reviewed in Fickers et al. (2005a).

Y. lipolytica as an Expression Host

The first report of transformation of *Y. lipolytica* was by Davidow et al. (1985). The method was based on the lithium acetate method of Ito et al. (1983), developed for *S. cerevisiae*. The *Y. lipolytica LEU2* gene was isolated by generating a *Y. lipolytica* library in a *leu2*$^-$ *E. coli* strain. The isolated *Y. lipolytica LEU2* gene was placed in the pBR322 plasmid, and the plasmid DNA transformed into a *leu2 Y. lipolytica* strain, either uncut or linearised at a unique restriction enzyme site. Transformations with DNA linearised at a site within the *Y. lipolytica*-derived DNA on the plasmid were the most successfully transformed, followed by plasmid linearised within the pBR22 DNA. Addition of carrier DNA (sonicated *E. coli* DNA) increased the transformation frequency up to 1,000-fold. The transformations were shown to have homologously integrated within the *leu2* site in the *Y. lipolytica* chromosome.

Two types of vectors can be used to express genes of interest: (1) episomal vectors, or (2) vectors integrated into the host's genome (Domínguez et al. 1998). Integration occurs preferentially by homologous recombination (Barth and Gaillardin 1996). Integration is stimulated by linearising the vector within the homologous region (100- to 1,000-fold enhancement of frequency) and results in high transformation frequencies of up to 10^6 transformants per µg DNA (Xuan et al. 1988, Chen et al. 1997). However, later techniques make use of non-homologous integration utilising the *zeta* region of a retrotransposon. The retrotransposon Ylt1 has been detected in the genome of some *Y. lipolytica* strains (Schmid-Berger et al. 1994). It is 9.6 kb long and can transpose in the genome. This retrotransposon is bounded by a long terminal repeat (LTR), the *zeta* element, which is 714 bp long, highly conserved, and can also exist as a solo element. Ylt1 and solo *zeta* regions are flanked by a 4 bp directly repeated genomic sequence. The copy number of Ylt1 and solo *zeta* are dependent on the strain examined, and up to 35 copies of Ylt1 and more than 30 copies of solo *zeta* per haploid genome have been observed, but they are not present in all strains. In *Y. lipolytica* strains where the Ylt1 and solo *zeta* regions are absent, having two *zeta* regions flanking the linearised DNA fragment for transformation stimulated random integration into the genome

(Mauersberger et al. 2001). In strains where there are *zeta* regions present in the genome, the *zeta* regions on the transforming DNA result in directed integration at the chromosomal *zeta* sites (Pignéde et al. 2000a). Other regions which are used to direct multi-copy integration include the rDNA units. There are a number of different clusters of different sizes located on various chromosomes, with more than 200 rDNA units available for integration (Casaregola et al. 2000). Integrated vectors exhibit a high stability (Hamsa and Chattoo 1994). "Auto-cloning" vectors have been developed to ensure no bacterial plasmid DNA is included in the integration (Nicaud et al. 2003; Pignéde et al. 2000a). The bacterial portion of the *E. coli* / *Y. lipolytica* shuttle vectors can be removed by restriction enzyme digestion prior to transformation of the linearised *Y. lipolytica* portion of the vector, containing the auxotrophic marker, the expression cassette and the flanking *zeta* regions for non-homologous (or *zeta*-targeted) integration.

Plasmids containing the 3 autonomously replicating sequences isolated from *Y. lipolytica*, *ARS18*, *ARS68(= ARS1)* and *ARS2,* transform with high frequency and are more mitotically stable than ARS plasmids in *S. cerevisiae* (Newlon 1988). Loss is between 1 – 4% per generation. This value is closer to the 1 – 3% observed in *S. cerevisiae* for artificial mini-chromosomes containing cloned centromeres than to the 5 – 50% found for ARS plasmids. The copy number of ARS plasmids in *Y. lipolytica* has been reported to be about 3 per cell, which is very different from the 5 – 50 copies for *S. cerevisiae* ARS plasmids, and closer to the 1 – 2 copies per cell described for centromeric plasmids. It has been found that *ARS18* and *ARS68* each carry functional centromeres, which do not contain any significant sequence similarity with the known *S. cerevisiae*, *K. lactis* or *S. pombe* sequences (Fournier et al. 1993). Both the centromere and origin of replication are necessary to establish the replicative plasmid in *Y. lipolytica* (Vernis et al. 1997).

Y. lipolytica is resistant to most antibiotics, which makes the use of dominant markers unattractive (Madzak et al. 2004). The most common markers for use in *Y. lipolytica* are complementary to host stain auxotrophies. *LEU2* and *URA3* are popular markers, especially as non-leaky non-reverting (*i.e.* carrying internal deletions) *leu2* and *ura3* recipient strains are available. Defective selection markers can be used to select for

multi-copy integrations. The *ura3d4* marker contains only 6 bp upstream of the ATG and can not confer the Ura^+ trait to the host when integrated as single copy, but can in multi-copy (Le Dall et al. 1994). The copy number of different clones with this defective marker tends to be around 10-13 copies, probably reflecting the optimum auxotrophy complementation (Juretzek et al. 2001). The efficiency of transformation in a Ylt1-free strain by a *zeta*-based vector is lower than with a classical integrative vector, and the use of a defective selection marker decreases the transformation frequency further by several orders of magnitude (Madzak et al. 2004). In *Y. lipolytica*, a very high frequency transformation can be obtained with a classical integrative vector (up to 10^5 to 10^6 transformants/µg of DNA). The transformation frequency of a Ylt1-carrying strain with a *zeta*-based vector carrying a defective selection marker (such as *ura3d4*) is much lower: around 10^2 transformants/µg DNA. The efficiency of the same vector in a Ylt1-free strain is still lower: less than 10 transformants per µg DNA. However, the non-homologously integrated copies appear to be more dispersed in the genome than the directed integrated copies, leading to better stability of high-copy integrants (Nicaud et al. 2003).

In *S. cerevisiae* and non-*S. cerevisiae* conventional yeasts, a number of promoters derived from tightly regulated or highly-expressed host genes have been used for the expression of heterologous proteins. However, in non-conventional yeasts, because of the relative lack of knowledge regarding these systems, very few of these promoters have been identified and used successfully for the expression of foreign proteins (Domínguez et al. 1998). The most commonly used native promoters for the expression of heterologous proteins in *Y. lipolytica* are derived from the promoters of the *XPR2* gene, encoding alkaline extracellular protease (AEP), and the *LIP2* gene, which encodes the major extracellular lipase, Lip2p (Pignéde et al. 2000a; Fickers et al. 2005b). The intact native *XPR2* promoter was initially used for expression of heterologous proteins (Hamsa and Chattoo 1994), but its complex regulation imposed a restriction on general industrial use (Gellissen et al. 2005). Regulation of AEP reflects the carbon, nitrogen, sulphur and pH status of the cell, among other things (Madzak et al. 1999). Induction occurs at pH above 6.0 on media lacking preferred carbon and nitrogen sources, and full induction requires

high levels of peptone in the culture medium, although the exact nature of the inducer is unknown (Ogrydziak et al. 1977). Two major upstream activation sequences (UAS1 and UAS2) of the *XPR2* promoter are essential for promoter activity under conditions of repression or full induction (Blanchin-Roland et al. 1994). Consequently, a hybrid promoter was constructed composed of four tandem copies of the UAS1, inserted upstream of the minimal *LEU2* promoter (reduced to its TATA box). The resulting recombinant hp4d promoter is almost independent of carbon sources and presence of peptones (Madzak et al. 2000a; Madzak et al. 2000b). It contains as-yet unidentified elements that drive growth phase-dependent gene expression, since hp4d-driven heterologous gene expression was found to occur at the beginning of the stationary phase. The hybrid promoter is no longer repressed by the preferred carbon or nitrogen sources, nor by acidic conditions (pH lower than 6.0). This allows the separation of biomass accumulation and protein production.

Inducible promoters are available for the expression of recombinant proteins in *Y. lipolytica*. These include the promoters from genes for key enzymes in the pathways for growth on hydrophobic substrates (Gellissen et al. 2005). Examples are the promoters for isocitrate lyase (*ICL1*), 3-oxo-acyl-CoA thiolase (*POT1*) and acetyl-CoA oxidases (*POX1, POX2* and *POX5*). They are highly inducible by fatty acids and repressed by glucose and glycerol. The *ICL1* promoter is also inducible by ethanol and acetate, but is not completely repressed in the presence of glucose and glycerol. However, industrial use of these promoters faces some problems. *ICL1* has a basal level of expression in the presence of certain carbon sources, and *POT1* and *POX2* rely on hydrophobic inducers. The presence of these compounds in a cultivation broth may be incompatible with efficient protein production or purification.

Y. lipolytica has been used extensively to study secretion because of its secretory capacity is greater when compared to those of *S. cerevisiae* or *S. pombe* (Titorenko et al. 1997). As in higher eukaryotes, protein secretion in yeasts is directed by an amino-terminal signal sequence which mediates co-translational translocation into the endoplasmic reticulum (Domínguez et al. 1998). Secretion signals from the AEP protein (*XPR2* gene)

and LIP2 have been used to direct secretion of heterologous proteins (Nicaud et al. 2002). *Y. lipolytica* strains grown in rich medium at pH 6.8 secrete large amounts (1 – 2 $g.l^{-1}$) of AEP (Ogrydziak 1993). AEP is synthesised as a prepro protein with a short pre-sequence followed by a stretch of 10 dipeptides (X-Ala or X-Pro) and a larger pro-region ending with the Lys-Arg recognition site of the Kex2-like endoprotease encoded by *XPR6*. The pro-region contains a glycosylation site and acts as the internal chaperone for AEP secretion. The *XPR2* secretion signal is now patented by the Pfizer Company (Davidow et al. 1996). The *LIP2* secretion signal has features similar to those of the *XPR2* signal: a short pre-sequence followed by four dipeptides, a short pro-region and the Lys-Arg cleavage site (Pignéde et al. 2000b).

The early steps of protein secretion from the cytoplasm to the ER can follow two pathways: co-translational or post-translational translocation (Madzak et al. 2004). The first was shown to be predominant in *Y. lipolytica* (Boisramé et al. 1998). This is also the case with higher eukaryotes (*i.e.* mammalian cells). This is a very important advantage of *Y. lipolytica* over *S. cerevisiae*, in which the post-translational pathway is predominant, particularly for the efficient production of complex heterologous proteins of higher eukaryotic origin.

An important modification that takes place in the secretory apparatus is the glycosylation of translated polypeptides. As described in the previous section, conventional yeasts only synthesise oligosaccharide chains of the high mannose type and are unable to synthesise oligosaccharide chains of the mammalian complex type (Romanos et al. 1992). Non-conventional yeasts such as *Y. lipolytica* have glycosylation patterns more similar to mammalian high-mannose type glycosylation. No hyper-glycosylation of heterologous proteins has been reported in *Y. lipolytica* (Madzak et al. 2004).

Examples of heterologous proteins expressed in *Y. lipolytica*

Table 4 illustrates the variety of proteins heterologously expressed in *Y. lipolytica*.

Table 4. Heterologous proteins expressed in *Y. lipolytica*

Gene	Promoter	Secretion signal	Copy number	Production Levels	Reference
Hepatitis B middle surface antigen	*XPR2*	Intracellular despite *XPR2* prepro	Single-copy at *LEU2*	85 mg/L Dane particles (2.35% total soluble protein)	Hamsa and Chattoo 1994
Rice α-amylase	*XPR*	Yes, via own secretion peptide	single-copy at *XPR2* promoter region	12 mg/L (batch) 350 mg/L (fed-batch)	Chang et al. 1997 Park et al. 1997
Zea mays cytokinin oxidase/ hydrogenase	hp4d	Yes, via *XPR2* pre-proregion	Single-copy at pBR322 docking platform of host strain Po1g	2,500 nkat/L	Kopečný et al. 2005
S. cerevisiae invertase	*XPR2*	*XPR2* pre, but only 18% secreted	Single copy	1400 U/L (flask)	Nicaud et al. 1989
T. reesei endoglucanase I	*XPR2*	Own secretion signal	Single copy	5-100 mg/L (flask vs. fed-batch)	Park et al. 2000
Bovine prochymosin	*XPR2 /LEU 2*	*XPR2* prepro			Franke et al. 1988
	hp4d	*XPR2* prepro	Single copy	20 -160 mg/L (flask vs. fed-batch)	Madzak et al. 2000a
Human proinsulin analogue	*XPR2*	*XPR2* prepro	Multicopy		James and Strick 1998

Examples in the table above show that the scale-up from flask to fed-batch cultivation can allow a 10 – 20 fold increase in production. Additionally, a 10 – 20 fold increase can be obtained using multi-copy vectors. Thus, for heterologous protein production, the increase obtained between a single-copy transformant grown in batch, and a multi-copy transformant grown in fed-batch, can be two orders of magnitude.

A.4. Fungal Expression Systems

Filamentous fungi

Filamentous fungi are a diverse group of lower eukaryotes that can be isolated from a wide variety of habitats. In nature many are found as soil-borne saprophytes, and play an important role in biodegradation and elemental recycling (Visser et al. 1994). They have also found a variety of industrial applications as producers of (1) fermented foods such as cheese, soy sauce, tempeh, and beverages such as sake, (2) primary metabolites such as organic acids and vitamins, (3) secondary metabolites such as antibiotics, alkaloids, gibberellins, and (4) a broad spectrum of extracellular enzymes.

Diverse in morphology, physiology and ecology, many of the best known fungi have a negative impact on humankind as agents of plant diseases (smuts, blights, wilts rusts) or as animal pathogens (as producers of toxins and mycoses) (Bennett 1998). Fungi range from microscopic moulds and yeasts, to macroscopic mushrooms and truffles. It is the microscopic species which are best known for their positive impact on humans.

Most of the filamentous fungi used in biotechnology are members of the Ascomycetes (Wainright 1992), which includes the approximately 200 species of *Aspergillus*. Aspergilli are ubiquitous in nature, and *A. niger* is a black fungus commonly called 'black mould', which often occurs as a contaminant of culture media. The asexual spore-forming stage of *Aspergillus* is the one most commonly encountered. The fungus also reproduces sexually by producing ascospores. Although many members of this genus have lost the ability to produce a sexual stage, the production of asci by some clearly shows its membership of the Ascomycetes. *A. niger* is able to grow in a wide variety of temperatures from 6°C to 47°C (Schuster et al. 2002), with temperature optima of 35-37°C. The water activity limit for growth is 0.88, which is relatively high compared to other *Aspergillus* species. *A. niger* is able to grow over an extremely wide pH range of pH 1.4 – pH 9.8.

As with other industrially exploited micro-organisms, filamentous fungi have gone through intricate strain improvement programmes. *A. niger* has been granted GRAS status, and considerable experience has been gained handling such filamentous fungi (Radzio and Kück 1997). It first gained practical importance in 1919 when its ability to produce citric acid was industrially exploited (Schuster et al. 2002). Since the 1960s, *A. niger* has become a source of a variety of enzymes well-established in the fruit processing, baking and starch industries. Filamentous fungi are commonly used in the fermentation industry for the large-scale production of proteins, mainly industrial enzymes (Nevalainen et al. 2005), with the main attraction of filamentous fungi their natural ability to secrete large amounts of proteins (mainly hydrolytic enzymes) into the growth medium.

Aspergillus niger as an Expression Host

Until the 1980s, *A. niger* industrial production strains were isolated through the use of classical mutagenesis followed by screening and selection (Schuster et al. 2002). Parasexual crossing was also used in strain improvement efforts. With the development of DNA-mediated transformation, initially in *Aspergillus nidulans* (Tilburn et al. 1983), and subsequently *A. niger* (Buxton et al. 1985), these fungal species could be modified to produce entirely new enzymes or improve the yield of existing products. The first report of DNA-mediated transformation of any fungal species came in 1973 from the laboratory of E. L. Tatum in Rockefeller University (Mishra and Tatum 1973). An inositol-requiring mutant (in1-) of *Neurospora crassa* was transformed with DNA isolated from the wild type (inl+). The DNA was supplied to growing cultures together with calcium, and from the conidia formed on such cultures it was possible to select inositol-independent strains. These early results were received with some scepticism. There was at the time a rather widespread viewpoint to regard the transformation of eukaryotes as difficult, if not impossible. It was not until fungal transformation was extended to other genes and other species that it became widely accepted as a working technique.

Transformation of *A. niger* with plasmids containing the expression cassette and the relevant markers results in the integration of the plasmid into the host's chromosome (Werners et al. 1985). Integration of a single plasmid can be followed by integration of additional plasmids to form tandemly repeated arrays (Tilburn et al. 1983). Random integration is favoured when using plasmids with little homology to the host genome, and directed transformation can occur when *A. niger* is transformed with plasmids containing DNA homologous to regions on the chromosome. Integrated plasmids are generally stable (Van Hartingsveldt et al. 1987). Transformation of *A. niger* using a vector containing the *A. niger pyrG* gene into a *pyrG* $^{-}$ strain resulted in 90% of the transformants with integration at the mutated chromosomal *pyrG* locus, with either replacement of the mutated allele by the wild-type allele (60%) or insertion of two copies of the vector which contained the *pyrG* gene. However, transformation of *A. niger* with a vector containing the *N. crassa pyrG* gave no integration at the *pyrG* locus and a much lower transformation frequency (2 transformants per µg DNA versus 40).

Because integrative plasmids are available to introduce foreign DNA into the chromosomes of filamentous fungi, the segregational instability found in bacteria and yeasts transformed with circular plasmid DNA is highly unlikely. However, deletions and substitutions in both foreign and native DNA have been known to occur (Withers et al. 1998). *A. niger* transformants expressing recombinant glucoamylase lost most of this glucoamylase activity when grown in chemostat culture. However, this only occurred in strains with a very high copy number of introduced genes (~30), whereas the lower copy number integrants did not show significant gene loss. It is therefore speculated that the higher copy number resulted in a higher metabolic load, which led to deletions in the highly selective chemostat environment. This maximal gene dosage effect was also seen by Verdoes et al. (1993). Up to 200 copies of the glucoamylase gene were inserted into *A. niger*, but the dosage-dependent glucoamylase activity was only seen up to about 20 copies. Attempts to introduce more copies by retransformation showed reductions in glucoamylase levels due to gene rearrangements (Verdoes et al. 1994). Different levels of heterologous expression of glucoamylase were attributed to copy number differences as well as to different sites of integration. Protein production per gene copy can show

considerable variation, indicating a clear effect of the site of integration on gene expression. This effect was also found in *S. cerevisiae* and *Lactococcus lactis* (Thompson and Gasson 2001). In *L. lactis,* there was a threefold difference in expression of the randomly integrated gene expressing β-glucuronidase (GUS), whereas in *S. cerevisiae*, a 14-fold variation was found in the expression of the *lacZ* reporter gene. This difference may reflect the greater complexity of the yeast genome. In 2D gel analysis, there was a two- to five-fold variation in the intensities in some of the fainter proteins. As *A. niger* is somewhat more complex than *S. cerevisiae*, it can be postulated that the effect may be even greater in *A. niger*.

Selectable markers for *A. niger* can be divided into three broad groups. There are genes that complement mutations in the host genome and alleviate auxotrophy, genes that provide drug resistance or growth on a previously non-utilisable nutrient source (dominant markers) and DNA that gives rise to selectable mutations when integrated into the genome of the host. Common selection markers for complementation include *trpC*, *argB*, *niaD* and *pyrG* (Goosen et al. 1989; Buxton et al. 1985; Campbell et al. 1989). The most used marker is the *pyrG* gene that encodes the enzyme orotidine-5'-phosphate decarboxylase, which catalyses the first enzymatic step in the *de novo* synthesis of uridine monophosphate. *pyrG*$^{-}$ mutants are auxotrophic for uracil or uridine, and transformation with *pyrG*-containing vectors remove the need for supplementation by uracil or uridine. Drug resistance markers that have been successfully used include resistance to pyrithiamine, hygromycin, oligomycin, phleomycin, and benomyl (Kubodera et al. 2002; Punt et al. 1987; Ward et al. 1988; Mattern et al. 1987; Orbach et al. 1986). When using these markers, the sensitivity of the host strain to the antibiotic needs to be determined as some fungi have low sensitivity to some antibiotics.

One of the most commonly used nutritive markers is the *amdS* gene from *A. nidulans* that encodes acetamidase, which hydrolyses acetamide to acetate and ammonium, allowing growth on acetamide as sole carbon source (Kelly and Hines 1985). *A. niger* grows poorly on acetamide as a nitrogen or carbon source and lack sequences homologous to the *A. nidulans amdS* gene. A plasmid containing the *amdS* gene was first used to

develop a transformation system for *A. nidulans* (Tilburn et al. 1983) and then applied to *A. niger* (Kelly and Hines 1985). The background growth of the poorly growing *A. nidulans* and *A. niger* on acetamide was suppressed by the addition of caesium chloride to the growing medium. The protoplast preparation and transformation developed for *A. nidulans* was applied to *A. niger* using a plasmid containing the *amdS* gene and regulatory elements. The transforming plasmid DNA was integrated in multiple tandem copies, with copy numbers ranging from 1-2 to >100 in different transformants. The production of acetamidase was dose-dependent, and the stability of the transformants was high, even in non-selective medium. Co-transformation with a non-selecting plasmid (*i.e.* no marker like *amdS*) and the *amdS* plasmid resulted in a high frequency of co-transformation. These results opened up the possibility of using the system for stable introduction of foreign genes into *A. niger* for production.

Fungal promoters that are used for recombinant protein production in *A. niger* can be divided into two broad groups (Radzio and Kück 1997): promoters from housekeeping genes with a high level of expression, and inducible promoters which are especially useful if the desired gene product is unstable or toxic to the host system. The most frequently used constitutive promoter for expression in *A. niger* is the *gpdA* promoter from *Aspergillus nidulans*, which drives gene expression of glyceraldehyde-3-phosphate dehydrogenase (Punt et al. 1991). Both homologous glyceraldehyde-3-phosphate dehydrogenase and recombinant *E. coli* β-galactosidase under the control of the *gpdA* promoter in *A. niger* were expressed intracellularly to levels as high as 10-25% of total protein (Punt et al. 1991). This promoter is also functional in several other species, including *Penicillium chrysogenum* and *A. nidulans*. Among the inducible promoters, the *A. niger* glucoamylase (*glaA*) promoter is most commonly used (Fowler et al. 1990). GlaA expression is upregulated by starch and down-regulated by glucose (Archer and Peberdy, 1997). When the expression of recombinant genes is under the control of *A. niger glaA* promoter, similar inducing and repressing profiles were found for protein production, *i.e.* starch is better than maltose, while maltose is better than glucose as the substrate (inducer) (Siedenberg et al. 1999; Mackenzie et al. 1994). The *A. nidulans alcA* promoter of the alcohol dehydrogenase I gene is tightly regulated (Romero et al. 2003) by

various substrates such as ethanol and threonine. It is also completely repressed by catabolite repression. Table 5 shows some examples of proteins expressed with the different promoters.

One strategy for the improvement of extracellular production of heterologous proteins in *A. niger* involves fusing the foreign gene to the partial or complete coding region of a strongly expressed, secreted homologous protein (Radzio and Kück 1997). The homologous protein serves as a carrier for the foreign protein. A recognition site for a kex2-like endopeptidase, which cleaves proteins at two adjacent basic amino acids, preferentially Lys-Arg, is inserted between the two coding sequences to allow separation of the two proteins. Typically, the *A. niger glaA* glucoamylase gene is used, with the catalytic domain of the *glaA* gene being fused to the heterologous protein, separated by the processing site in the form of Asn-Val-Ile-Ser-Lys-Arg (Punt et al. 2002). *T. reesei*'s cellobiohydrolase I gene has also been used as a carrier (Nyyssönen and Keränen 1995). Both glucoamylase and cellobiohydrolase I can be divided into three domains: an N-terminal catalytic domain, a C-terminal starch- or carbohydrate binding module (CBM) and a flexible *O*-glycosylated linker region that separates the two domains. It is mainly the CBM that has been used successfully, though fusions to full-length glucoamylase have also succeeded. It is postulated that the linker region permits the catalytic domain and the rest of the fusion protein to fold independently (Gouka et al. 1997). However, even with the use of gene-fusions, the levels of protein production for heterologous proteins are usually lower than those obtained for homologous proteins. There are also examples where the gene fusion approach completely fails to improve yields of a secreted protein (Conesa et al. 2000). Table 5 shows some examples of proteins expressed as fusions with domains of glucoamylase .

Like yeasts and mammalian cells, filamentous fungi are capable of both *N*- and *O*-linked glycosylation (Archer et al. 1994). The early event of *N*-linked glycosylation takes place in the ER and is identical to yeasts and higher eukaryotes (see Section 3). The main difference between yeast and filamentous fungal *N*-linked glycosylation occurs at the stage where yeasts add high mannose oligosaccharides to $Man_8GlcNac_2$ to form the high-

mannose glycosylation. In filamentous fungi, it appears that mannose residues are also removed at this stage, rather than added, which is closer to the situation in higher eukaryotes (Limongi et al. 1995; Maras et al. 1997; Wallis et al. 1999). There are also differences in the types of sugar residues added, and some types cannot be used (*e.g.* sialic acid). In fungi, *N*-linked oligosaccharides are primarily of the mannose type and may be modified with the addition of other sugars such as galactose, galactofuranose and charged residues such as phosphate and sulphate (Wallis et al. 1999). No complex or hybrid types are found in fungi. Fungal *O*-linked glycans are mainly composed of short mannose chains similar to those in yeasts. Ward et al. (2004) described the glycosylation of immunoglobulin antibodies produced as glucoamylase fusions in *A. niger* as a high mannose type, with the number of mannoses ranging from 6 to 15, with the most abundant structure being $Man_7GlcNAc_2$. However, both simpler and more complex structures were also present. Panchal and Wodenski (1998) investigated the glycosylation of phytase from *A. niger* and found similar structures, with only *N*-linked oligosaccharides present, of the type $Man_{5-10}GlcNAc_2$. Native glucoamylase and over-expressed recombinant glucoamylase were determined to have very similar glycosylation patterns, even with an 80-copy transformant producing 5-8-fold more protein and glucoamylase (Wallis et al. 1999). Short *N*-linked glycans were present ($Man_{7-8}GlcNAc_2$) and *O*-glycans comprised of short (1-3 residues) chains of mannose and galactose. The similar glycosylation patterns in strains with only a single copy of the *glaA* gene and up to 80 copies, indicates that the glycosylation of proteins is unlikely to create a bottleneck in strains producing high quantities of heterologous proteins.

In most cases, the expression rates that are achieved with heterologous genes are considerably lower than those obtained with homologous genes. For example, the production of hen egg white lysozyme was found to be five times less effective than the production of the homologous glucoamylase, although the same promoter was used and both genes were present in identical copy numbers (Archer et al. 1994). The reason for this discrepancy is most probably due to post-translational protein modification. Another recognised reason for lower production levels is the action of proteases (Papagianni and Moo-Young 2002). These proteases may be localised in the cell wall or secreted, or may

act intracellularly. The construction and use of protease-deficient strains has alleviated this problem to a certain extent (Mattern et al. 1992). Aspergillopepsin A is responsible for 80-85% of the total extracellular protease activity. Strains deficient in Aspergillopepsin A as well as Aspergillopepsin B have only 1-2% of the extracellular activity of the parent strain. However, heterologous proteins expressed in such strains are still proteolytically degraded, although at a much lower rate than the parent strain.

Low steady-state mRNA levels for heterologously-expressed genes can be another reason for low productivity (Gouka et al. 1997). These can result from either a slow transcription rate or low mRNA stability. Destabilisation and degradation of mRNA can be influenced by the mRNA structure or a high content of rare codons, which results in temporary translation arrest and subsequent degradation of the transcript. Incorrect processing of mRNA could also be a reason. Guar α-galactosidase was produced in *B. subtilis*, *H. polymorpha*, *S. cerevisiae, K. lactis* and *A. niger* (reviewed in Gouka et al. 1997), and only in *A. niger* does incorrect processing of the transcript occur. Incorrect processing can be prevented by the elimination of AT-rich regions, which can act as internal polyadenylation sequences. Gene fusions, for example with the *A. niger* glucoamylase, can also reduce mRNA instability.

Low levels of secreted protein may also be due to limitations at the translational level (Gouka et al. 1997). Intracellular degradation occurs, initiated by either inefficient translation/ translocation, incorrect glycosylation/folding or processing in the late Golgi. This is also partially solved by the gene-fusion strategy.

A feature of filamentous fungi is that proteins are secreted mainly at the tips of growing hyphae (Punt et al. 1994). They are tip-growing systems which grow by apical elongation with a flow of cytoplasm towards the hyphal tip (Wongwicharn et al. 1999). As time progresses, older hyphal regions become progressively more vacuolated, and may become metabolically less active, resulting in lower secretion. Thus, in a fungus in submerged culture, a range of hyphal compartments with differing metabolic capability

will normally be present. For optimal protein secretion, it is therefore important to maximise the number of actively growing hyphal tips in liquid culture.

Filamentous fungal cells exhibit two extreme types of morphology in submerged cultures: pelleted and free filamentous forms (Xu et al. 2000c). Reduced protease secretion has been found in pelleted growth. However, pelleted growth may result in reduced cell mass as a result of substrate limitation in the dense core of the pellet when pellets exceed a 'critical radius' and older hyphae within the core are metabolically less active. Therefore, the ability to obtain and control a certain size of pellet is important to balance biomass accumulation with protein secretion and protease production. Parameters influencing pellet formation include inoculum level, initial pH, agitation, medium composition, polymer additives and surfactants. Among them, inoculum level is regarded as the most important in determining pellet size. In the work of Xu et al. (2000c), an optimal pellet size of 1.6 mm was obtained with an inoculum level of 4×10^6 spores/ml, which gave one third of the protease activity compared to free mycelial growth, with three times as much specific Green Fluorescent Protein (GFP) yield (the GFP was fused in-frame with glucoamylase as a reporter gene).

pH also plays a major role in the protease levels. *A. niger* normally tends to acidify its medium when grown without pH control. *A. niger* protease activity peaks at pH 3 (O'Donnell et al. 2001). This means that the fungus creates an environment in which the proteases are at a maximal level. When a glucoamylase-GFP construct was cultivated with the pH controlled at 6, a significant 10-fold increase in recombinant protein was seen over the levels found in cultures with either no pH control or pH regulated at 3 (Xu et al. 2000c). The protease activity decreased 6-fold at pH 6.0. In a mutant strain of *A. niger* which acidifies less, as well as being protease-deficient, it was found that with an increase in regulated pH from 6.0 to 7.0, although there was a decrease in biomass, the production of glucoamylase-hIL-6 (human interleukin-6) was similar in absolute terms, with productivity going from 1.9 to 3.6 mg/g dry cell weight (Punt et al. 2002). This was ascribed to the fact that, with increasing pH, the fungal morphology was changing from larger to smaller pellets.

Examples of heterologous proteins expressed in *A. niger*

Table 5 illustrates a variety of proteins heterologously expressed in *A. niger*.

Table 5. Heterologous proteins expressed in *A. niger*

Gene	Promoter	Secretion signal peptide	Carrier protein	Production Levels	Reference
Human α-interferon	*A. niger* phosphate-repressible *aphA*	*A. niger aphA* signal peptide			Macrae et al. 1993
Hen egg white lysozyme	*A. niger glaA*	Own		50 mg/l	Jeenes et al. 1993
	A. niger glaA		*A. niger* $glaA_{1-498}$	1 g/l	Jeenes et al. 1993
Potato α-glucan phosphorylase	Synthetic p-NO8142	Intracellular		Up to 10% total intracellular protein	Koda et al. 2005
Equine lysozyme	*A. niger glaA*	Fused in-frame with *glaA*		150 mg/L	Spencer et al. 1999
Human mucus proteinase inhibitor	*A. niger glaA*		*A. niger* $glaA_{1-514}$	3 mg/L	Mikosch et al. 1996
Bovine enterokinase	*A. niger glaA*		*A. niger* $glaA_{1-614}$	5 mg/L	Svetina et al. 2000
Pleutorus ostreatus laccase	*A. nidulans glaA*		*A. niger* $glaA_{1-512}$	~0.8 ng/g dry wt[a] ~5.4 ng/g dry wt[b]	Weenink et al. 2006
Human interleukin-6 (hIL-6)	*A. niger glaA*	*A. niger glaA* or hIL-6		<0.1 mg/l	Carrez et al. 1990
	A. niger glaA or *A. nidulans gpdA*		*A. niger* $glaA_{1-614}$	5 mg/l	Contreras et al. 1991
	A. nidulans gpdA	*A. niger glaA*		<0.1 mg/l [a]	Broekhuijsen et al. 1993
				10 mg/l (batch) 50-150 mg/l (fed-batch)[c]	Punt et al. 2002
			A. niger $glaA_{1-514}$	2 – 15 mg/l (batch) [a]	Broekhuijsen et al. 1993

[a] protease deficient strain [b] protease deficient, hypersecreting mutant strain [c] protease deficient, non-acidifying strain

A.5. Summary of Expression Systems

On the next page is a table that summarises the different aspects to be taken into consideration when selecting an expression system for the production of a heterologous protein.

Table 6. Salient characteristics of expression systems used for the production of heterologous proteins (Marino 1989 Joosten et al. 2003; Nevalainen et al. 2005)

Characteristics	*E. coli*	Filamentous fungi	Yeasts	Insect cells	Mammalian cell culture	Plant cell culture	Transgenic plants
Cell growth	Hours to days	Days to 1 week	Days to 1 week	Days to 1 week	Weeks	Months	Weeks to months
Cost of growth medium	Low to medium	Low to medium	Low to medium	High	High	Medium to high	Low
Expression level	Low to high	Low to high	Low to high	Low to high	Low to moderate	High	Low to high
Secretion capability	Secretion to periplasm possible	Secretion to medium	Secretion to periplasm and medium	Secretion to medium	Secretion to medium	Secretion to medium	
Post-translational modifications:							
Protein folding	Refolding usually required	Refolding might be required	Refolding might be required	Possible proper folding	Proper folding	Proper folding	Proper folding
***N*-linked glycosylation**	None	Mammalian-type core, no sialic acid, non-human sugars added	High mannose, no sialic acid, non-human sugars added	Complex, no sialic acid, non-human sugars added	Complex, non-human sugars added, *e.g.* by murine cells	Complex, no sialic acid, non-human sugars added	Complex, no sialic acid, non-human sugars added
***O*-linked glycosylation**	None	Yes, though differs from mammalian	Yes, though differs from mammalian	Yes	Yes	Yes	Yes
Other modifications	Phosphorylation, acetylation (?)	Phosphorylation, acetylation, acylation, others (?)	Phosphorylation, acetylation, acylation, others (?)	Phosphorylation, acetylation, acylation, others (?)	Phosphoryl-ation, acetylation, acylation, γ-carboxylation	Phosphorylation, acetylation, acylation, others (?)	Phosphorylation, acetylation, acylation, others (?)
Ease of molecular cloning	Excellent	Excellent	Excellent	Good	Sufficient	Good	Good
Upscaling	Excellent	Excellent	Excellent	Sufficient	Poor	Poor	Excellent
Economic feasibility[1]	Excellent	Excellent	Excellent	Sufficient	Sufficient	Sufficient	Good
Pathogenic contaminants[2]	Excellent	Excellent	Excellent	Sufficient	Sufficient	Sufficient	Good

[1] a combination of time and cost of molecular cloning, upscaling and downstream processing

[2] viruses or pyrogens

B. Biocatalytic Enzymes

B.1. Epoxide Hydrolases

Epoxide hydrolases (EHs; EC 3.3.2.3) are hydrolytic enzymes that convert epoxides (three-membered ring cyclic ethers that are also known as oxiranes or alkylene oxides, containing an oxygen atom bonded to two other atoms, usually of carbon) to vicinal diols by ring-opening the epoxide, in the presence of water. They are present in mammals, vertebrates, invertebrates, plants, insects and micro-organisms (Orru and Faber 1999). Microbial EHs have been studied extensively in recent years for their potential application as biocatalysts for the production of optically active epoxides and vicinal diols. The use of EHs for the production of these enantiopure building blocks represents an alternative to asymmetric organic syntheses and may surpass the purely chemical processes in yield, enantiomeric purity of the product, and environmental friendliness (Kotik et al. 2005)

Epoxides are highly reactive valuable intermediates used by the pharmaceutical industry. Enantiopure epoxides are of high value in the production of pharmaceuticals such as pain-killers or protease-inhibitors. There are a number of ways to produce enantiopure epoxides, but nowadays an environmentally-friendly manner has a high preference. One such environmentally friendly method is the use of EHs. These enzymes are able to enantioselectively hydrolyse one epoxide-enantiomer to its vicinal diol. By this so-called kinetic resolution, it is possible to obtain both the epoxide and diol in an enantiopure form. Enantiopure diols are also of high value in the fine and pharmaceutical chemistry.

EHs also play crucial roles in the metabolism of organisms and as such are important drug targets. They are potentially important targets in the control of diseases of mammals and plants caused by parasites and micro-organisms, as well as in the control of insects, both as carriers of parasites infecting humans and to protect crops against insect pests.

Mechanism of Epoxide Hydrolases and Biocatalytic Importance

EHs catalyse the enantioselective hydrolysis of epoxides to the corresponding diols (Archelas and Furstoss 1997). Epoxides are highly reactive electrophiles because of the strain inherent in the three-membered ring and the electronegativity of the oxygen (Swaving and de Bont 1998), and react readily with various O-, N-, S-, and C-nucleophiles, acids, bases, and reducing and oxidizing agents. No co-factors or metal ions are involved in the EH-catalysed reaction.

In racemic mixtures, the EH-catalysed reaction can occur ***enentioselectively*** as shown in Figure 4, where one enantiomer in the racemic substrate mixture is preferentially hydrolysed to form the corresponding diol, leaving the opposite enantiomer behind. However, each enantiomer of an epoxide can theoretically be opened, following different kinetics, through attack at either or both carbon atoms of the oxirane ring (Moussou et al. 1998). Thus, the enantiomeric excess (ee) of the formed diol does not depend only on the enantioselectivity (the degree to which one enantiomer is preferentially produced in a chemical reaction) of the reaction, but also on its regioselectivity (the property of a chemical reaction of producing one structural isomer in preference to others that are theoretically possible) (Pedragosa-Moreau et al. 1996). The ee is a measure for how much of one enantiomer is present compared to the other. The enantioselectivity of the kinetic resolution can be described by the ratio of the individual reaction rates of the enantiomers, expressed as the 'enantiomeric ratio' (E) (Chen et al. 1982). During the *trans* opening of an epoxide by the addition of the single oxygen atom from water, the configuration of the substrate may be retained or inverted (see Figure 5). The pathway depends on the substitutional pattern of the carbon atom being attacked and the regioselectivity of the enzyme. For racemic mixtures, four pathways are possible, k_1 to k_4. The regioselectivity is determined as the ratio of retention versus inversion (k_1/k_2 and k_4/k_3) for each substrate enantiomer, and the enantioselectivity (E) is expressed as the ratio of the reaction rate of enantiomers: $(k_1+k_2)/(k_3+k_4)$.

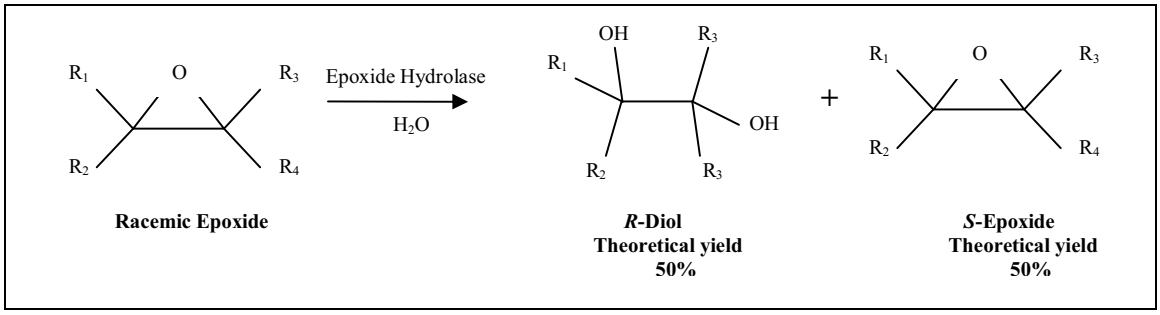

Figure 4. The stereochemistry behind most enzymatic hydrolyses of epoxides follows a kinetic, ***enantioselective*** resolution. From a racemic epoxide mixture, one enantiomer is preferentially hydrolysed to form the corresponding diol by leaving the opposite enantiomer behind. R_1 – R_4 can be any group. Taken from Orru and Faber (1999).

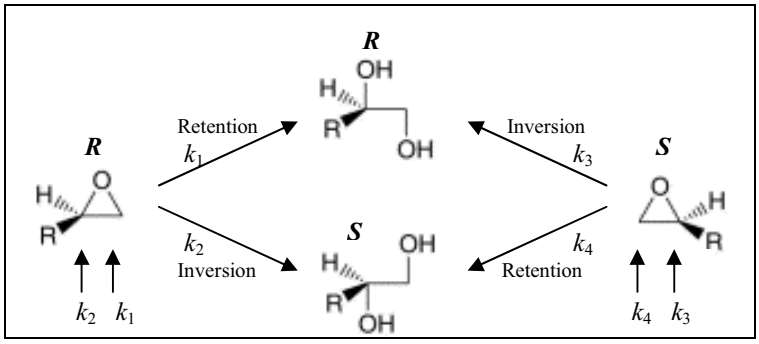

Figure 5. Stereochemical pathways of epoxide hydrolases (Taken from Steinreiber and Faber 2001)

In the conversion of an epoxide, three different reactions can occur: (1) a total regioselectivity on the same carbon atom, as shown in Figure 4; (2) a partial regioselectivity between the two carbon atoms of the oxirane ring, and (3) a total but opposite regioselectivity for each enantiomer, where one is retained and the other is inverted (Mousseau et al. 1998, Figure 6). This last case leads to a so-called 'enantioconvergent' process which can afford an optically pure diol in a theoretical yield of 100% from a racemic mixture. This is of value as racemic mixtures of epoxides can be cheaply prepared, and can be used for the production of a high value optically pure diol (Swaving and de Bont 1998).

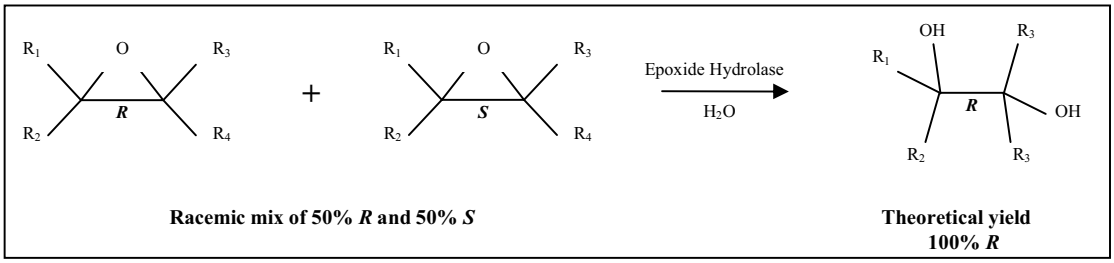

Figure 6. The stereochemistry of an enantioconvergent ***regioselective*** enzymatic hydrolysis of an epoxide. From a racemic epoxide mixture, both enantiomers are hydrolysed to form the same diol. R_1 – R_4 can be any group.

Major groups of substrate types that can be resolved enantiomerically by epoxide hydrolases include mono-substituted epoxides (type I), styrene oxide-type oxiranes (type II), di-substituted epoxides (type III), tri-substituted and tetra-substituted epoxides (type IV) (Figure 7) (Orru and Faber 1999). These substrates have enormous importance in the pharmaceutical, agrochemical and food industries. Enantiomerically pure epoxides are versatile fine chemical intermediates for use in the production of pharmaceuticals, agrochemicals, ferro-electric liquid crystals, and flavours and fragrances.

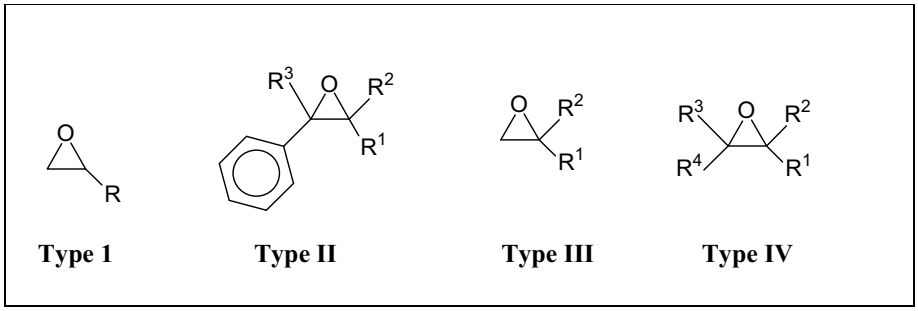

Figure 7. Different substrate types for epoxide hydrolases: mono-substituted epoxide (type I); styrene oxide-type epoxide (type II); 2,2, di-substituted epoxides (type III) and tri-substituted and tetra-substituted epoxides (type IV).

Epoxides can be used in the preparation of important drugs such as ibuprofen, nifenalol and eliprodil (Archelas and Furstoss 2001). Other examples include styrene oxide derivatives and phenyl glycidyl ethers that can be used in the synthesis of anti-diabetic and anti-obesity agents by the ring opening of chiral epoxides with an amine (Bloom et al. 1992). Epichlorohydrin can be used in the synthesis of food additives (Kabat et al. 1997), antibacterial agents (Schaus and Jacobsen 1996) and pharmaceuticals (Takano et al. 1993). In these instances, the epoxide is mainly used in a ring opening that occurs

with the retention of the configuration around the chiral centre of the epoxide (van Loo et al. 2004). It is therefore important in these cases either to start the conversion with an enantiopure epoxide, or catalyse the reaction with an enzyme capable of enantioconvergence.

Sources and Classification of Epoxide Hydrolases

Biologically, epoxides are generally formed from olefinic and aromatic compounds using cytochrome P450 mono-oxygenases (Swaving and de Bont 1998). These highly reactive epoxides react can readily with a great number of compounds within the cell, such as proteins and DNA. It is therefore important that organisms are able to immediately attack and eliminate these compounds, for example by hydrolysis with EHs. The conversion of the epoxides leads to more stable and less reactive intermediates. It is therefore not surprising that EHs are a ubiquitous family of enzymes. They are found widely in nature, and have been identified in mammals, plants, insects, fungi and bacteria, although detoxification is not their only biological function. Barth et al. (2004) conducted a systematic analysis of the sequences and structures of all known and putative EHs obtained from the NCBI-GenBank database. More than 100 EHs, including 56 putative EHs were sourced. Subsequent multiple alignments and phylogenetic analysis of 95 of these EHs separated the enzymes into two superfamilies: the microsomal (mEH) and cytosolic or soluble (sEH) families. The mEH family includes mammalian, insect, fungal and bacterial EHs, and the sEH family includes mammalian, plant and bacterial EHs. mEHs are membrane-bound and contain an N-terminal membrane anchor not found in sEHs. Figures 8 and 9 show selected members of the two superfamilies separated into their classes.

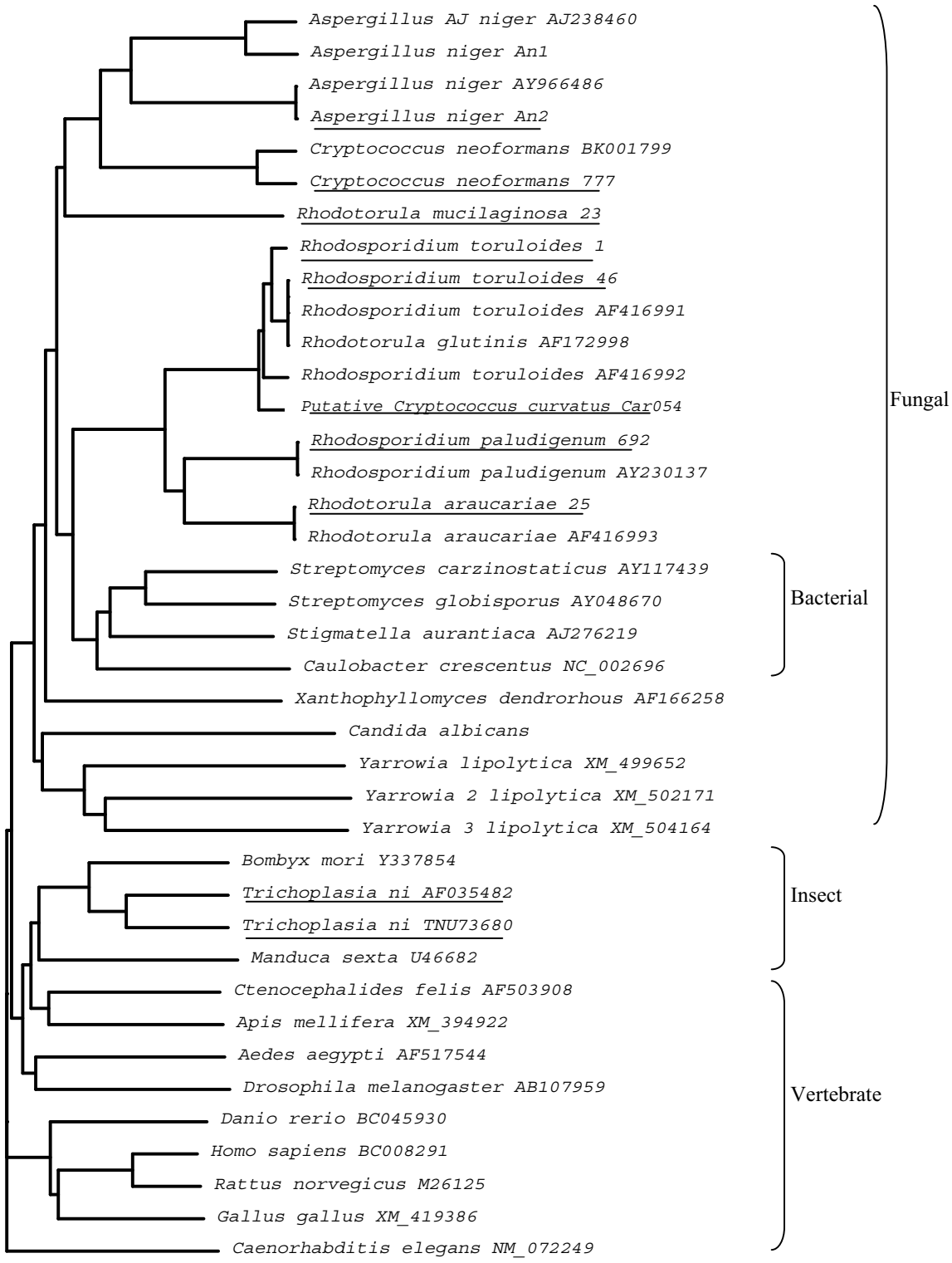

Figure 8. Phylogenetic analysis (performed using DNAMAN, Lynnon BioSoft, using observed divergency and 1000 Bootstrap trials) of DNA of selected microsomal EHs indicating the 4 major mEH groups of fungal (chequered), insect (yellow), vertebrate (pink) and bacterial (striped) origin. Sequences can be traced using the NCBI accession numbers. The underlined sequences are used in this work for expression in *Y. lipolytica* in Chapter 3.

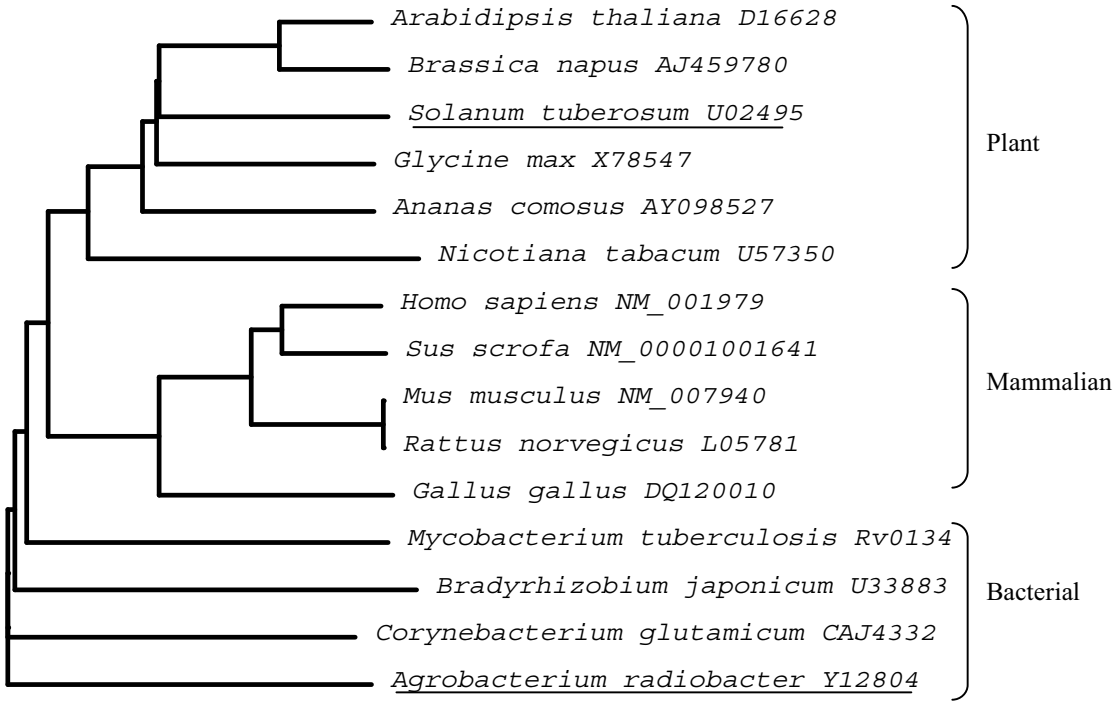

Figure 9. Phylogenetic analysis (performed using DNAMAN, Lynnon BioSoft, using observed divergency and 1000 Bootstrap trials) of DNA of selected soluble cytosolic EHs indicating the 3 major sEH groups of plant, mammalian and bacterial origin. Sequences can be traced using the NCBI accession numbers. The underlined highlighted are used in this work for expression in *Y. lipolytica* in Chapter 3. The phylogenetic tree of the deduced amino acid sequences is very similar.

sEHs in mammals are xenobiotic-metabolising enzymes that may also be involved in the metabolism of endogenously derived fatty acid epoxides (Meijer and DePierre 1988), whereas mEHs are principally involved with metabolism of xenobiotic compounds. Mammalian mEHs are present in the liver of animals (Weijers and de Bont 1999) and detoxification occurs by conversion of the undesired lipophilic substances into more water-soluble, readily excretable compounds.

Insect EHs are either part of the detoxification process, or are involved in the regulation of specific pheromones and hormones (Weijers and de Bont 1999). Plant EHs are specific for the hydrolysis of *cis* fatty acid epoxides, resulting in *threo* diols. They play a role in plant defence mechanisms against fungal and bacterial pathogens. Bacterial EHs can be divided into the constitutively-produced enzymes and enzymes that are produced in the metabolism of specific epoxides, which normally have very narrow substrate ranges. The *Mycobacterium tuberculosis* genome includes sequences for at least 20 EH

enzymes (Johansson et al. 2005). This unusually high number of EHs, combined with the potential toxicity of EH substrates in general, suggest a vital function for this enzyme family in the physiology of the pathogen. Blocking the EH activity in *M. tuberculosis* may represent a promising approach for anti-tubercular therapy.

Both sEHs and mEHs are members of the α/β hydrolase enzyme family (Fretland and Omiecinski 2000), and consist of two main domains: the α/β domain and a cap domain. The active site of the enzyme is located at the interface of the two domains and contains an Asp-His-Asp catalytic triad. Most EHs contain: (1) an oxyanion hole that consists of two residues which donate the backbone amide protons to stabilise the negative charge of the transition state, (2) an HGX-motif in which the X is the first oxyanion hole residue, (3) a nucleophile (Asp) which is the direct neighbour of the second residue of the oxyanion hole, (4) a catalytic histidine and (5) at least one tyrosine in the cap domain which is essential for activity by being involved in substrate binding and assisting in ring opening of the epoxide by acting as proton donor. Most also contain a second tyrosine. In order to avoid potential unfavourable steric interactions, the residues surrounding the nucleophiles have small side chains: Sm-X-Nu-Sm-Sm (Sm = small, x = any amino acid), with one or more of the small residues being a glycine (vanHook Harris et al. 1999).

Figure 10 shows the general mechanism of EHs. In the first step, the epoxide is attacked by the nucleophile Asp, forming a glycol-monoester-enzyme intermediate. In the second step, the glycol-monoester-enzyme intermediate is hydrolysed through the attack of OH^- from water, which is provided by the aid of the histidine residue, releasing the diol and liberating the enzyme.

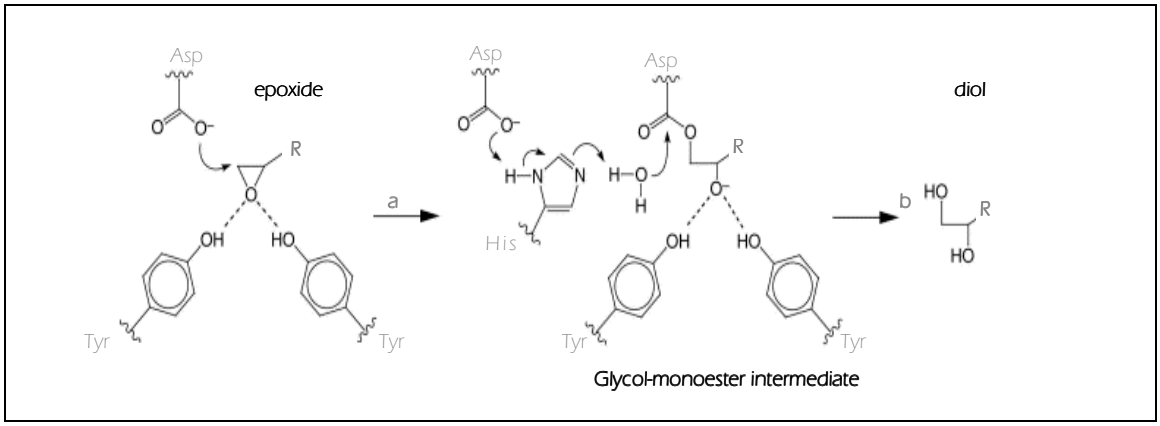

Figure 10. General mechanism of epoxide hydrolases. (a) The oxirane is attacked by a carboxylate nucleophile (aspartic acid) to form a glycol-monoester-enzyme intermediate. (b) OH^- derived from water and aided by a histidine residue, is used to hydrolyse the intermediate, yielding the vicinal diol and liberating the enzyme. R = any substituent. Adapted from Orru and Faber (1999) and Steinreiber and Faber (2001).

Three typical EH enzymes have been crystallised: those from *Agrobacterium radiobacter* (Nardini et al. 1999), mouse liver (Argiriadi et al. 1999) and *A. niger* (Zou et al. 2000). From these structures, backed up by site-directed mutagenesis data (Yamada et al. 2000; Rink et al. 1999), it was concluded that the two tyrosine residues located in the active site are also implicated in the epoxide ring opening, as can be seen in Figure 10. The primary sequence of the residues in the catalytic triad is always nucleophile (Asp) - acid (Asp or Glu) – base (His), a characteristic of the α/β hydrolase fold family (Ollis et al. 1992). Figure 11 shows the protein alignment of a number of different sEH and mEH enzymes, indicating the conserved regions.

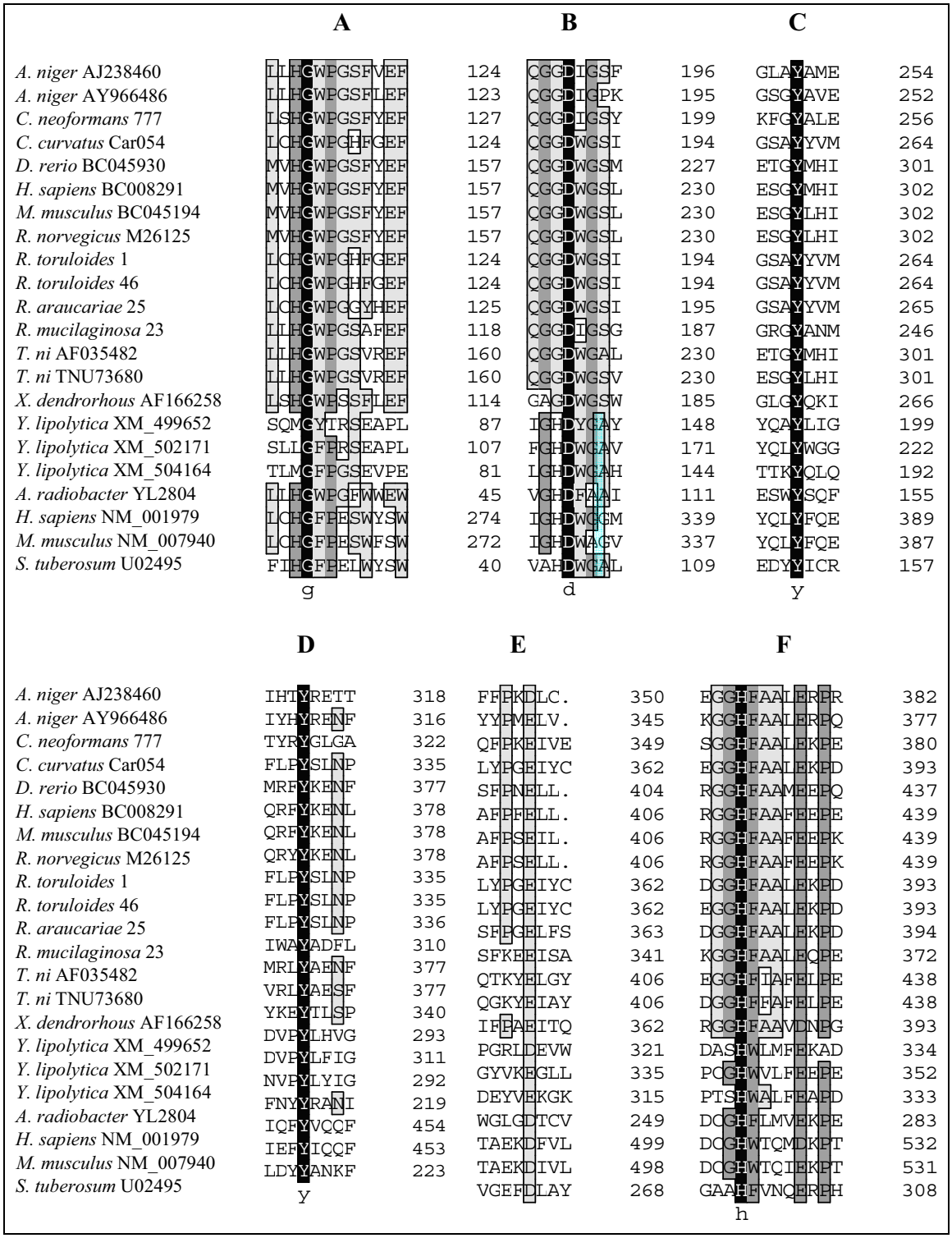

Figure 11. Partial protein sequence alignment (using DNAMAN, Lynnon BioSoft) between selected sEH and mEH enzymes. The bottom four sequences are sEH enzymes. The conserved regions include the HGXP motif (A), the nucleophile, Asp (B), the two Tyr residues (C) and (D), the acidic Asp or Glu (E) and basic catalytic His (F) residues of the charge relay system.

Heterologous Expression of Epoxide Hydrolases

EHs are found ubiquitously in nature, including in a variety of micro-organisms. In order to exploit this wide and ever increasing number of EHs for biocatalytic purposes and also to produce correctly-folded EHs for structure-function studies, a generic expression system is a necessity. No single enzyme is likely to be able to function optimally on all types of epoxides. The successful utilisation of EHs as biocatalytic tools will only be through the creation of a library of enzymes with differing activities. However, at present no single expression system has been developed that can express functionally-active EHs from the all the various animal, plant, insect and microbial sources currently available.

Isolated EH genes from a variety of origins have been expressed as recombinant proteins in several hosts, with *E. coli* as the major host for recombinant expression. For example, the EH from the bacterium *A. radiobacter* AD1, the mEH from the zebrafish (*Danio rerio*) and the sEH from the soybean (*Glyine max*) were all expressed in functional form in *E. coli* (Rink et al. 1997; Kim et al. 2005; Arahira et al. 2000). However, use of this prokaryotic expression host as a generic system suffers from a number of drawbacks:

- As a prokaryotic host, *E. coli* lacks the cellular machinery to perform glycosylation and post-translational modifications required for the correct folding of proteins of eukaryotic origin, which leads to altered 3-D structures of the recombinant protein. A true evaluation of the efficiency of epoxide hydrolases is thus not possible, as exemplified by the decrease in selectivity observed for yeast EHs expressed in *E. coli* (Visser et al. 2002.)
- Koschorreck et al. (2005) performed statistical calculations which predicted that the majority of the α/β-hydrolase fold enzymes would be expressed as insoluble aggregates in *E. coli*, making this system an obvious sub-optimal host for the production of EHs, especially those from eukaryotic origin. Most of the recombinant mEHs reportedly produced in this host were produced as insoluble aggregates in inclusion bodies despite efforts to maintain solubility. These efforts included maintaining the presence of several molecular chaperones, use of lower concentrations of IPTG and lower protein production temperature (Visser et al. 2003). The successful

expression of *A. radiobacter*'s and *A. niger*'s EH was achieved by cultivating the *E. coli* strain at 20°C in place of the usual 37°C (Rink et al. 1997; Arand et al. 1999a), and the expression of *D. rerio*'s EH was achieved by lowering the cultivation temperature to 15°C upon addition of IPTG (Kim et al. 2005).

- Loss of activity of recombinant EHs expressed in *E. coli* resulting from genetic instability of recombinant strains (Cedrone et al. 2003).
- Screening of DNA libraries with the *E. coli* host does not allow expression of eukaryotic EHs in which introns are often present, since this host is unable to perform splicing of introns to yield the mature mRNA corresponding to the ORF.

Recently, the yeasts *S. cerevisiae* and *P. pastoris* were also employed for the heterologous expression of EH-encoding genes. *S. cerevisiae* has been used for the functional expression of rat and human mEH (Arand et al. 1999b; Kelly et al. 2002). Unfortunately, no protein characterisation was performed in terms of protein size and enantioselectivity. The methylotrophic yeast, *P. pastoris*, has been used for the production of EHs of fungal (Lee et al. 2004) and plant origin (Bellevik et al. 2002a; 2002b). *S. cerevisiae* and *P. pastoris* have some limitations as an expression system such as hyper-glycosylation, low activity due to incorrect folding, and loss of selectivity of secreted proteins. *Baculovirus*-infected insect cells have also been employed for the heterologous expression of some EHs of plant origin, but performed poorly in the expression of functional EHs from insects such as *Trichoplusia ni* (VanHook Harris et al. 1999). This expression system is thus unsuitable for the general expression of EHs from diverse origins, and is also unsuitable for the practical large scale production of heterologous proteins. Table 7 is a comprehensive list of reported heterologous expression of EHs in different hosts.

Table 7. Examples of recombinant EHs produced in different expression hosts.

Source organism [a]	Host	Promoter used & assay location	Substrate	Specific Enzymatic Activity (V_{max}) [b]	Specific Enzymatic Activity (V_{max}) in source organism, if available	Rec. enantio-selectivity	Reference
Bacterial sEH							
A. radiobacter AD1 (Y12804)	*E. coli* BL31(DE3) (Novagen)	T7 promoter in fusion with *dhl*A, cell lysate	Epichlorohydrin	12 U (mg protein)$^{-1}$			Rink et al. 1997
			(*R*)-SO [c] (*S*)-SO	1.82 U (mg protein)$^{-1}$ 5.14 U (mg protein)$^{-1}$		(*R*)-SO	Lutje Spelberg et al. 1998
	E. coli TOP10 (Invitrogen)	*araB* promoter, soluble fraction	Epichlorohydrin SO p-NSO [d] p-NGE [e]	38 U (mg protein)$^{-1}$ 7.5 U (mg protein)$^{-1}$ 7 U (mg protein)$^{-1}$ 12 U (mg protein)$^{-1}$		E < 2 E = 16 E = 56 E = 3.4	Van Loo et al. 2004
	E. coli BL31(DE3)	T7 promoter, N-terminal His-tag, soluble fraction	SO (*R*)-SO (*S*)-SO	2 U (mg protein)$^{-1}$ 1.65 U (mg protein)$^{-1}$ 0.5 U (mg protein)$^{-1}$	 7.4 U (mg dcw)$^{-1}$ 18.5 U (mg dcw)$^{-1}$		Cao et al. 2006 Lee et al 2004
Mammalian sEH							
Rattus norvegicus (X65083)	*E. coli* JM109	T7 promoter, N-terminal His-tag, using whole cells	TSO	4 x 10^{-3} U (mg protein)$^{-1}$	8x less		Knehr et al. 1993
	Baculovirus: *T. ni* cells	Polyhedrin promoter, cytosolic fraction	TSO	1.8 U (mg protein)$^{-1}$			Yamada et al. 2000
	Baculovirus: *T. ni* cells	Polyhedrin promoter, cytosolic fraction	TSO CSO	0.1 U (mg protein)$^{-1}$ 8 x 10^{-3} U (mg protein)$^{-1}$			Morisseau et al. 2000
H. sapiens (EMBL L05779)	Baculovirus: Sf21 cells	Polyhedrin promoter, cytosolic fraction	TSO CSO	21.4 x 10^{-3} U (mg protein)$^{-1}$ 32.6 x 10^{-3} U (mg protein)$^{-1}$			Beetham et al. 1993
	Baculovirus: *T. ni* cells	Polyhedrin promoter, cytosolic fraction	TSO CSO	54 x 10^{-3} U (mg protein)$^{-1}$ 9 x 10^{-3} U (mg protein)$^{-1}$			Morisseau et al. 2000
Mus musculus (L05781)	COS-7 cells	SV40 expression, cell extracts	TSO	0.55-1.1 x 10^{-3} U (mg protein)$^{-1}$			Grant et al. 1993
	Baculovirus: *T. ni* cells	Polyhedrin promoter, cytosolic fraction	TSO CSO	0.48 U (mg protein)$^{-1}$ 68 x 10^{-3} U (mg protein)$^{-1}$			Morisseau et al. 2000
Plant sEH							
Arabidopsis thaliana (D16628)	*E. coli* XL1 Blue (Stratagene)	*lacZ* promoter, cytosolic fraction	TSO	52 x 10^{-3} U (mg protein)$^{-1}$			Kiyosue et al. 1994
	Baculovirus: *T. ni* cells	Polyhedrin promoter, cytosolic fraction	TSO CSO	2.1 U (mg protein)$^{-1}$ 5 x 10^{-3} U (mg protein)$^{-1}$			Morisseau et al. 2000
	P. pastoris GS115 (Invitrogen)	*AOX1* promoter, N-terminal His-tag, cytosolic fraction	TSO CSO	2 U (mg protein)$^{-1}$ 40 x 10^{-3} U (mg protein)$^{-1}$			Bellevik et al. 2002a

Source organism [a]	Host	Promoter used & assay location	Substrate	Specific Enzymatic Activity (V_{max}) [b]	Specific Enzymatic Activity (V_{max}) in source organism, if available	Rec. enantio-selectivity	Reference
Glycine max (X78547)	*E. coli* BL21(DE3)	T7 promoter, cytosolic fraction	SO	1.3 U (mg protein)$^{-1}$			Arahira et al. 2000
Solanum tuberosum (U02495)	Baculovirus: sf9 cells	Polyhedrin promoter, cytosolic fraction	TSO	63 x 10^{-3} U (mg protein)$^{-1}$	1.4 x 10^{-3} U (mg protein)$^{-1}$		Stapleton et al. 1994
	Baculovirus: *T. ni* cells	Polyhedrin promoter, cytosolic fraction	TSO CSO	0.42 U (mg protein)$^{-1}$ 4.5 x 10^{-3} U (mg protein)$^{-1}$			Morriseau et al. 2000
	E. coli XL1 Blue	Tac promoter, His-tag, cytosolic fraction	TSO *R,R*-TSO *S,S*-TSO p-NSO	6.1 U (mg protein)$^{-1}$ 43 U (mg protein)$^{-1}$ 2.7 U (mg protein)$^{-1}$ 1.6 U (mg protein)$^{-1}$			Elfström and Widersten 2005
	E. coli TG1	*lac* promoter, using whole cells	SO	0.16 U (mg protein)$^{-1}$			Cao et al. 2006
	E. coli BL21-CodonPlus (DE3)-RIPL (Stratagene)	T7 promoter, N-terminal His-tag, soluble fraction	SO (*R*)-SO (*S*)-SO	22 U (mg protein)$^{-1}$ 2.3 U (mg protein)$^{-1}$ 27 U (mg protein)$^{-1}$			Cao et al. 2006
Brassica napus (AJ459780	*P. pastoris* GS115	*AOX1* promoter, cell lysate	TSO CSO	0.46 U (mg protein)$^{-1}$ 15 x 10^{-3} U (mg protein)$^{-1}$			Bellevik et al. 2002b
Nicotiana tabacum (U57350	*E. coli* DH5α	T7 promoter, in fusion with maltose binding protein, soluble fraction	TSO	44 x 10^{-3} U (mg protein)$^{-1}$			Guo et al. 1998
Fish mEH							
Dania rerio (AAH45930)	E. coli BL21(DE3)	*cpsA* cold shock promoter, His-tag, whole cells	(*R*)-SO (*S*)-SO	11.4 U (mg dcw)$^{-1}$ 1.1 U (mg dcw)$^{-1}$		(*R*)-SO	Kim et al. 2005
Mammalian mEH							
Rattus norvegicus (P07687)	*E. coli* TG1	*lpp-lac* fusion promoter, *ompA* N-terminal fusion for secretion, membrane fraction	SO Benzo(*a*)pyrene-4,5-oxide	0.42 U (mg protein)$^{-1}$ 0.73 U (mg protein)$^{-1}$			Bell and Kasper 1993
	Baculovirus: *Spodoptera frugiperda* sf9 cells	Polyhedrin promoter, solublised cell lysate	phenanthrene 9,10-oxide	61 U (mg protein)$^{-1}$			Lacourciere et al. 1993
	S. cerevisiae W3031-1B	*GAL* promoter, microsomal fraction	SO TSO [f]	0.365 U (mg protein)$^{-1}$ 8.42 U (mg protein)$^{-1}$ for Glu^{404}→Asp mutant No activity			Arand et al. 1999b
H. sapiens (X07936)	Baculovirus: *T. ni* insect cells	Polyhedrin promoter, cytosolic fraction	CSO [g]	80 x 10^{-3} U (mg protein)$^{-1}$			Yamada et al. 2000

Source organism [a]	Host	Promoter used & assay location	Substrate	Specific Enzymatic Activity (V_{max}) [b]	Specific Enzymatic Activity (V_{max}) in source organism, if available	Rec. enantio-selectivity	Reference
Insect mEH							
Ctenocephalides felis (AF503908)	*E. coli* HB101 (GibcoBRL)	*trc* promoter, N-terminal His-tag, in insoluble fraction					Keiser et al. 2002
Trichoplusia ni (U73680)	Baculovirus: sf9 cells	AcMNPV polyhedrin promoter, mostly insoluble fraction	JH-III [h] TSO CSO	6×10^{-6} U(mg protein)$^{-1}$ 0.4×10^{-6} U(mg protein)$^{-1}$ 0.2×10^{-6} U(mg protein)$^{-1}$	1.1×10^{-3} U (mg protein)$^{-1}$		VanHook Harris et al. 1999
Bombyx mori (AY377854)	Baculovirus: sf9 insect cells	Polyhedrin promoter, microsomal fraction	JH-III	46×10^{-3} U(mg protein)$^{-1}$			Zhang et al. 2005
Manduca sexta	Baculovirus: *T. ni* and sf21 cells	Polyhedrin promoter, microsomal fraction	JH-III	70×10^{-3} U(mg protein)$^{-1}$			Debernard et al. 1998
Drosophila melanogaster (AB107959)	Baculovirus: *T. ni* cells	Polyhedrin promoter, microsomal fraction	JH-III TSO CSO	No activity Not tested 23×10^{-6} U (mg protein)$^{-1}$	0.28×10^{-6} U (mg protein)$^{-1}$ 2.6×10^{-6} U (mg protein)$^{-1}$ 0.13×10^{-3} U (mg protein)$^{-1}$		Taniai et al. 2003
Fungal mEH							
Xantho-phyllomyces dendrorhous (AF166258)	*E. coli* XL1-Blue-MRF' (Stratagene)	T7 promoter, whole cells	1, 2-epoxyhexane MCHO [i]	31×10^{-3} U (mg protein)$^{-1}$ 1×10^{-3} U (mg protein)$^{-1}$	4.8×10^{-3} U (mg protein)$^{-1}$ 0.17×10^{-3} U (mg protein)$^{-1}$	Not select-ive	Visser et al. 1999
Aspergillus niger (AJ238459)	*E. coli* BL21(DE3)	*lac* promoter, cell lysate	4-NSO	98 U (mg protein)$^{-1}$			Arand et al. 1999a
	E. coli DH5α (Stratagene)	T5 promoter, C-terminal His-tag, cell lysate	pNP-EB [j]	23 U (mg protein)$^{-1}$ 31 U (mg protein)$^{-1}$ – mutant obtained via error-prone PCR			Cedrone et al. 2003
Aspergillus niger (AY966486)	*E. coli* BL21(DE3)	*lac* promoter, N-terminal His-tag cell lysate	pNSO	12.3 U (mg protein)$^{-1}$ (2.5 g.L^{-1})		(*R*)-pNSO	Liu et al. 2007
Rhodosporidium toruloides CBS 14 (AF16992)	*E. coli* BL21(DE3)	T7 promoter, whole cells	T-1-P-1,2-EP [l]	0.97 U (mg dcw)$^{-1}$	0.2×10^{-3} U (mg dcw)$^{-1}$ (E = 6)	1*S*, 2*S* (E = 100)	Visser et al. 2002
R. toruloides CBS 349 (AF16991)	*E. coli* BL21(DE3)	T7 promoter, whole cells	T-1-P-1,2-EP	0.81 U (mg dcw)$^{-1}$	3.2×10^{-3} U(mg dcw)$^{-1}$ (E = 21)	1*S*, 2*S* (E = 61)	Visser et al. 2002

Source organism [a]	Host	Promoter used & assay location	Substrate	Recombinant Activity (V_{max}) [b]	Activity (V_{max}) in source organism, if available	Rec. enantio-selectivity	Reference
Rhodotorula glutinis ATCC 201718 / CIMW 147 (AF172998)	*E. coli* XL1-Blue-MRF	T7 promoter, whole cells	1,2-EH [k]	0.2 U(mg protein)$^{-1}$	0.12 U(mg protein)$^{-1}$ (Kronenberg et al. 1999)		Visser et al. 2000
	E. coli BL21(DE3)	T7 promoter, N-terminal His-tag, most of protein inactive in insoluble fraction (inclusion bodies)	1,2-EH	Cells: 14 U (mg dcw)$^{-1}$ Soluble fraction: 23 U (mg protein)-^{1}	Cells: E= 84 50 x 10^{-3} U (mg dcw)$^{-1}$ Cell-free extract: E = 40 0.11 U (mg protein)$^{-1}$	E = 48 E = 30	Visser et al. 2003
	E. coli BL21(DE3)•, BLR(DE3)♦, Rosetta$^{\nabla}$ (Novagen)	T7 promoter C-terminal His-tag, whole cells	(*R*)-SO	•840 x 10^{-3} U (mg dcw)$^{-1}$ ♦515 x 10^{-3} U (mg dcw)$^{-1}$ $^{\nabla}$2.3 U (mg dcw)$^{-1}$	10.5 x 10^{-3} U (mg dcw)$^{-1}$	(*R*)-SO	Kim et al. 2006
			(*S*)-SO	•390 x 10^{-3} U (mg dcw)$^{-1}$ ♦176 x 10^{-3} U (mg dcw)$^{-1}$ $^{\nabla}$1.44 U (mg dcw)$^{-1}$	34.5 x 10^{-3} U (mg dcw)$^{-1}$		
	P. pastoris GS115	*AOX1* promoter, whole cells	(*R*)-SO (*S*)-SO	0.36 U (mg dcw)$^{-1}$ 0.11 U (mg dcw)$^{-1}$	0.035 x 10^{-3} U (mg dcw)$^{-1}$ 0.01 x 10^{-3} U (mg dcw)$^{-1}$	(*R*)-SO	Lee et al. 2004
		AOX1 promoter, whole cells	(*R*)-SO (*S*)-SO	0.4 U (mg dcw)$^{-1}$ 0.135 U (mg dcw)$^{-1}$	10.5 x 10^{-3} U (mg dcw)$^{-1}$ 34.5 x 10^{-3} U (mg dcw)$^{-1}$	(*R*)-SO	Kim et al. 2006
	S. cerevisiae EBY100 (Invitrogen)	*GAL* promoter, cell surface displayed	(*R*)-SO (*S*)-SO	27.2 x 10^{-3} U (mg dcw)$^{-1}$ 6.2 x 10^{-3} U (mg dcw)$^{-1}$	10.5 x 10^{-3} U (mg dcw)$^{-1}$ 34.5 x 10^{-3} U (mg dcw)$^{-1}$	(*R*)-SO	Kim et al. 2006
Rhodotorula araucariae CBS 6031 (AF16993)	*E. coli* BL21(DE3)	T7 promoter, whole cells	T-1-P-1,2-EP	52 x 10^{-3} U(mg dcw)$^{-1}$	0.3 x 10^{-3} U(mg dcw)$^{-1}$ (E = 5)	1*S*, 2*S* (E = 20)	Visser et al. 2002
Rhodoturula mucilaginosa	*Y. lipolytica* Po1h	*TEF* promoter, whole cells	(2,3-epoxy-propyl)benzene			(S)-SO ee_s = 97%	Labuschagne and Albertyn 2007

[a] Genbank accession number in brackets

[b] 1 unit is defined as 1 µmol of diol formed per minute

[c] Styrene oxide

[d] *para*-nitrostyrene oxide

[e] *para*-nitrophenyl glycidyl ether

[f] *Trans*-stilbene oxide

[g] *Cis*-stilbene oxide

[h] Juvenile Hormone III

[i] 1-methylcyclohexene oxide

[j] 4-(p-nitrophenoxy)-1,2-epoxybutane

[k] 1,2-epoxyhexane

[l] Trans-1-phenyl-1,2-epoxypropane

A number of interesting observations can be made from the data in Table 7. For example, when comparing the native EH from *R. toruloides* CSB 14 and CSB 349, and the recombinant enzymes produced in *E. coli*, there was a 4,850- and 253-fold difference in expression levels, respectively. The enantioselectivity for the recombinant enzymes was also superior. However, in the native producers, CBS 349 was superior to CBS 14 in both activity and enantioselectivity, whereas in the recombinant producers, CBS 14 out-performed CBS 349 on both fronts. This indicates that heterologous expression of an EH may have an impact on the structure and functionality of the enzyme, meaning that it is impossible to directly extrapolate the level and type of activity directly from the known function in the wild-type host to the heterologous host.

Microsomal EHs from yeast species are of particular interest as they have broad substrate ranges, high enantioselectivities and reaction rates (Weijers and de Bont 1999). However, in most cases the wild-type yeasts do not meet the requirements for specific epoxide conversions in industrial processes. The cultivation is not optimal in terms of growth rates, enzyme production levels and ease of enzyme purification. It is therefore essential that a generic expression system be developed for the production of these valuable enzymes. It has also been found that detection of enantioselective EH activity during screening of wild-type yeast species depends on the presence of an enzyme with high activity towards the substrate being assessed (Smit 2004). Enzymes with desired functionality are therefore missed if the levels of activity are not sufficient. Having a generic expression system can therefore assist in screening EHs for different enantioselectivities with different substrates. sEHs are phylogenetically completely removed from mEHs. If a host strain can be found that is capable of expressing EHs from both families, with retention or improvement of their kinetic properties compared to the endogenous hosts, it would prove the general applicability of this host for the expression of EHs from all sources.

The development of a single highly efficient expression system for producing functional heterologous EHs from any source, including animal, insect, plant and microbial origins, would be a significant advantage given the limitations and constraints of the current

options available. A microbial whole-cell biocatalyst that is able to functionally express a broad range of EHs from diverse origins as active intracellular proteins is commercially sought after. Furthermore, an expression system that is capable of producing high-levels of high quality heterologous proteins from diverse origins without, for example, loss of the desired characteristics identified in the wild-type, is highly desired. This is particularly useful for the development of an array of chirally specific EH biocatalysts to provide a range of chiral epoxide substrates useful as building blocks in chiral pharmaceutical syntheses. High-level functional expression of EHs from diverse origins is also required to produce high-quality proteins in sufficient quantities to allow structure-function studies. In addition, a eukaryotic expression system capable of splicing introns that can be transformed with high efficiency at a specified locus in the genome is required for evaluation and direct comparison of the characteristics of clones generated by protein engineering, screening of environmental libraries containing eukaryotic EHs with introns present, as well as for the evaluation of biocatalyst activity and selectivity from diverse sources. Recently, a newly-isolated mEH from *Rhodotorula mucilaginosa* was successfully expressed in *Y. lipolytica*, using a single-copy random integration method (Labuschagne and Albertyn 2007). The activity and selectivity for the epoxide substrate (2,3-epoxypropyl)benzene when using the recombinant *Y. lipolytica* as a whole-cell biocatalyst was far superior to any other activity and selectivity reported in literature using wild-type organisms (Labuschagne and Albertyn 2007).

Research presented in this dissertation

The following chapter will explore the applicability of *Y. lipolytica* as an expression system for EHs. Two fungal *eph* genes were isolated The *eph* gene from *Rhodosporidium toruloides* NCYC3181 displays a 92% similarity with the *R. toruloides* CBS349 *eph* gene (Genbank accession number AF416991), The *eph* gene from a strain named Car054, originally identified as *Cryptococcus curvatus* (NCYC 3158), was isolated. A sequence analysis showed that this gene was more similar to the *eph* genes of the *Rhodosporidium* and *Rhodotorula* species than the known *eph* gene from *Cryptococcus neoformans* (Genbank accession number AY466857). The identification

of this strain is therefore suspect. A number of additional EHs from different sources, including bacteria, yeasts, fungi and plants, were also chosen for expression in *Y. lipolytica*, in order to determine its suitability as the expression system of choice for the production of EHs. The EH from *Solanum tuberosum* (potato) was be used as an example of a sEH from plant origin, due to the good selectivity of this enzyme reported for styrene oxide (Monterde et al. 2004). Furthermore, the *S. tuberosum* enzyme was reported to display selectivity for styrene oxide opposite to those of *A. niger* and yeast EHs. The EH from *A. radiobacter* was selected as an example of a sEH from bacterial origin, due to the good selectivity of this enzyme reported for styrene oxide (Lutje Spelberg et al. 1998). However, this enzyme reportedly became unstable if epoxide concentrations exceeded the solubility limit (*i.e.* formed a second phase), due to interfacial deactivation. The kinetic characteristics of this enzyme was thus only reported at very low concentrations (5 mM) by Lutjie Spelberg et al. (1999) while it is shown that the *Y. lipolytica*-expressed *A. radiobacter* EH can perform the biotransformation at substrate concentration of 100 mM. Evaluation of the use of a secretion signal for extracellular expression will be done using selected EHs. The efficacy of *Y. lipolytica* versus other EH expression systems previously reported in literature will be discussed. The broad applicability of *Y. lipolytica* as an effective expression system for EHs will be highlighted, with a number of different EHs from various sources, with highly different enantioselectivities and activities being successfully expressed and used for biotransformations. *S. cerevisiae* is used as host for the expression of the mEH from *Rhodotorula araucariae*[1], and its functionality as a whole-cell biocatalyst is evaluated for comparison purposes.

[1] The *S. cerevisiae* strain was produced by Dr N. Rohitllal of CSIR Biosciences, with assistance from the author.

B.2. Halohydrin dehalogenase

Halohydrin dehalogenases (HHdHs), also referred to as haloalcohol dehalogenases or halohydrin hydrogen-halide lyases, catalyse the nucleophilic displacement of the halogen ion in various aliphatic and aromatic halohydrins (= haloalcohols) by a vicinal hydroxyl group, yielding an epoxide, a proton, and a halide ion (Van Hylckama Vlieg et al. 2001) (Figure 12, Reaction A). These enzymes also efficiently catalyse the reverse reaction, *i.e.* the halogenation of epoxides, as well as the enantioselective and β-regioselective ring-opening of epoxides by pseudohalides such as azide and cyanide (de Jong et al. 2005). They have been shown to be capable of utilising these alternative nucleophiles such as N_3^-, NO_2^- and CN^- and inserting them into the resultant product (Figure 12, Reaction B).

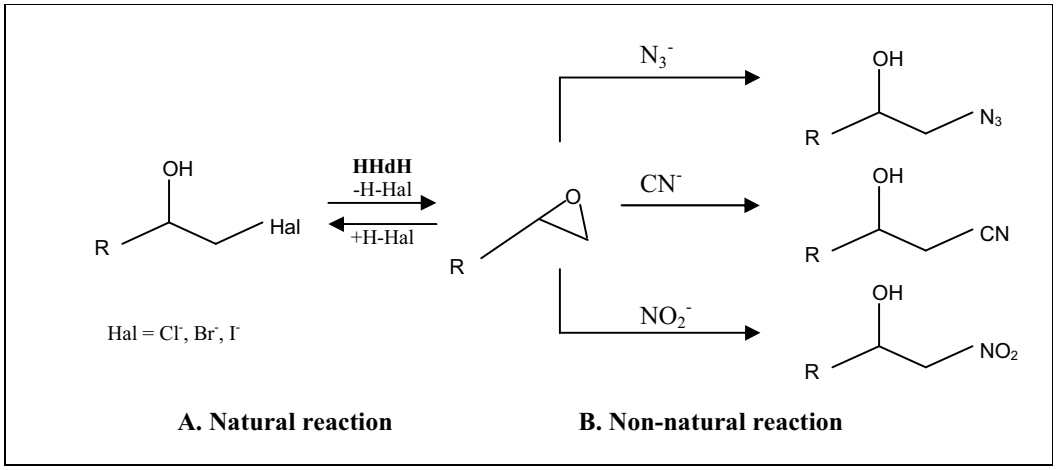

Figure 12. Reversible intra-molecular substitution of halo-alcohol to the corresponding epoxide (reaction A) and alternative reverse reactions catalysed by HHdHs - the halogenation of epoxides using alternative nucleophiles (reaction B) (Faber and Kroutil, 2005).

The interest in HHdHs increased when it was found that the dehalogenation of halohydrins may proceed with high enantioselectivity, making these enzymes useful catalysts to prepare various optically active epoxides, as well as halohydrins and other β-substituted alcohols. The production of single enantiomers of these chiral intermediates has become increasingly important in the pharmaceutical industry. Single enantiomers can be produced by chemical synthesis. The advantage of biocatalysis over chemical synthesis is that the enzyme-catalysed reactions are often highly enantio- and regioselective, and can be carried out at ambient temperature and pressure, thus avoiding

the use of extreme conditions that can cause problems with isomerisation, racemisation, epimerisation and rearrangement. Microbial cells and enzymes can be immobilised and reused for many cycles, and enzymes can be over-expressed to make the biocatalytic process economically efficient.

The use of HHdHs and EHs in tandem allows for the production of compounds with higher enantioselectivity. By adding an excess of EH, the reversible conversion catalysed by HHdH can be drawn to completion (Lutje Spelberg et al. 1999). The EH is added to draw the kinetic resolution of the halohydrin to conclusion by removing the inhibiting epoxide. The EH itself has no influence on the enantioselectivity of the conversion. Halohydrins such as (*S*)-2,3-dichloro-propanol (E>100) and (*S*)-2-chloro-1-phenylethanol (E=73) were obtained with an enantiomeric excess higher than 99%. Figure 13 depicts the conversion of racemic 2,3-dichloro-propanol (Lutje Spelberg et al. (1999).

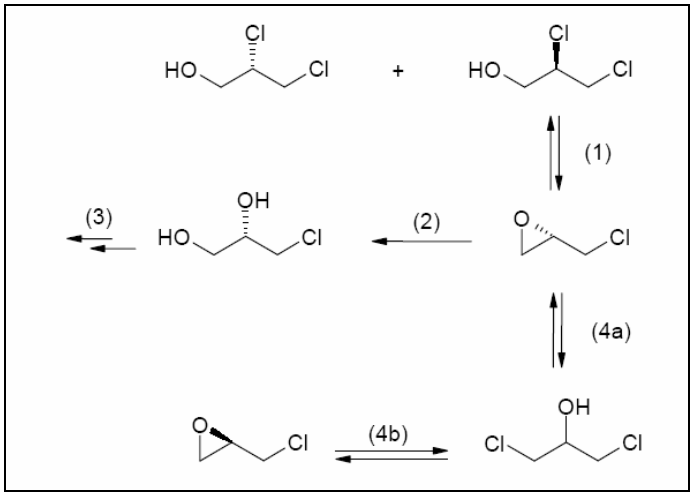

Figure 13. Conversion of 2,3-dichloro-propanol using a combination of HHdH and EH, reproduced from Lutje Spelberg et al. (1999). HHdH catalyses steps **1**, **3**, **4a** and **4b**. EH catalyses steps **2** and **3** (Lutje Spelberg et al. 1999). The (*R*)-enantiomer is initially converted to (*R*)-epichlorohydrin (**1**), with the yield of the remaining (*S*)-enantiomer 49.5% (maximum theoretical yield is 50%). Instead of enantiomerically pure (*R*)-epichlorohydrin, racemic epichlorohydrin and prochiral 1,3-dichloro-2-propanol are formed. This is explained by the reverse reaction, the ring opening of (*R*)-epichlorohydrin, where the favoured attack by the Cl^- was at the β-position (**4a**) to create 1,3-dichloro-2-propanol rather than the α-attack (**1**) to reform the (*R*)-epichlorohydrin. The prochiral 1,3-dichloro-2-propanol is then converted to both enantiomers of epichlorohydrin by HHdH (**4a**, **4b**). Addition of EH results in the formed (*S*)-epichlorohydrin being converted immediately to (*S*)-3-chloro-1,2-propanediol (**2**). This is then further converted via glycidol to glycerol (**3**). In this case, the formation of 1,3-dichloro-2-propanol and (*S*)-epichlorohydrin are not observed (Lutje Spelberg et al. 1999).

Halohydrin dehalogenase of *Agrobacterium radiobacter* AD1

The halohydrin dehalogenase (encoded by the gene *HheC*) of the 1,3-dichloro-2-propanol-utilising bacterium *Agrobacterium radiobacter* (also known as *A. tumefaciens*) AD1 is a homo-tetrameric protein (Tang et al. 2002), structurally and mechanistically related to the short-chain dehydrogenase/reductases (SDR) enzyme family (Jörnvall et al. 1995; Van Hylckama Vlieg et al. 2001). The typical SDR catalytic triad of Ser-Tyr-Lys/Arg is largely conserved, with the third residue being an Arg in HHdHs (de Jong et al. 2003). The halohydrin substrate binds near the Ser^{132}-Tyr^{145}-Arg^{149} catalytic triad with its hydroxyl group interacting with Ser^{132} and Tyr^{145} (de Jong et al. 2005). The halide binding site can also accommodate other small negatively charges ions such as azide and nitrite. These ions replace the halide ion in the reverse reaction.

This HHdH exhibits remarkable enantioselectivity with a broad range of aliphatic and aromatic halohydrins. It is highly enantioselective with aromatic halohydrins such as 1-(*para*-nitrophenyl)-2-chloro-ethanol and can catalyse not only the ring-closure of halohydrins but also the reverse reaction: the nucleophilic ring opening of an epoxide, as shown in Figure 13 above (reaction 4a) (Lutje Spelberg et al. 2002). HheC-catalysed ring opening of various styrene oxides with azide as the nucleophile results in highly enantioselective and regioselective production of azido-alcohols (Figure 14). Nitrite can also be used as the nucleophile to form unstable hydroxyl-nitrite ester intermediates, which can be further hydrolysed to the corresponding diols (Hasnaoui et al. 2005) Under oxidising conditions, the enzyme is susceptible to inactivation, which can be prevented by the addition of β-mercaptoethanol or glycerol (Tang et al. 2002).

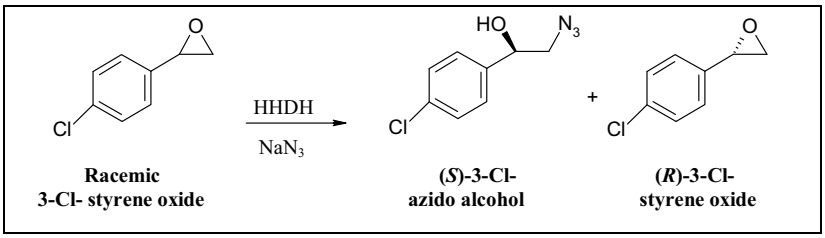

Figure 14. Enantioselective production of 3-chloro-azido-alcohol from a racemic mix of 3-chloro-styrene oxide using azide as nucleophile (Lutje Spelberg et al. 2002).

The absolute enantioselectivity is illustrated in Figure 15, which shows the binding and catalysis of (*S*)- and (*R*)-*para*-nitrostyrene oxide in the active site of HheC (de Jong et al. 2005). In contrast to the (*R*)-enantiomer of pNSO, the (*S*)-enantiomer binds in a non-productive conformation that does not permit protonation of the epoxide's O-atom and the concurrent attack of its C_β atom by the nucleophilic anion (X^-) in the halide binding site. Therefore, only the (*R*)-enantiomer can be converted to the corresponding β-substituted alcohol.

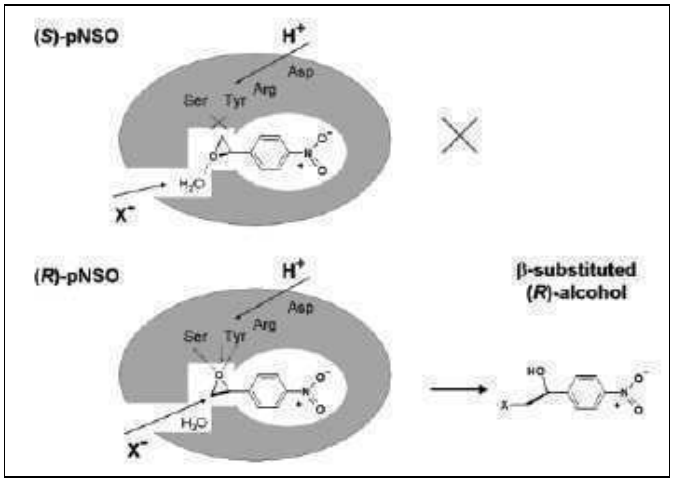

Figure 15. Binding of the (*R*)- and (*S*)-enantiomers of *para*-nitrostyrene oxide (pNSO) to the halide-binding site of the *A. radiobacter* AD1 HheC, indicating the non-productive binding of the (*S*)-enantiomer (Reproduced from de Jong et al. 2005).

Heterologous expression of *HheC*

The *HheC* gene from *A. radiobacter* AD1 was expressed in *E. coli* by van Hylckama Vlieg et al. (2001), along with the HHdH genes from *Arthrobacter* AD2 (*HheA*) and *Mycobacterium* GP1 (*HheB*). All three proteins were expressed in soluble and active form, at up to 15 – 25% of the total cellular protein, in *E. coli* BL21(DE3). All three were active with all brominated and chlorinated C_2 and C_3 vicinal halohydrins tested.

The related *hheA* and *hheB* genes from *Corynebacterium* sp. strain N-1074, which can transform 1,3-dichloro-2-propanol to the optically active 3-chloro-1,2-propanediol, were also cloned and expressed in *E. coli* (Yu et al. 1994). The *hheA* and *hheB* gene products are functional isozymes which catalyse the interconversion of 1,3-dichloro-2-propanol to

epichlorohydrin, the first step in the production of 3-chloro-1,2-propanediol. They catalyse the conversion of several halohydrins to the corresponding epoxides with the liberation of hydrogen halide, as well as the reverse reactions (Nakamura et al. 1994).

Research presented in this dissertation

In this study the intention was to conduct ring-closure reactions using HheC from *A. radiobacter* AD1, which was cloned and over-expressed in *Y. lipolytica*. This would expand the use of *Y. lipolytica* as a host strain for heterologous production of enzymes of biocatalytic importance. Figure 16 illustrates an example of the ring-closure reactions conducted with recombinant HheC.

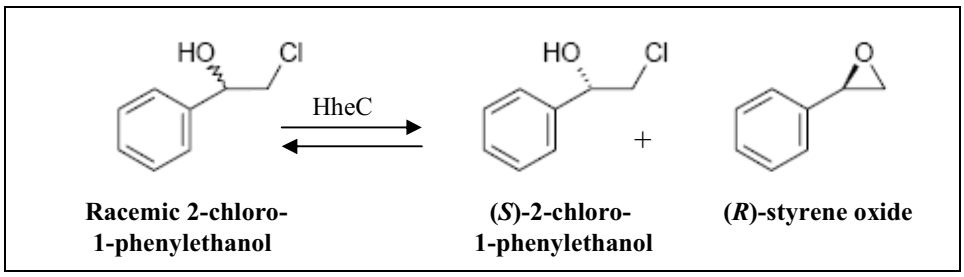

Figure 16. Enantioselective ring-closure of racemic 2-chloro-1-phenylethanol by HheC, encoded by *A. radiobacter* AD1.

Optimisation of expression levels was attempted by increasing the number of expression cassettes (consisting of promoter – *HheC* – terminator) integrated into the genome of *Y. lipolytica*. The *ura3d4* selection marker should ensure selection of transformants with 10 – 13 copies, probably reflecting the optimum auxotrophy complementation (Juretzek et al. 2001). To increase the copy number, vectors were constructed which contained up to five expression cassettes per single *ura3d4* selection marker, compared to the usual single expression cassette per *ura3d4* gene. Theoretically, the same number of integration events should occur, resulting in up to five times as many expression cassettes integrating into the genome. As is shown in Chapter 4, the expression levels in recombinant *Y. lipolytica* strains were successfully increased, in a dose-dependent manner, when tested using ring closure reactions of 2-chloro-1-phenylethanol enantiomers, while retaining the selectivity of the native enzyme.

B.3. Endo-β-1,4-Mannanase

Endo-β-1,4-mannanases (β-D-mannanase, E.C. 3.2.1.78) cleave randomly within the β-1,4-D-mannan backbones of galactomannan, glucomannan, galactoglucomannan and mannan (McCleary 1988). They are important for the enzymatic digestion of hemicelluloses, one of the most abundant groups of polymers in nature. Mannan is one of the major constituents of hemicellulosic materials in softwoods (Stålbrand et al. 1993), with a basic molecular structure of a linear backbone comprised of β-1,4-linked D-mannopyranose residues which, depending on the origin and method of extraction, may be substituted with branches containing mainly acetyl and galactosyl residues (Gübitz et al. 1996; Singh et al. 2003). Linear β-1,4-polymers are found in certain plant species, such as ivory nut (*Phytelephas macrocarpa*), and date (*Phoenix dactylofera*) (Meier, 1958). Glucomannans and galactomannans are present in the endosperm of many plants as storage polysaccharides. Galactoglucomannan has a hetero-β-1,4-linked backbone of mannose and glucose residues, substituted with α-1,6-linked galactosyl side groups, while galactomannan is composed of a homogenous backbone of β-1,4-linked mannose residues. The hydrolysis of galactomannan and galactoglucomannan is affected by the degree and pattern of substitution of the main chain by α-D-galactosyl residues (McCleary 1988). The complete cleavage of complex structures of β-1,4-mannan requires the combined action of β-mannanase as well as β-mannosidase and β-glucosidase, with branching enzymes such as α-galactosidase and acetyl esterase (Gübitz et al. 1996). β-mannanases hydrolyse linear mannan to yield short chain manno-oligosaccharides (Arcand et al. 1993). These sugars can then be further hydrolysed to mannose by β-mannosidases.

β-mannanases are primarily produced by bacteria (Veith et al. 2004; Bicho et al. 1991; Braithwaite et al. 1995; Cann et al. 1999; Ethier et al. 1998; Marga et al. 1996, Hatada et al. 2005) and hemicellulolytic fungi (Tang 2001; Ferreira and Filho 2004; Hägglund et al. 2003; Ademark et al. 1999; Stålbrand et al. 1993). A few mannanases have been purified and characterised from higher organisms such as mollusca (Xu et al. 2002a). β-

mannanases are classified in either the family 5 (GH5) or family 26 (GH26) glycosyl hydrolases, according to the classification of Henrissat and Bairoch (1993). Members of GH26 are exclusively prokaryotic, whereas GH5 are derived from both bacteria and fungi (Hogg et al. 2003). The recently isolated β-mannanase from the blue mussel *Mytilus edulis* shows significant sequence identity to GH5 mannanases (Xu et al. 2002b)

Many mannanases are modular, containing an N-terminal catalytic module and a C-terminal carbohydrate binding module (CBM) connected by a linker region (Stålbrand et al. 1995; Sunna et al. 2001; Stoll et al. 2000). These CBM's have also been found in other types of hemicellulolytic enzymes (Black et al. 1996; Millward-Sadler et al 1994). Most research on the effect of CBM's has been carried out with xylanases, and it has been postulated that the presence of the CBM in the *Trichoderma reesei* β-mannanase is part of the reason for its superior performance in pulp bleaching experiments, when compared to some other β-mannanases (Suurnäkki et al. 1996). The CBM of this enzyme had been found to bind to cellulose (Hägglund et al. 2003). The CBM does not affect the hydrolysis of soluble mannan-polymers, but seems to play an important role in the hydrolysis of mannan/cellulose complexes. Intact β-mannanase binds to the complex, but β-mannanase with the CBM removed does not. It therefore appears that the CBM facilitates hydrolysis through some interaction with the cellulose component of the complex. Since mannanase and cellulose are closely associated in plant cell walls, it is plausible that the binding of the β-mannanase to cellulose facilitates the degradation of the mannose/cellulose complex by increasing the substrate proximity.

β-mannanases are used in the food and pharmaceutical industry (Christgau et al. 1994a; McCleary 1990), as well as in the paper industry (Paice and Jurasek 1984). The β-mannanase from *T. reesei* has been shown to facilitate bleaching (Buchert et al. 1993). Mannanase can act synergistically with xylanases as biological pre-bleaching agents for softwood pulp, allowing a significant reduction of environmental pollutants, compared to the use of chlorine-based chemical agents (Suurnäkki et al. 1996) The enzymatic saccharification of lignocellulosic biomass has potential applications in fuel production, solid waste disposal, animal feed and in the textile industry (Hägglund et al. 2003; Wong

and Saddler 1993). Mano-oligosaccharides produced by the enzymatic hydrolysis of β-mannan are used in food and feed-processing industries (Hossain et al. 1996). These mano-oligosaccharides are utilised selectively by intestinal *Bifidobacterium* species, which cause a reduction in serum cholesterol and triglyceride levels as well as a lowering of blood pressure (Mitsuoka 1990).

β-mannanases can reduce the viscosity of instant coffee (Wong and Saddler 1993; Sachslehner et al. 2000). Mannan is the main polysaccharide component of coffee extract, which along with arabinogalactan and cellulose, comprises almost half its dry weight. The major polysaccharide is a water-insoluble mannan that forms approximately 20-30% of the dry weight of Arabica and Robusta coffees. The mannan present after roasting negatively affects the processing of instant coffee, as it causes high viscosity of the coffee extract when it is concentrated before spray- or freeze-drying. The reduction of viscosity by cleaving the mannan portion with mannanases would simplify the production of instant coffee by improving the effectiveness with which the extracts can be concentrated and by reducing energy costs.

Heterologous expression of mannanases

Table 8 summarises some of the examples from literature of β-mannanases that have been successfully expressed heterologously.

Table 8. Examples of heterologously expressed endo-1,4-β-mannanase genes

Source	Host	Enzyme Activity	Production levels (mg/L)	Comments	Reference
Aspergillus aculeatus KSM510	*A. oryzae* A 1560	Spec act 60 µmol/ min/mg enzyme		Extracellular	Christgau et al. 1994b
A. aculeatus Iizuka	*S. cerevisiae* Y294	521 nkat/ml ($ADH2_p$) 379 nkat/ml ($PGKI_p$)	118 86	Extracellular	Setati et al. 2001
Trichoderma reesei Rut30	*S. cerevisiae* DBY746	0.22 nkat/ml	150	Encodes 2 isozymes, pI = 4.6, 5.4	Stålbrand et al. 1995
T. reesei Rut30	*T. reesei* QM9414	20-30 nkat/ml	nd [1]	CBM removed, activity similar on locust bean gum, reduced activity of mannan/cellulose complex	Hägglund et al. 2003

Source	Host	Enzyme Activity	Production levels (mg/L)	Comments	Reference
Mytilus edulis (blue mussel)	*P. pastoris* KM71H	4.1 U.ml	900	Extracellular, *S. cerevisiae* MF-α secretion signal	Xu et al. 2002b
Bacillus sp. JAMB-750	*B. subtilis* ISW1214	Spec act 36.3 U/mg	nd	Alkaline: optimal pH 10, extracellular	Hatada et al. 2005
B. subtilis Z-2	*E. coli* BL21(DE3)	20 U/ml	nd	Both intra- & extracellular, *pel*B signal sequence	Zhang et al. 2006
Bacillus stearo-thermophilus ATCC12016	*E. coli* BL21	Spec act 384 U/mg	25	Intracellular, his-tagged protein	Ethier et al. 1998
Caldocellum saccharolyticum	*E. coli* RR28	Spec act 6.8 U/mg,	nd	intracellular, thermophilic; temperature optimum 80°C	Lüthi et al 1991
	E. coli RR28	9.8 U/ml	nd		Bicho et al. 1991
	E. coli PB1427	Spec act 27 U/mg	nd	Intracellular	Gibbs et al. 1992
Streptomyces lividans 66 strain 1326	*S. lividans* IAF10-164	Spec act 876 U/mg 87 U/ml	nd	extracellular	Arcand et al. 1993
	S. lividans 66 strain 1326	115 U/ml	nd	Replacement of natural promoter of above construct with *lac* promoter	Marga et al 1996
Pseudomonas fluorescens subsp. *cellulose*	*E. coli* XL1-Blue	Spec act 4 U/mg	nd	Intracellular	Braithwaite et al. 1995
Thermoanaero-bacterium polysaccharo-lyticum	*E. coli* JM109	Spec act 1412 U/mg	nd	Intracellular	Cann et al. 1999

[1] not determine

Research presented in this dissertation

In Chapter 5, the *Aspergillus aculeatus man1* gene, encoding the β-mannanase Man5A (Setati et al. 2001), was expressed in *Y. lipolytica* and *A. niger*[2]. Extracellular β-mannanase activities were compared and shake flask cultivations, as well as batch and fed-batch fermentations were carried out with both to determine the best heterologous Man5A producer.

[2] The construction of the Man1-producing *A. niger* strain was done by Dr Shaunita Rose of the University of Stellenbosch, South Africa. The construction of the *Y. lipolytica* Man1 production strains was done by the author. Further optimisation of the Man1 production by these strains was done by the team at CSIR.

B.4. Laccase

Laccase (benzenediol: oxygen oxidoreductase; *p*-diphenol oxidase, EC 1.10.3.2) was first discovered in 1883 in the Chinese lacquer tree (*Rhus vernicifera*), where it was found in the white latex produced by these trees. In the presence of this enzyme, the phenols in the latex are rapidly oxidised to radicals, which spontaneously polymerise to form the black lacquer (Burges 1963). The blue copper-containing oxidase enzyme was named 'laccase' about 10 years later after isolation and purification of the responsible catalyst.

Laccase is one of a small group of enzymes called the large blue copper proteins or the blue copper oxidases. These proteins contain multinuclear copper moieties that result in the characteristic blue colour (Thurston 1994). Other members of this protein group are cytochrome C oxidase, the mammalian plasma protein ceruloplasmin and plant ascorbate oxidases (Thurston 1994; Yaropolov et al. 1994).

Laccase is widely distributed in plants and fungi (Messerschmidt and Huber 1990). In fungi, laccase is present in Ascomycetes, Basidiomycetes and Fungi Imperfecti, and is particularly abundant in white-rot fungi that degrade lignin (Mayer and Harel 1979). Their postulated biological functions include polymerisation and depolymerisation of lignin and its monomers, as well as a plant defence mechanism to protect against pathogens. Because of the non-specific action of these enzymes, they have been investigated for use in the degradation of certain xenobiotic compounds. Phenolic-based xenobiotics possess many structural similarities to lignin (Boominathan and Reddy 1992). This makes it possible to use enzymes that degrade lignin for the degradation of pollutants that may be resistant to other means of degradation. There are also other possible uses for these enzymes and the organisms that produce them. These include biopulping and biobleaching in the pulp and paper industry, upgrading agricultural residues as feedstocks and detoxification of lignocellulosic hydrolysates. Among the best characterised laccases are those of the white-rot fungus *Trametes versicolor*. *T. versicolor* laccases have been shown to be capable of degrading polycyclic aromatic hydrocarbons (PAHs), polychlorophenols, anthracene, benzo[*a*]pyrene and other phenolic

compounds (Ullah et al. 2000; Majcherczyk 1998; Johannes et al. 1996; Collins et al. 1996; Roy-Arcand and Archibald 1991).

Biotechnological and environmental applications require large amounts of enzymes. Laccases secreted from wild-type fungal organisms may not be suitable for commercial purposes; therefore, it is thought that their heterologous expression in different hosts could be a valuable approach to solve these problems.

Biological role

Lignin is a unique biopolymer. Unlike other naturally occurring biopolymers such as starch, cellulose, proteins and nucleic acids, it does not contain identical, readily hydrolysable, repeating linkages that occur at regular intervals. Instead, it is a highly irregular, three-dimensional polymer that has no precise chemical structure, but which contains a series of substructures that occur at random and that are different in lignins from various sources (Boominathan and Reddy 1992). These structural features, together with the large size of the lignin polymer (a molecular weight of 10^5 to 10^6 kDa) mean that lignin-degrading enzymes must be extracellular and relatively non-specific.

Plant tissues are a major source of organic matter in the biosphere, and it is thus not surprising that the ability to degrade lignin, cellulose and hemicellulose is wide-spread in fungi and bacteria. Wood-degrading fungi are the primary contributors to the degradation of wood in nature, secreting extracellular enzymes that degrade the polymeric components of wood cell walls (Glazer and Nikaido 1995). Laccases are thought to be nearly ubiquitous among fungi and are produced in multiple isoforms, depending on the fungal species and environmental conditions. Laccases have a broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups, and as such have the ability to react with the phenolic hydroxyl groups found in lignin (Youn et al. 1995). The rate of laccase production has been correlated to the rate of lignin degradation in *Pleurotus* species cultivated on ligninolytic media, and *Pleurotus* mutants lacking laccase activity have been found to degrade lignin poorly. It is likely that laccase co-operates

with other ligninolytic enzymes to degrade lignin (Youn et al. 1995). Youn et al. (1995) suggested that the initial attack on lignin by lignin-degrading enzymes may be enabled by other enzymes, *i.e.* cellulase and hemicellulase, allowing laccase (and lignin peroxidases and manganese peroxidase) to move progressively into cells. Tsujiyama et al. (1993) suggested a reaction scheme in which laccase can assist in the process of separating the carbohydrate moieties, allowing for subsequent degradation of the lignin (Figure 17).

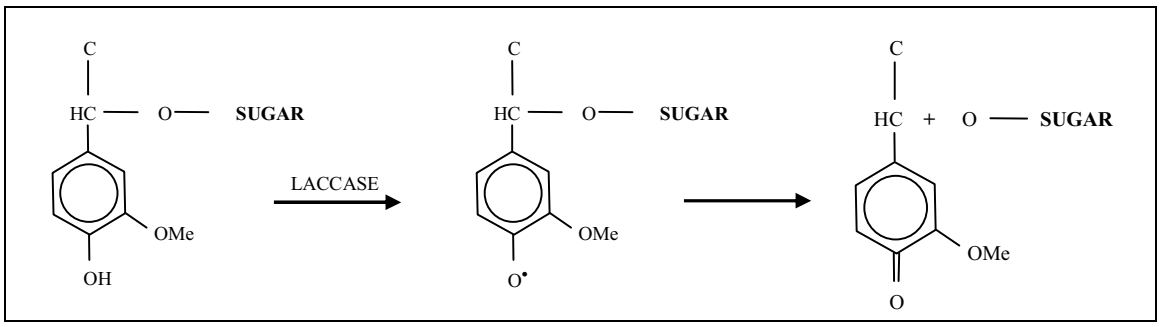

Figure 17. Reaction scheme of splitting covalent bonds between lignin and hemicellulose by laccase treatment (Tsujiyama et al. 1993).

The great abundance of laccases in wood-rotting basidiomicetous fungi seems to indicate that the main role of fungal laccases is to depolymerise lignin. However, this function contrasts with that of laccases in plants. Evidence has shown that laccases in plants are associated with the process of lignin polymerisation. Several studies indicated that laccases and laccase-like activities are closely correlated with lignin deposition in developing xylem (Davin et al. 1992, Dean and Eriksson 1994). Studies conducted *in vitro* on small lignin model compounds (Hatakka 1994; Youn et al. 1995) showed that the first step of laccase-mediated lignin degradation is an oxidative reaction with the loss of one electron from phenolic hydroxyl groups of lignin to produce phenoxy radicals (similar to the reaction mechanism shown in Figure 17 above). The radicals may spontaneously reorganise and give rise to the cleavage of alkyl side chains of the polymer. Concurrently, due to the polymerising activity of the enzyme, the low-molecular-weight products may form polymeric compounds as well. These two contrasting actions suggest that lignin biodegradation by laccase-producing fungi probably occurs in nature through more complicated enzymatic mechanisms, involving the synergistic effects of other enzymes (peroxidases, glyoxal oxidase, glucose oxidase,

aryl alcohol oxidase) and non-enzymatic components that interact to establish equilibrium between enzymatic polymerisation and lignin depolymerisation. Use of a mediator enhanced the delignification process using the laccase from *Trametes villosa*, using a mechanism as shown in Figure 18, for the oxidation of model lignin substrate (Barraca et al. 2003; Bourbonnais et al. 1995).

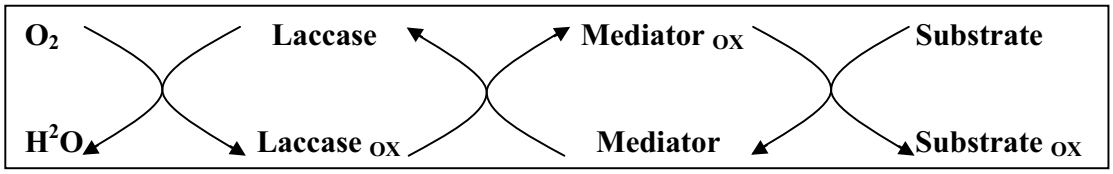

Figure 18. The role of a mediator in laccase activity.

Structure and mechanism of action of laccases

Both plant and fungal laccases are glycosylated enzymes, the former ones showing a higher extent of glycosylation (22-45%) than the latter (10-25%) (Brown et al. 2002). The range of substrates laccases can oxidize is very wide. Reports on molecular weight, pH optima, substrate specificity and other properties of laccases show extreme diversity (Hoshida et al. 2001; Niku-Paavola et al. 2004; Sulistyaningdyah et al. 2004; Shleev et al. 2004). Baldrian (2006) conducted an analysis of known fungal laccase enzymes and summarised the properties of laccases. The molecular weight varies from 43 to 383 kDa with a median of 66 kDa, temperature optima in the range of 25ºC to 80ºC with an average of 55ºC, pH optima of 2.0 – 5.0 with ABTS (2,2'-azino-*bis*(3-ethylbenz-thiazoline-6-sulphonic acid) as substrate (average 3.0) and 3.5 to 7.0 with syringaldazine (average 6.0). Laccases have been isolated as monomeric, dimeric or even as tetrameric proteins in their active holoenzyme form, showing generally an acidic pI, and usually containing four copper atoms per monomer (Solomon et al. 1996). The copper centres are differentiated as the type 1 (T1) or blue copper centre, type 2 (T2) or normal copper, and type 3 (T3) or coupled binuclear copper centres. The four coppers are distributed between a mononuclear site (one type-1 copper) and a trinuclear site (one type-2 and two type-3 coppers). The type 1 copper centres are responsible for intense electronic absorption band near 600 nm, which is responsible of the deep blue colour of the

enzymes. However, the occurrence of laccase enzymes which lack the typical absorption around 600 nm has been reported. For example, a ‘‘white’’ laccase (containing 1 copper, 1 iron and 2 zinc atoms) has been purified from *Pleurotus ostreatus* (Palmieri et al. 1997), while ‘‘yellow’’ laccases (containing copper but in an altered oxidation state) have also been reported (Leontievsky et al. 1997a; 1997b).

A multiple sequence alignment by Kumar et al. (2003) of more than 100 fungal and plant laccases showed four sequence regions (L1-L4), distinguishing laccases within the broader class of multi-copper oxidases (see Table 9). L2 and L4 are signature sequences of multi-copper oxidases, and L1 and L3 are distinctive to laccases. The 12 amino acid residues in the enzymes serving as the copper ligands are housed within these conserved regions. The amino acid ligands of the trinuclear cluster (eight histidines) occur in a highly conserved pattern of four HXH motifs. In one of these motifs (L4), X is the cysteine bound to the T1 copper while each of two histidines is bound to one of the two type 3 coppers.

Table 9. Copper signature sequences (Kumar et al. 2003)

Nomenclature	Observed signature sequences in laccases[a]
L1	$^{2}\mathbf{H}$-W-$^{3}\mathbf{H}$-G-X_9-D-G-X_5-Q-C-P-I
L2	G-T-X-W-Y-$^{3}\mathbf{H}$-$^{3}\mathbf{S}$-$^{3}\mathbf{H}$-X_3-Q-Y-C-X-D-G-L-X-G-X-(FLIM)
L3	$^{1}\mathbf{H}$-P-X-$^{2}\mathbf{H}$-$^{3}\mathbf{L}$-$^{3}\mathbf{H}$-G-H
L4	G-(PA)-W-X-(LFV)-$^{3}\mathbf{H}$-$^{1}\mathbf{C}$-$^{3}\mathbf{H}$-I-D-A-E-X-$^{1}\mathbf{H}$-X_3-$^{1}\mathbf{G}$-(LMF)-X_3-(LFM)

[a] Conserved regions that are at least eight residues in length are shown. Each region houses at least one residue that is ligated with copper. Residues associated with the different types of copper are indicated with 1,2, or 3 above the relevant residue.

Reduction of oxygen to water by laccase is accompanied by the oxidation of a substrate, typically a phenolic substrate (Thurston 1994). The T1 Cu site functions as the primary electron acceptor, extracting electrons from the reducing phenol substrate, and delivering them to the trinuclear site. The trinuclear centre, the binding site for the second substrate (O_2), accepts electrons from the T1 site for reduction.

Laccases are remarkably non-specific as to their reducing substrates, and the range of substrates oxidised varies from one laccase to another. They can oxidise polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds, but not tyrosine (Thurston 1994). Laccase is joined with catechol oxidase (*o*-diphenol: oxygen oxidoreductase, EC 1.10.3.1) as EC 1.14.18.1 (monophenol monooxygenase) (Mayer and Harel 1979). This nomenclature can be misleading as catechol oxidase is quite distinct from laccase. Catechol oxidase is also referred to as phenolase, polyphenol oxidase, tyrosinase (because of its ability to oxidise tyrosine), catecholase or cresolase. Laccase oxidises a wide range of substrates including monophenols, triphenols and ascorbic acid, as well as *o*- and *p*-diphenols. The name *p*-diphenol oxidase for laccase can therefore be misleading. However, its ability to oxidise these *p*-diphenols is diagnostic. Catechol oxidase cannot oxidise *p*-diphenol under any circumstances.

Laccases catalyse the four one-electron reduction of O_2 to water with four concomitant one-electron oxidations of a reducing substrate. A general reaction scheme has been proposed (Holm et al. 1996):

$$4\ RH + O_2 \rightarrow 4\ R^{\cdot} + 2H_2O$$

The oxidation of a reducing substrate typically involves the formation of a free (cation) radical after the transfer of a single electron to laccase. The radical can undergo further laccase-catalysed oxidation (*e.g.*, to form quinone from phenol) or non-enzymatic reactions (*e.g.*, hydration, polymerization or disproportionation [undergoing both oxidation and reduction]) (Figure 19).

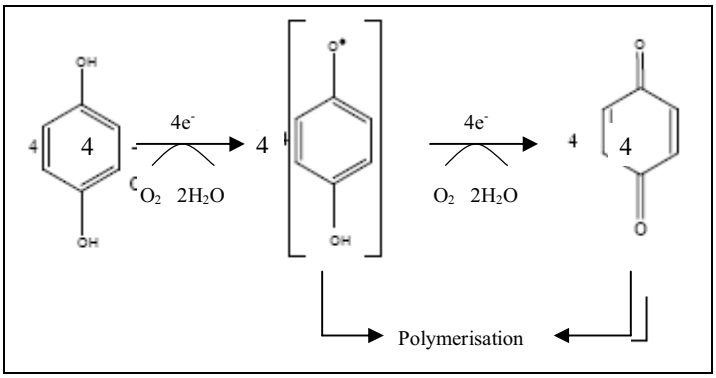

Figure 19. A typical laccase-catalysed reaction involves the formation of a free radical after the transfer of a single electron to laccase. The radical can undergo further laccase-catalysed oxidation (*e.g.*, to form quinone from phenol) or non-enzymatic reactions such as polymerisation.

The optimal conditions for laccase depend on the source of the enzyme. Laccase from the fungus *Coriolus sanguinea* has an optimum of pH 5.0 (Nishizawa et al. 1995), *Coriolus hirsutus* has a reported pH optimum of 3.5-4.5 (Yarolopov et al. 1994). The pH optimum of laccases may also vary depending on the substrate used. Laccase produced by *P. ostreatus* had an optimal pH range of 3.0-3.5 when ABTS was used, and 5.0-6.0 and 6.0-7.0 when guaiacol and syringaldazine, respectively, were used (Palmieri et al. 1993). Similarly, laccase from *Coriolus villosa* had optimal activity at pH 5.0-5.5 with syringaldazine, and at pH ≤2.5 with ABTS as the substrate (Yaver et al. 1996). Most laccases from fungi are unstable at alkaline pH (Mayer and Harel 1979). In contrast, the laccase from the plant *Rhus vernicifera* was found to have a pH optimum between pH 6.8-7.4 (Benfield et al. 1964). The differences in pH optima between plant and fungal laccases may be point to the difference in the function of the enzyme, where the fungal laccase may be a mechanism for removing toxic phenols from the medium in which the fungi grow under natural conditions, while the plant enzyme may be involved in synthetic processes like lignin formation.

Various laccase enzymes also differ with regard to their optimum temperatures. Most laccases have relatively high temperature optima (>37°C). *N. crassa* laccase exhibits maximum activity at 60°C (Nishizawa et al. 1995), and the laccase in *P. ostreatus* is almost fully active in the temperature range 40-60°C, with maximum activity at 50°C (Palmieri et al. 1993). A laccase from an basidiomycetous isolate from the Eastern Cape

province of South Africa exhibited an optimum temperature of 70°C and was stable at 60°C for 9 hours (Jordaan and Leukes 2003; Jordaan et al. 2004). These thermostable enzymes are potentially important for industrial purposes.

Biocatalytic relevance of laccase

Although oxidation reactions are essential in several industries, most of the conventional oxidation technologies have various drawbacks: non-specific or undesirable side-reactions and use of environmentally hazardous chemicals (Rodriguez Couto and Toca Herrera 2006). This has hastened the search for new oxidation technologies based on biological systems with advantages over chemical oxidation, such as enzymes which are specific and biodegradable catalysts, with reactions carried out in mild conditions. Laccases are increasingly of interest for their oxidative properties in a variety of practical applications ranging from hair dyeing processes (Onuki et al. 2000; Pruche et al. 2000) to the generation of wood adhesives (Peshkova and Kaichang 2003). The reported redox potentials of laccases are lower than non-phenolic compounds, so the enzymes cannot oxidise such substances. However, it was shown that in the presence of small compounds capable of acting as electron transfer mediators, laccases are able to oxidise non-phenolic structures (Bourbonnais and Paice 1990; Call and Mücke 1997; Srebotnik and Hammel 2000), expanding the range of compounds that can be oxidized by such enzymes.

Biopulping: About 25% of the wood pulp produced in the world is created using a mechanical pulping method, which has twice the yield of chemical pulping. In 2000, the world wood pulp production has been estimated at 135,852,000 metric tons, from which 6.3% was mechanical pulp, 15.6% thermomechanical pulp, 3.6% semimechanical pulp, and 74.5% chemical pulp (Food and Agriculture Organization, 2001). Mechanical pulping has two disadvantages: it is energy intensive, and yields paper that is not as strong as paper produced from chemical processing. In many cases, chemical (Kraft) pulp is blended with mechanical pulp to add strength to the paper. However, chemical pulp is expensive and produces excessive amounts of air and water pollutants. Enzyme applications have been proposed for pulp and paper manufacture to enhance pulp

bleaching, pulp refining, deinking, cellulose purification, deposit control and papermaking (Wong and Mansfield 1999). One of the approaches to delignification of wood fibres for preparation of pulp has been the use of laccases (with or without a mediator) for this purpose (known as biopulping).

Ethanol production: To improve the production of fuel ethanol from renewable raw materials, a laccase from the white rot fungus *T. versicolor* was expressed in *S. cerevisiae* to increase its resistance to phenolic inhibitors present in lignocellulose hydrolysates (Larsson et al. 2001). The laccase-producing transformant had the ability to convert coniferyl aldehyde at a very fast rate, which enabled faster growth and ethanol formation, showing a definite advantage of using laccase-expressing yeast strains for producing ethanol from lignocellulose.

Bioremediation: Laccases have been reported to oxidize many recalcitrant substances, such as chlorophenols (Fahr et al. 1999; Grey et al. 1998; Ricotta et al. 1996; Roy-Arcand and Archibald 1991), lignin-related structures (Bourbonnais et al. 1996; Boyle et al. 1992), organophosphorous compounds (Amitai et al. 1998), non-phenolic lignin model compounds (Kawai et al. 1999; Majcherczyk et al. 1999) and phenols (Bollag et al. 1988; Xu et al 1996). Interestingly, the 'yellow' laccase of *P. tigrinus* was capable of oxidizing non-phenolic substrates without a mediator (Leontievsky et al. 1997a), whereas most of the blue laccase are reported to require the presence of a mediator to catalyse the oxidation. Laccases are also able to act on polycyclic aromatic hydrocarbons (PAHs) (Majcherczyk et al. 1998), which are common, persistent and recalcitrant environmental contaminants with a tendency to accumulate in the environment (Crawford and Crawford 1996; Harvey 1997). Many PAHs are toxic, carcinogenic and/or mutagens. They are generated by incomplete combustion of fossil fuels and other organic substances in petroleum refinery, manufactured gas production, wood treatment facilities and car exhausts as waste by-products (Durrant et al. 1999; Sayler et al. 1999).

Textile and food industries: Large amounts of structurally diverse dyes are used for textile, leather, paper or food dyeing. Azo dyes are commercially the most important

group of dyes, making up about half of the total amount used (Chivukula and Ranganathan 1995). The textile industry is by far the largest sector for dye utilisation (7 x 10^5 tons per annum) (Riu et al. 1998; Zollinger 2002). During processing, up to 15% of the used dyes are released into the process water (Vaidya and Datye 1982). All dyes used in the textile industry are designed to resist fading upon exposure to sweat, light, water, many chemicals including oxidizing agents and microbial attack. Dye-containing effluents are hardly decolourised by conventional biological wastewater treatments (Shaul et al. 1991; Willmott et al. 1998). In addition to their visual effect and their adverse impact in terms of chemical oxygen demand, many synthetic dyes are toxic, mutagenic and carcinogenic (Michaels and Lewis 1985; Chung et al. 1992). The class of micro-organisms most efficient in breaking down these synthetic dyes is the white-rot fungi (Wesenberg et al. 2003). This is based on their capacity to produce one or more extracellular lignin-modifying enzymes, which, due to their lack of substrate specificity, are also capable of degrading a wide range of xenobiotics. Recently, *Trametes hirsuta*, and a laccase purified from the fungus, were used to degrade triarylmethane, indigoid, azo and anthraquinonic dyes used in dyeing textiles (Abadulla et al. 2000), as well as 23 industrial dyes (Rodriguez et al. 1999).

Exploiting the same characteristic of the enzyme, laccases can be applied to processes that enhance or modify the colour appearance of food or beverages (Rodrigues Couto and Toca Harrera 2006). These include the elimination of undesirable phenolics responsible for browning, haze formation and turbidity in clear fruit juice, beer and wine.

Heterologous expression of laccase

Given the wide variety of applications of laccases, with just a few of those highlighted above, it is important for the exploitation of these capabilities of the enzyme that sufficient quantities of the purified enzyme or an enzyme-producing organism are available. This has lead to the cloning and functional expression of many different laccases in different expression hosts. Table 10 summarises some of the examples from literature for laccases that have been successfully expressed heterologously.

Table 10. Examples of heterologously expressed laccase genes from different sources

Source	Laccase	Host	Comments	References
C. cinereus	Lcc1	*A. oryzae*	Secreted, 8.0 - 135 mg.L^{-1}	Yaver et al. 1999
C. hirsutus	PO1, PO2	*S. cerevisiae*	Secreted	Kojima et al. 1990
C. subvermispora	lcs-1	*A. nidulans*	Secreted, 0.23 U.ml^{-1} (ABTS)	Larrondo et al. 2003
F. lignosus	Lcc	*P. pastoris*	Secreted, 9 U.ml^{-1} (ABTS)	Liu et al. 2003
G. arboreum	Lac1	*A. thaliana*	Secreted, conferring resistance to phenolic compounds of plant origin.	Wang et al. 2004
L. tulipifera	lcc2	Tobacco cells	Intracellular	LaFayette et al. 1999
M. albomyces	Lac1	*S. cerevisiae*	Secreted, 2.8 nkat.ml^{-1} (ABTS)	Kiiskinen and Saloheimo 2004
		T. reesei	160 nkat.ml^{-1}/230 mg.L^{-1} (flask), 250 nkat.ml^{-1}/920 mg.L^{-1} (fed-batch)	Kiiskinen et al. 2004
M. mediterranea	PpoA	*E. coli*	Production of recombinant protein, most activity located in the membrane fraction rather than in the soluble one.	Sanchez-Amat et al. 2001
M. thermophila	MtL	*A. oryzae*	Secreted, 0.85 U.ml^{-1} (SGZ)	Berka et al. 1997
		S. cerevisiae	Secreted, 0.6 U.L^{-1} (ABTS). Total activity was enhanced 170-fold by directed evolution.	Bulter et al. 2003
P. cinnabarinus	Lac1	*P. pastoris*	Secreted, 8.0 mg.L^{-1} of hyperglycosylated laccase (ABTS)	Otterbein et al. 2000
		A. niger	Secreted, ~7,000 U.L^{-1} (ABTS) with GLA prepro sequence	Record et al. 2002
			Secreted, ~90 U.L^{-1} (ABTS) with *lac1* signal sequence	
			Secreted, 7,118 U.L^{-1} (ABTS)	Sigoillot et al. 2004
		A. oryzae	Secreted, 8,061 U.L^{-1} (ABTS)	Sigoillot et al. 2004
		Y. lipolytica	Secreted,7 nkat.ml^{-1} (ABTS)	Madzak et al. 2005
P. ostreatus	PoxC	*K. lactis*	Secreted, 0.067 U.ml^{-1} (ABTS)	Piscitilli et al. 2005
		S. cerevisiae	No detectable activity	Piscitilli et al. 2005
	POX 1b	*K. lactis*	Secreted, 3.9 U.ml^{-1} (ABTS)	Piscitilli et al. 2005
		S. cerevisiae	Secreted, 0.2 U.ml^{-1} (ABTS)	Piscitilli et al. 2005
P. radiata	PrL	*T. reesei*	Secreted, 7.7 nkat.ml^{-1}, 20 mg.L^{-1} (ABTS)	Saloheimo and Nikku-Paavola. 1991
P. sajor-caju	Lac4	*P. pastoris*	Secreted, 4.85 mg.l^{-1}	Soden et al. 2002
R. solani	Lcc1, lcc2, lcc4	*A. oryzae*	Secreted, lcc2 low activity suggesting instability	Wahleithner et al. 1996
S. commune	LacA	*A. sojae*	Secreted, 774 U.ml^{-1} (Gallic acid).	Hatamoto et al. 1999
S. lavendulae	STSL	*E. coli*	Intracellular, 30 mg.L^{-1}, low activity due to aggregation and precipitation.	Suzuki et al. 2003
S. tuberosum	PPO	*L. esculentum*	Resistance conferred to pathogen *P. syringae*	Li and Steffens 2002

Source	Laccase	Host	Comments	References
T. versicolor	Lcc1	*P. pastoris*	Secreted, 24 $U.L^{-1}$ (ABTS)	Jönsson et al. 1997; O'Callaghan et al. 2002
		P. pastoris	Secreted, 39 $mU.L^{-1}$	Gelo-Pujic et al. 1999
		Z. mays High II	50 ppm dry weight (up to 2% TSP), remainder immobilised in seed	Bailey et al. 2004
T. versicolor	lccα	*S. cerevisiae*	Secreted, 35 $\mu U.ml^{-1}$ (ABTS)	Necochea et al. 2005
	lcc2	*S. cerevisiae*	Secreted, 0.0022 $nkat.ml^{-1}$ (ABTS)	Cassland and Jönsson 1999; Larsson et al. 2001
	LacIIIb	*Y. lipolytica*	Laccase secreted activity of 0.23 $U.ml^{-1}$ (ABTS) 2.5 $mg.L^{-1}$	Jolivalt et al. 2004
	LccIII (*cvl3*)	*Nicotiana tabacum*	Secreted, in cell-free extracts of roots	Sonoki et al. 2004
		T. versicolor	Secreted,1 $mU.ml^{-1}$ (Guaiacol)	Kajita et al. 2004
	LccIV	*P. pastoris*	Secreted, ~1,500 $U.L^{-1}$ (ABTS).	Brown et al. 2002
		A. niger	Solid-state fermentation: 592 $U.L^{-1}$ Submerged ferm: 13 $U.L^{-1}$ (ABTS)	Téllez-Jurado et al. 2006
	lcc1	*P. methanolica*	Secreted, 12,600 $U.L^{-1}$	Guo et al. 2006
T. villosa	Lcc1	*A. oryzae*	Secreted activity	Yaver et al. 1996
Trametes sp. strain C30	Lac1	*S. cerevisiae*	Activity barely detectable in medium.	Klonowska et al. 2005
	Lac2	*S. cerevisiae*	Undetectable laccase activity in medium.	Klonowska et al. 2005
	Lac3	*S. cerevisiae*	Secreted, 0.5 $U.L^{-1}$ (SGZ), 2 $mg.L^{-1}$	Klonowska et al. 2005
Trametes sp. 420	LacD	*P. Pastoris*	8.3 x 10^4 $U.L^{-1}$ (ABTS)	Hong et al. 2007

Research presented in this dissertation

A deduction that can be made from the table above is that different expression systems and different expression control mechanisms within the same expression system can produce different levels of laccase. For example, the *P. cinnabarinus* laccase 1 (encoded by the *lac1* gene), is expressed at levels varying from 90 to 7,000 $U.L^{-1}$ in the same *A. niger* system, but with different secretion signals: 7,000 $U.L^{-1}$ when using the *A. niger* glucoamylase signal sequence versus 90 $U.ml^{-1}$ when the *lac1* signal peptide is present (Record et al. 2002). Sigoillot et al. (2004) also reported levels of 7,118 $U.L^{-1}$. LccIV from *T. versicolor* was expressed at a level of ~1,500 $U.L^{-1}$ in *P. pastoris* (Brown et al.

2002) and 13 $U.L^{-1}$ in submerged culture of *A. niger* (Téllez-Jurado et al. 2006), but reached levels of almost 600 $U.L^{-1}$ when cultivated in a solid-substrate fermentation.

Based on the information in the table, it can be postulated that *A. niger* gives the best expression of fungal laccases, and this theory is tested in the research presented in Chapter 5. The *lcc2* gene from *T. versicolor* was expressed in *A. niger* and *P. pastoris*[3], and levels of activity compared. The Lcc2 enzyme from the recombinant *A. niger* was subsequently purified and characterised in terms of molecular weight and glycosylation, and compared to the wild-type enzyme purified from *T. versicolor*. The results confirm the supposition that *A. niger* is a superior laccase producer. Expression of laccase from *T. versicolor* was optimised by Rancaño et al. (2003) in an airlift fermenter, using 2,5-xylidine as inducer. Activity levels of 1,500 $U.L^{-1}$ were reached. In solid-state fermentations, levels of 3,500 $U.L^{-1}$ were obtained (Rodriguez Couto et al. 2003). It will be shown that a level of 2,700 $U.L^{-1}$ was reached for the heterologous expression of the *T. versicolor* laccase in *A. niger* in shake flasks, implying further optimisation could push this figure even higher.

[3] The expression of *T. versicolor* laccases in *P. pastoris* was done by Christina Bohlin of Karlstad University

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Chapter 3.

Isolation of epoxide hydrolases, functional expression in *Yarrowia lipolytica*, evaluation of recombinant strains as whole-cell biocatalysts and comparison to recombinant *Saccharomyces cerevisiae* strains

by

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The construction of the *Y. lipolytica* strains was done by the author.
The *S. cerevisiae* strain was produced by Dr N. Rohitlall.
Mrs N. Abrahams kindly assisted with the assays.
Drs A. Botes and R. Mitra provided guidance and advice.

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1. Recombinant yeasts for synthesizing epoxide hydrolases.

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   Inventors:
   BOTES, Adriana Leonora; LABUSCHAGNE, Michel; ROTH, Robyn; MITRA, Robin Kumar; LOTTER, Jeanette; LALLOO, Rajesh; RAMDUTH, Deepak; ROHITLALL, Neeresh; SIMPSON, Clinton; VAN ZYL, Petrus.

2. Methods for obtaining optically active epoxides and diols from 2,3-disubstituted and 2,3-trisubstituted epoxides.

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   Inventors:
   BOTES, Adriana Leonora; MITRA, Robin Kumar; LOTTER, Jeanette; LABUSCHAGNE, Michel; ROTH, Robyn; ABRAHAMS, Nasreen; SIMPSON, Clinton; VAN DER WESTHUIZEN, Christopher; VISSER, Daniel.

Where relevant, results shown in this chapter that also appear in these patents are indicated.

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Abstract

Epoxide hydrolases (EHs; EC 3.3.2.3) are hydrolytic enzymes that convert epoxides to vicinal diols by ring-opening the epoxide in the presence of water. They are present in mammals, vertebrates, invertebrates, plants, insects and micro-organisms. Microbial EHs have been studied extensively in recent years for their potential application as biocatalysts for the production of optically active epoxides and vicinal diols. In general, wild-type microbial cells as biocatalysts have intrinsic limitations as they have only small amounts of EHs in the cells. Purification of enzymes to homogeneity may be costly and time-consuming. A compromise between the advantages and drawbacks of wild-type whole-cell and purified enzyme biocatalysts is the use of recombinant whole-cell biocatalysts expressing heterologous proteins at a high level.

Here, the isolation of two new EH genes from *Rhodosporidium toruloides* NCYC 3181 and *Cryptococcus curvatus* NCYC 3158, and the expression of other EHs from a variety of sources in the yeast *Yarrowia lipolytica* is described. EHs that cluster into the microsomal epoxide hydrolase family along with mammalian EHs include the fungal enzymes from *Rhodotorula* and *Rhodosporidium* genera, as well as *Aspergillus niger*, *Cryptococcus neoformans* and the cabbage looper, *Trichoplusia ni*. The soluble, cytosolic EH superfamily is represented by the enzymes from *Solanum tuberosum* and *Agrobacterium radiobacter*. Kinetic resolution of a number of different classes of epoxide substrates was achieved using *Y. lipolytica* transformants as whole-cell biocatalysts. The presence of a secretion signal was investigated, and wass found to affect both enantioselectivity and activity of the biocatalysts. The use of *Sacchromyces cerevisiae* as a whole-cell biocatalyst was also investigated and compared to *Y. lipolytica*. The broad applicability of *Y. lipolytica* as an effective expression system for EHs is shown, with a number of different EHs from various sources being successfully expressed and used for biotransformations.

Introduction

Epoxide hydrolases (EHs; EC 3.3.2.3) are hydrolytic enzymes that convert epoxides to diols by ring-opening, in the presence of water. They are present in mammals, vertebrates, invertebrates, plants, insects and micro-organisms (Orru and Faber 1999). Microbial epoxide hydrolases have been studied extensively in recent years for their potential application as biocatalysts for the production of optically active epoxides and diols. The application of enzymes for the production of these enantiopure building blocks represents an alternative to asymmetric organic syntheses and may surpass the purely chemical processes in yield, enantiomeric purity of the product and environmental friendliness (Kotik et al. 2005)

EHs from a variety of sources have been expressed in a number of hosts, including *Escherichia coli*, *Saccharomyces cerevisiae* and *Pichia pastoris*, with varying success. There are potential problems associated with the expression of EHs in some of these expression systems. As a prokaryotic host, *E. coli* lacks the cellular machinery to perform glycosylation and post-translational modifications required for the correct folding of proteins of eukaryotic origin, which leads to altered 3-D structures of the recombinant protein. A true evaluation of the efficiency of epoxide hydrolases is thus not possible, as exemplified by the decrease in enantioselectivity observed for yeast epoxide hydrolases expressed in this host (Visser et al. 2002). Koschorreck et al. (2005) performed statistical calculations, which predicted that the majority of the α/β-hydrolase fold enzymes, which include EHs, would be expressed as insoluble aggregates in *E. coli*. Most of the recombinant microsomal EHs (mEHs) produced in this host were produced as insoluble aggregates in inclusion bodies, despite efforts to maintain solubility. These efforts included the presence of chaperones, different induction protocols and lower protein production temperature (Visser et al. 2003, Rink et al. 1997a; Arand et al. 1999a; Kim et al. 2005).

The yeasts *S. cerevisiae* and *P. pastoris* were also employed for the heterologous expression of EH-encoding genes. *S. cerevisiae* has been used for the functional

expression of rat and human mEH (Arand et al. 1999b; Kelly et al. 2002), while the methylotrophic *P. pastoris* has been used for the production of EHs of fungal (Lee et al. 2004) and plant origin (Bellevik et al. 2002a; 2002b). Fungal mEHs from *Xanthophyllomyces dendrorhous, Aspergillus niger, Rhodotorula* and *Rhodosporidium* have been expressed in *E. coli*, *S. cerevisiae* or *P. pastoris* with varying success (Visser et al. 1999; 2000; 2002; 2003; Arand et al. 1999a; Lee et al. 2004). These conventional yeasts have some limitations as expression systems, such as hyperglycosylation, low activity due to incorrect folding, and loss of enantioselectivity of the secreted protein.

Baculovirus-infected insect cells have also been employed for the heterologous expression of some cytosolic EHs (soluble, sEHs) from plant origin, but performed poorly in the expression of functional EHs from insects such as *Trichoplusia ni* and *Drosophila melanogaster* (VanHook Harris et al. 1999; Taniai et al. 2003). This expression system is also unsuitable for the practical large scale production of heterologous proteins. The sEHs from the plants *Arabidopsis thaliana* and *Solanum tuberosum* have been produced in *E. coli* and insect cells (Kiyosue et al. 1994; Morriseau et al. 2000; Elfström and Widersten 2005; Cao et al. 2006).

Yarrowia lipolytica is a dimorphic yeast that belongs to the non-conventional class of yeasts, which has been used in GRAS industrial processes, and for which a large number of molecular tools are available (Madzak et al. 2004). *Y. lipolytica* diverges greatly from other ascomycetous yeasts in its: (i) high GC content, (ii) high frequency of introns, often of a relatively large size, (iii) unusual structure of its rDNA genes, (iv) relatively low level of similarity of its genes with their counterparts in other yeasts, and (v) unusual types of transposable elements (Kurtzman and Phaff 1987; van Heerikhuizen et al. 1985; Naumova et al. 1993). It has been used successfully for the expression of a number of different enzymes, and shows great secretion capacity (Titorenko et al. 1997). Secretion signals from the alkaline extracellular protease (AEP, encoded by the *XPR2* gene) and LIP2 (lipase) proteins have been used to direct secretion of heterologous proteins in *Y. lipolytica* (Nicaud et al. 2002). The intact native *XPR2* promoter was initially used for expression of heterologous proteins (Hamsa and Chattoo 1994) but its complex regulation

imposed a restriction on general industrial use (Gellissen et al. 2005). Consequently, a hybrid hp4d promoter ($hp4d_P$) was constructed, which drives growth phase-dependent gene expression, since $hp4d_p$-driven heterologous gene expression was found to occur at the beginning of the stationary phase (Madzak et al. 2000a; Madzak et al. 2000b). This promoter is no longer repressed by carbon or nitrogen sources, nor by acidic conditions. This allows the separation of biomass accumulation and protein production. Multi-copy vectors containing the defective *ura3d4* marker, is required in multiple copies to complement the uracil auxotrophy of host strain *Y. lipolytica* Po1h, allowing for selection of transformants with multiple inserts. Recently, an epoxide hydrolase from *Rhodotorula mucilaginosa* was expressed in *Y. lipolytica* as a single-copy integrant, with the resultant recombinant whole-cell activity and selectivity for the epoxide substrate (2,3-epoxypropyl)benzene far superior to any other activity and selectivity reported in literature using wild-type organisms (Labuschagne and Albertyn 2007).

This chapter covers the isolation of a mEH gene (*eph*) from the yeasts *Rhodosporidium toruloides* 1 (NCYC 3181), which displays 92% similarity on a nucleotide level to *R. toruloides* CBS349 *eph* (Genbank accession number AF416991) and a strain originally identified as *Cryptococcus curvatus* Car054 (NCYC 3158). On sequence analysis, the gene's sequence was more similar to those of the *R. toruloides* species, rather than known *Cryptococococcus eph* genes. The identification of this strain is therefore uncertain. Also covered is the expression of a variety of different mEHs and sEHs in *Yarrowia lipolytica*, and screening for hydrolysis of several classes of epoxides using the recombinant *Y. lipolytica* strains. It will be shown that *Y. lipolytica* is a particularly useful whole cell biocatalyst for the hydrolysis of epoxides, as it can functionally express both mEHs and sEHs from bacterial, yeast, fungal, insect and plant origins. A single yeast mEH from *Rhodotorula araucariae* was expressed intracellularly in *S. cerevisiae*[1], and the functionality of the recombinant strain as a whole-cell biocatalyst was evaluated for comparison purposes.

[1] The *S. cerevisiae* strain was produced by Dr N. Rohitlall of CSIR Biosciences, with assistance from the author.

Materials and Methods

Strains and Media

The genotypes of the microbial strains and plasmids used in the present study are summarised in Table 1.

Table 1. Microbial strains and plasmids used in the present study

Strain or plasmid	Relevant genotype	Source or reference
Strains		
Crytoptococcus curvatus Car054		NCYC 3158
Rhodosporidium toruloides 1		UOFS Y-0517/ NCYC 3181
Rhodotorula araucariae 25		UOFS Y-0473/ NCYC 3183
Rhodosporidium toruloides 46		UOFS Y-0471
Escherichia coli TB1	F$^-$ *ara* Δ(*lac-proAB*) [Φ*80dlac* Δ(*lacZ*)*M15*] *rpsL*(*Str^R*) *thi hsdR* = JM83 *hsdR*	New England Biolabs, USA
Yarrowia lipolytica Po1h	*MATA,leu2-270, ura3-302 (= ura3::pXPR2:SUC2), uxpr2-322, axp1-2*. Phenotype: Leu$^-$, Ura$^-$, ΔAEP, ΔAXP, Suc$^+$	Madzak (2003) CLIB 882
Y. lipolytica Po1g	*MATA, leu2-270, ura3-302::URA3, xpr2-322, axp1-2* Phenotype: Leu$^-$, ΔAEP, ΔAXP, Suc$^+$. pBR docking platform	Madzak et al. (2000a) CLIB 725
S. cerevisiae INVSc1	*MATa ura3-52 his3Δ1 leu2 trp1-289 / MATα ura3-52 his3Δ1 leu2 trp1-289*	Invitrogen, USA
Y. lipolytica Po1h transformants*:* all contain multi-copy integrations of hp4d$_P$ – *eph* gene - *LIP2*$_T$		
YL-Car054-HmA	*eph* from *C. curvatus* Car054	This work
YL1HmA	*eph* from *R. toruloides* UOFS Y-0517	This work
YL23HmA	*eph* from *Rhodotorula mucilaginosa* UOFS Y-0198 (CBS 8596)	This work
YL25HmA	*eph* from *R. araucariae* UOFS Y-0473	This work
YL46HmA	*eph* from *R. toruloides* UOFS Y-0471	This work
YL692HmA	*eph* from *Rhodosporidium paludigenum* UOFS Y-0482	This work
YL777HmA	*eph* from *Cryptococcus neoformans* CBS132	This work
YL-Ar-HmA	*eph* from *Agrobacterium radiobacter* AD1	This work
YL-St-HmA	*Solanum tuberosum* synthetic *eph*, codon-optimised for *Y. lipolytica*	This work
YL-An-HmA	*eph* from *Aspergillus niger* similar to AY966486	This work
YL-Tn1-HmA	*eph* from *Trichoplusia ni* TNU73680 = Tn1	This work
YL-Tn2-HmA	*eph* from *T. ni* AF035482 = Tn2	This work
YL25HmL	*eph* from *R. araucariae* UOFS Y-0473, *LIP2* secretion signal	This work
YL46HmL	*eph* from *R. toruloides* UOFS Y-0471, *LIP2* secretion signal	This work
YL692HmL	*eph* from *R. paludigenum* UOFS Y-0482, *LIP2* secretion signal	This work
S. cerevisiae InvSc1 transformants*:*		
S. cerevisiae Sc25	*GAL1*$_P$ - *R. araucariae* UOFS Y-0473 *eph* – *CYC1*$_T$	This work

Strain or plasmid	Relevant genotype	Source or reference
Plasmids		
pBluescript SK+	*bla*	GenBank X52328
pGEM®-T Easy	*bla*	Promega, USA
pYES2	*E. coli* / *S. cerevisiae* shuttle vector. *URA3*, *bla*, $GAL1_P$ $-CYC1_T$	Invitrogen, USA
pINA1291	*E. coli* / *Y. lipolytica* vector. *ura3d4* (*Y. lipolytica*), Kan^R, *ura3d4*, $hp4d_P$-$LIP2_T$, *zeta*, multi-copy random integration vector for Po1h	Nicaud et al., 2002
pINA1293	As pINA1291, with *Y. lipolytica LIP2* secretion signal	Nicaud et al., 2002
pGEM-T:23	*R. mucilaginosa* UOFS Y-0198 *eph* (*Bgl*II, *Bln*I)	Labuschagne and Albertyn 2007
pGEM-T:25	*R. araucariae* UOFS Y-0473 *eph* (*Bam*HI, *Bln*I)	Labuschagne 2003
pGEM-T:46	*R. toruloides* UOFS Y-0471 *eph* (*Bam*HI, *Bln*I)	Labuschagne 2003
pGEM-T:692	*R. paludigenum* UOFS Y-0482*eph* (*Bam*HI, *Nhe*I)	Labuschagne 2003
pGEM-T:777	*C. neoformans* CBS132 *eph* (*Bam*HI, *Nhe*I)	Labuschagne 2006
pGEM-T:Ar	*A. radiobacter* AD1 *echA* (*Bam*HI, *Bln*I)	Labuschagne 2006
pPCR-Script:St	*S. tuberosum* Lemhi Russet *eph*, codon-optimised for *Y. lipolytica*, in pPCR-Script, based on STU02497	GENEART AG, Germany
pGEM-T:An	*A. niger eph* (similar to AY966486) (*Bam*HI, *Bln*I)	Labuschagne 2006
pGEM-T:Tn1	*T. ni* TNU73680 *eph* Tn1 (*Bam*HI, *Bln*I)	Labuschagne 2006
pGEM-T:Tn2	*T. ni* AF035482 *eph* Yn2 (*Bgl*II, *Bln*I)	Labuschagne 2006
pBSK:Car054	*C. curvatus* Car054 *eph* cDNA gene	This work
pBSK:1	*R. toruloides* UOFS Y-0517 *eph* cDNA gene	This work
pYES2:25	$GAL1_P$ – *R. araucariae* UOFS Y-0198 *eph* – $CYC1_T$, *URA3*	This work
pINA1291 constructs:	all contain $hp4d_P$ – *eph* gene - $LIP2_T$, *ura3d4*, Kan^R	
pYL-Car054-HmA	*C. curvatus* NCYC 3158 *eph*	This work
pYL1HmA	*R. toruloides* UOFS Y-0517 *eph*	This work
pYL23HmA	*R. mucilaginosa* UOFS Y-0198 *eph*	This work
pYL25HmA	*R. araucariae* UOFS Y-0473 *eph*	This work
pYL46HmA	*R. toruloides* UOFS Y-0471 *eph*	This work
pYL692HmA	*R. paludigenum* UOFS Y-0482 *eph*	This work
pYL777HmA	*C. neoformans* CBS132 *eph*	This work
pYL-Ar-HmA	*A. radiobacter* AD1 *echA*	This work
pYL-St-HmA	*S. tuberosum* soluble synthetic *eph* gene	This work
pYL-An-HmA	*eph* gene from *A. niger*, similar to AY966486	This work
pYL-Tn1-HmA	Tn1 *eph* gene from *T. ni* (TNU73680)	This work
pYL-Tn2-HmA	Tn2 *eph* gene from *T. ni* (AF035482)	This work
pINA1293 constructs:	all contain $hp4d_P$ - *LIP2* secretion signal – *eph* gene - $LIP2_T$, *ura3d4*, Kan^R	
pYL25HmL	*R. araucariae* UOFS Y-0473 *eph*	This work
pYL46HmL	*R. toruloides* UOFS Y-0471 *eph*	This work
pYL692HmL	*R. paludigenum* UOFS Y-0482 *eph*	This work

Nomenclature

Wild-type yeasts are referred to as 'WT' followed by the strain number (*e.g. R. araucariae* 25 → WT-25). *S. cerevisiae* recombinant strains are labelled 'SC-' followed by the original strain number of the wild-type gene source (*e.g. S. cerevisiae* containing the EH of *R. araucariae* 25 → SC-25). *Y. lipolytica* recombinant strains created by cloning *eph* genes into *Y. lipolytica* Po1h, are labelled 'YL-', followed by:

- The original strain number of the wild-type source strain (*e.g. R. araucariae* 25 → YL-25…);
- The promoter symbol: H (hp4d promoter) (*e.g.* H → YL-25H…);
- Copy status: m (multi-copy) (*e.g.* m → YL-25Hm…);
- Secretion signal status: L (*LIP2* present) or A (*LIP2* absent) (*e.g.* A → YL-25HmA).

Recombinant plasmids were constructed and amplified in *Escherichia coli* TB1. *E. coli* was cultivated at 37°C in LM (1% yeast extract, 1% tryptone, 0.5% NaCl) liquid medium on a rotary shaker at 200 rpm, or on LM agar (15 g/L agar), supplemented with the relevant antibiotics at a final concentration of 100 µg/ml ampicillin or 50 µg/ml kanamycin. 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 µg/ml 5-bromo-4-chloro-3-indolyl-β- D-galactopyranoside (X-GAL) were added for blue-white selection where necessary.

Cultivation

C. curvatus Car054 and *R. toruloides* 1 were cultivated in YPD (2% peptone, 2% glucose, 1% yeast extract) and the *Y. lipolytica* strains were cultivated in either YPD medium for nucleic acid isolation or on selective YNB_{casa} medium (2% glucose, 0.4% NH_4Cl, 0.2% casamino acids, 0.17% yeast nitrogen base without amino acids and without ammonium sulphate, 0.03% leucine) for transformant selection and heterologous protein production, with agar (15 g/l) for solid media. Yeast strains were cultured in 250 ml Erlenmeyer flasks containing 50 ml medium at 28°C on a rotary shaker at 200 rpm, unless otherwise indicated. Wild type strains were grown in GPP medium (50 mM phosphate buffer pH 7.5, 1% glycerol, 0.34% proteose peptone, 50 mg/l adenine and

0.34% yeast nitrogen base without amino acids and ammonium sulphate). *S. cerevisiae* transformants were cultivated in SC^{-URA} minimal media (0.67% yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 1% glucose, 0.01% of each of adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan and 0.005% of each of aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine and valine).

DNA Manipulations

Standard protocols were followed for DNA manipulations (Sambrook et al. 1989). The enzymes for DNA cleavage and ligation were purchased from Fermentas. Restriction endonuclease-digested DNA was eluted from agarose using the GFX™ PCR DNA and Gel Band Purification Kit. Dephosphorylation of vector DNA was carried out using New England Biolab's Antarctic Phosphatase prior to ligation. *E. coli* transformations were carried out using the Bio-Rad Gene-Pulser. Genomic DNA was isolated from *Y. lipolytica* strains using Promega's Wizard® Genomic DNA Purification Kit. Polymerase Chain Reaction (PCR) was done using an Eppendorf Mastercycler Gradient PCR machine. PCR products were purified using Roche's High Pure PCR Product Purification kit.

Genomic DNA Isolation from wild-type yeasts

C. curvatus Car054 and *R. toruloides* 1 were cultivated for 48 hours in YPD. The biomass from 2 ml culture was resuspended in 500 µl DNA lysis buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 1% SDS), and 200 µl glass beads (200 µm) were added. The tubes were vortexed for 4 min, with cooling on ice after each min. The liquid phase was mixed with 275 µl 7 M ammonium acetate (pH 7.0) and incubated for 5 min at 65°C, followed by 5 min on ice. 500 µl chloroform was subsequently added, the tubes vortexed briefly and centrifuged for 5 min at 13,000 rpm at 4°C. The DNA was precipitated from the supernatants by adding one volume of isopropanol, incubating for 5 min at room temperature, then harvesting the DNA by centrifugation for 5 min, 13,000 rpm at 4°C.

The DNA pellet was washed with 1 ml 70% ethanol and the DNA harvested as above. The pellet was dried in a vacuum drier and resuspended in 50 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) supplemented with 50 µg/ml RNase A.

C. curvatus Car054 *eph* PCR using Genomic DNA as Template

Because no sequence information for epoxide hydrolase genes from any *Cryptococcus* strains was available at the time, a selection of known *eph* sequences were used to design primers for PCR, and were used with genomic DNA of *C. curvatus* Car054 as the template. These primers used are shown in Table 2. Based on these results, the most suitable primers could be used to amplify the gene from cDNA.

Table 2. DNA sequences of the oligodeoxynucleotide primers used in the amplification of *C. curvatus* Car054 *eph* gene. Restriction enzyme sites are underlined in bold

Organisms on which primers were based	Name	5' -3' sequence	Genbank Accession numbers
R. toruloides CBS 349	EH1	GT**GGATCC**ATGGCGACACACA	AF416991
	EH2	GA**CCTAGG**CTACTTCTCCCACA	
R. paludigenum CBS 6565	EH3	GT**GGATCC**ATGGCGCCCA *Bam*HI	AY230137
	EH4	GA**GCTAGC**TCAGGCCTGG *Nhe*I	
R. araucariae CBS 6031	EH5	GT**GGATCC**ATGAGCGAGCA *Bam*HI	AF416993
	EH6	GA**CCTAGG**TCACGACGACAG *Bln*I	
A. niger LCP521	EH9	GA**GGATCC**ATGTCCGCTCCG	AJ238460
	EH10	GA**CCTAGG**CTACTTCTGCCAC *Bln*I	
X. dendrorhous CBS 6938	EH11	GA**GGATCC**ATGACGTCTGCG	AF166258
	EH12	GA**CCTAGG**TTAAAGCTCGGAAT	

PCR reactions for the amplification of the genomic *eph* gene from *C. curvatus* Car054 contained 200 µM dNTPs, 250 nM of each primer, 2 mM of $MgCl_2$, 100 ng genomic DNA and ExTaq (TaKaRa). The PCR profile was: 95ºC for 5 min, followed by 30 cycles of: denaturation at 95ºC for 1 min, primer annealing at 50ºC for 1 min, extension at 72ºC for 2 min, then a final extension of 72ºC for 10 min.

RNA Isolation, cDNA Synthesis and *eph* PCR

Total RNA from *C. curvatus* Car054 and *R. toruloides* 1 was isolated using Trizol® (Invitrogen). Biomass was harvested from 50 ml YPD cultures by centrifugation for 10 min at 4ºC, 13,000 rpm and the pellets frozen at -70ºC. The frozen biomass was ground to a fine powder under liquid N_2. 0.5 ml powder was thawed by the addition of 1 ml Trizol®. The manufacturer's instructions were followed for the isolation of total RNA. The RNA was resuspended in 50 µl formamide and stored at -70ºC.

Roche's mRNA Capture kit was used to isolate mRNA from the total RNA, according to the manufacturer's instructions. Expand Reverse Transcriptase (Roche) was used for the amplification of cDNA. Using this synthesized cDNA of *R. toruloides* 1 and *C. curvatus* Car054, PCR reactions were set up with primers EH1 and EH2. The PCR profiles were as described in the previous section.

The amplified 1.3 kb DNA fragments were digested with *Bam*HI and *Bln*I and ligated to pBSK+, pre-digested with *Bam*HI and *Xba*I. pBSK:Car054 and pBSK:1 were sequenced to confirm their gene structure.

Expression Vector Construction

The *C. curvatus* Car054 and *R. toruloides* 1 cDNA *eph* genes were amplified from pBSK:Car054 and pBSK:1 by PCR using EH1 and EH2 as primers, with PCR conditions as described in the previous section. The PCR products were digested with *Bam*HI and *Bln*I, and ligated to pINA1291 similarly digested.

All other *eph* genes were isolated by digesting the cloning plasmids (pGEM-T Easy and pPCR-Script) containing the genes with the relevant restriction enzymes (as indicated in Table I). The DNA fragments were separated on agarose gels and the *eph* genes purified from the agarose. The *eph* fragments were ligated into the *Bam*HI and *Bln*I sites of

pINA1291. The *eph* genes from *R. araucariae* 25, *R. toruloides* 46 and *R. paludigenum* 692 were similarly sub-cloned into pINA1293.

The *eph* gene from *R. araucariae* 25 was expressed in *S. cerevisiae* for comparison to *Y. lipolytica*-expressed EHs. The *eph* gene was amplified from pYL25HmL using primers EH13 (5'-GTGGATCCATGAGCGAGCA-3', *Bam*HI underlined) and EH14 (5'-GAGAATTCTCACGACGACAG-3', *Eco*RI underlined).

The PCR reaction and amplification conditions were as described above. The amplicon was purified from an agarose gel, digested with *Bam*HI and *Eco*RI and ligated to pYES2 (Invitrogen), which had been similarly digested.

Y. lipolytica Po1h Transformation and Identification of *Y. lipolytica* Transformants

Large-scale isolations of each of the pINA1291 and pINA1293 constructs were carried out using Qiagen's Plasmid MIDI kit, from 50 ml overnight cultures. 5 µg of plasmid DNA was digested with *Not*I to release the ~4 kb expression cassettes, which were purified from agarose gels. The cassettes are bounded by the *zeta* regions and contain the *ura3d4* marker and either the hp4d$_P$ - *LIP2*$_S$ - *eph* gene - *LIP2*$_T$ fragment for the constructs based on pINA1293 or the hp4d$_P$ - *eph* gene - *LIP2*$_T$ fragment for the constructs based on pINA1291. The method of Xuan et al. (1988) was used for the transformation of *Y. lipolytica* Po1h. Colonies appearing on the YNB$_{casa}$ selective plates after 7 – 21 days were transferred onto fresh plates.

Transformants were subjected to genomic DNA isolation from overnight cultures grown in YPD. The genomic DNA was used as the template for PCR screening, where the presence of a product would indicate the presence of the expression cassette within the genome. PCR was carried out using either gene-specific primers or pINA-1 and pINA-2, which are designed as universal screening primers for pINA1291 and pINA1293 transformants.

pINA-1 (20-mer): 5'-CAT ACA ACC ACA CAC ATC CA-3'
pINA-2 (21-mer): 5'-TAA ATA GCT TAG ATA CCA CAG-3'

These primers anneal up- and downstream of the *Bam*HI and *Bln*I recognition sites of both plasmids pINA1291 and pINA1293, the sites where the *eph* genes were inserted. Amplification with these primers would not be influenced by the sequence of the specific *eph* gene within the expression cassette.

Each 10 µl PCR reaction contained 200 µM dNTPs, 250 nM of each primer, 2 mM of $MgCl_2$, template genomic DNA and 0.25 U of BioTaq (Bioline). The PCR profile was: 95ºC for 5 min, followed by 30 cycles of: denaturation at 95ºC for 1 min, primer annealing at 50ºC for 1 min, extension at 72ºC for 1 min.

S. cerevisiae Transformation

S. cerevisiae INVScI was transformed with pYES2:25 by the lithium acetate/DMSO method (Hill et al. 1991). The transformed cells were plated onto selective SC^{-URA} and incubated for 48 hours at 30°C.

Copy number determinations

Real time PCR (RT-PCR) was used to estimate the copy number of the integrated expression cassettes. This was only carried out subsequent to the initial screening and selection of the best clone for each transformant type. Genomic DNA was extracted from *Y. lipolytica* transformants and used as template for the RT-PCR reactions. Primers as shown in Table 3 were designed. The *S. cerevisiae SUC2* gene had previously been introduced as a marker gene in *Y. lipolytica*, and is present in a single copy of the genome of *Y. lipolytica* Po1h (Nicaud et al. 1989. The *SUC2* set of primers anneal to this single copy of the *S. cerevisiae SUC2* gene within the chromosome, generating the internal control signal in each strain. The *URA3* primers target the *ura3d4* marker gene present on each integration cassette, and can be compared to the control signal. *Y. lipolytica*

Po1g was used as a control organism with a single copy of both the *SUC2* and *URA3* target sequences (Madzak et al. 2000a).

Table 3. DNA sequences of the oligodeoxynucleotide primers used in the real-time PCR copy number estimations

Signal	Name	5' -3' sequence
Control	SUC2-fwd	5'-CTACGGTTCAGCATTAGGTATTG-3'
	SUC2-rev	5'-GACCAGGGACCAGCATTAC-3'
Target	URA3-fwd	5'-GTGCTTCTCTGGATGTTACC-3'
	URA3-rev	5'-CAATATCTGCGAACTTTCTGTC-3'

Genomic DNA was diluted to a standard concentration of 25 pg.µl^{-1}. Reaction mixes (25 µl) consisted of 30 pg template DNA, 1 X iQ SYBR Green Supermix (Bio-Rad), and 300 nM of each primer. Each reaction was run in duplicate in a Bio-Rad MiniOpticon Real Time PCR machine. The profile used was: 95°C for 3 min; 40 cycles of: 95°C for 30 sec, 48°C for 30 sec, 72°C for 30 sec; followed by a final extension of 5 min at 72°C. A plate read was done after each 72°C step. A melt curve analysis was conducted after the amplification, from 45°C to 95°C.

Analysis was done using MJ Opticon Monitor Analysis software (Bio-Rad, version 3.1.32). The threshold was set to a standard deviation of 10 over the cycle range for each run, and the trend baseline subtraction setting was used. Maximum smoothing was applied. Average Ct values of the 2 profiles (*SUC2* and *URA3*) were used to estimate the relative copy numbers for each transformant strain. The Livak and Schmittgen (2001) equation was used to determine the copy number of the *URA3* target sequences (and therefore the integrated expression cassettes), in comparison to the *SUC2* copy number:

$$\Delta\Delta Ct = [\ Ct(URA3) - Ct(SUC2)\ ]_{sample} - [\ Ct(URA3) - Ct(SUC2)\ ]_{calibrator}$$

$$2^{-\Delta\Delta Ct} = \text{relative copy number of URA compared to SUC2 in sample.}$$

where 'sample' in each case is the relevant transformant strain, and 'calibrator' is the *Y. lipolytica* Po1g strain. Ct is the cycle number where the relevant curve intersects the threshold.

This value was compared to the relative copy number determined by the Pfaffl method (Pfaffl, 2001), as the Livak and Schmittgen method is only valid when the amplification efficiencies of the target (*URA3*) and reference (*SUC2*) genes are similar. The efficiency of each *URA3* PCR reaction was determined, the average E-value calculated and used in the Pfaffl equation. Similarly, an average *SUC2* E-value was determined. The Pfaffl equation is:

$$\text{Ratio} = \frac{(E_{URA3})^{\Delta Ct(URA3)}}{(E_{SUC2})^{\Delta Ct(SUC2)}}$$

where 'Ratio' is the relative copy number of *URA3* compared to SUC2, E_{URA3} is the average E-value (efficiency) of all the *URA3* amplifications (determined with the Opticon Monitor Analysis software at the conditions described), E_{SUC2} is the average E-value of all the *SUC2* amplifications, and $\Delta Ct = Ct_{\text{calibrator}} - Ct_{\text{sample}}$ for either *URA3* or *SUC2*.

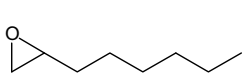
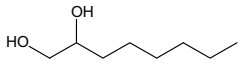
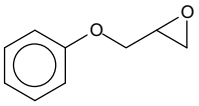
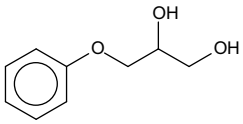
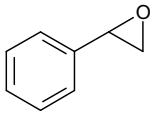
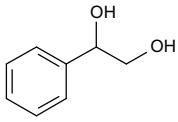
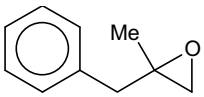
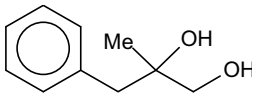
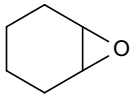
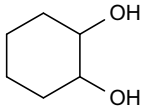
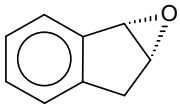
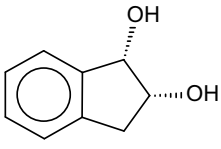
Screening for EH activity in *Y. lipolytica* Transformants by Biotransformation of Epoxides

Y. lipolytica transformants were grown in 50 ml liquid media in a 250 ml Erlenmeyer flask for 3 days at 28°C shaking at 200 rpm. The cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C and the pellets resuspended to a concentration of 10 - 50% (m/v) in chilled 50 mM potassium phosphate buffer (pH 7.5) for immediate evaluation of enzyme activity without further storage, or with the addition of 20% (m/v) glycerol to the buffer for storage at -20°C for later use.

For evaluation of epoxide hydrolase characteristics of whole cell biocatalysts, biotransformation screens were started by the addition of the selected epoxide to 1 ml cell suspension, to the desired concentration. Table 4 indicates the substrates used. Addition of the substrate to either cell suspension or culture supernatant (see next section) was followed by incubation at room temperature with vortex mixing, in sealed glass vials. The epoxide was added directly (*e.g.* 1,2-epoxyoctane, styrene oxide) or as a stock

solution in ethanol (*i.e.*, indene oxide, 2-methyl-3-phenyl-1,2-epoxypropane, cyclohexene oxide).

Table 4. Epoxide substrates for the evaluation of heterologously-expressed EHs in *Y. lipolytica* transformants

Class	Substrate	Illustrative compound for each class	Product
Mono-substituted epoxides (Type I)	1,2-Epoxyoctane Epichlorohydrin Epibromohydrin	 1,2-epoxyoctane	 1,2-octanediol
Glycidyl ethers	Phenyl glycidyl ether Benzyl glycidyl ether	 Phenyl glycidyl ether 1,2-epoxy-3-phenoxypropane	 3-phenoxy- 1,2-propanediol
Styrene oxide-type (Type II)	Styrene oxide 4-Nitrostyrene oxide 3-Chlorostyrene oxide	 Styrene oxide	 Phenylethanediol
2,2-Disubsituted epoxides (Type III)	2-Methyl-3-phenyl-1,2-epoxypropane	 2-methyl-3-phenyl- 1,2-epoxypropane	 2-Methyl-3-phenyl- 1,2-propanediol
***meso*-Epoxides**	Cyclohexene oxide Cyclopentene oxide	 Cyclohexene oxide	 Cyclohexanediol
***cis*-2,3-Disubstituted epoxides**	Indene oxide *cis*-2,3-Epoxybutane	 (*1S, 2R*)-Indene oxide	 (*1S,2R*)-indandiol

After suitable incubation, samples were removed and extracted with ethyl acetate, or the reactions were stopped by the addition of ethyl acetate to 60% of the reaction volume. The samples were vortexed for 1 min, and centrifuged at 13,000 rpm for 5 min. The solvent layer was dried over anhydrous magnesium sulphate and analysed by Thin Layer Chromatography (TLC) for an initial indication of EH activity (presence of epoxide and/or diol) and High Pressure Liquid Chromatography (HPLC) or Gas Chromatography (GC) for chiral analysis of remaining substrate and/or formed diol. See "Analytical Methods".

Evaluation of *LIP2* Secretion Signal for Expression of EHs in *Y. lipolytica*

Y. lipolytica recombinant strains with the *LIP2* secretion signal were tested using different epoxides. Whole-cell-associated activity was determined as above and compared to the extracellular activity found in culture supernatant from a 25 ml shake flask concentrated by ultra-filtration to 10 ml reaction volume (*i.e.* 2.5x concentrated). Whole-cell activities of the wild type yeasts *R. araucariae* 25 (WT-25) and *R. toruloides* 46 (WT-46) were determined, measured in the same manner as *Y. lipolytica* recombinant strains.

Screening for EH activity in *S. cerevisiae* expressing *R. araucariae* 25 EH

S. cerevisiae SC-25 was grown in 50 ml SC^{-URA} containing 2% glucose, in 250 ml Erlenmeyer flasks for 24 hrs at 30°C, followed by induction of protein expression by the addition of 2% galactose and allowed to continue for a further 24 hrs. This extended period of induction was determined experimentally. Cells were harvested by centrifugation, suspended in 50 mM phosphate buffer (pH 7.5) to a concentration of 50% (m/v) for immediate evaluation of enzyme activity without further storage or with the addition of 20% (m/v) glycerol to the buffer for storage at -20°C for later use.

Whole-cell EH activity of *S. cerevisiae* SC-25 was determined on 100 mM styrene oxide, indene oxide, 2-methyl-3-phenyl-1,2-epoxypropane and cyclohexene oxide, using 10 -

20% (m/v) biomass loading, and compared to the whole-cell activity determined with *Y. lipolytica* YL25HmA.

Analytical methods

Initial Activity Screens

Non-chiral TLC was performed using commercially available silica gel plates (Merck 5554 DC Alufolien 60 F_{254}) as the stationary phase and chloroform: ethyl acetate [1:1 (v/v)] as the mobile phase. Ceric sulphate (saturated with 15% H_2SO_4) or vanillin stain [2% (w/v) vanillin, 4% (v/v) H_2SO_4 dissolved in absolute ethanol] was used as a spray reagent to visualize the residual epoxide and formed diol.

Stereoselectivity Assays

Chiral GC was performed on a Hewlett Packard 5890-series II gas chromatograph equipped with a FID detector and an Aligent 6890-series autosampler-injector, using hydrogen as a carrier gas at a constant column head pressure of 140 kPa.

Quantitative analysis of the enantiomers of 1,2-epoxyoctane and 1,2-octanediol was achieved using a Chiraldex A-TA chiral fused silica cyclodextrin capillary column (supplied by Supelco) at oven temperatures of 40˚C and 115˚C, respectively. Analysis of cyclohexane diol was achieved using GC using a β-DEX 225™ fused silica cyclodextrin capillary column (Supelco) (30 m length, 25 mm id, 25 μm film thickness). Analysis of styrene oxide and 3-chlorostyrene oxide was achieved with GC using a β-DEX 225 ™ fused silica cyclodextrin capillary column (Supelco) (30 m length, 25 mm id, 25um film thickness) oven temperatures of 90 ˚C and 100˚C, respectively. Analysis of 2-methyl-3-phenyl-1,2-epoxypropane, *cis*-2,3-epoxybutane and 2-methyl-3-phenyl-propanediol was performed using a fused silica β-DEX 110 cyclodextrin capillary column (Supelco) (30 m length, 25 mm ID and 25 μm film thickness). The initial temperature of 80°C was maintained for 22 min, increased at a rate of 4°C per min to 160°C, and maintained at this temperature for 1 min. The retention times (min) were as follows: R_t (*S*)-epoxide = 31.9, R_t (*R*)-epoxide = 32.1, R_t (*S*)-diol = 47.7, R_t (*R*)-diol = 48.0.

Chiral HPLC was performed on a Hewlett Packard HP1100 equipped with UV detection. Quantitative chiral HPLC analysis of indene oxide enantiomers was achieved using Chiracel OB-H, 5u 20 cm X 4.6 mm, S/N OBHOCE-DK024, using hexane: ethanol (90: 10) mobile phase, at a constant flow rate of 1 $ml.min^{-1}$. HPLC analysis of glycidyl ether epoxides was achieved using Chiracel OD, using hexane: isopropanol mobile phase (80: 20 for phenyl glycidyl ether and 85:15 for benzyl glycidyl ether), at a constant flow rate of 1 $ml.min^{-1}$. Analysis of glycidyl ether diols was achieved using Chiracel OJ-R. Other GC and HPLC analyses were carried out essentially as described in Botes et al. (1999).

Results

Cloning of *C. curvatus* Car054 and *R. toruloides* 1 *eph* cDNA genes

PCR primers from known *eph* gene sequences were used to screen genomic DNA of *C. curvatus* Car054. This was done in order to determine whether any of these primer pairs could be used for the amplification of the cDNA copy of the *eph* gene from Car054. The PCR products from reactions with genomic DNA from *C. curvatus* Car054 as template, using the different PCR primer pairs in Table 2 were run on a 0.8% agarose gel (Figure 1). Most known microbial sequences have genomic *eph* gene sizes of approximately 1.6 kb, so PCR products of this size were sought. Primer pairs EH7/8, EH9/10 and EH11/12 gave no PCR products, and are not shown in the figure.

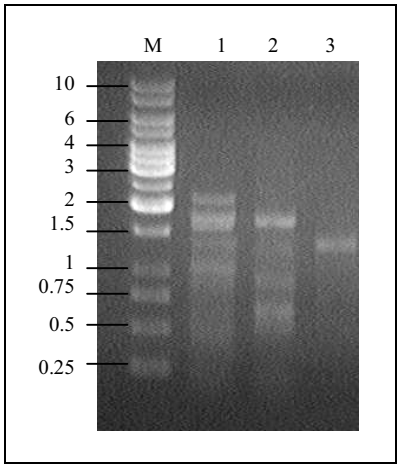


Figure 1. Putative *eph* PCR products from *C. curvatus* Car054 with genomic DNA as template, using primer pairs EH1/2 based on *R. toruloides* (lane 1), EH3/4 based on *R. paludigenum* (lane 2), and EH5/6 based on *R. araucariae* (lane 3). M = 1 kb GeneRuler (Fermentas) with sizes indicated on the left in kb.

As can be seen in Figure 1, both EH1/2, and EH3/4 primer sets gave PCR products which were in the expected 1.6 kb size range. Based on these results, primers EH1 and EH2 were used to isolate a cDNA copy of the putative *eph* gene from *C. curvatus* Car054.

The *C. curvatus* Car054 and *R. toruloides* 1 cDNA *eph* genes were subsequently successfully isolated from synthesised first strand cDNA, using these primers designed

from the *eph* sequence of *R. toruloides* CBS14 and CBS349. The PCR products were cloned into pBluescript SK+ for sequencing purposes.

Nucleotide sequences of the *eph* cDNA genes from *C. curvatus* Car054 and *R. toruloides* 1

The nucleotide and deduced amino acid sequences of the *R. toruloides* 1 and *C. curvatus* Car054 cDNA *eph* genes are given in Figures 2 and 3.

```
1          ATGGCGACACACACATTCGCTTCGCCTCCCACGCGCTTCACCGTCGACATCCCACAGTCAGAACTCGACGAACTCGACTTCCGACTCGAC
1           M  A  T  H  T  F  A  S  P  P  T  R  F  T  V  D  I  P  Q  S  E  L  D  E  L  D  F  R  L  D

91         AAGACCCGCTGGCCGGCGACAGAGATCGTTCCAGAGGATGGGGCGGACGACCCGACGGCGTTTGGGCTCGGAGCAGGGCCGACGCTGCCG
31          K  T  R  W  P  A  T  E  I  V  P  E  D  G  A  D  D  P  T  A  F  G  L  G  A  G  P  T  L  P

181        CTCATGAAGGAACTGGCGAAGGGTTGGCGCGAGTTCGACTGGAAGAAGGCGCAGGACCACCTCAACACCTTTGAGCACTACACGGTCGAA
61          L  M  K  E  L  A  K  G  W  R  E  F  D  W  K  K  A  Q  D  H  L  N  T  F  E  H  Y  T  V  E

271        ATCGAGGACCTCTCCATCCACTTCCTCCACCACCGCTCGACTCGCCCGAATGCTGTTCCGCTCATCCTCTGCCACGGCTGGCCAGGCCAC
91          I  E  D  L  S  I  H  F  L  H  H  R  S  T  R  P  N  A  V  P  L  I  L  C  H  G  W  P  G  H

361        TTCGGCGAGTTCCTGAACGTCATACCGCTCTTGACGGAGCCGTCGGACCCGTCCGCCCAGGCGTTCCACGTCGTCGCGCCTTCGATGCCC
121         F  G  E  F  L  N  V  I  P  L  L  T  E  P  S  D  P  S  A  Q  A  F  H  V  V  A  P  S  M  P

451        GGTTATGCTTGGTCTTCGCCTCCTCCGTCCTCCAAGTGGAGCATGCCTGATACCGCGAGGGTCTTCGACAAGCTCATGACCGGGCTTGGC
151         G  Y  A  W  S  S  P  P  P  S  S  K  W  S  M  P  D  T  A  R  V  F  D  K  L  M  T  G  L  G

541        TACGAGAAGTACATGGCGCAGGGCGGAGACTGGGGCAGCATCGCTGCTCGCTGCCTTGGATCGCTTCACAAAGACCACTGCAAAGCCGTC
181         Y  E  K  Y  M  A  Q  G  G  D  W  G  S  I  A  A  R  C  L  G  S  L  H  K  D  H  C  K  A  V

631        CACCTCAACTTCCTCCCCGTCTTCCCACCCGTCCCGATGTGGCTTATCAACCCGCACACGCTCCTTGCCTGGGCACCGCGCTTCCTCGTG
211         H  L  N  F  L  P  V  F  P  P  V  P  M  W  L  I  N  P  H  T  L  L  A  W  A  P  R  F  L  V

721        CCGGAGAAGCAGGCTGCGCGTATGAAGCGCGGGTTGGCGTACCTTGAGAAGGGCTCCGCCTACTACGTCATGCAGCAGTTGACGCCTCGC
241         P  E  K  Q  A  A  R  M  K  R  G  L  A  Y  L  E  K  G  S  A  Y  Y  V  M  Q  Q  L  T  P  R

811        ACGCCTGCGTACGGCCTGACCGACAGTCCCGTCGGCTTGCTGGCCTGGATCGGCGAGAAGTTCGAGCCGACCATTCAGGAGGCGAGCAAG
271         T  P  A  Y  G  L  T  D  S  P  V  G  L  L  A  W  I  G  E  K  F  E  P  T  I  Q  E  A  S  K

901        CAAGCCCAGCCGACCCTGACTCGCGACGAGCTCTACTTCACCTGCTCGCTCTACTGGTTCACCCGCTCAATCGGCACCTCCTTCCTTCCC
301         Q  A  Q  P  T  L  T  R  D  E  L  Y  F  T  C  S  L  Y  W  F  T  R  S  I  G  T  S  F  L  P

991        TACTCGCTCAACCCGCACTTCACCACCTTCCTGACCGACAGCAGGTACCACCTGCCCAACTTTGCCCTGTCCCTCTACCCGGGCGAGATC
331         Y  S  L  N  P  H  F  T  T  F  L  T  D  S  R  Y  H  L  P  N  F  A  L  S  L  Y  P  G  E  I

1081       TACTGCCCGGCAGAACGGGACGCCAAGCGTACCGGCAACCTCAAGTGGATCAAGGACGCGCCCGATGGAGGACACTTTGCTGCGCTCGAG
361         Y  C  P  A  E  R  D  A  K  R  T  G  N  L  K  W  I  K  D  A  P  D  G  G  H  F  A  A  L  E

1171       AAGCCCGATGTGTTTGTCGAGCATCTCAGGGAGGCGTTTGGCGTCATGTGGGAGAAGTAG
391         K  P  D  V  F  V  E  H  L  R  E  A  F  G  V  M  W  E  K  *
```

Figure 2. Nucleotide and deduced amino acid sequences of the *eph* cDNA gene from *R. toruloides* 1. The deduced amino acid sequence is shown in bold below the nucleotide sequence.

```
1       ATGGCGACACACACATTCGCTTCGCCTCCCACCCGCTTCACCGTCGACATTCCGCAGTCGGAAGTTGACCAACTTCACTCGCGACTCGAT
1        M  A  T  H  T  F  A  S  P  P  T  R  F  T  V  D  I  P  Q  S  E  V  D  Q  L  H  S  R  L  D

91      AAGACTCGCTGGCCAGGGACAGAGATCGTTCCAGAGGATGGGGCGGACGACCCGACGGCGTTTGGACTCGGAGCAGGACCAACGCTGCCG
31       K  T  R  W  P  G  T  E  I  V  P  E  D  G  A  D  D  P  T  A  F  G  L  G  A  G  P  T  L  P

181     CTCATGAAGGAACTGGCGAAGGGTTGGCGCGACTTCGACTGGAAGAAGGCGCAGGACCACCTCAACACCTTCGAGCACTACACGGTGGAG
61       L  M  K  E  L  A  K  G  W  R  D  F  D  W  K  K  A  Q  D  H  L  N  T  F  E  H  Y  T  V  E

271     ATCGAGGACCTCTCGATCCACTTCCTCCACCATCGCTCGACTCGTCCGAATGCTGTTCCCCTCATCCTCTGCCACGGCTGGCCAGGACAC
91       I  E  D  L  S  I  H  F  L  H  H  R  S  T  R  P  N  A  V  P  L  I  L  C  H  G  W  P  G  H

361     TTCGGCGAGTTTCTACACGTTATACCACTCTTGACGGAGCCGTCGGACCCGTCCGCTCAGGCGTTTCACGTCGTCGCGCCTTCGATGCCT
121      F  G  E  F  L  H  V  I  P  L  L  T  E  P  S  D  P  S  A  Q  A  F  H  V  V  A  P  S  M  P

451     GGTTATGCTTGGTCTTCGCCTCCTCCTACCTCCAAGTGGAATATGCCTGACACCGCAAGGGTGTCCGACAAGCTTATGAACGGACTCGGC
151      G  Y  A  W  S  S  P  P  P  T  S  K  W  N  M  P  D  T  A  R  V  S  D  K  L  M  N  G  L  G

541     TACGAGAAGTACATGGCGCAGGGCGGAGACTGGGGCAGCATCGCCGCTCGCTGCCTCGGAGCGCTGCACAAAGATCACTGCAAAGCCGTC
181      Y  E  K  Y  M  A  Q  G  G  D  W  G  S  I  A  A  R  C  L  G  A  L  H  K  D  H  C  K  A  V

631     CACCTCAACTTCCTCCCCGTCTTCCCGCCCGTCCCTATGTGGCTCATCAACCCACACACACTCCTCGCATGGGCTCCGCGCTTCCTTGTG
211      H  L  N  F  L  P  V  F  P  P  V  P  M  W  L  I  N  P  H  T  L  L  A  W  A  P  R  F  L  V

721     CCGGAGAAGCAGGCTGCGCGCATGAAGCGCGGGTTGGCGTACCTCGAAAAGGGCTCCGCCTACTACGTCATGCAGCAATTGACGCCTCGC
241      P  E  K  Q  A  A  R  M  K  R  G  L  A  Y  L  E  K  G  S  A  Y  Y  V  M  Q  Q  L  T  P  R

811     ACGTCTGCGTACGGCCTTACTGACAGTCCCGTCGGCTTGCTGGCCTGGATCGGCGAGAAGTTTGAGCCGACCATTCAGGAAGCGAGCAAG
271      T  S  A  Y  G  L  T  D  S  P  V  G  L  L  A  W  I  G  E  K  F  E  P  T  I  Q  E  A  S  K

901     CAAGCCCAGCCGACCCTCACTCGCGACGAGCTCTACTTCACCTGCTCCTTGTACTGGTTCACCCGCTCAATCGGCACCTCCTTCCTGCCC
301      Q  A  Q  P  T  L  T  R  D  E  L  Y  F  T  C  S  L  Y  W  F  T  R  S  I  G  T  S  F  L  P

991     TACTCGCTCAACCCGCACTTCACGACCTTCCTGACCGACAGCAAGTATTACCTGCCCAATTTTGCCCTCTCCCTGTACCCGGGCGAGATC
331      Y  S  L  N  P  H  F  T  T  F  L  T  D  S  K  Y  Y  L  P  N  F  A  L  S  L  Y  P  G  E  I

1081    TACTGCCCTGCCGAGCGGGACGCCAAGCGCACTGGCAACCTCAAGTGGATCAAGGACGCGCCTGAGGGAGGACACTTTGCGGCGCTCGAG
361      Y  C  P  A  E  R  D  A  K  R  T  G  N  L  K  W  I  K  D  A  P  E  G  G  H  F  A  A  L  E

1171    AAGCCCGACGTGTTTGTCGACCATCTCAGGGAGGCATTTGGCGTTATGTGGGAGAAGTAG
391      K  P  D  V  F  V  D  H  L  R  E  A  F  G  V  M  W  E  K  *
```

Figure 3. Nucleotide and deduced amino acid sequences of the *eph* cDNA gene from *C. curvatus* Car054. The deduced amino acid sequence is shown in bold below the nucleotide sequence.

Figure 4 indicates the homology of the newly isolated genes with the sequences of *R. toruloides* CBS 349 and the other genes used in this research. Sequence analysis of the Car054 gene, in comparison to other known *eph* genes, indicated that the Car054 gene is most similar to the *eph* genes of *Rhodotorula* and *Rhodosporidium* (Figure 4), and not the subsequently isolated *Cryptococcus neoformans eph* gene, which had been expected due to strain being identified as a *Cryptococcus* species. This was not due to any cross-contamination during the cDNA synthesis or gene amplification processes, as no other strain which was screened at the same time, gave amplification products of a similar size or sequence. The potential exists that the strain was either mis-identified or contamination had occurred during the storage process. In order to confirm the origin of this gene, the strain would need to be re-identified. However, for continuity, the Car054 label is continued throughout this chapter.

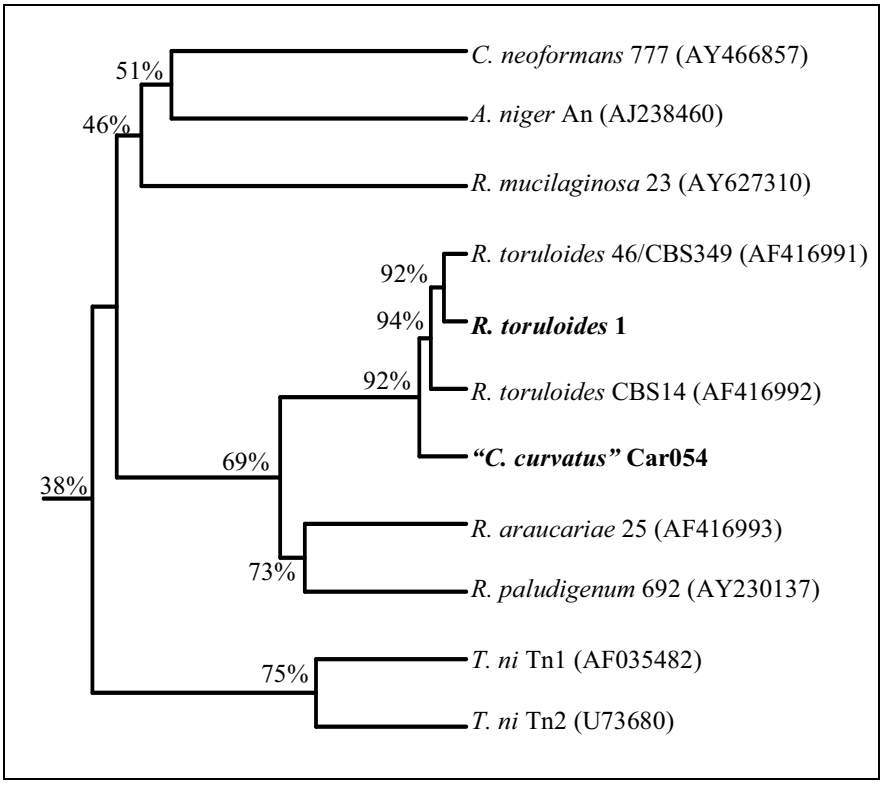


Figure 4. Homology of isolated *eph* cDNA genes from *C. curvatus* Car054 and *R. toruloides* 1 with *R. toruloides* CBS 349 and other *eph* genes (Analysed using the full sequence alignment function, DNAMAN, Lynnon Biosoft). Sequence analysis of *C. curvatus* Car054 indicates the source of this gene is probably not a *Cryptococcus* species, being more similar to the *eph* gene sequences of *Rhodosporidium* and *Rhodotorula*. This strain is therefore further referred to as only "Car054" rather than "*C. curvatus* Car054".

Expression vector construction

All *eph* genes were cloned into the *Bam*HI and *Bln*I restriction enzyme sites of pINA1291. The *eph* genes from *R. araucariae* 25, *R. toruloides* 46 and *R. paludigenum* 692 were sub-cloned into pINA1293, in-frame with the *LIP2* secretion signal ($LIP2_S$) on the vector. All are under control of the growth-phase inducible hybrid hp4d promoter. They all also contain the defective *ura3d4* marker gene, which is required in multiple copies to alleviate the auxotrophy of the host.

In order to compare expression levels, the *eph* gene from *R. araucariae* 25 was also expressed in *S. cerevisiae*, under control of the inducible *GAL1* promoter. The *eph* gene was amplified from pYL25HmL and cloned into pYES2, forming pYES2:25.

Y. lipolytica transformations

Subsequent to the transformation of *Y. lipolytica* Po1h with the *Not*I-linearised expression cassettes from the different constructs using the method of Xuan et al. (1988), transformant colonies were subjected to PCR screening using genomic DNA and gene-specific primers or pINA-1 and pINA-2, which are designed as universal screening primers for pINA1291 and pINA1293 transformants. The presence of PCR products of the expected size was taken as confirmation that integration of the relevant expression cassette had occurred in the genome of *Y. lipolytica* Po1h.

Copy number determination

RT-PCR amplifications were carried out on *Y. lipolytica* transformants selected for analysis, subsequent to the TLC-based selection of the best clone for each transformant type. The copy numbers of the recombinant *Y. lipolytica* strains with the *eph* genes downstream of the *LIP2* secretion signal were not deter mined. Experimental analysis had shown that these strains were inefficient for expressing epoxide hydrolases, and no further work was carried out with them after the initial activity determinations. Copy numbers were only determined for those strains which were included in the broad screening programme.

Standardised DNA concentrations were used in all reactions. The equation $\Delta\Delta Ct = [\ Ct(URA) - Ct(SUC2)\ ]_{sample} - [\ Ct(URA) - Ct(SUC2)\ ]_{control}$ (Livak and Schmittgen 2001) was used to estimate the copy number of the integrated expression cassette in each sample strain, where the 'control' is Po1g, with single copy of each of the two targets (*URA3* and *SUC2* genes). The Ct values of the two profiles are also the same, and the $[\ Ct(URA) - Ct(SUC2)\ ]_{control}$ value can therefore be assumed to be 0. This was also experimentally shown (Figure 5).

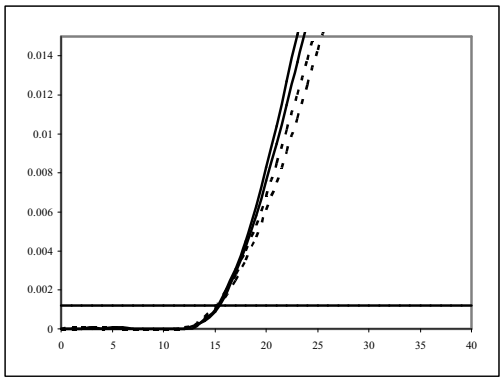


Figure 5. RT-PCR profile of the control organism *Y. lipolytica* Po1g showing the fluorescence values versus cycle number. The solid lines indicate the average *SUC2* amplifications, and dotted lines represent the *URA3* amplifications. The horizontal line indicates the Ct threshold set manually. All curves intersect the threshold at the same cycle number, indicating the copy numbers of the two targets are equivalent.

The equation could therefore be simplified to $\Delta\Delta Ct = [\ Ct(URA) - Ct(SUC2)\ ]_{sample}$. This value can then be inserted into the equation $N = 2^{-\Delta\Delta Ct}$, where there are N-fold copies of *URA3* compared to *SUC2*. As it is known that there is only a single copy of *SUC2* in *Y. lipolytica* Po1h (Nicaud et al. 1989), it can be stated that there are N-copies of the *URA3* target present. Table 4 shows the Ct values obtained for each strain, and the estimated copy numbers, using the Livak and Schmittgen equation.

In order to determine whether the use of this equation was justified, the copy numbers were checked against values obtained with the Pfaffl method (Pfaffl, 2001). The average E-value (efficiency of the amplification) for the *SUC2* PCR reactions was determined to be 1.97 and the *URA3* E-value was calculated as 1.94. In a perfect PCR reaction, the E-value is 2, indicating a perfect doubling of amplicons per round of PCR. This is presumed in the Livak equation and Schmittgen. The slightly lower E-values indicate slightly less than perfect doubling. Using the Ct values as shown in Table 4, the Pfaffl equation was used to determine the copy numbers for the various transformants. This value is shown in the final column in Table 4, indicating a good agreement with the Livak-determined copy number. Figure 6 is a representative set of data.

Table 4. Copy numbers of integrated expression cassettes in different *Y. lipolytica* Po1h transformants.

Transformant / Strain	Ave Ct(URA3)	Ave Ct(SUC2)	ΔΔCt = [Ct(URA)-Ct(SUC2)]	Livak Copy Number: $N = 2^{-\Delta\Delta Ct}$	Pfaffl Copy Number
Control					
Y. lipolytica Po1g	15.17 ± 0.18	15.17 ± 0.11	0.00	1	1
mEH-expressing strains*					
YLCar054HmA	10.08 ± 0.69	14.77 ± 0.28	-4.69	26	21
YL1HmA	14.91±0.24	16.69±1.01	-1.8	3	3
YL23HmA	13.67±0.2	15.86±0.09	-2.2	5	4
YL25HmA #1	11.89 ± 0.08	15.78 ± 0.13	-3.89	15	13
YL25HmA #2	14.43 ± 0.04	14.75 ± 0.45	-0.32	1	1
YL46HmA	11.84 ± 0.43	15.38 ± 0.23	-3.55	11	10
YL692HmA	14.09±0.08	16.95±0.35	-2.9	7	6
YL777HmA	15.26 ± 0.18	18.13 ± 0.21	-2.88	7	7
YL-An-HmA	10.98 ± 0.06	15.11 ± 0.28	-4.13	17	15
YL-Tn1-HmA	11.45 ± 0.71	14.26 ± 0.67	-2.81	7	6
YL-Tn2-HmA	12.71 ± 0.02	16.05 ± 0.42	-3.35	10	9
sEH-expressing strains*					
YL-Ar-HmA	3 ± 0.23	16.53 ± 0.52	-4.23	18	16
YL-St-HmA	79 ± 0.19	18.65 ± 0.22	-3.86	15	12

*1 = *R. toruloides* UOFS Y-0517, 23 = *R. mucilaginosa*, 25 = *R. araucariae*, 46 = *R. toruloides* UOFS Y-0471, 692 = *R. paludigenum*, 777 = *C. neoformans*. An = *A. niger* AY966486, Tn1 = *T. ni* TNU73680, Tn2 = *T. ni* AF035482, Ar = *A. radiobacter*, St = *S. tuberosum.*

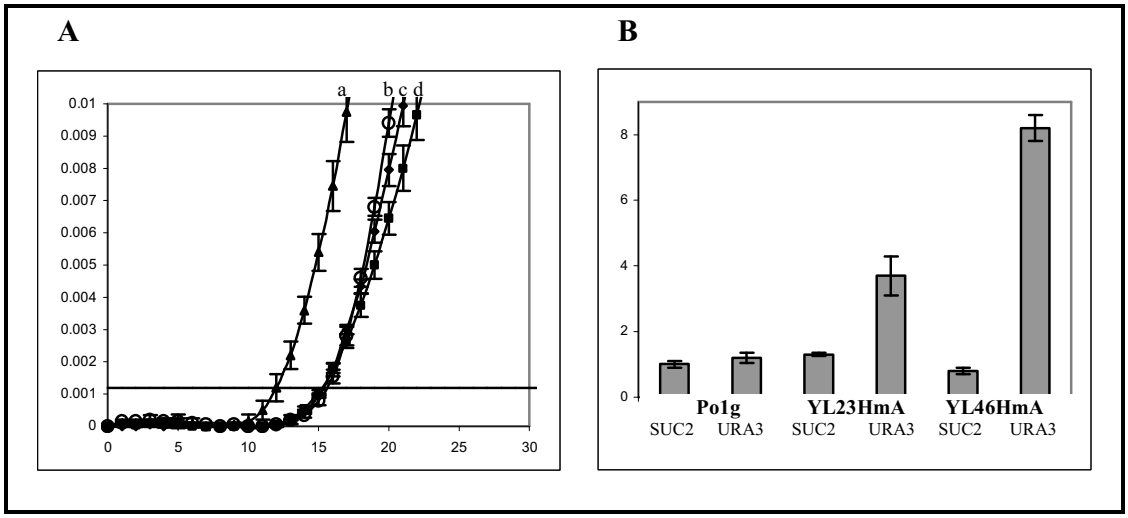


Figure 6A - B. Representative relative copy number determination of selected *Y. lipolytica* Po1h transformants. **(A)** depicts fluorescence versus cycle number, enlarged to show the curves clearly. -▲- (a) is the *URA3* amplification from YL46HmA, containing the *R. toruloides eph* gene. The -○- (b) curve is the *SUC2* amplification of YL46HmA. The -♦- (c) and -■- (d) curves are *URA3* and *SUC2* amplifications from Po1g, respectively. The values are the average of duplicate reactions. The standard deviations are shown by the vertical ticks. As can be seen, the *URA3* amplification of YL46HmA results in a much lower Ct value (intersection point with the threshold, depicted by the horizontal line). The Ct values of the *URA3* amplification of Po1g and *SUC2* amplifications of both samples all result in similar Ct values, indicting similar copy numbers. **(B)** is a representation of the relative copy numbers of the different amplicons, in Po1g, YL23HmA (*R. mucilaginosa eph*) and YL46HmA (*R. toruloides eph)*.

Evaluation of the *LIP2* secretion signal for expression of epoxide hydrolases in *Y. lipolytica*

In order to evaluate the efficiency of the *LIP2* secretion signal, biotransformations were performed to compare the activity and enantioselectivity of different EHs expressed in *Y. lipolytica,* with and without the signal peptide, across a range of different epoxide classes. Extracellular (supernatant) and cell-bound activity levels were determined.

Whole-cell biotransformations, with 50 mM substrate and 20% (w/v) cell loading, were carried out using the wild-type *R. toruloides* 46 (-▲-), and compared to the cell-bound (-■-) and fully secreted activity of YL46HmL (Figure 7). The racemic mono-substituted 1,2-epoxyoctane (Type 1 epoxide) was used as substrate. The recombinant *Y. lipolytica* strain did not secrete any measurable EH into the supernatant, with all the detectable activity remaining cell-bound. However, the expression of the recombinant enzyme with a signal peptide caused a slight decrease in the enantioselectivity of the enzyme over time compared to the wild-type, though both hydrolysed the (*R*)-isomer preferentially. Initial enantioselectivities were similar.

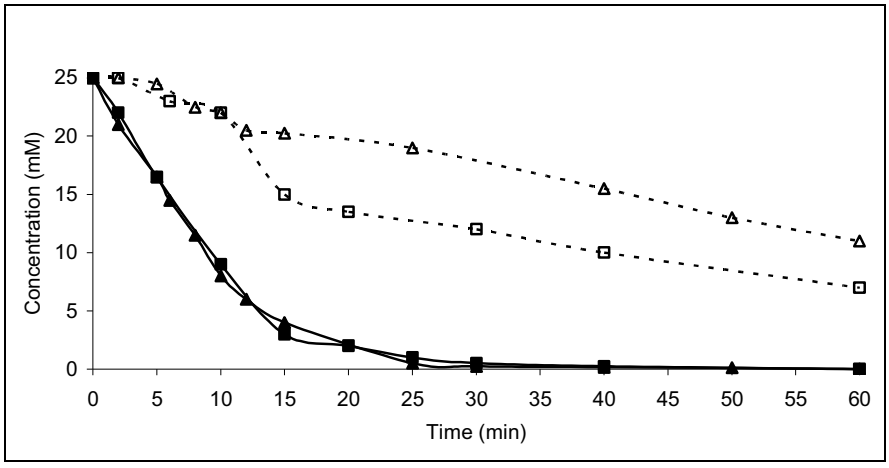


Figure 7. Comparison of the kinetic resolution of 50 mM 1,2-epoxyoctane by *R. toruloides* 46 (WT-46), and the recombinant EH enzyme expressed in *Y. lipolytica* with the *LIP2* signal peptide (YL46HmL). Both strains tested at 20% (w/v) cells. Solid icons indicate (*R*)-1,2-epoxyoctane, open icons indicate (*S*)-1,2-epoxyoctane. -▲- = WT-46, -■- = YL46HmL cell-bound activity. No extracellular activity was detected. (PCT WO 2007/010403).

Figure A1 in Appendix 1 depicts the results using *R. araucariae* WT-25 and *Y. lipolytica* YL25HmL for biotransformation of the same substrate. Some extracellular activity was detected in this recombinant strain, but this lost the enantioselectivity displayed with the WT-25 and cell-bound conversion.

Based on these results, with either no or little extracellular activity detected, it was decided to concentrate on cell-bound activity for future experiments rather than the extracellular activity. The effect of the presence of the *LIP2* signal peptide on the cell-bound activity was further investigated by determining the activity and initial enantioselectivity of a number of recombinant EHs on a number of different substrates, in the presence and absence of the *LIP2* signal peptide, *i.e.* in YL-HmL and YL-HmA recombinant *Y. lipolytica* strains.

Figures 8 shows the hydrolysis of 1,2-epoxyoctane with YL692HmA and YL692HmL. Even with a biomass loading of 5% (w/v) for YL692HmA versus 10% (w/v) loading used for the YL692HmL, the YL692HmA transformant (-■-) was significantly superior to YL692HmL (-▲-), both in terms of the activity and enantioselectivity, indicating that the presence of the secretion signal negatively affected the use of these recombinant *Y. lipolytica* strains as whole-cell biocatalysts.

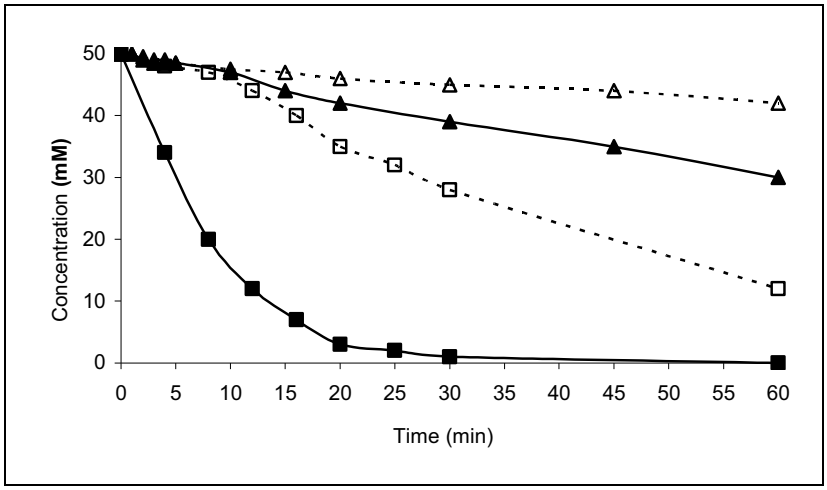


Figure 8. Comparison of kinetic resolution of 100 mM racemic 1,2-epoxyoctane by YL692HmL versus YL692HmA (*R. paludigenum eph*). YL692HmA was tested using 5% (w/v) cell loading, whereas YL926HmL was done using 10% (w/v). Solid icons indicate (*R*)-1,2-epoxyoctane, open icons indicate (*S*)-1,2-epoxyoctane. -■- = YL692HmA, -▲- = YL692HmL (PCT WO 2007/010403).

YL25HmA and YL25HmL were also tested and gave a very similar result (not shown). Further results are shown in Figures A2 – A5 in Appendix 1.

Whole-cell biocatalysis using *Y. lipolytica* strains expressing fungal and insect EHs

In order to establish that the *Y. lipolytica* expression host is a universal strain for the production of EHs, various epoxides from the different epoxide substrate groups were tested in whole-cell biotransformations with different recombinant *Y. lipolytica* strains. Based on the data showing that the presence of a signal sequence is detrimental to the activity and enantioselectivity of whole-cell biocatalysts, only YL-HmA constructs were tested. Not all strains tested are shown in the following sections, representative strains showing good enantioselectivity and/or activity are reported. Figures A6 – A12 in Appendix 1 depict further substrates and recombinant *Y. lipolytica* strains tested and found to be effective.

Class: Mono-substituted epoxides (Type 1)

Figure 9 depicts the hydrolysis of 1,2-epoxyoctane by different *Y. lipolytica* recombinant strains. None of the strains are highly selective, though a number of them show very good activity. YL-692-HmA (-■-, Figure 9B) in particular shows extremely high levels of activity, though both enantiomers are hydrolysed at almost the same rate. Y777HmA (-▲-, Figure 9B) displays enantioselectivity combined with good activity under the conditions tested, as initially there is a difference in the rate of hydrolysis of the two enantiomers, with (*S*)-1,2-epoxyoctane very poorly hydrolysed at first, though both are almost completely hydrolysed over time. (See also Figures A6 and A7 in Appendix 1). Yl-Car054-HmA did not exhibit particularly good activity, though the reactions were enantioselective.

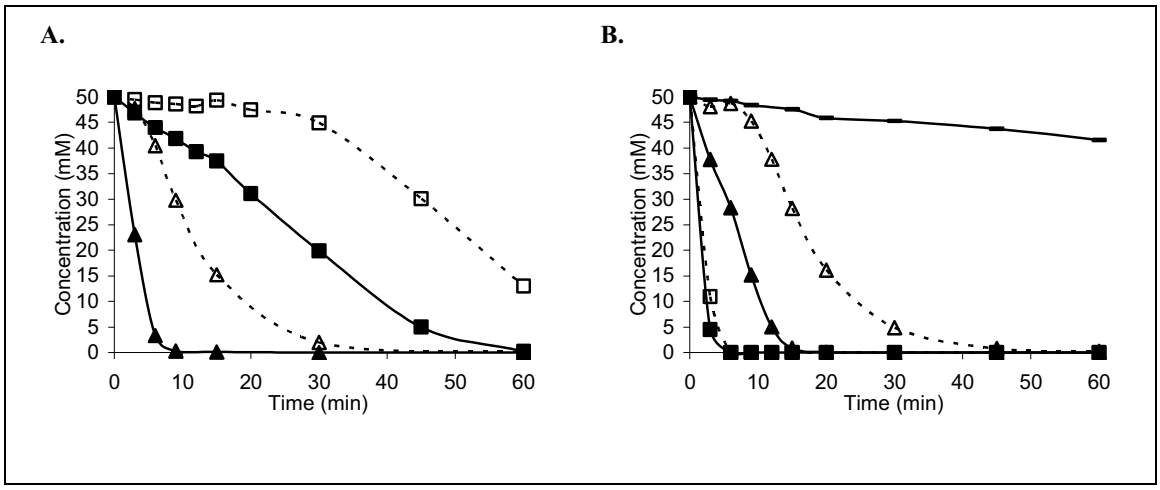


Figure 9. Kinetic resolution of 100 mM 1,2-epoxyoctane by the recombinant EHs expressed in *Y. lipolytica* using 10% (w/v) cell loading. Solid icons indicate (*R*)-1,2-epoxyoctane, open icons indicate (*S*)- ,2-epoxyoctane . (A) -■- = YL23HmA (*R. mucilaginosa eph*), -▲- = YL-An-HmA (*A. niger eph*). (B) -▲- = YL777HmA (*C. neoformas eph*), -■- = YL692HmA (*R. paludigenum eph*), - ▬ - = *Y. lipolytica* Po1h

Results can also be given in initial rates of hydrolysis, displaying the conversion in nmol.(mg wet weight)$^{-1}$ over time. The slope of the trend lines in the graphs in Figure 10 (the *m* value in the y=*m*x trend equations), is the conversion per min, *i.e.* nmol.(mg wet weight)$^{-1}$.min^{-1}. This corresponds to the specific activity of the enzyme towards each enantiomer, and towards the total racemic solution. The closer the specific activity for a single enantiomer is to the specific activity towards the total racemic mixture, the more enantioselective the enzyme is for that specific enantiomer. As can be seen in Figure 10, there is a preferred activity for (*R*)-1,2-epoxyoctane (-●-) in YL777HmA and YL23HmA, but little enantioselectivity in YL-An-HmA. However, these are initial rates and cannot be compared directly to the results as shown in Figure 9, which display the conversions over an extended period. Even initially, enantioselective enzymes may hydrolyse the less preferred enantiomer over time. In future graphs, these initial rates are generally not given. Comparisons about enantioselectivity of a specific enzyme can be made by comparing the *difference* between slopes of the linear early portions of the graphs of the two enantiomers, and activity can be deduced by the gradient of the slopes.

The results shown in Figure 9 and 10 are under non-optimised conditions, which could be optimised to ensure a more enantioselective process over time, *e.g.* through a lower cell loading. This is common to all of the reactions where initial rates are selective, though

over time the reaction becomes less selective as the second enantiomer is hydrolysed. Altering the cell loading or the substrate concentration is a way to manipulate the enantioselectivity of the entire process. That is why is it necessary to consider only the initial linear portions of the graphs in these non-optimised reactions when determining the possible enantioselectivity of the process.

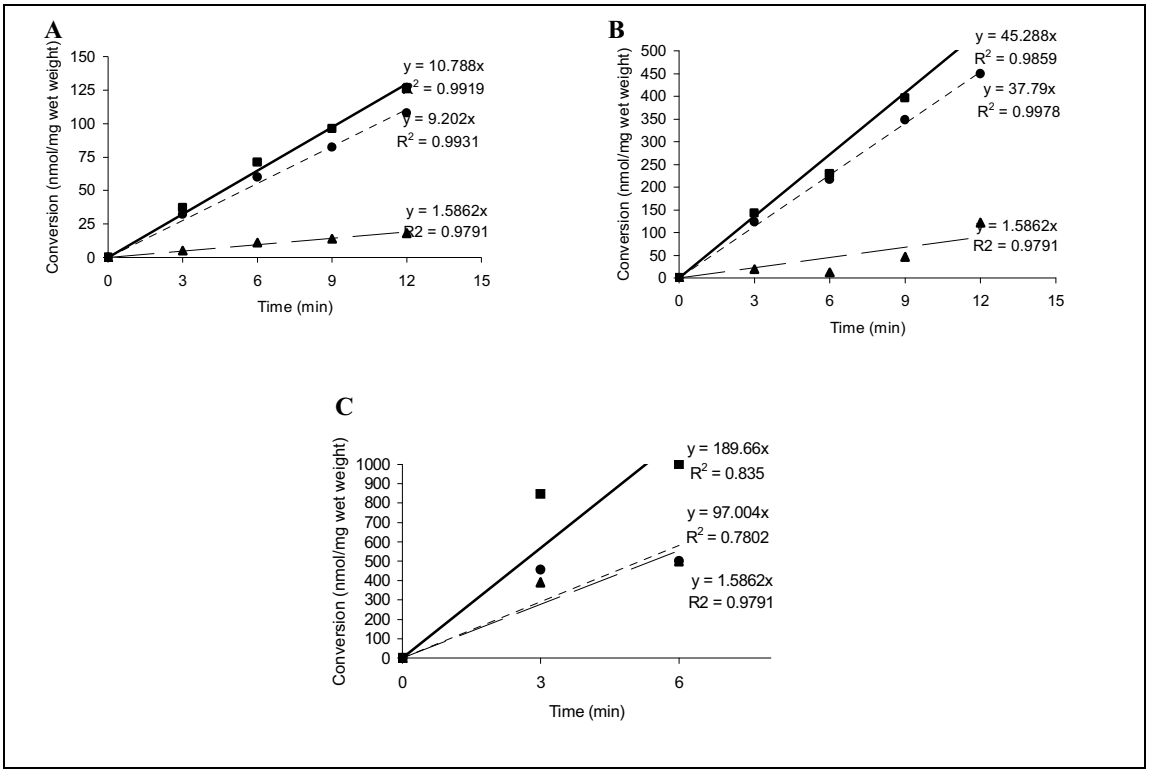


Figure 10A - C. Initial rates of hydrolysis of 100 mM 1,2-epoxyoctane by **(A)** YL777HmA (*C. neoformans eph*), **(B)** Y23HmA (*R. mucilaginosa eph*) and **(C)** YL-An-HmA (*A. niger eph*). -▲- = (*S*)-1,2-epoxyoctane, -●- = (*R*)-1,2-epoxyoctane, -■- = total 1,2-epoxyoctane.

Class: glycidyl ethers

Figure 11 depicts the hydrolysis of phenyl glycidyl ether by the newly isolated EHs from *R. toruloides* 1 (NCYC 3181) and *C. curvatus* Car054 (NCYC 3158). YL25HmA and YL46HmA both display the same hydrolysis pattern (results not shown). The recombinant enzymes are highly enantioselective for the (*S*)-enantiomer of the substrate.

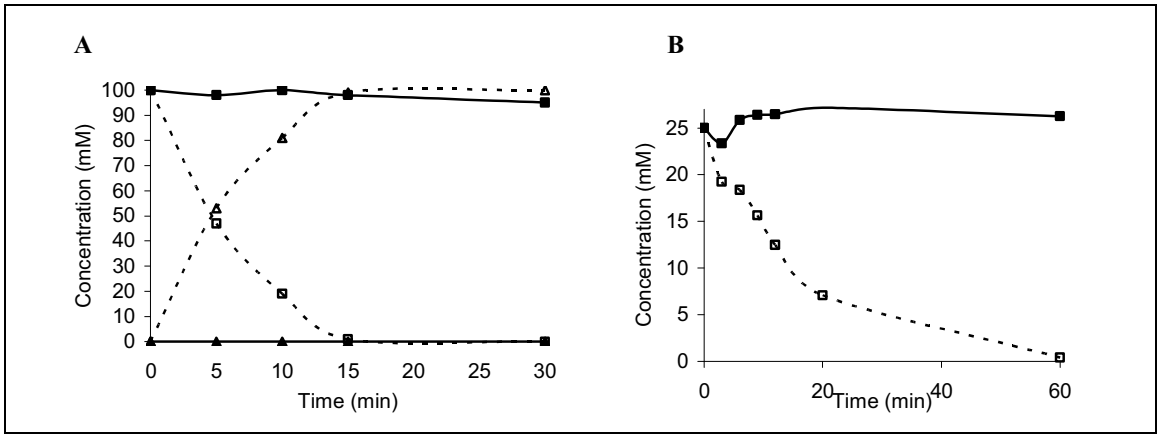


Figure 11A – B. Kinetic resolution of phenyl glycidyl ether by **(A)** YL1HmA (*R. toruloides eph* UOFS Y-0517) using 200 mM substrate and **(B)** YL-Car054-HmA using 50 mM substrate, both 5% (w/v) cell loading. -■- indicates (*R*)-phenyl glycidyl ether, and -□- indicates (*S*)-phenyl glycidyl ether. -▲- = (*R*)-diol and -Δ- = (*S*)-diol. *Note the differences in time (x-axis) and the difference in initial substrate concentration (y-axis).*

Figure A8 in Appendix 1 illustrates conversions using benzyl glycidyl ether.

Class: Styrene oxide-type epoxides (Type II)

Figure 12A shows a range of whole-cell biocatalyst-driven styrene oxide biotransformations using 100 mM substrate with different EHs expressed in *Y. lipolytica*. As can be seen, all the shown recombinant EHs preferentially hydrolyse the (*R*)-enantiomer over the (*S*)-enantiomer, though none are completely enantioselective. Optimisation of cell loading may help to improve the enantioselectivity of the complete reaction, as described previously. By analysing the initial rates of hydrolysis (Figure 12B, 12C), it can be seen that YL25HmA is initially the most enantioselective of the examples, but the activity of the enzyme from YL1HmA (-■-) is the highest. YL23HmA is neither particularly active nor particularly enantioselective.

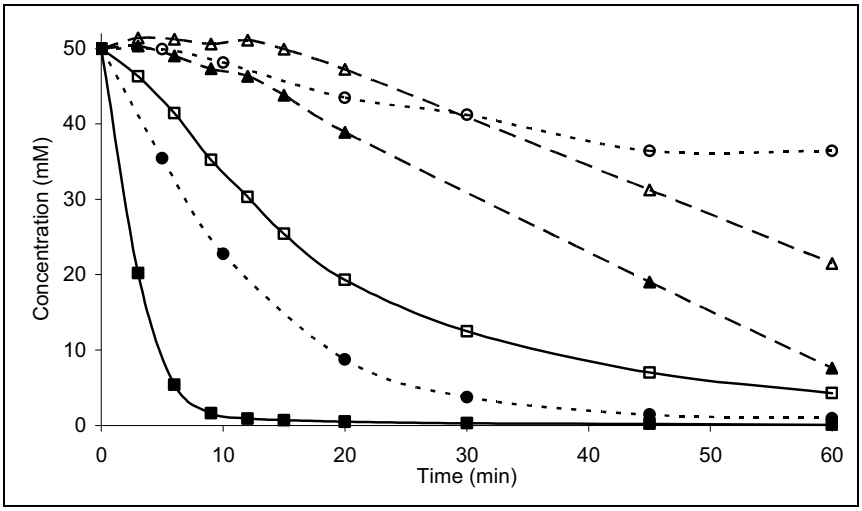


Figure 12A. Comparison of the kinetic resolution of 100 mM styrene oxide by recombinant EHs expressed in *Y. lipolytica*. Solid icons indicate (*R*)-SO, open icons indicate (*S*)-SO. -■- = YL1HmA (*R. toruloides eph* UOFS Y-0517), -▲- = YL23HmA (*R. mucilaginosa eph*), -●- = YL25HmA (*R. araucariae eph*). YL1HmA and YL23HmA were tested using 10% (w/v) cell loading, whereas YL25HmA was done with 25% (w/v).

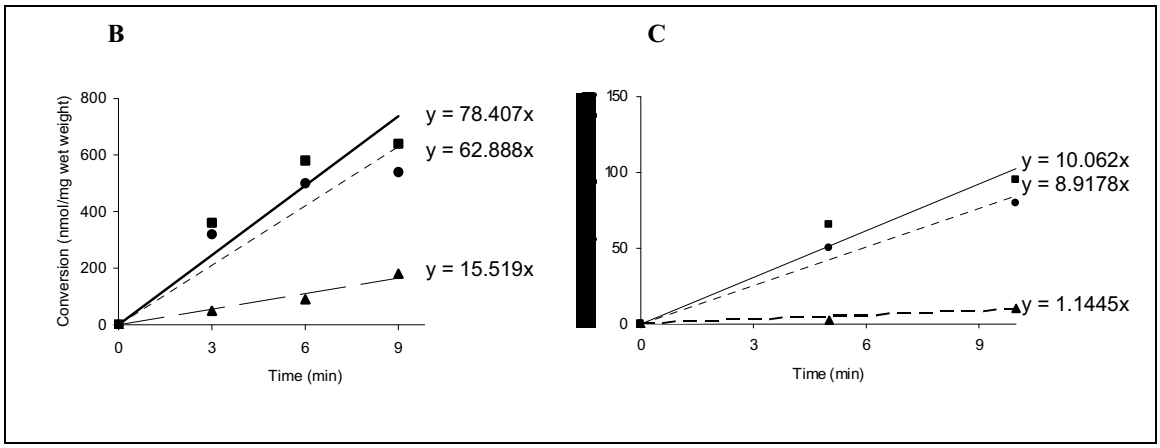


Figure 12B – C. Initial rates of hydrolysis of styrene oxide by **(B)** YL1HmA (*R. toruloides eph* UOFS Y-0517) and **(C)** YL25HmA (*R. araucariae eph*). -▲- = (*S*)-SO, -●- =(*R*)-SO, -■- = racemic SO.

Figures A9 and A10 demonstrate the hydrolysis of 4-nitrostyrene oxide and 3-chlorostyrene oxide.

Class: 2,2-disubstituted epoxide (Type III)

Figure 13 indicates that the 2,2-disubstituted epoxide, 2-methyl-3-phenyl-1,2-epoxypropane, is efficiently hydrolysed by a number of different recombinant strains. YL23HmA (23A) and YL777HmA (not shown) give similar profiles, with both enantiomers very quickly utilised. Interestingly, in both cases the (*R*)-diol is preferentially formed. 50 mM of each of the (*R*)- and (*S*)-epoxide is converted to approximately 60 mM of (*R*)-diol and 40 mM of (*S*)-diol. This indicates that these enzymes are somewhat enantioconvergent for these substrates. This is also the case for YL1HmA (Figure 13B) and YL692HmA (not shown), although the conversion is slower.

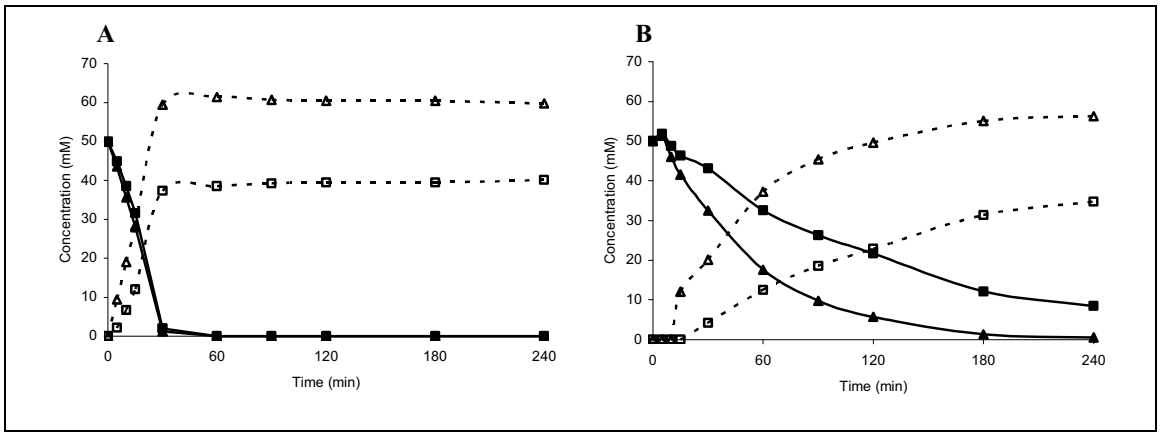


Figure 13A – B. Kinetic resolution of 100 mM 2-methyl-3-phenyl-1,2-epoxypropane (MPEP) by **(A)** YL23HmA (*R. mucilaginosa eph*) and **(B)** YL1HmA (*R. toruloides* UOFS Y-0517) using 10% (w/v) cell loading. -■- = (*S*)-epoxide/diol, -▲- = (*R*)-epoxide/diol, closed icons indicate epoxide utilisation, open icons indicate diol formation.

Class: meso-epoxides

Cyclohexene oxide is converted, prochirally, by epoxide hydrolases to (*R,R*)-cyclohexanediol (Figures 14A and 14B), utilising the substrate (-▲-) almost completely. (See also Figure A3 in Appendix 1). Virtually no (*S,S*) diol (-□-) is produced. YL1HmA shows the same profile as YL23HmA (*R. mucilaginosa eph*), YL46HmA (*R. toruloides* UOFS Y-0471 *eph*) and YL692HmA (*R. paludigenum eph*). YL777HmA shows a much lower level of activity, although its enantioselectivity is the same.

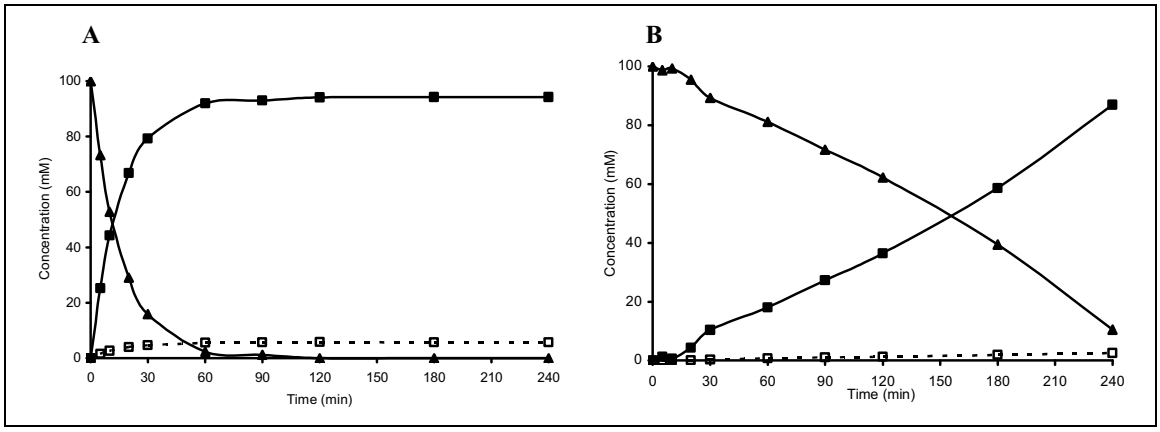


Figure 14A – B. Production of diols by kinetic resolution of 100 mM cyclohexene oxide by **(A)** YL1HmA (*R. toruloides* UOFS Y-0517 *eph*) and **(B)** YL777HmA (*C. neoformans eph*), using 10% (w/v) cell loading. -▲- = CHO, -■- indicates (*R,R*)-cyclohexanediol, -□- indicates (*S,S*)-cyclohexanediol.

Figure A11 in Appendix 1 shows the resolution of chloropentene oxide by YL25HmA and YL46HmA.

Class: cis-2,3-disubstituted epoxides

Hydrolysis of indene oxide is shown in Figure 15. Most strains tested show a certain level of activity, but none of the strains tested display high enantioselectivity levels. Interestingly, the fungal EHs of YL23HmA (-■-), YL1HmA (-▲-) and YL692HmA (data not shown) show opposite enantioselectivity to the insect EHs of YL-Tn2-HmA (-●-) and YL-Tn1-HmA (data not shown), the epoxide hydrolases from the cabbage looper, *T. ni*. This may be biocatalytically relevant, as a different biocatalyst can be used, depending on the desired result.

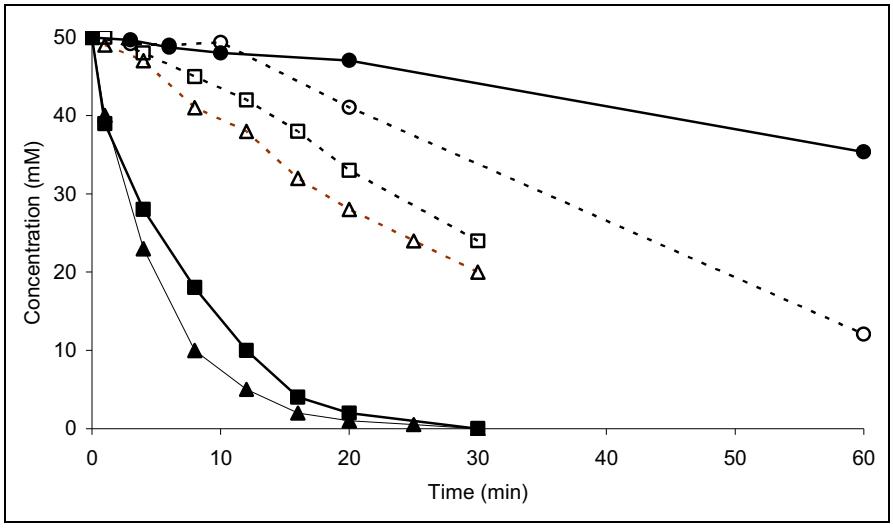


Figure 15. Kinetic resolution of 100 mM indene oxide (IO) by recombinant EHs expressed in *Y. lipolytica*. Solid icons indicate (*1S,2R*)-IO, open icons indicate (*1R,2S*)-IO. -▲- = YL1HmA (*R. toruloides* UOFS Y-0517 *eph*) and -■- = YL23HmA (*R. mucilaginosa eph*), both using 5% (w/v) cell loading (PCT WO 2007/069079) , -●- =YL-Tn2-HmA (*T. ni* AF035482), 10% (w/v) cell loading.

Figure A 12 in Appendix 1 demonstrates the absolute enantioselectivity for *cis*-2,3-epoxybutane by YL25HmA and YL46HmA.

Cytosolic EHs

The codon-optimised, synthesised *S. tuberosum* sEH gene was cloned into *Y. lipolytica* and the YL-St-HmA transformant was used for the hydrolysis of styrene oxide. Activity was determined in whole-cell reactions, with a 10% (w/v) cell loading and 100 mM racemic styrene oxide as substrate. The activity and enantioselectivity of the recombinant potato EH enzyme was compared to that of YL692HmA containing the yeast *R. paludigenum* EH (Figure 16). The YL-St-HmA transformant displayed the same excellent enantioselectivity for (*S*)-SO as reported for the native gene (Monterde et al. 2004), which is opposite to that of yeast epoxide hydrolases, as can be seen by the preferential utilisation of (*R*)-SO by YL692HmA. Activity was essentially identical to that obtained for YL692HmA.

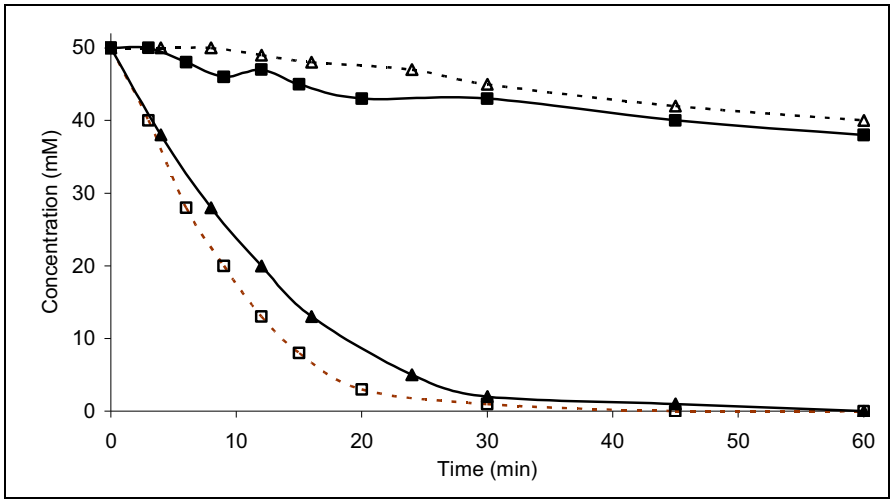


Figure 16. Kinetic resolution of 100 mM styrene oxide by YL-HmA transformants harbouring the sEH *eph* gene from *S. tuberosum* and the EH gene from *R. paludigenum* 692, using 10% (w/v) cell loading. Solid icons indicate (*R*)-SO, open icons indicate (*S*)-SO. -■- = YL-St-HmA -▲- = YL692HmA (PCT WO 2007/010403).

The bacterial sEH *echA* gene of *A. radiobacter* was expressed in *Y. lipolytica* and the YL-Ar-HmA transformant used for the hydrolysis of styrene oxide (Figure A13 in Appendix 1). The YL-Ar-HmA transformant displayed essentially the same enantioselectivity as reported for the native gene in *A. radiobacter* (Rink and Janssen 1998). Unlike the sEH from *S. tuberosum* above, the enantioselectivity is not opposite to that of the fungal EHs.

Whole-Cell Biocatalysis using Recombinant EH of *R. araucariae* 25 Expressed in *S. cerevisiae*

To compare the results obtained in whole-cell biotransformations using recombinant EHs expressed in *Y. lipolytica* to other commonly-used yeast heterologous protein expression hosts, similar hydrolyses were carried out with whole-cell biocatalysts comprising *S. cerevisiae* expressing the EH from *R. araucariae* 25. Expression of the recombinant EH was induced by the addition of galactose to the growth media of *S. cerevisiae* SC-25. Whole-cell EH activity was determined on 100 mM styrene oxide (Figure 17), as well as cyclohexene oxide and indene oxide (Figures A14 and A15 in Appendix 1), using 10 - 20% (w/v) biomass loading, and comparing the activity to YL25HmA.

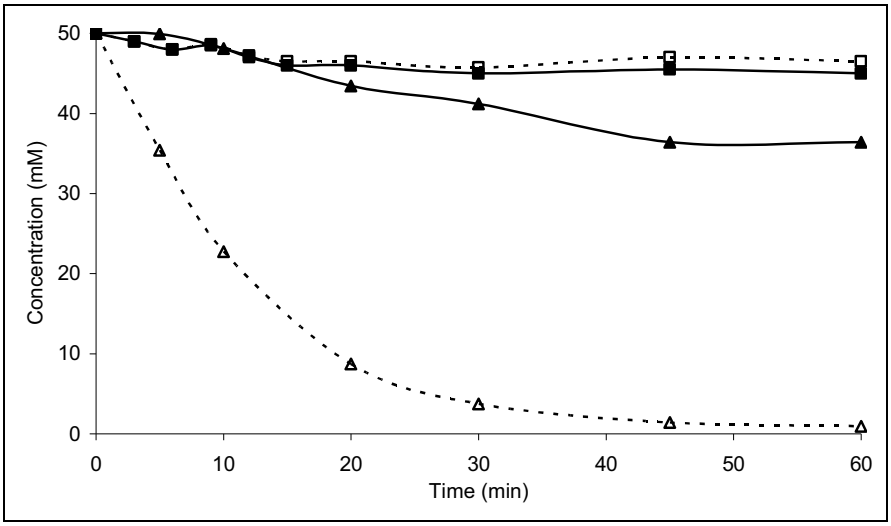


Figure 17. Comparison of the kinetic resolution of 100 mM styrene oxide by the recombinant enzyme from *R. araucariae* 25 expressed in *S. cerevisiae* (SC-25), and *Y. lipolytica* (YL25HmA). Solid icons indicate (*R*)-SO, open icons indicate (*S*)-SO. -■- = SC-25, -▲- = YL25HmA.

While the kinetic properties of the enzyme expressed in *Y. lipolytica* (YL25HmA) remained substantially unchanged when compared to the wild-type enzyme from *R. araucariae* 25 (see Figure A1 in Appendix 1), activity as well as enantioselectivity of the recombinant enzyme expressed in *S. cerevisiae*, used as a whole-cell biocatalyst, decreased compared to the recombinant *Y. lipolytica* for all epoxides tested, indicating that *S. cerevisiae* may not be a suitable host for the production of whole cell biocatalysts expressing EHs.

However, a caution here is necessary. Only one *eph* gene was expressed in *S. cerevisiae*, and a definitive conclusion should only be drawn after further testing. This data does not allow one to draw conclusions regarding the actual production of recombinant EHs in *S. cerevisiae*, only the use of *S. cerevisiae* as a whole-cell biocatalyst with the intracellular production of the *R. araucariae* EH, as a direct comparison to the use of *Y. lipolytica* with intracellular production of the same enzyme.

Discussion

In general, wild-type microbial cells as biocatalysts for the resolution of epoxides have intrinsic limitations, as they tend to express sub-optimal amounts of EHs. Various EH enzymes have been purified and used as biocatalysts for enantioselective hydrolysis reactions. However, purification of enzymes to homogeneity is time-consuming, and requires additional costs. In some cases, purified enzymes may lose their activity during the isolation procedure. Baldascini and Janssen (2005) described the inactivation of purified EH enzymes at a high product concentration. A compromise between the advantages and drawbacks of wild-type whole-cell and purified enzyme biocatalysts is the use of recombinant whole-cell biocatalysts expressing heterologous proteins at a high level. The use of recombinant whole-cell biocatalysts can also stabilise activity of the EHs during reactions at high substrate levels, with the epoxide constituting a separate phase by themselves (Lee et al. 2004).

For all recombinant *Y. lipolytica* strains and all epoxide substrates tested, the presence of the *LIP2* signal peptide caused a decrease in both the activity and enantioselectivity of the recombinant EHs, compared to the wild-type yeasts and the same *Y. lipolytica* strains without the *LIP2* secretion signal. It can be postulated that, because the fungal EHs which were tested with the *LIP2* secretion signal are similar to the eukaryotic microsomal enzymes and cluster into the mEH superfamily (as discussed in Chapter 2), they are not successfully processed through the secretory pathway. The EH enzymes from *R. glutinis* and *R. toruloides* are known to be membrane-associated (Botes 1999; Kronenburg et al. 1999). This could result in the enzymes being caught up in the secretory apparatus within the cell, resulting in loss of function. While *Y. lipolytica* is widely used as an expression host for the extracellular production of recombinant enzymes due to its inherent capability to secrete proteins with very high efficiency, it was found that the desired characteristics of EHs such as activity and enantioselectivity are adversely affected and that the enzymes are still predominantly retained within the cell when secretion signals are linked to them.

Botes et al. (1999) reported the enantioselective resolution of 1,2-epoxides, specifically by the genera *Rhodotorula* and *Rhodosporidium.* *R. toruloides* UOFS Y-0471 (WT-46) and *R. araucariae* UOFS Y-0473 (WT-25) were highlighted as strains with broad substrate ranges and good enantioselectivity. The expression of the EHs from these strains, as well as the EH from *R. paludigenum* UOFS Y-0482 (WT-692) and the newly isolated EH from *R. toruloides* 1 (NCYC 3181) in *Y. lipolytica,* resulted in the retention of this enantioselectivity. The 1,2-epoxides included in this study included 1,2-epoxyoctane, epibromohydrin and epichlorohydrin, as well as the glycidyl ethers, phenyl glycidyl ether and benzyl glycidyl ether. The activity towards these substrates was extremely high, especially for transformant YL1HmA, containing the *eph* gene from *R. toruloides* 1 (NCYC 3181).

Styrene oxide and its derivatives, 4-nitro-styrene oxide and 3-chloro-styrene oxide, were efficiently and enantioselectively hydrolysed by the *Y. lipolytica* strains tested. Kim et al. (2006) used styrene oxide to test the whole-cell biocatalysis with *P. pastoris*, *S. cerevisiae* and *E. coli* expressing the EH from the anamorph of *R. toruloides, R. glutinis* ATCC 201718, whose sequence is 100% identical to that of *R. toruloides* CBS349 (Visser et al. 2002). Kim et al. (2006) used 20 mM styrene oxide as substrate, whereas the *Y. lipolytica* transformants shown here efficiently hydrolysed 100 mM substrate. YL1HmA, whose EH was amplified using primers designed to the EH from CBS349, was a particularly efficient strain. It showed high levels of activity and initial enantioselectivity, but direct comparisons were not possible between our data and that of Kim et al., as different units were used. The EH from "*C. curvatus"* Car054 also displayed high enantioselectivity with 3-chloro-styrene oxide.

The EH from *R. mucilaginosa* UOFS Y-0198 in YL23HmA converted 50 mM of each of the (*R*)- and (*S*)-enantiomers of the 2-methyl-3-phenyl-1,2-epoxypropane to approximately 60 mM of (*R*)-diol and 40 mM of (*S*)-diol. This was also the case with the EH from *C. neoformans* CBS132 (YL777HmA). YL1HmA and YL692HmA (*R. paludigenum eph*) also preferentially formed the (*R*)-diol, although the conversion

was slower. These results illustrate the enantioconvergence of these enzymes towards this substrate, indicating opposite regioselectivities towards the two enantiomers.

YL1HmA also showed very good activity and enantioselectivity towards cyclohexene oxide, while YL46HmA (*R. toruloides* UOFS Y-0471) demonstrated the same for both the *meso*-epoxides, cyclohexene and cyclopentene oxide. All strains tested showed high levels of enantioselectivity in the prochiral conversion of these substrates to the (*R,R*)-diols.

The two insect EHs (Tn1 and Tn2) from the cabbage looper, *T. ni*, were successfully expressed in *Y. lipolytica.* Resolution of indene oxide revealed an interesting characteristic of the insect EHs: while all the recombinant fungal EHs screened utilised the (*1S,2R*)-enantiomer, Tn1 and Tn2 displayed a preference for the (*1R,2S*)-enantiomer. Although no strains exhibited an absolute preference for their preferred substrate, this difference in resolution may be important. Hydrolysis of 4-nitrostyrene oxide by Tn1 and Tn2 revealed levels of activity and enantioselectivity that are lower than the recombinant fungal EHs with which they were compared although, in this case, the preference was for the same enantiomer as the fungal EHs.

The plant sEH from *S. tuberosum* also displays interesting properties from a biocatalytic perspective. The kinetic resolution of styrene oxide proceeds with the opposite enantioselectivity to the hydrolysis catalysed by most fungal EHs, or by the bacterial sEH from *A. radiobacter*. Morriseau et al. (2000) also speculated that there were structural differences in the sEH enzymes from plants and mammals, with different orientations relative to their respective hydrophobic substrate binding pockets. Industrial demands for '100% yield, 100% enantiomeric excess' reactions starting from a racemic mixture, may be answered by taking advantage of this 'stereochemical flexibility', setting up a single process combining the complementary enantioselectivities of different EH enzymes. Recent reports on *S. tuberosum* sEH (Mateo et al. 2007, Monterde et al. 2004) have claimed that this enzyme is capable of performing such an enatioconvergent process on its own, in two steps, with the initial attack on the α (benzylic) carbon atom of (*S*)-styrene

oxide to form (*R*)-phenylethanediol, followed by the hydrolysis of the β (terminal) carbon atom of (*R*)-styrene oxide to form the same product. However, the rate of reaction with (*R*)-styrene oxide is 300 times slower. Cao et al. (2006) used the opposite enantioselectivities of the *S. tuberosum* sEH and a mutated *A. radiobacter* sEH in a combination to convert racemic styrene oxide to pure (*R*)-phenylethanediol, in a simultaneous reaction.

The activity and enantioselectivity of the recombinant enzyme *S. tuberosum* sEH could not be directly compared to published data (Morriseau et al. 2000; Stapleton et al. 1994; Elfström and Widersten 2005), since purified enzyme was used in literature. However, the enantioselectivity compared well to published data, and no inactivation occurred when expressed intracellularly in *Y. lipolytica* as host. The recombinantly-expressed EH was highly active. It is thus clear that highly active and selective EHs from very diverse origins can be expressed with retention of the kinetic properties in *Y. lipolytica*, but at much higher levels of expression.

Expression of a bacterial EH without any codon optimisation is important as this highlights the efficacy of *Y. lipolytica* as a versatile expression host for these biocatalytic enzymes. The sEH *echA* gene of *A. radiobacter* was expressed in *Y. lipolytica* and the YL-Ar-HmA transformant used for the hydrolysis of styrene oxide. The YL-Ar-HmA transformant displayed essentially the same enantioselectivity as reported for the native gene in *A. radiobacter* (Rink and Janssen 1998). This enzyme has only previously been expressed in *E. coli* (Rink et al. 1997b). Lutje Spelberg et al. (1998) determined the enantioselectivity of the partially purified recombinant *E. coli*-expressed enzyme and demonstrated the highly preferential hydrolysis of the (*R*)-enantiomer of styrene oxide, converting it to (*R*)-phenylethanediol, as was found here with the recombinant sEH from YL-Ar-HmA. After the complete hydrolysis of (*R*)-styrene oxide, Lutje Spelberg et al. (1998) noted initiation of the hydrolysis of the (*S*)-enantiomer. A possible explanation was the preferential binding of the (*R*)-enantiomer, preventing the hydrolysis of the (*S*)-enantiomer until there was no further (*R*)-styrene oxide to inhibit its binding. Another recently identified bacterial sEH from *Sphingomonas* sp HXN-200 displayed a similar

correlation between the completion of the hydrolysis of (*R*)-styrene oxide and the onset of the hydrolysis of (*S*)-styrene oxide (Liu et al 2006). Figure A12 (in Appendix 1) illustrates a comparable relationship with YL-Ar-HmA, with the onset of hydrolysis of the (*S*)-styrene oxide coinciding with the depletion of the (*R*)-enantiomer after 25 minutes. This implies the configuration of the *Y. lipolytica*-produced enzyme retains its integrity.

Expression of microsomal EHs in *S. cerevisiae* has been reported for the mEH from *R. norvegicus* (Arand et al. 1999b) and *R. glutinis* (Kim et al. 2006). Expression of the rat mEH was limited to 0.2% of the microsomal protein fraction. *R. glutinis* EH was expressed on the surface of *S. cerevisiae* in an effort to reduce the mass transfer limitations caused by the substrate having to diffuse across the cell membrane. However, expression levels were very low, indicating that the recombinant *S. cerevisiae* exhibited a low whole-cell EH activity per unit cell mass. Kim et al. (2006) concluded that the cell surface expression of EH did not enhance the catalytic activity. The results presented here also indicate that only low levels of activity were obtained in the *S. cerevisiae* whole-cell biocatalysts expressing the EH from *R. araucariae*. However, only one *eph* gene was expressed in *S. cerevisiae*, and a definitive conclusion should only be drawn after further testing. A disadvantage of the use of whole-cell biocatalysts is the requirement for the substrates to diffuse across the cell membrane, which can cause mass transfer limitations. In general, K_m values are much lower in purified enzymes, indicating that the mass transfer resistance conferred by cell walls is high. This may be the case with the expression of EHs in *S. cerevisiae*. It is also known that *S. cerevisiae* can hyper-glycosylate foreign proteins (Buckholz and Gleeson 1991), which may sterically hinder the epoxide hydrolase. Extracellular expression may improve the levels of production of functional EH, but this would be at the expense of the advantages of using whole-cell biocatalysts.

The copy numbers of the integrated EH expression cassettes in the *Y. lipolytica* transformants varied greatly. According to Juretzek et al. (2001), the presence of the defective *ura3d4* marker should ensure selection of transformants that contain 10 – 13

copies of the *ura3d4* gene, which seemed to be the minimum to alleviate the host's uracil auxotrophy. However, as shown in Table 4, some transformants, selected as the best of their type via epoxide hydrolysis detected by TLC screening, appear to have only three to five copies of the *ura3d4* gene, and therefore also the relevant EH gene. Chiral analyses of the kinetic resolutions catalysed by these strains indicate they are highly functional, and they were repeatedly cultivable on media maintaining selective pressure. A possible explanation is that the expression cassette integrated at a site in the genome that is highly active, and the expression of the *ura3d4* selection marker, as well as the *eph* gene, was influenced by regulatory elements in the vicinity of the site of integration. The copy numbers of two different YL25HmA (*R. araucariae eph*) transformant are shown, one displaying the expected 15 copies of the *ura3d4* gene, and the other with only one copy. In this case, differences in the transformants' copy numbers and poor activity of the single copy transformant against various substrates (not shown), may be as a result of unwanted recombination (gene conversion) between the partially deleted *ura3* genomic locus (containing an internal deletion, so both upstream and downstream regions of gene are still intact) and the *ura3d4* marker gene on the expression cassette. This results in an intact version of the marker gene that is sufficiently transcriptionally active, due to the wild type promoter that is strong enough to restore the $URA3^{+}$ phenotype on media lacking uracil.

The development of a single highly efficient expression system for producing functional heterologous EHs from any source, including animal, insect, plant and microbial origins, is a significant benefit, given the limitations and constraints of the current options available. The results shown here, where kinetic resolutions of epoxides from all classes tested were successfully achieved, even under non-optimised conditions, permits speculation that the use of *Y. lipolytica* as a generic expression system for EHs holds considerable potential.

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Chapter 4.

Functional expression of *Agrobacterium radiobacter* AD1 halohydrin dehalogenase in *Yarrowia lipolytica*

by

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The expression of *A. radiobacter* AD1 halohydrin dehalogenase in *Y. lipolytica* was done by the author.
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Abstract

Halohydrins can be considered direct precursors of epoxides. Halohydrin dehalogenases catalyse the nucleophilic displacement of a halogen ion in various aliphatic and aromatic halohydrins by a vicinal hydroxyl group, yielding an epoxide, a proton and a halide ion. They can also efficiently catalyse the reverse reaction, the halogenation of epoxides. The interest in halohydrin dehalogenases increased when it was found that the dehalogenation of halohydrins may proceed with high enantioselectivity, making these enzymes useful catalysts to prepare various optically active epoxides. Ring closure of an optically pure halohydrin generally yields an optically pure epoxide.

The halohydrin dehalogenase of the 1,3-dichloro-2-propanol-utilising bacterium *Agrobacterium radiobacter* (also known as *A. tumefaciens*) AD1 exhibits remarkable enantioselectivity with a broad range of aliphatic and aromatic halohydrins. It is encoded by the *HheC* gene. Here, the successful expression of *A. radiobacter*'s *HheC* in the yeast *Yarrowia lipolytica* is described. Both single copy and multi-copy transformants are generated. Optimisation of expression levels is attempted by constructing vectors which contain up to five expression cassettes (hp4d promoter - *HheC* - *LIP2* terminator) per single multi-copy *ura3d4* selection marker, compared to the usual single expression cassette per *ura3d4* gene. Theoretically, the same number of integration events could occur, resulting in up to five times as many expression cassettes integrating into the genome. Results of ring-closure reactions with aromatic 2-chloro-1-phenylethanol enantiomers indicate HHeC is functionally expressed in a dose-dependent manner, retaining the enantioselectivity of the native enzyme.

Introduction

Halohydrin dehalogenases (HHdHs) catalyse the reversible conversion of a vicinal halo-alcohol to its corresponding epoxide, by the nucleophilic substitution of the halogen by the vicinal hydroxyl group, thereby releasing a proton and a halide ion. They are key enzymes in the bacterial degradation of vicinal halo-propanols and structurally related nematocides (Van Hylckama Vlieg et al. 2001). Substrates include chlorinated and brominated C_2 and C_3 alcohols such as 2-chloro-ethanol, 1,3-dichloro-2-propanol and 2,3-dichloro-1-propanol and their brominated analogues, as well as aromatic halohydrins such as 1-chloro-2-phenylethanol.

The halohydrin dehalogenase HheC (encoded by the gene *HheC*) of the 1,3-dichloro-2-propanol-utilising bacterium *Agrobacterium radiobacter* (also known as *A. tumefaciens*) AD1 is a homo-tetrameric protein containing three cysteines per 28 kDa subunit (Tang et al. 2002). *A. radiobacter* AD1's HheC exhibits remarkable enantioselectivity with a broad range of aliphatic and aromatic halohydrins. It is highly enantioselective with aromatic halohydrins such as 1-(*para*-nitrophenyl)-2-chloro-ethanol and can catalyse not only ring closure of a halohydrin but also the reverse reaction of the nucleophilic ring opening of an epoxide, using nucleophiles such as azide, cyanide and nitrite (Nakamura et al. 1994; Lutje Spelberg et al. 2001; Hasnaoui et al. 2005). The opening of various styrene oxide rings by HheC with azide as the nucleophile results in the highly enantioselective and regioselective production of azido-alcohols (Lutje Spelberg et al. 2002). Under oxidising conditions, the enzyme is susceptible to inactivation, which can be prevented by the addition of β-mercaptoethanol or glycerol (Tang et al. 2002).

These properties of halohydrin dehalogenases make them promising candidates to become tools in the synthesis of enantiopure aliphatic and aromatic epoxides and halo-alcohols. The production of single enantiomers of these chiral intermediates has become increasingly important to the pharmaceutical industry. Single enantiomers may also be produced by chemical or chemo-enzymatic synthesis, but the advantage of using a biological system is that enzyme-catalysed reactions may be highly enantioselective and regio-selective.

The *HheC* gene from *A. radiobacter* AD1 was previously expressed in *Escherichia coli* by Lutje Spelberg et al. (1999), as well as by Van Hylckama Vlieg et al. (2001), who expressed it along with the HHDH-encoding genes from *Arthrobacter* AD2 (*HheA*) and *Mycobacterium* GP1 (*HheB*). The *hheA* and *hheB* genes from *Corynebacterium* sp. strain N-1074, were also cloned and expressed in *E. coli* (Yu et al. 1994). The *hheA* and *hheB* gene products are functional isozymes which catalyse the inter-conversion of 1,3-dichloro-2-propanol to epichlorohydrin, the first step in the production of 3-chloro-1,2-propanediol.

Yarrowia lipolytica is one of the most promising 'non-conventional' yeasts available as hosts for heterologous protein production (Müller et al. 1998), with a large number of molecular tools available for heterologous protein expression in *Y. lipolytica* (Madzak et al. 2004). Low copy vectors are available that contain the non-defective *ura3d1* selection marker, which complements the *Y. lipolytica* Po1h host strain's auxotrophy in single or low copy numbers. Multi-copy vectors contain the *ura3d4* marker, which is required in multiple copies to complement, allowing for selection of transformants with multiple inserts. The *ura3d4* selection marker should ensure selection of transformants with 10 - 13 copies of the integrated cassette (Juretzek et al. 2001). *Y. lipolytica* Po1g contains a pBR322 docking platform to direct single-copy integration of pBR322-based vectors (Madzak et al. 2000). This allows direct comparison of activities of transformants, as there is no variation due to the site of random integration, as is found with the Po1h strain, when transformed with either *ura3d4*- or *ura3d1*-containing DNA.

In this study the intention was to conduct ring-closure reactions using HheC from *A. radiobacter* AD1 that had been cloned and over-expressed in *Y. lipolytica*. This expanded the use of *Y. lipolytica* as a host strain for heterologous production of enzymes of biocatalytic importance. Figure 1 illustrates the ring-closure reaction by HheC used in this study.

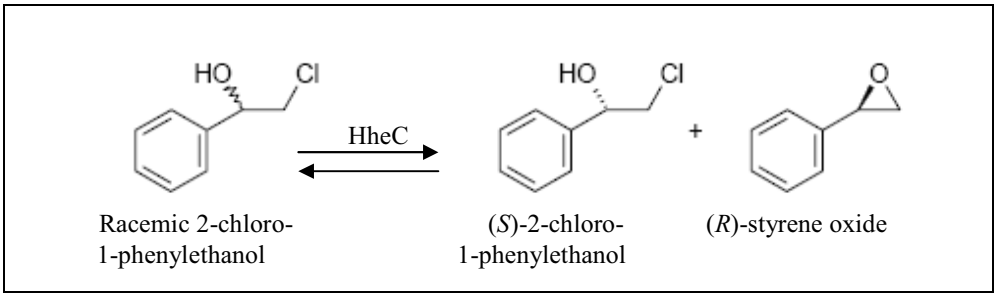

Figure 1. Enantioselective ring-closure of racemic 2-chloro-1-phenylethanol by HheC, encoded by *A. radiobacter* AD1.

The native *A. radiobacter HheC* gene sequence was codon-optimised to match the optimal codon usage in *Y. lipolytica* (Dujon et al. 2004). Expression was controlled in *Y. lipolytica* with the growth-phase dependent synthetic hybrid promoter hp4d (hp4d$_P$, Nicaud et al. 2002) and the *Y. lipolytica LIP2* terminator (*LIP2*$_T$, Pignède et al. 2000). Single–copy transformants were generated in *Y. lipolytica* Po1g using the pBR322 docking system with the *ura3d1* marker, while multiple copy integrants were created in *Y. lipolytica* Po1h, using *ura3d4* as the selection marker. In addition, the copy number of the integrated expression cassettes was optimised by creating tandem cassettes for multi-copy integration in *Y. lipolytica* Po1h to maximise expression. These contained from two to five copies of the expression cassette (hp4d$_P$-s*Hhe*C–*LIP2*$_T$) per auxotrophic *ura3d4* marker copy.

Materials and Methods

Strains, Plasmids and PCR primers

The genotypes of the microbial strains and plasmids used in the present study, and the deoxyoligonucleotide primers used are summarised in Tables 1 and 2.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference/Source
Strains		
Escherichia coli DH5αMCR	F⁻ *mcr*A Δ(*mrr-hsdRMS-mcrBC*) Φ*80dlac* Δ*lacZM15* Δ (*lacZYA-argF*)U169 *endA1 recA1 deoR thi-1 aupE44* λ^- *gyrA96 relA1*	Gibco BRL
Yarrowia lipolytica Po1h	*MATA, leu2-270, ura3-302 (= ura3::pXPR2:SUC2), uxpr2-322, axp1-2*. Phenotype: Leu^-, Ura^-, ΔAEP, ΔAXP, Suc^+	Madzak (2003) CLIB 882
Yarrowia lipolytica Po1g	*MATA, leu2-270, ura3-302::URA3, xpr2-322, axp1-2* Phenotype: ΔAEP, ΔAXP, Suc^+, pBR docking platform	Madzak et al. (2000) CLIB 725
Y. lipolytica Po1h transformants		
Y. lipolytica [s*HheC*]	$hp4d_P$-s*HheC-LIP2*$_T$, *ura3d4,* Kan^R, multi-copy	This work
Y. lipolytica [s*HheC*-2]	($hp4d_P$-s*HheC-LIP2*$_T$) x 2, *ura3d4,* Kan^R, multi-copy	This work
Y. lipolytica [s*HheC*-5]	($hp4d_P$-s*HheC-LIP2*$_T$) x5, *ura3d4,* Kan^R, multi-copy	This work
Y. lipolytica Po1g transformants		
Y. lipolytica [s*HheC*-single]	$hp4d_P$-s*HheC-LIP2*$_T$ integrated in single copy into the Po1g genome at the pBR322 docking site	This work
Plasmids		
pGEM-T® Easy	*bla*	Promega
pINA1291	*E. coli / Y. lipolytica* vector. Kan^R, *ura3d4*, $hp4d_P$-*LIP2*$_T$, *zeta*, multi-copy random integration vector for Po1h	Nicaud et al. (2002)
pKOV136	*E. coli / Y. lipolytica* vector. *ura3d1, bla*. pBR322-based integrative vector for site-directed integration at pBR322 docking platform of Po1g. *TEF* promoter-*LIP2*$_T$	Labuschagne and Albertyn (2007)
pYL-sHsA	pKOV136 with *TEF*$_P$ replaced with $hp4d_P$	This work
s*HheC*:pPCR-Script	Synthetic *A. radiobacter* AD1 *HheC* gene (*sHheC*), with *Y. lipolytica*-optimised codon usage	GeneArt GmbH
pYL-HmA constructs		
pYL-s*HheC*-HmA	$hp4d_P$-s*HheC-LIP2*$_T$, *ura3d4,* Kan^R	This work
pYL-s*HheC*2-HmA	($hp4d_P$-s*HheC-LIP2*$_T$) x2, *ura3d4,* Kan^R	This work
pYL-s*HheC*5-HmA	($hp4d_P$-s*HheC-LIP2*$_T$) x5, *ura3d4,* Kan^R	This work
pYL-sHsA constructs		
pYL-s*HheC*-sHsA	$hp4d_P$-s*HheC-LIP2*$_T$, *ura3d1, bla*	This work

Recombinant plasmids were constructed and amplified in *Escherichia coli* DH5αMCR. *E. coli* was cultivated at 37°C in LM (l^{-1}: 10 g yeast extract, 10 g tryptone, 5 g NaCl) liquid medium on a rotary shaker at 200 rpm, or on LM agar (15 $g.l^{-1}$), supplemented with the relevant antibiotics at a final concentration of 100 µg/ml ampicillin or 50 µg/ml kanamycin. 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) were added for blue-white selection where necessary.

Y. lipolytica Po1h transformants were selected on YNB_{casa} medium (l^{-1}: 20 g glucose, 4 g NH_4Cl, 2 g casamino acids, 1.7 g yeast nitrogen base without amino acids and without ammonium sulphate, 0.3 g leucine), with agar (15 $g.l^{-1}$) for solid media. *Y. lipolytica* Po1g transformants were plated on YNB_{N5000} (l^{-1}: 10 g glucose, 1.7 g yeast nitrogen base without amino acids and without ammonium sulphate, 5 g ammonium sulphate). *Y. lipolytica* strains were cultivated in either rich non-selective YPD (l^{-1}: 10 g yeast extract, 20 g peptone, 20 g glucose) medium for nucleic acid isolation, or the required selective YNB-based medium for heterologous protein expression. Yeast strains were cultured in 250 ml Erlenmeyer flasks containing 50 ml medium at 28°C on a rotary shaker at 180 rpm, unless otherwise indicated.

DNA manipulations

Standard protocols were followed for DNA manipulations (Sambrook et al. 1989). The enzymes for DNA cleavage and ligation were purchased from Fermentas. Restriction endonuclease-digested DNA was eluted from agarose using the GFX™ PCR DNA and Gel Band Purification Kit. Dephosphorylation of vector DNA prior to ligation was carried out using New England Biolab's Antarctic Phosphatase. *E. coli* transformations were performed using Bio-Rad's Gene-Pulser. Genomic DNA was isolated from *Y. lipolytica* strains using Promega's Wizard® Genomic DNA Purification Kit, according to the manufacturer's recommended protocol for yeasts. Polymerase Chain Reaction (PCR) was done using an Eppendorf Mastercycler Gradient PCR machine.

Construction of pYL-sHsA

The hp4d promoter region was isolated from pINA1291 using *Bam*HI and *Cla*I. The *TEF* (translation elongation factor-1α) promoter from pKOV136 was removed using the same enzymes, and the 440 bp hp4d promoter fragment was inserted in its place, creating pYL-sHsA.

Expression vector construction

A synthetic gene constructed, based on the native *HheC* gene from *A. radiobacter* AD1 (Genbank accession number AF397296), with optimal codon usage for *Y. lipolytica* (Dujon et al. 2004). Figure 2 shows the original *A. radiobacter HheC* gene (n*HheC*) compared to the synthetic gene sequence (s*HheC*).

Gene synthesis was performed by GeneArt, GmBH, and the synthetic *A. radiobacter* s*HheC* gene was provided in the cloning vector pPCR-Script, with flanking *Bam*HI and *Bln*I restriction enzymes sites, to enable excision of the s*HheC* gene. This 774 bp *Bam*HI/*Bln*I DNA fragment was ligated into vectors pINA1291 and pYL-sHsA, creating pYL-s*HheC*-HmA and pYL-s*HheC*-*s*HsA, respectively.

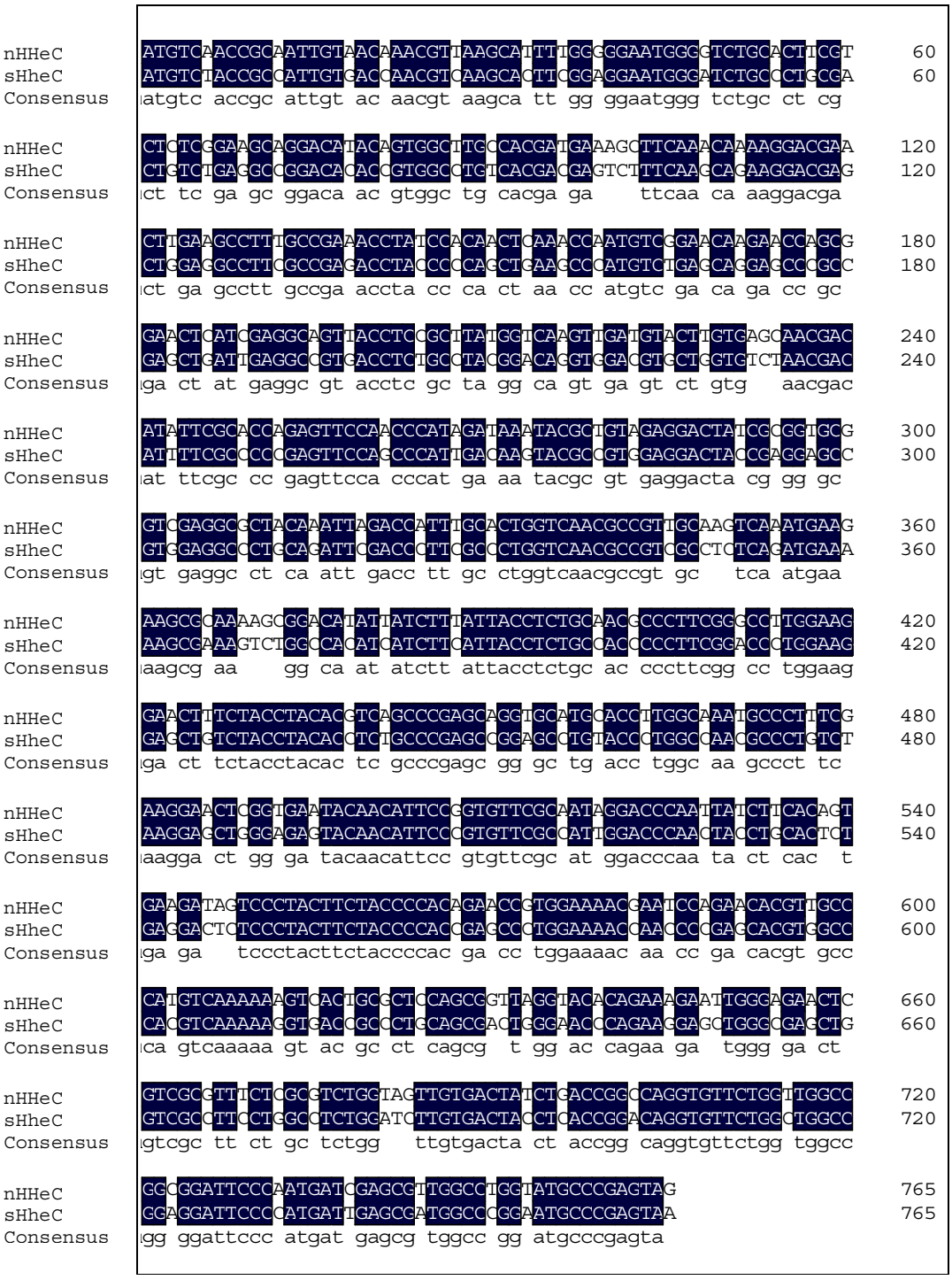

Figure 2. Gene sequences of the native *A. radiobacter* n*HheC*, NCBI accession number AF397296 (upper line), and *Y. lipolytica*-codon optimised s*HheC* (lower line). Black indicates 100% similarity.

Tandem Repeats of expression cassette

In order to increase the yield of heterologous protein, vectors were constructed, based on pINA1291, containing multiple (two – five) tandem repeats of the expression cassette (hp4d$_p$-s*Hhe*C–*LIP2*$_T$) per single *ura3d4* selection marker, compared to the pINA1291's single expression cassette per *ura3d4* gene. Theoretically, the same number of integration events could occur, resulting in five times as many expression cassettes integrating into the genome.

Primers Tandem-1F and Tandem-1R (Table 2) were used with pYL-s*HheC*-HmA as template, to amplify the hp4d$_p$-s*Hhe*C–*LIP2*$_T$ cassette. Reactions were set up containing 200 µM dNTPs, 250 nM of each primer, an aliquot of plasmid DNA as template and 0.005 U/µl of TaKaRa ExTaq. The programme used was: 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, 72°C extension for 2 min 30 sec; then a final extension of 72°C for 10 min. The 1,740 bp DNA fragment was then ligated to pGEM-T Easy (Promega). This construct is designated p-s*Hhe*C-TA (Figure 4). p-s*Hhe*C-TB was constructed in a similar manner, using primers Tandem-3F2 and Tandem-1R to amplify a 1,522 bp product from pYL-s*HheC*-HmA

p-s*HheC*-TB was digested with *Eco*RV and *Dra*I, to release the 1,510 bp expression cassette. This DNA fragment was inserted at the *Eco*RV site of p-s*Hhe*C-TA to generate p-s*Hhe*C-TC (Figure 3). The procedure was repeated to generate expression cassettes p-s*Hhe*C-TD, -TE and -TF, as illustrated in Figure 3.

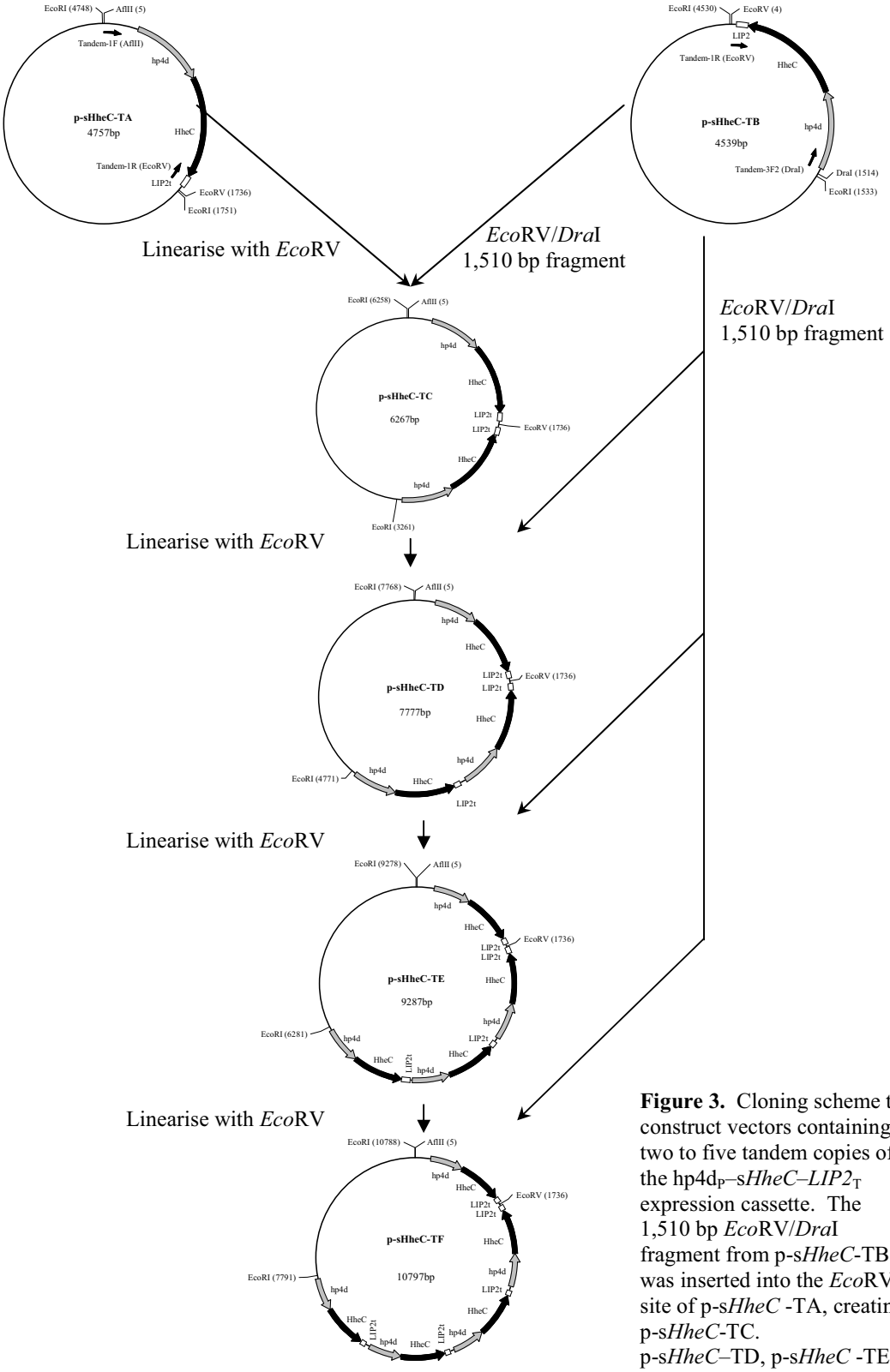

Figure 3. Cloning scheme to construct vectors containing two to five tandem copies of the hp4d$_P$–s*HheC*–*LIP2*$_T$ expression cassette. The 1,510 bp *Eco*RV/*Dra*I fragment from p-s*HheC*-TB was inserted into the *Eco*RV site of p-s*HheC* -TA, creating p-s*HheC*-TC. p-s*HheC*–TD, p-s*HheC* -TE and p-s*HheC*-TF were similarly constructed.

In order to insert two copies of the expression cassette into pINA1291, p-s*HheC*-TC (Figure 4) was digested with *Afl*II and *Eco*RI to liberate the 3,256 bp DNA fragment containing two expression cassettes, which was inserted into the corresponding sites of pINA1291, to form pYL-s*HheC*2-HmA. To insert five copies of the expression cassette, p-s*HheC*-TF (Figure 4) was similarly digested to release the 7,786 bp DNA fragment containing the five expression cassettes, which was also ligated into pINA1291, to create pYL-s*HheC*5-HmA.

Y. lipolytica transformation and identification of *Y. lipolytica* transformants

Large-scale isolations of each of the pINA1291 and pYL-sHsA constructs were carried out using Qiagen's Plasmid MIDI kit, from 50 ml overnight cultures. 5 µg of plasmid DNA was digested with *Not*I. The resultant DNA fragments from pINA1291 constructs are bounded by the *zeta* regions to stimulate random integration and contain the *ura3d4* marker and the expression cassettes, without any bacterial plasmid DNA. The DNA fragment from pYL-sHsA is flanked by regions of the pBR322 plasmid, which integrates at the homologous docking platform integrated in the chromosome of Po1g (Madzak et al. 2000). The method of Xuan et al. (1988) was used for the transformation of *Y. lipolytica* Po1h with the pINA1291 constructs, and *Y. lipolytica* Po1g with the pYL-sHsA constructs. Po1h colonies appearing on the YNB_{casa} selective plates after 7-14 days, and Po1g transformants on the YNB_{N5000} plates after 2 – 5 days, were transferred onto fresh plates and re-grown.

Transformants appearing on the selective plates were subjected to genomic DNA isolation. The genomic DNA was used as the template for PCR screening, which was carried out with the *HheC* gene-specific primers HheC-F and HheC-R (Table 2). Each 10 µl PCR reaction contained 200 µM dNTPs, 250 nM of each primer, 2 mM of $MgCl_2$, template DNA and 0.25 U of BioTaq (Bioline). The PCR profile was: 95ºC for 5 min, 30 cycles of: denaturation at 95ºC for 1 min, annealing at 50ºC for 1 min, extension at 72ºC for 1 min; followed by a final extension at 72°C for 5 min.

Table 2. DNA sequences of the oligodeoxynucleotide primers used in this study. Restriction enzyme sites are underlined in bold

Primer name	Sequence (5'-3')	Restriction enzyme site
Tandem-1F	CGTG**CTTAAG**AGCAAGTTCCTTGAG	*Afl*II
Tandem-1R	G**GATATC**GTCTTAGAGGAACGCATATACAGTAATCATAGAG	*Eco*RV
Tandem-3F2	GAGAC**TTTAAA**AGCTTCGTAGGAGGGCATTTTG	*Dra*I
HheC-F	GA**GGATCC**ATGTCAACCGCAAT	*Bam*HI
HheC-R	GA**CCTAGG**CTACTCGGGCAT	*Bln*I

Copy number determinations for *Y. lipolytica* transformants

Real-time PCR (RT-PCR) was used to estimate the copy number the integrated expression cassettes. This was carried out as described in Chapter 3.

Heterologous expression of HheC in *Y. lipolytica*

Ring closure reactions using 2-chloro-1-phenylethanol

To determine whether functional HheC was expressed from the various *Y. lipolytica* transformants containing *sHHeC*, ring closure reactions using 2-chloro-1-phenylethanol (CPE) as substrate were carried out. The (*R*)- and (*S*)-enantiomers of CPE were tested separately. The *Y. lipolytica* transformants were tested as whole-cell biocatalysts. Three selected transformants of each of the multi-copy strains *Y. lipolytica* [s*HheC*], [s*HheC*-2] and [s*HheC*-5], as well as the single copy *Y. lipolytica* [s*HheC*-single], were cultivated for 6 days. The extended period was chosen as experimentation with the epoxide hydrolase-producing *Y. lipolytica* transformants, subsequent to the research shown in Chapter 3, had indicated that enzyme activity peaked later than the original 3 days' of cultivation. Cells were harvested by centrifugation, suspended in 200 mM phosphate buffer (pH 7.5) to a concentration of 50% (m/v), for immediate evaluation of enzyme activity. *Y. lipolytica* [s*HheC*], [s*HheC*-2] and [s*HheC*-5] transformants were used at a final concentration of 10% cells (m/v), while *Y. lipolytica* [s*HheC*-single] was used at 20% cells (m/v). Mercaptoethanol (3 mM) was used in all reactions. Reactions were started by addition of 100 mM substrate, and were conducted for 150 mins at room temperature. Sampling was done after 15, 40, 80 and 150 min, when 200 µl samples were removed and extracted in 900 µl ethanol.

Analytical Methods

Chiral Gas chromatography (GC) was performed on a Hewlett Packard 5890 series II gas chromatograph equipped with Flame Ionisation Detector (FID) and Agilent 6890 series autosampler injector, using hydrogen as a carrier gas at a constant column head pressure at 12psi.

Quantitative analysis of the enanatiomers of 2-choro-1-phenylethanol (CPE) and styrene oxide (SO) was achieved using a chiral capillary GC column Rt-Beta Dex sm (30 m length x 0.32 mm internal diameter x 0.25 µm film thickness, supplied by Restek). The injection temperature was maintained constant at 220°C, detector temperature at 250°C, and split ratio at 50:1. The oven temperature programme was as follows: the initial temperature of 80°C was maintained for 1 min, increased at a rate of 5°C per min to 180°C, and maintained at this temperature for 3 mins. The retention times (Rt) (min) were as follows: Rt (*R*)-SO = 14.115, Rt (*S*)-SO = 14.615, Rt (*S*)-CPE = 23.700, Rt (*S*)-CPE = 23.830.

Results

Construction of expression vectors

The *A. radiobacter* AD1 s*HheC* gene was synthesised with codon usage optimised for *Y. lipolytica*. This s*HheC* DNA sequence has a 77.4% similarity to the original gene, but the amino acid sequence remains 100% homologous. Analysis of the original gene by GeneArt GmBH indicated that this sequence used rare codons with high frequency. A number of *cis*-acting motifs which could potentially negatively affect expression were also detected. These were also removed during the codon optimisation process. The new sequence was determined using GeneArt's GeneOptimizer® software tool.

The synthetic s*HheC* gene was sub-cloned into pINA1291, forming pYL-s*HheC*-HmA. pYL-sHsA was constructed by replacing the *TEF* promoter of pKOV136 with the synthetic hp4d promoter. The synthetic s*HheC* gene was inserted, forming pYL-s*HheC*-sHsA. Plasmids p-s*HheC*-TA to p-s*HheC*-TF were constructed successfully, as shown in Figure 4. The hp4d$_P$-s*HheC*-*LIP2*$_T$ expression cassettes were liberated from p-s*HheC*-TC and p-s*HheC*-TF, containing two and five copies of the expression cassette respectively, with *Afl*II and *Eco*RI, and inserted into pre-digested pINA1291. This resulted in constructs pYL-s*HheC*2-HmA and pYL-s*HheC*5-HmA.

Y. lipolytica transformations

*Not*I-linearised expression cassettes from pINA1291 constructs were transformed into *Y. lipolytica* Po1h, and that from pYL-s*HheC*-sHsA was transformed into *Y. lipolytica* Po1g. Subsequent to the transformation of *Y. lipolytica* with the different constructs, transformant colonies were subjected to genomic DNA isolation and PCR screening using *HheC* gene-specific primers. The presence of PCR products of the expected size (780 bp) was taken as confirmation that integration of the relevant expression cassette had occurred in the genome of *Y. lipolytica* Po1h or Po1g.

Y. lipolytica Po1h transformed with pYL-s*HheC*-HmA, pYL-s*HheC*2-HmA and pYL-s*HheC*3-HmA were designated *Y. lipolytica* [s*HheC*], [s*HheC*-2] and [s*HheC*-5], respectively. *Y. lipolytica* Po1g transformed with pYL-s*HheC*-sHsA was designated *Y. lipolytica* [s*HheC*-single].

Copy number determination for *Y. lipolytica* transformants

Real-time PCR amplifications were carried out on all *Y. lipolytica* transformants selected for analysis. *Y. lipolytica* Po1g was chosen as the control organism as the two targets (*URA3* and *SUC2*) are of known copy number, with a single copy of each. Standard DNA concentrations were used in all reactions.

The equation $\Delta\Delta Ct = [\ Ct(URA) - Ct(SUC2)\ ]_{sample} - [\ Ct(URA) - Ct(SUC2)\ ]_{control}$ (Livak and Schmittgen 2001) was used to estimate the copy number of the integrated expression cassette, where the control strain is Po1g. In this case, the $[\ Ct(URA) - Ct(SUC2)\ ]_{control}$ value was assumed to be 0, as the copy numbers of the 2 genes are the same, and the Ct values of the profiles are therefore also the same. This was also experimentally shown (Figure 5, Chapter 3).

The equation could therefore be simplified to $\Delta\Delta Ct = [\ Ct(URA) - Ct(SUC2)\ ]_{sample}$. The $\Delta\Delta Ct$ value can then be inserted into the equation $N = 2^{-\Delta\Delta Ct}$, where there are N-fold copies of *URA3* compared to *SUC2*. As it is known that there is only a single copy of SUC2 in *Y. lipolytica* Po1h, it can be stated that there are N-copies of the *URA3* target present. Table 4 shows the Ct values obtained for each strain, and the estimated copy numbers, using the Livak equation.

Table 4. Relative copy numbers of URA3 target sequence in different *Y. lipolytica* transformants

Transformant	Ave Ct(URA3)	Ave Ct(SUC2)	ΔΔCt = [Ct(URA)-Ct(SUC2)]	Livak Copy Number: $N = 2^{-\Delta\Delta Ct}$ [1]	Pfaffl Copy Number [1]
Y. lipolytica Po1g	15.17 ± 0.18	15.17 ± 0.11	0.00	1	1
Y. lipolytica [s*HheC*-5] 1	14.48 ± 0.23	17.32±0.35	-2.8	7	8
Y. lipolytica [s*HheC*-5] 2	16.05 ± 0.58	16.25 ± 0.23	-0.2	1	1
Y. lipolytica [s*HheC*-5] 3	13.40 ± 0.11	16.40 ± 0.52	-3.0	8	10
Y. lipolytica [s*HheC*-2] 1	13.40 ± 3.1	17.50 ± 0.43	-4.1	17	22
Y. lipolytica [s*HheC*-2] 2	13.43 ± 0.18	17.48 ± 0.45	-4.1	17	22
Y. lipolytica [s*HheC*-2] 3	13.51 ± 0.00	17.66 ± 0.21	-4.2	18	22
Y. lipolytica [s*HheC*] 1	13.37 ± 1.12	16.76 ± 0.19	-3.4	11	13
Y. lipolytica [s*HheC*] 2	13.68 ± 0.07	17.67 ± 0.51	-4.0	16	20
Y. lipolytica [s*HheC*] 3	13.71 ± 0.47	17.99 ± 0.47	-4.3	20	25
Y. lipolytica [s*HheC*-single]	16.06 ± 0.12	16.76 ± 0.46	-0.7	2[1]	2[1]

[1] rounded off to nearest whole number.
[2] the single copy integrant *Y. lipolytica* [s*HheC*-single] shows a copy number of 2 due to the additional presence of the intact *URA3* gene in the host Po1g.

In order to determine whether the use of this equation was justified, the copy numbers were checked against values obtained with the Pfaffl method (Pfaffl, 2001). The average E-value for the *SUC2* PCR reactions was determined to be 2.08 and the *URA3* E-value was calculated as 2.17. In a perfect PCR reaction, the E-value is 2, presumed in the Livak equation. The slightly higher E-values are potentially due to the smoothing process applied to the curves. Using the Ct values as shown in Table 4, the Pfaffl equation was used to determine the copy numbers for the various transformants. This value is shown in the final column in Table 4. Figure 4 shows some representative data.

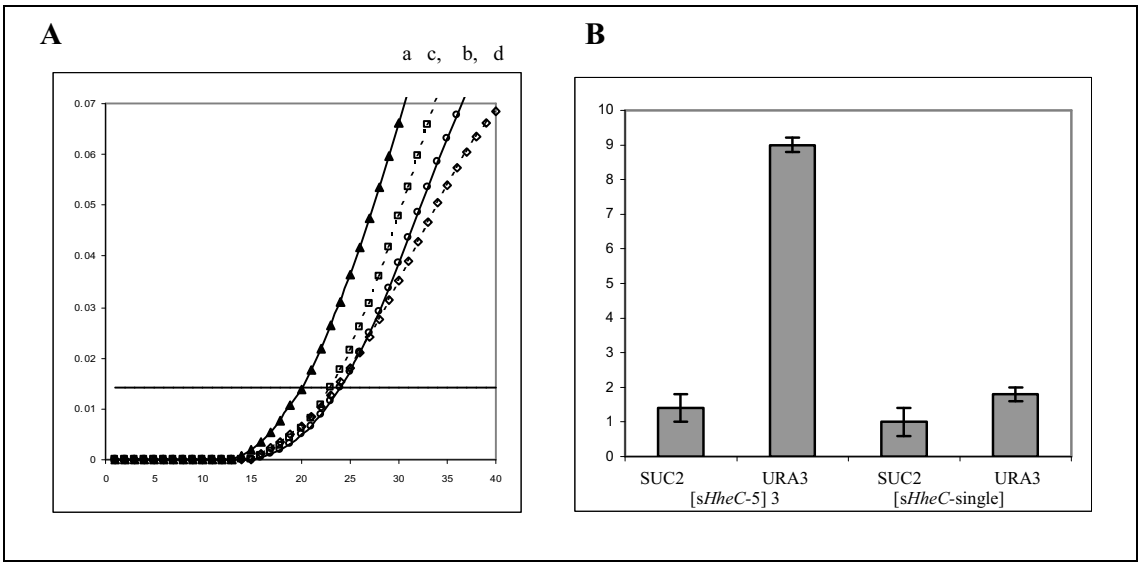

Figure 4A - B. Representative relative copy number determination of selected *Y. lipolytica* Po1h transformants. **(A)** depicts fluorescence versus cycle number, enlarged to show the curves clearly. -▲- (a) is the *URA3* amplification from *Y. lipolytica* [s*HheC*-5] 3. The -○- (b) curve is the *SUC2* amplification of the same strain. The -♦- (c) and -■- (d) curves curves are *SUC2* and *URA3* amplifications from *Y lipolytica* [s*HheC*-single], respectively. The values are the average of duplicate reactions. The standard deviations are shown by the vertical ticks. As can be seen, the URA3 amplification of *Y. lipolytica* [s*HheC*-5] 3 results in a much lower Ct value (intersection point with the threshold, depicted by the horizontal line). The Ct values of the *SUC2* amplifications of both samples result in similar Ct values, indicting similar copy numbers. Only a small difference between the *URA3* and *SUC2* of *Y. lipolytica* [s*HheC*-single] is observed. **(B)** is a graphical representation of the relative copy numbers of the different amplicons in *Y. lipolytica* [s*HheC*-5] 3 and *Y. lipolytica* [s*HheC*-single].

HHDH activity in whole-cell biocatalysis using *Y. lipolytica* transformants

Ring-closure reactions using 2-chloro-1-phenylethanol

Ring-closure reactions were carried out with the (*R*)- and (S)-enantiomers of 2-CPE, separately. Figure 5 indicates the resultant epoxide: halohydrin ratio for all strains tested, using 10 mM (*R*) 2-CPE as substrate, with the addition of 3 mM mercaptoethanol to minimise enzyme inactivation under oxidising conditions. The total epoxide value corresponds to the total concentrations of the produced (*R*)- and (*S*)-styrene oxide, including the corresponding diol. The diol is formed by an endogenous epoxide hydrolase-like activity of *Y. lipolytica* (A. Botes, personal communication). Figure 6 shows only the best strains for each transformant type, demonstrating the dose-dependent activity of the different transformants. Figure 7 gives details of the epoxide and diol

products formed for these best strains. The (*R*)-epoxide is preferentially formed in each case. *Y. lipolytica* [*sHheC*-5] transformant 2 indicates no or very little activity in any of the assays. Its determined copy number of *ura3d4* marker gene is also only one, much lower than the copy numbers for the other 2 transformants of the type. This strain was presumed to be a false positive and discarded.

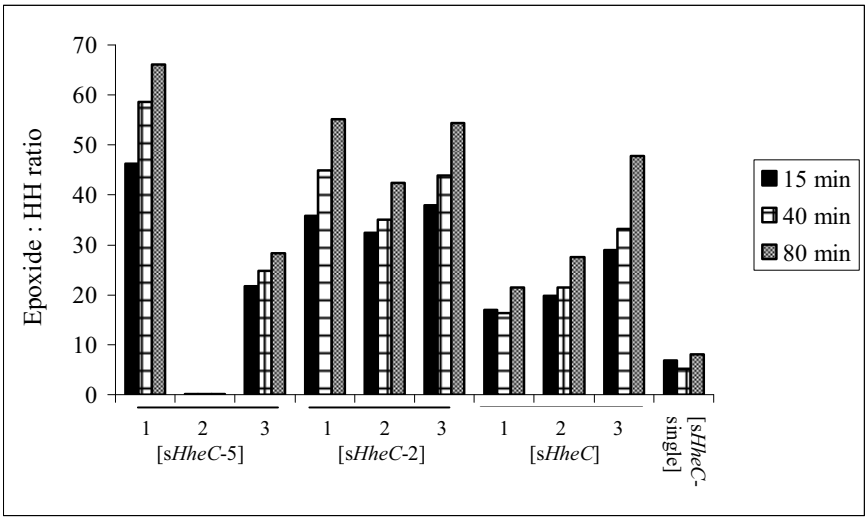

Figure 5. Results of the ring-closure of (*R*)-2-chloro-1-phenylethanol, indicating the increasing epoxide: halohydrin (HH) over time.

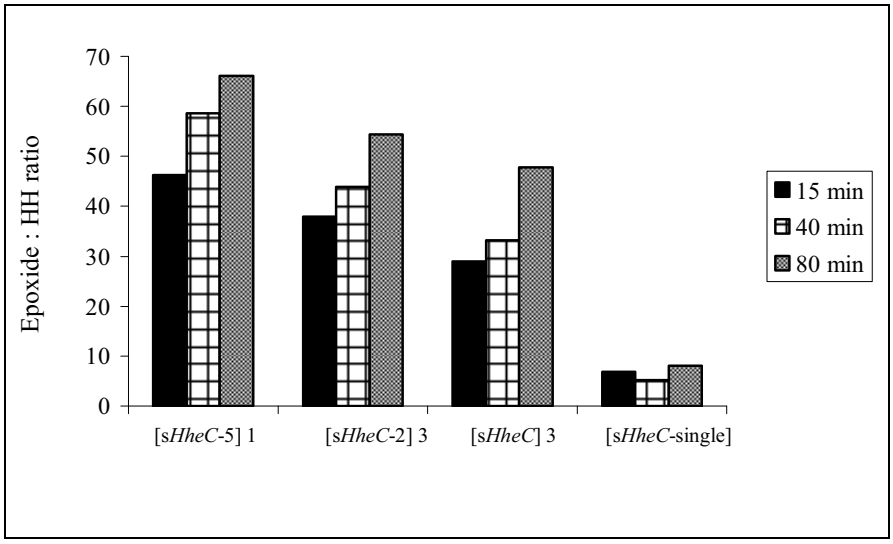

Figure 6. Results of ring-closure of (*R*)-2-chloro-1-phenylethanol, highlighting the dose-dependent relationship between copies of the s*HheC* gene integrated into *Y. lipolytica* and the detected HheC activity.

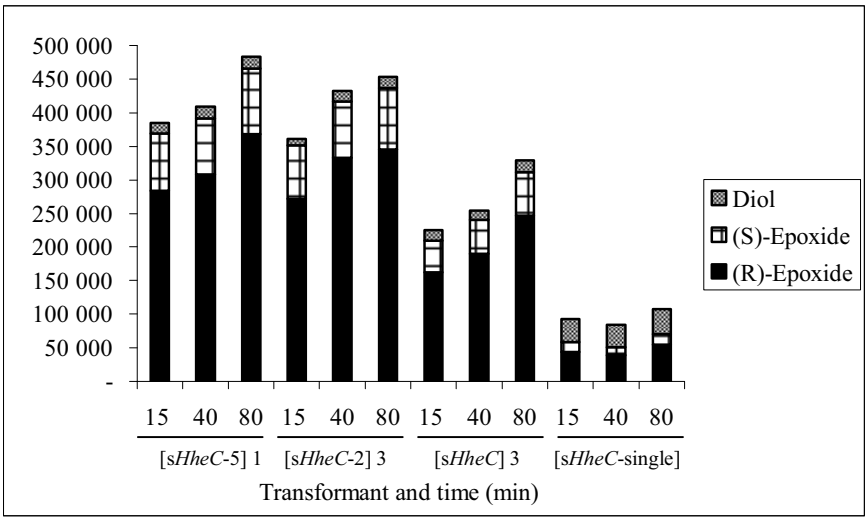

Figure 7. Products of the ring-closure reaction with (*R*)-2-chloro-1-phenylethanol. The (*R*)-epoxide is preferentially formed in each case.

Figures 8 and 9 indicate the resultant epoxide: halohydrin ratio of the ring closure of (*S*)-CPE as substrate. Figure 10 gives details of the epoxide and diol products formed for the best strains. The styrene oxide is produced at a much lower rate using (*S*)-CPE than when the (*R*)-enantiomer is used as substrate, indicating a preference towards the (*R*)-enantiomer. The styrene oxide produced is predominantly (*R*)-styrene oxide. However, the dose-dependent response is also evident with the (*S*)-enantiomer.

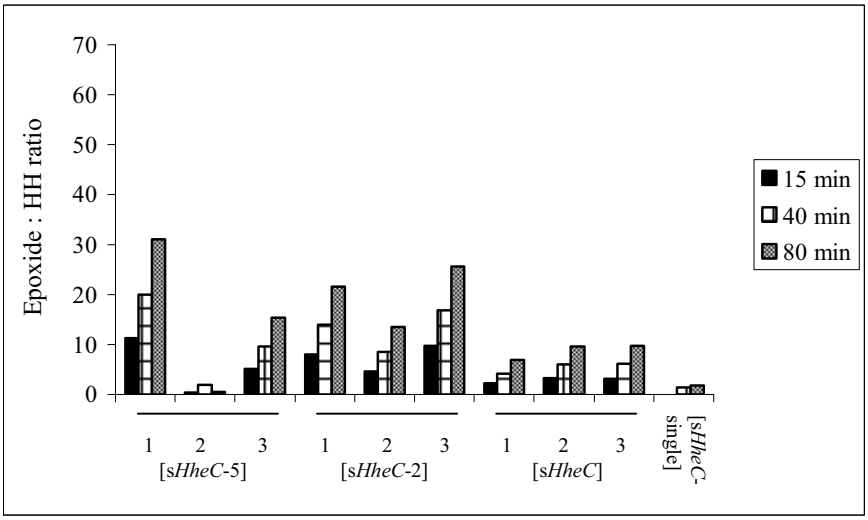

Figure 8. Results of the ring-closure of (*S*)-2-chloro-1-phenylethanol, indicating the increasing epoxide: halohydrin (HH) over time.

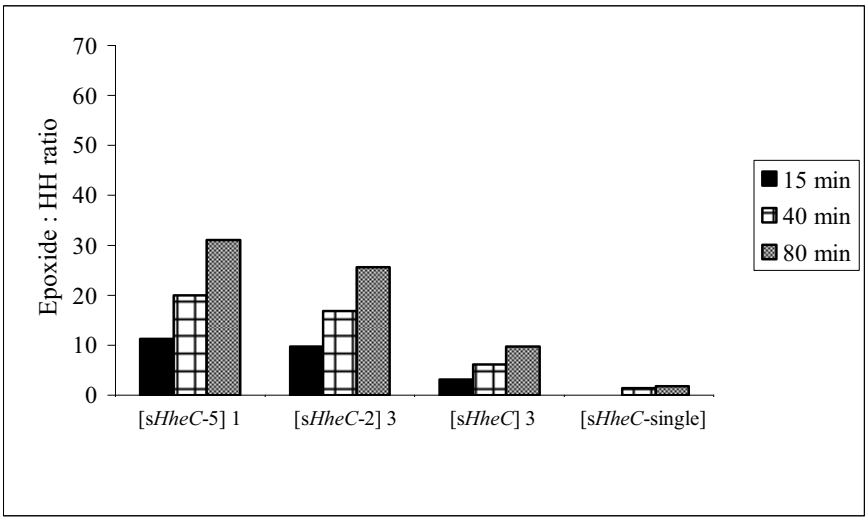

Figure 9. Results of the ring-closure of (*S*)-2-chloro-1-phenylethanol, highlighting the dose-dependent relationship between copies of the *sHHeC* gene integrated into *Y. lipolytica* and the HheC activity.

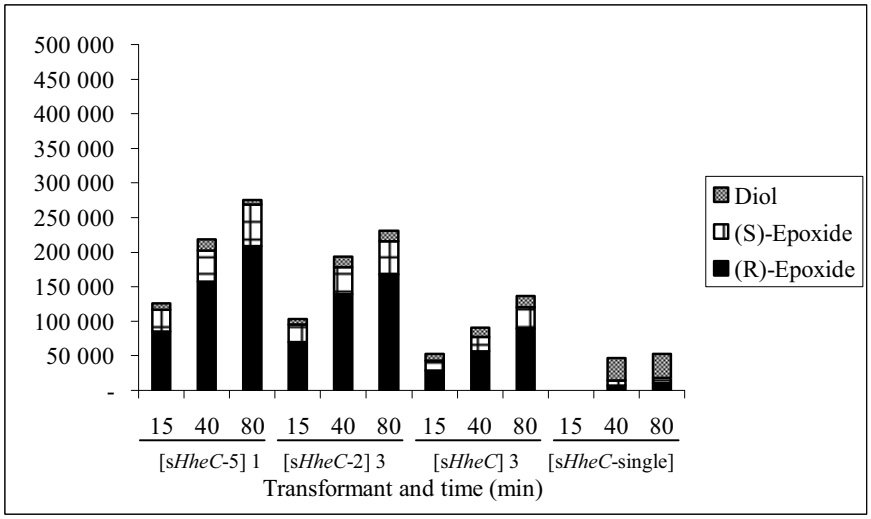

Figure 10. Products of the ring-closure reaction with (*S*)-2-chloro-1-phenylethanol. The (*R*)-epoxide is preferentially formed in each case.

For both (*R*)- and (*S*)-CPE, *Y. lipolytica* [s*HheC*-5] transformant 1, *Y. lipolytica* [s*HheC*-2] transformant 3 and *Y. lipolytica* [s*HheC*] transformant 3 give the best conversion of their transformant groups.

Discussion

A. radiobacter's AD1 halohydrin dehalogenase, HheC, was successfully expressed in *Y. lipolytica*, following codon-optimisation to counteract negative effects of possible inefficient translation due to the differences in the codon usage of the prokaryotic gene source and eukaryotic heterologous expression host. Various constructs were generated, ranging from a single-copy targeted integrant. *Y. lipolytica* [s*HheC*-single], through traditional multiple copy integrants, *Y. lipolytica* [s*HheC*], to tandem multiple copy integrants, *Y. lipolytica* [s*HheC*-2] and [s*HheC*-5]. Multiple copy integrants were all generated by random integration. Random integration at different sites in the genome can result in a variety of HheC activities for transformants containing the same number of transgene copies. This has also been observed in *Aspergillus niger* transformants expressing glucoamylase (Verdoes et al. 1993), as well as in *Saccharomyces cerevisiae* and *Lactococcus lactis* (Thompson and Gasson 2001). In *L. lactis,* there was a three-fold difference in expression of randomly integrated β-glucuronidase (GUS) genes, whereas in *S. cerevisiae*, a 14-fold variation was found in the expression of the *lacZ* reporter gene. This underlines the need to screen all transformants obtained for activity, as genetic screening is not able to be directly extrapolated into function. Based on the Livak copy numbers determined for the target *URA3* sequence, the actual numbers of the s*HheC* gene in the various transformants can be extrapolated, as is shown in Table 5.

Table 5. Relative copy numbers of integrated expression cassettes in different *Y. lipolytica* transformants

Transformant	Livak Copy Number: URA3 target seq.	No. of s*Hhe*C copies per URA3 target seq.	Total number of s*Hhe*C copies
Y. lipolytica [s*HheC*-5] 1	7	5	35
Y. lipolytica [s*HheC*-5] 2	1	5	5
Y. lipolytica [s*HheC*-5] 3	8	5	40
Y. lipolytica [s*HheC*-2] 1	17	2	34
Y. lipolytica [s*HheC*-2] 2	17	2	36
Y. lipolytica [s*HheC*-2] 3	18	2	36
Y. lipolytica [s*HheC*] 1	11	1	11
Y. lipolytica [s*HheC*] 2	16	1	16
Y. lipolytica [s*HheC*] 3	20	1	20
Y. lipolytica [s*HheC*-single]	1[1]	1	1

[1] Chromosomal *Y. lipolytica* Po1h *URA3* copy subtracted

The relationship between the copy number and the activity seen in Figures 5-10 is apparent. The relationship is not absolute as the region of the genome where the random integration occurs also plays a role. All three *Y. lipolytica* [s*HheC*-2] strains show very similar copy numbers, but transformants 1 and 3 display better activity than transformant 2. *Y. lipolytica* [s*HheC*-5] 1 and 3 are similar in copy number, but transformant 1 shows better activity. However, they both show better activities than the *Y. lipolytica* [s*HheC*-2] transformants, which in turn display higher levels than the *Y. lipolytica* [s*HheC*] transformants. It is notable that, in general, the copy numbers of the *Y. lipolytica* [s*HheC*-2] and [s*HheC*] transformants are higher than the *Y. lipolytica* [s*HheC*-5] transformants. This is likely due to the longer length of the expression cassettes in the *Y. lipolytica* [s*HheC*-5] transformants. These transformants have the lowest permissible copy numbers of the marker gene that are capable of alleviating the host's uracil auxotrophy.

The preferential production of the corresponding (*R*)-epoxide from both (*R*)- and (*S*)-2-chloro-1-phenylethanol (CPE) by the recombinant HheC expressed in *Y. lipolytica* is in agreement with the published literature. Purified HheC expressed in *E. coli* by Van Hylckama Vlieg et al. (2001) displayed an enantioselectivity of 73 for the (*R*)-substrate, similar to the value calculated by Lutje Spelberg et al. (1999). Lutje Spelberg et al. (1999) reported that (*R*)-styrene oxide was formed with an ee of 90% from a racemic mixture of CPE. It is not possible to compare the results obtained here directly with these values, as the results shown here are for whole-cell biocatalysts rather than purified protein, but the native enantioselectivity appears to be retained when expressed in *Y. lipolytica*.

The results obtained indicate that small amounts of (*S*)-styrene oxide are formed from (*R*)-CPE, alongside the majority product of (*R*)-styrene oxide. This was a surprising result, since epimerisation of CPE under the mild conditions used was not expected. A possible explanation is based on the regioselectivity of the attack in the ring-*opening* mode (also catalysed by HheC). The initial reaction is the ring-closure of (*R*)-CPE to form (*R*)-styrene oxide. This compound is now available for ring-*opening*. The

nucleophile (Cl^-) may attack at either of the epoxide carbons. If it attacks at the terminal carbon, the (*R*)-substrate will undergo ring-opening with the retention of its configuration, and the (*R*)-styrene oxide will be converted to back (*R*)-CPE. However, attack can also occur at the benzylic carbon (due to the influence of the aromatic ring), which would result in the (*R*)-styrene oxide being opened with inversion of configuration to (*S*)-CPE, which is then fully converted back to the (*S*)-styrene oxide at the early stages of the reaction, without noticeable accumulation of (*S*)-CPE. Later in the reactions (80 min), the accumulation of low levels of (*S*)-CPE is also seen, alongside both the (*R*)- and (*S*)-styrene oxide (not shown). A comparable rationalisation can be made for the production of low levels of (*R*)-styrene oxide from (*S*)-CPE, where the initially formed (*S*)-styrene oxide is subjected to ring-*opening* to form both (*R*)- and (*S*)-CPE, after which the (*R*)-CPE is subsequently preferentially closed to (*R*)-styrene oxide. A similar reaction mechanism was described by Lutje Spelberg et al. (1999) in the conversion of 2,3-dichloro-1-propanol (DCP). The (*R*)-enantiomer was initially converted to (*R*)-epichlorohydrin, with the yield of the remaining (*S*)-enantiomer was 49.5% (maximum theoretical yield is 50%). Instead of enantiomerically pure (*R*)-epichlorohydrin, racemic epichlorohydrin and prochiral 1,3-DCP were formed. This was explained by the reverse reaction, the ring opening of (*R*)-epichlorohydrin, where the favoured attack by the Cl^- was at the β-position to create 1,3-DCP rather than the α-attack to reform the (*R*)-epichlorohydrin. The prochiral 1,3-DCP was then converted to both enantiomers of epichlorohydrin by HHdH.

The successful expression of a tetrameric protein such as *A. radiobacter* AD1 HHeC in *Y. lipolytica* is important. The recombinant activity in these strains is evidence that the heterologous HheC is successfully folded and aggregates into a functional tetramer. This is further confirmation of the utility of *Y. lipolytica* as an expression host for a variety of different biocatalysts.

Acknowledgements

Y. lipolytica Po1h and Po1g and vector pINA1291 were kindly provided by the Institut National de la Recherche Agronomique (INRA, France).

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Chapter 5.

Heterologous expression of *Aspergillus aculeatus* endo-1,4-β-mannanase in *Yarrowia lipolytica* and *Aspergillus niger*

by

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The construction of the *Y. lipolytica* Man1 production strains was done by the author. The construction of the Man1-producing *A. niger* strain was done by Dr Shaunita H. Rose of the University of Stellenbosch.
Further optimisation of the Man1 production by these strains was done by the team at CSIR.

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Abstract

Endo-β-1,4-mannanase catalyses the random hydrolysis of the β-1,4-mannan main chains of mannans and heteromannans. β-mannanases are useful in a variety of industrial processes, including extraction of vegetable oils, the reduction of viscosity of coffee extracts during the manufacture of instant coffee, and biobleaching of softwood Kraft pulps.

The β-mannanase gene (*man1*) from *Aspergillus aculeatus* MRC11624 (Izuka) was originally cloned and expressed in *S. cerevisiae* (Setati et al. 2001). Here, the successful expression of β-mannanase from *A. aculeatus* MRC11624 in *Aspergillus niger*[1] and *Yarrowia lipolytica* is reported. Following optimisation with copy numbers and culture conditions, maximal activity levels of 26,139 nkat.ml^{-1} and 240 nkat.(mg DCW)$^{-1}$ were obtained for *Y. lipolytica*. Maximal activity levels of 16,596 nkat.ml^{-1} and 574 nkat.(mg DCW)$^{-1}$ were obtained for *A. niger*.

The *A. aculeutus man1* gene was cloned and expressed in *Y. lipolytica* under control of the growth phase-inducible hybrid hp4d promoter. Both single and multi-copy transformants were constructed and the secretion of the enzyme was evaluated as an in-frame fusion with the *LIP2* secretion signal, as well as with its natural secretion signal. The constructs were evaluated in shake flasks, where the highest volumetric and specific enzyme activity was obtained with the multi-copy integrant utilising the β-mannanase's own secretion signal. The best β-mannanase-producing strain was subsequently evaluated in batch fermentation and resulted in maximum volumetric enzyme activity of 6,719 nkat.ml^{-1}. Fed batch fermentations resulted in a 3.9-fold increase in volumetric enzyme activity compared to batch fermentation, and a maximum titre of 26,139 nkat.ml^{-1} was reached.

[1] The construction of the Man1-producing *A. niger* strain was done by Dr Shaunita Rose of the University of Stellenbosch. The construction of the *Y. lipolytica* Man1 production strains was done by Robyn Roth of CSIR Biosciences. Further optimisation of the Man1 production by these strains was done by the team at CSIR.

The *A. aculeutus man1* gene was also expressed in *A. niger* under control of the *A. niger* glyceraldehyde-3-phosphate dehydrogenase promoter (gpd_P). The recombinant enzyme displayed pH and temperature optima of pH 4.8 and 75°C, respectively. Initial shake flask cultivation yielded 6,800 nkat ml^{-1}. The effect of supplementation by organic nitrogen, as well as glucose and inorganic nitrogen concentration, for *A. niger* cultivation was investigated, and maximal activity levels were increased to 16,596 nkat.ml^{-1} and 574 nkat.(mg DCW)$^{-1}$.

Introduction

Endo-β-1,4-mannanase (β-mannanase, E.C. 3.2.1.78) belongs to the glycosyl hydrolase family and has been cloned from bacterial and fungal origins (Arcand et al. 1993; Stålbrand et al. 1995; Setati et al. 2001; Zhang et al. 2006). β-mannanases catalyse the random hydrolysis of the β-1,4-mannan main chains of mannans and heteromannans. Mannans are one of the major constituents of hemicellulosic soft woods and are also found as a minor portion of hemicelluloses in hardwoods as glucomannans (Stålbrand et al. 1995). Other types of heteromannans are found as storage polysaccharides in plant seeds, *e.g.* locust bean gum, a galactomannan polymer with a main chain of β-1,4-mannan with α-1,6-bound galactosyl side groups. β-mannases are useful in several industrial processes, such as extraction of vegetable oils from leguminous seeds, and the reduction of viscosity of coffee extracts during the manufacture of instant coffee (Sachslehner et al. 2000). They can also be used for biobleaching of softwood Kraft pulps to enhance extractability of lignin (Suurnäkki et al. 1996; Montiel et al. 1999).

Filamentous fungi such as *Aspergillus niger* have the ability to produce and secrete exceptionally large amounts of properly folded proteins and can produce proteins that contain *O*- and *N*-linked glycans without extensive hyperglycosylation (Hombergh et al. 1997; Maras et al. 1999; Punt et al. 1994). Filamentous fungi are commonly used in the fermentation industry for the large-scale production of proteins, mainly for industrial enzymes (Nevalainen et al. 2005). The main attraction of filamentous fungi is their natural ability to secrete large amounts of proteins into the growth medium. *A. niger* has

been granted GRAS status, and considerable experience has been gained handling such filamentous fungi (Radzio and Kück 1997).

Yarrowia lipolytica is a yeast species that has been classified as Generally Regarded As Safe (GRAS) by the American Food and Drug Administration (FDA) for citric acid production (Fickers et al. 2005), and has been widely used in industrial applications. It is one of the most promising 'non-conventional' yeasts available as hosts for heterologous protein production (Müller et al. 1998). A large number of molecular tools are available for heterologous protein expression in *Y. lipolytica* (Madzak et al. 2004), including low copy integration vectors that contain the non-defective *ura3d1* selection marker, which complements the *Y. lipolytica* Po1h host strain's auxotrophy in single or low copy numbers. Multi-copy vectors contain the defective *ura3d4* marker, which is required in multiple copies to complement, allowing for selection of transformants with multiple inserts. The *ura3d4* selection marker should ensure selection of transformants with 10 - 13 copies of the integrated expression cassette (Juretzek et al. 2001).

The β-mannanase gene (*man1*) from *Aspergillus aculeatus* MRC11624 (Izuka) was originally cloned and expressed in *S. cerevisiae* (Setati et al. 2001). The alcohol dehydrogenase (*ADH2*) and phosphoglycerate kinase (*PGK1*) promoters and terminators were used, and β-mannanase with activities of 521 nkat.ml^{-1} and 379 nkat.ml^{-1} respectively, was secreted into the medium. In this chapter, the expression of β-mannanase from *A. aculeatus* MRC11624 in *A. niger* and *Y. lipolytica* is reported. The Man1 enzyme was efficiently secreted by *Y. lipolytica,* using its own secretion signal, under control of the growth-phase dependent synthetic hybrid promoter hp4d ($hp4d_P$, Nicaud et al. 2002) and the *Y. lipolytica LIP2* terminator ($LIP2_T$, Pignède et al. 2000). *man1* was also successfully expressed in *A. niger*, under control of the *A. niger* glyceraldehyde-3-phosphate dehydrogenase promoter (gpd_P) and the *Aspergillus awamori* glucoamylase terminator (gla_T). The effect of supplementation by organic nitrogen, as well as glucose and inorganic nitrogen concentration, for *A. niger* cultivation was investigated. Maximal activity levels were obtained of 16,596 nkat.ml^{-1} and 574

nkat.(mg DCW)$^{-1}$ for *A. niger* D15[*man1*], and 26,140 nkat.ml^{1} and 240 nkat.(mg DCW)$^{-1}$ for *Y. lipolytica* [*man1*], respectively.

Materials and Methods

The genotypes of the microbial strains and plasmids used in the present study are summarised in Table 1, and the oligodeoxynucleotide primers used are shown in Table 2.

Table 1. Microbial strains, plasmids used in the present study

Strain or plasmid	Relevant genotype	Reference
Strains		
Escherichia coli JM109	*end*A1 *rec*A1 *gyr*A96 *thi hsd*R17 ($r_k^-m_k^+$) *rel*A1 *sup*E44 Δ(*lac-pro*AB) [F' *tra*D36 *pro*AB *lacI*^{q}ZΔM15]	Promega
E. coli DH5α	*supE44 ΔlacU169 (∅80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi*$^{-1}$ *relA1*	Sambrook et al. 1989
Yarrowia lipolytica Po1h	*MATA,leu2-270, ura3-302 (= ura3::pXPR2:SUC2), uxpr2-322, axp1-2*. Phenotype: Leu$^-$, Ura$^-$, ΔAEP, ΔAXP, Suc$^+$	Madzak 2003
Y. lipolytica Po1g	*MATA, leu2-270, ura3-302::URA3, xpr2-322, axp1-2* Phenotype: Leu$^-$, ΔAEP, ΔAXP, Suc$^+$. pBR docking platform	Madzak et al. 2000
Y. lipolytica [HmA]	Po1h with hp4d$_P$-*LIP2$_T$* integrated into the chromosome, multi-copy	This study
Y. lipolytica [*man1*- low]	Po1h with hp4d$_P$-*man1-LIP2$_T$* integrated into the chromosome, low-copy	This study
Y. lipolytica [*man1- LIP2*]	Po1h with hp4d$_P$-*man1-LIP2$_T$* integrated into the chromosome, multi-copy, under control of *LIP2* secretion signal	This study
A. niger D15	*pyrG prtT phmA* (non-acidifying)	Wiebe et al. 2001
A. niger D15[pGT]	*A. niger* D15 with *gpd$_P$-glaA$_T$* integrated into the chromosome	Rose and Van Zyl 2002
A. niger D15[*man1*]	*A. niger* D15 with *gpd$_P$-man1-glaA$_T$* integrated into the chromosome	This study
Plasmids		
pBLUESCRIPT SK	*bla*	Stratagene
pINA1291	*E. coli / Y. lipolytica* vector. Kan^R, *ura3d4*, hp4d$_P$-*LIP2$_T$, zeta*, multi-copy random integration vector	Nicaud et al. 2002
pINA1313	*E. coli / Y. lipolytica* vector. Kan^R, *ura3d1*, *LIP2$_s$*-hp4d$_P$-*LIP2$_T$, zeta*, low-copy random integration vector	Nicaud et al. 2002
pINA1293	*E. coli / Y. lipolytica* vector. Kan^R, *ura3d4*, *LIP2* secretion signal, hp4d$_P$-*LIP2$_T$, zeta*, multi-copy random int. vector	Nicaud et al. 2002
pYL- *man1*-HsL	hp4d$_P$-*LIP2$_s$-man1-LIP2$_T$, ura3d1,* Kan^R	This study
pYL- *man1*-HmL	hp4d$_P$-*LIP2$_s$-man1-LIP2$_T$, ura3d4,* Kan^R	This study
pYL-*man1*-HmA	hp4d$_P$-*man1-LIP2$_T$, ura3d4,* Kan^R	This study

Strain or plasmid	Relevant genotype	Reference
pGT	*E. coli / A. niger* vector, bla, GPD_P-GLA_T	Rose and Van Zyl 2002
pBS-pyrGamdS	*bla*, $pyrG_P$-*pyrG*-$pyrG_T$, $amdS_P$-*amdS*-$amdS_T$	Plüddeman 2002
pBS-*man1*	*bla, man1*	Setati et al. 2001
pGT-*man1*	*bla*, gpd_P-*man1*-$glaA_T$	This study

Table 2. DNA sequences of the oligodeoxynucleotide primers used in this study. Restriction enzyme sites are underlined in bold

Primer name	Sequence (5' - 3')	Restriction enzyme site
Man-1	GA**GGATCC**ATGAAGCTTTCTCA	*Bam*HI
Man-2	GA**CCTAGG**CTACTTCGACTG	*Bln*I
Man-3	GA**GGATCC**AAGCTTTCTCACATG	*Bam*HI
YL-FWD	GATCCCCACCGGAATTG	
YL-REV	GGAGTTCTTCGCCCAC	

Recombinant plasmids were constructed and amplified in *E. coli* JM109 or DH5α. *E. coli* was cultivated at 37°C in LM (per litre: 10 g yeast extract, 10 g tryptone, 5 g NaCl) liquid medium on a rotary shaker at 200 rpm, or on LM agar (15 $g.l^{-1}$ agar), supplemented with the relevant antibiotics at a final concentration of 100 $\mu g.ml^{-1}$ ampicillin or 50 $\mu g.ml^{-1}$ kanamycin.

Untransformed *Y. lipolytica* Po1h was cultivated in YPD media (per litre: 10 g yeast extract, 20 g peptone, 20 g glucose). *Y. lipolytica* transformants were selected on YNB_{casa} medium (per litre: 20 g glucose, 4 g NH_4Cl, 2 g casamino acids, 1.7 g yeast nitrogen base without amino acids and without ammonium sulphate, 0.3 g leucine). *Y. lipolytica* [*man1*], [*man1-LIP2*] and [*man1*-low] transformants were cultivated in YM media (per litre: 5 g yeast extract, 20 g malt extract, 10 g peptone, 15 g glucose; pH 5.5) for initial heterologous protein production and best transformant selection.

The *A. niger* fungal strains were cultivated at 30°C in minimal media (MM) containing (per litre): 5 g yeast extract, 2 g casamino acids, 10 g glucose, 6 g $NaNO_3$, trace elements and 0.01 M uridine, prior to spheroplast harvesting (Rose and van Zyl 2002; Punt and van den Hondel 1992). Transformants were selected on minimal medium containing 10 mM acetamide and 15 mM CsCl, but lacking casamino acids, uridine and $NaNO_3$. Media were inoculated to a final concentration of $1x10^6$ $spores.ml^{-1}$. *A. niger* D15 transformants

were cultivated in double strength minimal media (2xMM) containing 100 $g.l^{-1}$ glucose for initial transformant selection and heterologous enzyme characterisation. For media optimisations, the flasks were inoculated to a final concentration of 3 x 10^5 $spores.ml^{-1}$ and incubated at 30ºC on a rotary shaker at 220 rpm. Data shown are from triplicate flasks in all cases.

DNA manipulations

Standard protocols were followed for DNA manipulations (Sambrook et al. 1989). The enzymes for DNA cleavage and ligation were purchased from Fermentas. Restriction endonuclease-digested DNA was eluted from agarose using the GFX™ PCR DNA and Gel Band Purification Kit. Dephosphorylation of vector DNA prior to ligation was carried out using New England Biolab's Antarctic Phosphatase. Ligations were done using T4 DNA ligase (Fermentas). *E. coli* transformations were carried out using Bio-Rad's Gene-Pulser. Genomic DNA was isolated from *Y. lipolytica* strains using Promega's Wizard® Genomic DNA Purification Kit, according to the manufacturer's recommended protocol for yeasts. Polymerase Chain Reaction (PCR) was done using an Eppendorf Mastercycler Gradient PCR machine. DNA concentrations were determined using a Qubit Fluorometer (Invitrogen). PCR products were purified and concentrated using Roche's High Pure PCR Product Purification Kit. Real-time PCR (RT-PCR)

Expression Vector Construction

The 1.1 kb *A. aculeatus* MRC11624 *man1* gene was amplified from pBS-*man1* (Setati et al. 2001) with Man-1 or Man-3 (Table 2) as forward primers, with Man-2 as the reverse primer in both cases. Each 50 µl PCR reaction contained 200 µM dNTPs, 250 nM of each primer, 2 mM of $MgCl_2$, template DNA and 2.5 U of ExTaq (TaKaRa). The PCR profile was: 95ºC for 5 min; followed by 30 cycles of: denaturation at 95ºC for 1 min, primer annealing at 50ºC for 1 min, extension at 72ºC for 2 min; then a final extension of 72ºC for 10 min. For expression in *Y. lipolytica*, the PCR products were ligated into the

*Bam*HI and *Bln*I sites of pINA1313, pINA1293 and piNA1291, generating pYL-*man1*-HsL, pYL-*man1*-HmL and pYL-*man1*-HmA, respectively.

Plasmid pGT (Rose and Van Zyl 2002) was used as expression vector for the constitutive expression of the recombinant *man1* gene in *A. niger*. The *man1* gene was retrieved from pBS-*man1* (Setati et al. 2001) as a *Not*I-*Xho*I fragment and cloned into the *Not*I and *Sal*I sites of pGT, generating pGT-*man1*. The gene product of *man1* was designated Man1.

Y. lipolytica Po1h Transformations and Identification of Transformants

The DNA fragments from pYL-*man1*-HsL, pYL-*man1*-HmL and pYL-*man1*-HmA, containing either the *ura3d4* or *ura3d1* selection markers and the hp4d$_P$-(*LIP2*$_s$)-*man1*-*LIP2*$_T$ expression cassettes and bounded by *zeta* regions to stimulate random chromosomal integration in *Y. lipolytica* Po1h, were amplified in PCR reactions using primers YL-FWD and YL-REV (Table 2). Each 100 µl PCR reaction contained 250 µM dNTPs, 250 nM of each primer, 2 mM of $MgCl_2$, template DNA and 2.5 U of ExTaq (TaKaRa). The PCR profile was: 95ºC for 5 min, followed by 30 cycles of: denaturation at 95ºC for 1 min, primer annealing at 50ºC for 1 min, extension at 72ºC for 4 min, then a final extension of 72ºC for 10 min.

The method of Xuan et al. (1988) was used for the transformation of *Y. lipolytica* Po1h, using approximately 1 µg of linear DNA per transformation. Colonies appearing on the YNB_{casa} selective plates after 7-14 days were transferred onto fresh YNB_{casa} plates.

Genomic DNA was isolated from transformants and used as template for PCR screening. PCR was carried out using Man-1 and Man-2 primers, with each 10 µl PCR reaction containing 200 µM dNTPs, 250 nM of each primer, 2 mM of $MgCl_2$, template DNA and 0.25 U of BioTaq (Bioline). The PCR profile was: 95ºC for 5 min, followed by 30 cycles of: denaturation at 95ºC for 1 min, primer annealing at 50ºC for 1 min, extension at 72ºC for 1 min.

Real-time PCR (RT-PCR) was used to estimate the copy number the integrated expression cassettes. This was carried out as described in Chapter 3.

Y. lipolytica [*man1*-low], [*man1-LIP2*] and [*man1*] Heterologous Expression

Selected *Y. lipolytica* transformants and untransformed *Y. lipolytica* Po1h was cultivated in 100 ml YM at 30°C on a rotary shaker at 220 rpm. Samples (2 ml) were taken at regular time intervals and the residual sugar concentration determined using Accutrend$^{@}$ (Boehringer Mannheim). After glucose depletion, approximately 3 days after inoculation, a 2 ml sample was taken, centrifuged and the supernatant used to determine enzyme activity in triplicate. The pellet was used for dry cell weight determination by drying to constant weight at 110ºC.

The intracellular enzyme activity was determined in triplicate by harvesting 2 ml culture and washing the pellet. The wet weight was determined and 1 ml Y-PER reagent (Pierce) per gram of wet weight added. The resuspended cells were incubated for 30 mins at 22 - 25°C, and the volume made up to 2 ml, before re-centrifugation. The supernatant used for determination of intracellular β-mannanase activity.

Supernatants from *Y. lipolytica* [*man1*-low], [*man1-LIP2*] and [*man1*] were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook et al. 1989) to estimate the size of the produced protein, using the PageRuler molecular weight marker (Fermentas) to estimate the size of the produced protein. Coomassie brilliant blue was used to visualize the protein bands (Ausubel et al. 1998).

Y. lipolytica [*man1*-low] and *Y. lipolytica* [*man1*] Fermentations

2-L Fernbach flasks containing 200 ml YM medium (pH 5.5) were inoculated with the selected transformants, incubated at 220 rpm for 24 hrs and the culture used to inoculate the Braun B (2-L) or C (15-L) fermenters. The fermentation medium consisted of (per litre): 2.5 g citric acid, 0.88 g $CaCl_2.2H_2O$, 8.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 11.3 g

KH_2PO_4, 58 g $(NH4)_2SO_4$, 20 g glucose, 15 g yeast extract, 16.3 ml H_3PO_4 (85%), 1.7 ml trace element solution, 1 ml antifoam and 1.7 ml vitamin solution.

The trace element solution consisted of (per litre): 50 ml HCl, 35 mg $FeSO_4.7H_2O$, 7.5 mg $MnSO_4.7H_20$, 11 mg $ZnSO_4.7H_2O$, 1 mg $CuSO_4.5H_2O$, 2 mg $CoCL_2.6H_2O$, 1,3 mg $Na_2MoO4.2H_2O$, 1.3 mg $Na_2B_4O_7.10H_2O$, 0.35 mg Kl, and 0.5 mg $Al_2(SO_4)_3$. The vitamin stock solution was filter-sterilised and added to the inoculum flask before inoculating the fermenter and consisted of (per litre): 0.7 mg $NaH_2PO_4.2H_2O$, 0.3 mg $Na_2HPO_4.7H_2O$, 170 mg *meso*-inositol, 8.5 mg nicotinic acid, 0.3 mg biotin, 8.5 mg thiamine HCl, 34 mg Ca panthothenate, 6.8 mg ascorbic 8.5 mg pyridoxine HCl, 1.7 mg *para*-amino benzoic acid, 0.3 mg folic acid, 0.3 mg riboflavin and 0.3 mg ascorbic acid.

The fermentations were run at 28°C at an initial stirrer speed of 500 rpm. The pH was maintained at 5.5 with either 33% NH_4OH or 25% H_2SO_4. The airflow rate was set at 2 slpm for the Braun B and 10 slpm for the Braun C fermenters. The PO_2 was controlled at 30% by adjusting the stirrer speed. Samples for the batch fermentations in 2-L Braun B fermenters were taken every 3 hours after depletion of the glucose. For the fed-batch fermentations in Braun C fermenters, glucose feed was initiated once the initial glucose was depleted and was fed at a rate to maintain the residual glucose concentration below 20 $g.l^{-1}$. Samples were taken every 3 hours.

A. niger D15[*man1*] Heterologous Expression

The supernatants of *A. niger* D15[pGT] and D15[*man1*] were collected after 7 days of cultivation in 2XMM and freeze-dried before determining the specific activity at pH 3.0 to 6.0 and at 60 to 95°C. Supernatant from *A. niger* D15[*man1*] cultivated on CSLM (see "Media Composition") was separated by 10% SDS-PAGE using the PageRuler molecular weight marker (Fermentas) to estimate the size of the produced protein. Coomassie brilliant blue was used to visualize the protein bands (Ausubel et al. 1998).

Optimisation of *A. niger* D15[*man1*] Man1 Expression by Media Composition

Inoculum train, sampling and dry cell weight determination

A. niger D15[*man1*] was cultivated on selective agar medium containing (per litre): 18 g agar, 2 g neopeptone (Difco), 1 g yeast extract, 0.4 g $MgSO_4.7H_2O$, 10 g glucose and 2 g casamino acids. 20 ml 50X AspA+N and 1 ml 1000X trace element stock solutions were added aseptically after sterilisation. The 50X AspA+N stock solution contained (per litre): 300 g $NaNO_3$, 26 g KCl, 76 g and KH_2PO_4, pH 6.5. The 1000X trace element stock solution contained (per litre): 22 g $ZnSO_4.7H_2O$, 11 g H_3BO_3, 5 g $MnCl_2.4H_2O$, 5 g $FeSO_4.7H_2O$, 1.7 g $CoCl_2.6H_2O$, 1.6 g $CuSO_4.5H_2O$, 1.5 g $Na_2MoO_4.2H_2O$ and 50 g EDTA. The plates cultivated at 30ºC for 3-5 days, until the spores covered 80% of the culture surface, when the spores were harvested and resuspended in 10–15 ml 0.9% (m/v) NaCl. Flasks containing 200 ml of the different media, pH- adjusted to pH 5 with 33% $(NH_4)OH$ and 20% H_2SO_4, were inoculated to a final concentration of 3 x 10^5 spores.ml^{-1}. Where included, glucose, casamino acids, AspA+N and trace elements were added aseptically after sterilisation. After inoculation the flasks were incubated for 7 days at 30ºC on a rotary shaker at 220 rpm.

Flasks were sampled daily, samples filtered and the supernatant used for sugar and enzyme analysis. Residual glucose was determined using Accutrend@ (Boehringer Mannheim). The fungal biomass was dried at 100°C to constant dry weight

Medium Composition

Different media formulations were prepared, including different corn steep liquor sources, different AspA+N and glucose concentrations, and inorganic nitrogen concentrations. The effect of the presence of yeast extract was also determined.

1. ***The effect of CSL and yeast extract on the production of Man1***

   Yeast Extract Medium (YEM) had the following composition (per litre): 10 g yeast extract, 2 g $MgSO_4.7H_2O$, 100 g glucose, 2 g casamino acids, 40 ml 50X AspA+N

and 2 ml 1000X trace elements. Cornsteep Liquor Medium (CSLM) had the following composition (per litre): 4.12 g Roquette CSL^{SD} (spray-dried), 5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 100 g glucose, 2 g casamino acids, 40 ml 50X AspA+N and 2 ml 1000X trace elements. Phytase-treated CSL medium (PCSLM) had the following composition (per litre): 13 ml Roquette CSL^{SP} (phytase-treated, spray-dried), 5 g $KH_2.PO_4$, 0.5 g $MgSO_4.7H_2O$, 100 g glucose, 2 g casamino acids, 40 ml 50X AspA+N and 2 ml 1000X trace elements.

2. ***The effect of AspA+N salts and glucose concentrations on the production of Man1***
   The CSL^{SD} medium (CSLM) was prepared as above with different combinations of glucose and AspA+N concentrations (the different concentration are shown in Table 10 in Results).

3. ***The combined effect of CSL^{SD} and yeast extract on the production of Man1***
   Basal medium consisted of (per litre): 5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 100 g glucose, 2 g casamino acids, 40 ml 50X AspA+N and 2 ml 1000X trace elements. Different concentrations of CSL^{SD} and/or yeast extract were added to the medium (the different concentration are shown in Table 11 in Results).

4. ***The effect of inorganic nitrogen concentration on the production of Man1***
   Different concentrations of $NaNO_3$ were added to a basic medium containing (per litre): 4.12 g Roquette CSL^{SD}, 5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 100 g glucose, 2 g casamino acids and 2 ml 1000X trace elements (the different concentration are shown in Table 12 in Results).

Enzymatic Assays

The temperature and pH optima of the Man1 enzyme was determined by using 0.25% galactomannan (Sigma) in 50 mM citrate phosphate buffer, as described by Bailey et al. (1992). The amount of reducing sugars released during the degradation of mannan was determined by the dinitrosalicylic acid method using mannose as standard (Miller et al.

1960). One unit of enzyme was defined as the activity producing 1 µmol reducing sugar per minute in mannose equivalents under the optimal assay conditions. After pH and temperature optima determination, further assays were carried out at 75°C, in 50 mM citrate phosphate buffer, pH 3.8.

Results

Construction of expression vectors and recombinant strains

The *A. aculeutus* MRC11624 *man1* gene was amplified by PCR and the *man1* PCR products cloned into pINA1313, pINA1293 and pINA1291, creating pYL-*man1*-HsL, pYL-*man1*-HmL and pYL-*man1*-HmA, all under control of the growth-phase inducible hybrid hp4d promoter. pINA1291 and pINA1293 contain the defective *ura3d4* gene, which is required in multiple copies to alleviate the auxotrophy of the host, while pINA1313 contains the non-defective *ura3d1* gene.

DNA fragments bound by the *zeta* regions were amplified from all constructs, and the linear PCR products introduced into *Y. lipolytica* Po1h by the method of Xuan et al. (1988). Transformant colonies were subjected to PCR screening of genomic DNA. The presence of PCR products of the expected 1,134 bp was taken as confirmation that integration of the relevant expression cassette had occurred in the genome of *Y. lipolytica* Po1h. Integration of the expression cassettes from pYL-*man1*-HsL, pYL-*man1*-HmL and pYL-*man1*-HmA generated recombinant strains *Y. lipolytica* [*man1*-low], [*man1-LIP2*], and [*man1*], respectively. The empty expression cassette from pINA1291 was introduced into *Y. lipolytica* Po1h to generate reference strain *Y. lipolytica*[HmA].

Plasmids pGT and pGT-*man1* were individually co-transformed with plasmid pBS-pyrGamdS (Plüddeman 2002) into *A. niger* D15, resulting in transformants *A. niger* D15[pGT] and *A. niger* D15[*man1*]. Plasmid pBS-pyrGamdS contains the *amdS* and the *pyrG* marker genes that were used for the double selection of the transformants. Introduction of the *pyrG* gene enabled the transformants to grow in the absence of uridine, whereas *amdS* allowed the utilisation of acetamide or acrylamide as the sole carbon and nitrogen source. Transformant *A. niger* D15[pGT] was used as reference strain in all experiments.

The *A. niger* D15[*man1*] transformant that produced the highest level of activity was selected for further study. Chromosomal DNA was isolated from *A. niger* D15[pGT] and *A. niger* D15[*man1*]. The DNA was digested overnight with *Eco*RV, an enzyme which does not cut within the coding regions of the *man1* gene. The Southern blot analyses revealed the presence of at least one copy of the *man1* expression cassette integrated into the genome of *A. niger* D15 (Figure 1).

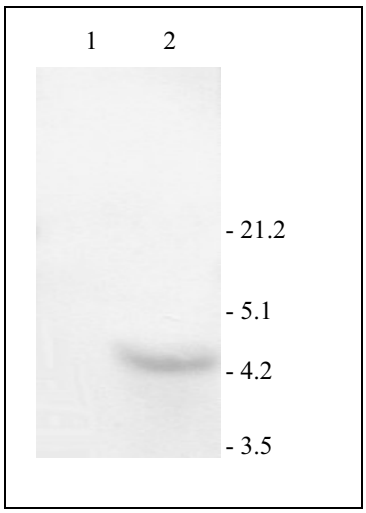

Figure 1. Southern blot analysis to determine the copy number of the *man1* gene present in the genome of *A. niger* D15[*man1*]. The total DNA was isolated and digested overnight with *Eco*RV. Lane 1 contains the DNA isolated from *A. niger* D15[pGT] and lane 2 the DNA isolated from *A. niger* D15[*man1*]. The entire 1.2 kb coding region of *man1* was labelled with [α-^{32}P]ATP and used as DNA-probe. The marker sizes are indicated on the right in Kb. The ^{32}P-highlighted DNA species represents a copy of the *man1* gene.

RT-PCR amplifications were carried out on all *Y. lipolytica* transformants selected for analysis. The equation $\Delta\Delta Ct = [\ Ct(URA) - Ct(SUC2)\ ]_{sample} - [\ Ct(URA) - Ct(SUC2)\ ]_{control}$ (Livak and Schmittgen 2001) was used to estimate the copy number of the integrated expression cassette, where the control is *Y. lipolytica* Po1g. Po1g was chosen as the two targets (*URA3* and *SUC2*) are of known copy number, with a single copy of each. In this case, the value of the second term, $[\ Ct(URA) - Ct(SUC2)\ ]_{control}$, was assumed to be 0, as the copy numbers of the 2 genes are the same, and the Ct values of the profiles are also the same. This was also experimentally shown (Figure 4, Chapter 3).

The equation could therefore be simplified to $\Delta\Delta Ct = [\ Ct(URA) - Ct(SUC2)\ ]_{sample}$. The $\Delta\Delta Ct$ value can then be inserted into the equation $N = 2^{-\Delta\Delta Ct}$, where there are N-fold

copies of the *URA3* target, compared to the *SUC2* target. As it is known that there is only a single copy of *S. cerevisiae SUC2* in *Y. lipolytica* Po1h (Nicaud et al. 1989), it can be stated that there are N-copies of the *URA3* target present. Table 5 shows the Ct values obtained for each strain, and the estimated copy numbers, using the Livak equation.

In order to determine whether the use of this equation was justified, the copy numbers were checked against values obtained with the Pfaffl method (Pfaffl, 2001). The average E-value for the *SUC2* PCR reactions was determined to be 2.25 ± 0.14 and the *URA3* E-value was calculated as 2.06 ± 0.04. In a perfect PCR reaction, the E-value is 2. This is presumed in the Livak equation. The slightly higher values are presumably a result of the manipulation of the curves by smoothing. Using the Ct values as shown in Table 5, the Pfaffl equation was used to determine the copy numbers for the various transformants. This value is shown in the final column in Table 5, indicating a good agreement with the Livak-determined copy number. Figure 2 shows some representative data.

Table 5. Relative copy numbers of integrated expression cassettes in different *Y. lipolytica* Po1h transformants, determined by real-rime PCR.

Transformant	**Ave Ct(URA3)**	**Ave Ct(SUC2)**	**ΔΔCt = [Ct(URA)-Ct(SUC2)]**	**Livak Copy Number: $N = 2^{-\Delta\Delta Ct}$ ***	**Pfaffl Copy Number ***
Y. lipolytica Po1g	15.05 ± 0.18	15.17 ± 0.11	-0.13	1	1
Y. lipolytica [*man1*- low] 3	14.20 ± 0.02	15.39 ± 0.20	-0.58	2	2
Y. lipolytica [*man1-LIP2*] 4	15.67 ± 0.10	16.35 ± 0.01	-1.20	2	2
Y. lipolytica [*man1*] 3	11.59 ± 0.22	14.61 ± 0.07	-3.03	8	8
Y. lipolytica [*man1*] 13	14.62 ± 0.33	17.63 ± 0.37	-3.01	8	10

* rounded off to nearest whole number

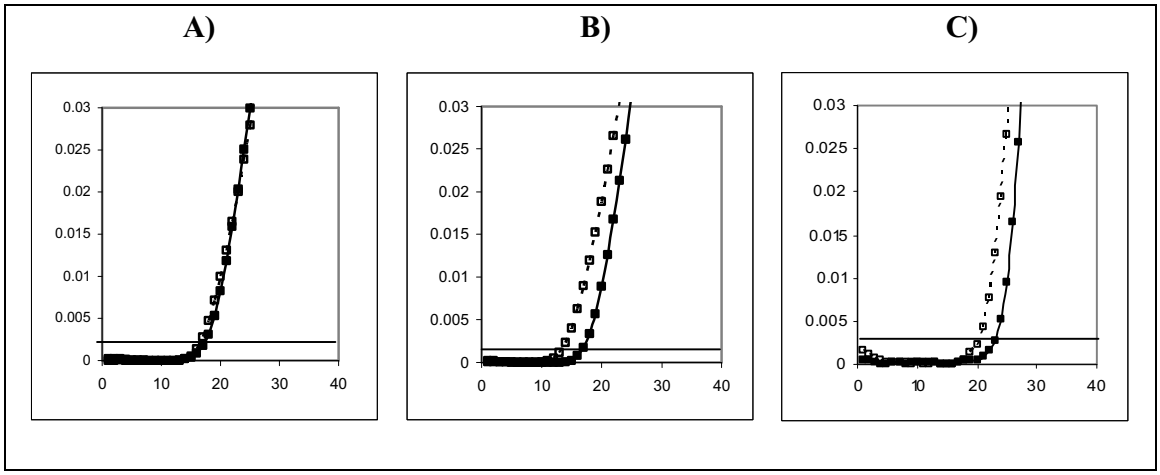

Figure 2. Graphs of fluorescence versus cycle number for representative *Y. lipolytica* recombinant strains, indicating differences in copy number. (A) is the graph of *Y. lipolytica* [*man1-LIP2*] (4), (B) is *Y. lipolytica* [*man1*] (3) and (C) is *Y. lipolytica* [*man1*] (13). -■- represents *SUC2* and -□-, *URA3*. The distinct difference in copy number between the two targets in both (B) and (C) can be seen in the different crossing points of the two curves, where they intersect the threshold, indicated by the horizontal dotted line. The graphs are the average of two amplifications, and the standard deviations are shown by the vertical tick marks

Heterologous Man1 Enzyme Production in *Y. lipolytica*

Shake flask analysis

The production of heterologous Man1 by *Y. lipolytica* [*man1*-low] transformants was determined after 4 days of cultivation in YM medium in Erlenmeyer flasks. Glucose was consumed within three days. Table 6 indicates the maximum extracellular and intracellular volumetric and specific enzyme activities of these low copy constructs.

Table 6. Maximum volumetric and specific Man1 activities of selected *Y. lipolytica* [*man1*-low] transformants in shake flasks

***Y. lipolytica* [*man1*-low] transformant**	**Enzyme activities (nkat.ml^{-1})[a]**		**Specific enzyme activities (nkat.mg$^{-}_{1}$)[a]**	
	Extracellular	**Intracellular**	**Extracellular**	**Intracellular**
2	68.1 (21.2)	0	4.2 (1.2)	0
3	**123.1 (10.3)**	1.93 (1.5)	**6.4 (0.5)**	0.05 (0.02)
8	36.5 (3.9)	11.3 (16.1)	1.9 (0.2)	0.6 (0.43)
Po1h	1.9 (1.3)	0	0.58	0

[a] Values are the average activities obtained by triplicate assays on duplicate flasks. Standard deviations are reported in brackets

The maximum extracellular volumetric and specific enzymatic activity of 123 nkat.ml^{-1} and 6.4 nkat.(mg DCW)$^{-1}$ respectively, was obtained from *Y. lipolytica* [*man1*-low]

transformant 3, with 1.5% of the total β-mannanase activity found to be intracellular. The host strain *Y. lipolytica* Po1h was found to have low levels of endogenous extracellular activity that shows up in the assays. All intracellular enzyme levels were low, and further enzyme assays were only done on extracellular fractions. However, the expression levels observed in low copy transformants were not very high, and multi-copy transformants were constructed, using either the *LIP2* or the native *Man1* secretion signal.

The production of heterologous Man1 by the multi-copy *Y. lipolytica* [*man1*] and [*man1-LIP2*] transformants was determined after 5 days of cultivation in YM medium in shake flasks (Table 7). As with the low copy transformants, activity levels varied greatly, indicating a potential difference in copy number and region of integration.

Table 7. Maximum volumetric and specific Man1 activities of selected *Y. lipolytica* [*man1*] and [*man1-LIP2*] transformants in shake flasks

Y. lipolytica **transformants**	**Volumetric enzyme activities (nkat.ml^{-1})[a]**	**Specific enzyme activities (nkat.mg^{-1})[a]**
***Y. lipolytica*[*man1*]**		
1	7,048 (799)	655 (66)
3	8,619 (149)	846 (76)
8	4,055 (387)	398 (31)
11	10,746 (79)	890 (40)
12	10,288 (534)	801 (22)
13	**13,073 (1,027)**	**1,020 (100)**
***Y. lipolytica*[*man1-LIP2*]**		
4	**496.2 (22.4)**	**32.1 (12.5)**
8	389.4 (54)	31.1 (1.2)
9	349.3 (37)	32.4 (18.2)

[a] Values are the average activities obtained by triplicate assays on duplicate flasks. Standard deviations are reported in brackets

The highest volumetric and specific enzyme activity of 13,073 nkat.ml^{-1} and 1,020 nkat.(mg DCW)$^{-1}$ respectively was obtained by *Y. lipolytica*[*man1*] (13) after 5 days of cultivation. The use of the *LIP2* secretion signal resulted in a 26-fold lower β-mannanase activity, compared to activities obtained using β-mannanase's own secretion signal, with the highest volumetric β-mannanase activity of 496.2 nkat.ml^{-1} obtained with *Y. lipolytica* [*man1-LIP2*] (4).

SDS-PAGE Analysis of Man1 produced by Y. lipolytica[man1]

The supernatants of *Y. lipolytica* [*man1*-low] (3), *Y. lipolytica* [*man1*] (3) and (13), *Y. lipolytica* [*man1-LIP2*] (4) and *Y. lipolytica*[HmA], cultivated in YNB_{casa} for 96 hours, were separated by 10% SDS PAGE (Figure 3). The recombinant Man1 protein is present in *Y. lipolytica*[*man1*] at approximately 70 - 80 kDa, larger than the 50 kDa of Man1 expressed in *S. cerevisiae* (Setati et al. 2001). This increased size is could be due to increased glycosylation, but does not seem to affect the activity of the enzyme.

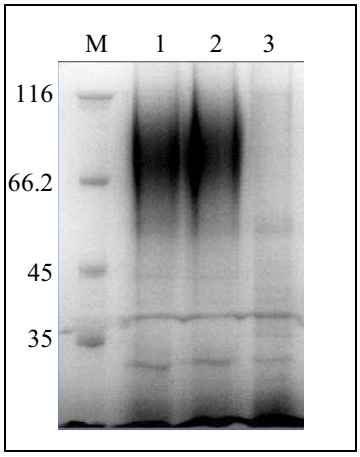

Figure 3. Separation of the total extracellular protein fractions (30 µl) of *Y. lipolytica*[*man1*] (lanes 1 and 2) and *Y. lipolytica*[HmA] (lane 3) on 10% SDS-PAGE. Lane M contains the molecular weight marker with the sizes depicted on the left hand side in kDa. The recombinant Man1 is present at approximately 70 kDa, larger than the Man1 expressed in *S. cerevisiae* (Setati et al. 2001).

Batch and fed-batch fermentations of Y. lipolytica [man1-low] and Y. lipolytica [man1]

The best performing low-copy construct *Y. lipolytica*[*man1*-low] (3) and multi-copy constructs *Y. lipolytica*[*man1*] (3) and (13), were compared in 2-L batch fermentations in Braun B fermenters. A maximum volumetric enzyme activity of 685 $nkat.ml^{-1}$ was reached after 48 hours by *Y. lipolytica* [*man1*-low], compared to 6,719 $nkat.ml^{-1}$ by *Y. lipolytica* [*man1*] (13) for the same time period (Table 8). The enzyme activity obtained of 685 $nkat.ml^{-1}$ by *Y. lipolytica*[*man1*-low] was 5.5-fold higher in batch fermentations than in shake flask production (Table 6, 123 $nkat.ml^{-1}$). The integration of multi-copy gene inserts into the host organism had a major effect on the biomass production, with the biomass concentration for both *Y. lipolytica* [*man1*] transformants approximately half that of *Y. lipolytica* [*man1*-low].

Table 8. Comparison of β-mannanase production in 2-L batch fermentations by low- (*Y. lipolytica* [*man1*-low]) and multi-copy (*Y. lipolytica* [*man1*]) constructs of *Y. lipolytica*

Constructs	***Y. lipolytica* [*man1*-low]**	***Y. lipolytica* [*man1*] 3**	***Y. lipolytica* [*man1*] 13**
Age (Hours)	48	48	48
Volumetric enzyme activity (nkat.ml^{-1})	685	5,776	6,719
Specific enzyme activity (nkat.(mg DCW)$^{-1}$)	12	182	305
Dry cell weight (g.l^{-1})	59	32	22
Yield (nkat.g^{-1} glucose)	0.03	0.29	0.33
Productivity (nkat.ml^{-1}.h^{-1})	14	120	140

The lower biomasses and the higher volumetric enzyme activities obtained for the two *Y. lipolytica* [*man1*] strains compared to *Y. lipolytica* [*man1*-low], resulted in a 15-fold increase in the specific enzyme activity to 182 nkat.(mg DCW)$^{-1}$ for *Y. lipolytica*[*man1*] (3) and a 25-fold increase to 305 nkat.(mg DCW)$^{-1}$ for *Y. lipolytica*[*man1*] (13), compared to 12 nkat.(mg DCW)$^{-1}$ for *Y. lipolytica*[*man1*-low]. The productivities of enzyme production of 120 and 140 nkat.ml^{-1}.h^{-1} for *Y. lipolytica*[*man1*] (3) and (13) respectively, were 8.6- and 10-fold higher than the productivity of 14 nkat.ml^{-1}.h^{-1} for *Y. lipolytica* [*man1*-low].

The major difference between the growth of the two constructs *Y. lipolytica* [*man1*] (3) and (13) was the biomass obtained (Figure 5). *Y. lipolytica* [*man1*] (3) achieved 34 (g DCW).l^{-1} after 24 h, while *Y. lipolytica* [*man1*] (13) reached 22 (g DCW).l^{-1} during the same period of time, with yields of 1.0 and 0.7 g.g^{-1} respectively. Although the volumetric rate of glucose utilisation was similar for *Y. lipolytica* [*man1*] (3) and (13), at 1.4 and 1.3 g.l^{-1}.h^{-1} respectively, the specific glucose utilisation rate was 1.2-fold higher for *Y. lipolytica* [*man1*] (13) (1.01 g.g^{-1}.h^{-1}) than *Y. lipolytica* [*man1*] (3) (0.87 g.g^{-1}.h^{-1}).

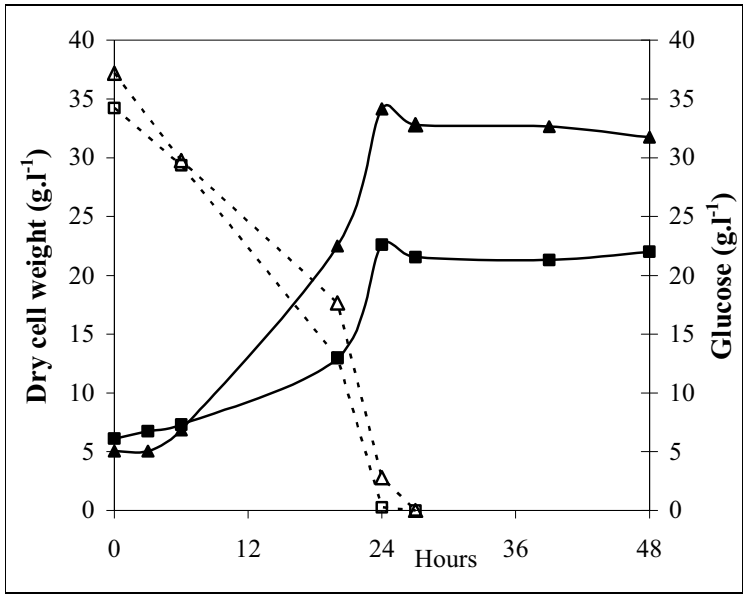

Figure 4. Growth (g.l^{-1} dry cell weight, in closed symbols) and residual glucose concentration (g.l^{-1}, in open symbols) of *Y. lipolytica* [*man1*] (3) and (13) in batch fermentations. *Y. lipolytica* [*man1*] (3) is represented by Δ and *Y. lipolytica* [*man1*] (13) by □.

As glucose interfered with the β-mannanase enzyme assay, samples were only taken for enzyme analyses once the initial concentration of glucose was depleted. The hp4d promoter is not inhibited by residual glucose, as can be observed by the high volumetric enzyme activities of 2,964 nkat.ml^{-1} for *Y. lipolytica* [*man1*] (3) and 4,599 nkat.ml^{-1} for *Y. lipolytica* [*man1*] (13), observed at the time of sugar depletion (Figure 5).

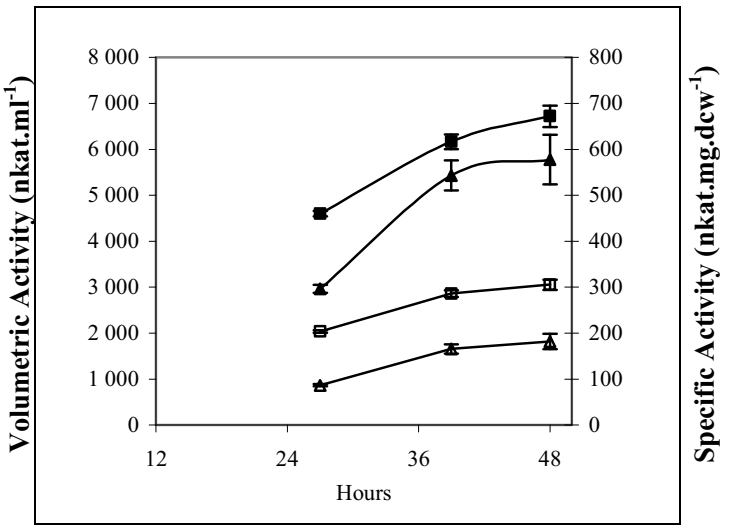

Figure 5. Volumetric (closed symbols) and specific (open symbols) β-mannanase activities of *Y. lipolytica* [*man1*] (3) (Δ) and *Y. lipolytica* [*man1*] (13) (□) in 2-L Braun B fermenters over time. Standard deviation of triplicate assays given in error bars.

Assuming no enzyme was produced during the first 6 hours of the fermentation, it was calculated that *Y. lipolytica* [*man1*] (3) grew at a μ of 0.09 h-1 and produced β-mannanase at a specific rate of 2.7 nkat.mg-1.h-1, whereas *Y. lipolytica* [*man1*] (13), growing at a μ of 0.06 h-1, produced β-mannanase at a specific rate of 8 nkat.mg-1.h-1

The maximum volumetric enzyme activity of 6,719 nkat.ml^{-1} was obtained with *Y. lipolytica*[*man1*] (13) after 48 h. This was 1.2-fold higher than 5,776 nkat.ml^{-1} achieved by *Y. lipolytica*[*man1*] (3). As can be expected from the higher volumetric enzyme activities and lower biomass obtained by *Y. lipolytica*[*man1*] (13), the specific enzyme activity was 1.7-fold higher than that of *Y. lipolytica*[*man1*] (3). The maximum specific enzyme activities were 182 and 305 nkat.(mg DCW)$^{-1}$ for *Y. lipolytica*[*man1*] (3) and *Y. lipolytica*[*man1*] (13), respectively, after 48 hours.

Y. lipolytica[*man1*] (13) was subsequently evaluated in 15-L fed-batch fermentations in an attempt to increase the β-mannanase production. Glucose feed was started at the point of sugar depletion and an attempt was made to maintain the residual glucose concentration below 20 g.l^{-1}. The maximum growth rate in fed-batch fermentation was 0.19 h^{-1} compared to 0.06 h^{-1} in batch fermentation. A maximum biomass of 128 g.l^{-1} was reached at a productivity of 2.27 g.l^{-1}.h^{-1}.

The fermentation was scaled up to 15-L fed-batch with the objective of increasing the biomass while maintaining high specific enzyme activities obtained in the 2-L fermenters, and subsequently increasing the volumetric enzyme activity. *Y. lipolytica*[*man1*] (13) produced more β-mannanase in fed-batch fermentations than in batch fermentations, with a 3.9-fold increase compared to batch fermentation, reaching a maximum titre of 26,139 nkat.ml^{-1} after 70 h of fermentation (Figure 6). The overall enzyme productivity achieved by *Y. lipolytica*[*man1*] (13) in fed-batch was 373 nkat.ml^{-1}.h^{-1} compared to 140 nkat.ml^{-1}.h^{-1} in batch fermentation. However, the specific enzyme activity of 240 nkat.(mg DCW)$^{-1}$ obtained in fed-batch fermentation was lower than the 305 nkat.(mg DCW)$^{-1}$ obtained in batch fermentation (Figure 5), and the shake flask activity of 1,020 nkat.(mg DCW)$^{-1}$, as shown in Table 6. In comparison to the shake

flask trial, the volumetric enzyme activity obtained in the Braun C 15-L fermenters increased by 100%, reaching a maximum of 26,139 nkat.ml^{-1} after 70 hours, compared to 13,073 nkat.ml^{-1}.

Heterologous Enzyme Production in *A. niger* D15[*man1*]

pH and temperature optima of Man1 in A. niger D15[man1]

The activity of Man1 was determined using 0.25% galactomannan (Sigma) (pH 4.8) at a temperature of 75°C. The pH optimum of Man1 was determined around pH 4.8 (Figure 6A) which differs slightly from that observed with expression of the native enzyme (pH 3), as well as with expression of the *man1* gene in *S. cerevisiae* (Setati et al. 2001). The temperature optimum of Man1 was determined between 75 and 80°C (Figure 7B), which differs significantly from the 60°C optimum achieved with the native enzyme as well as with expression in *S. cerevisiae* (Setati et al. 2001).

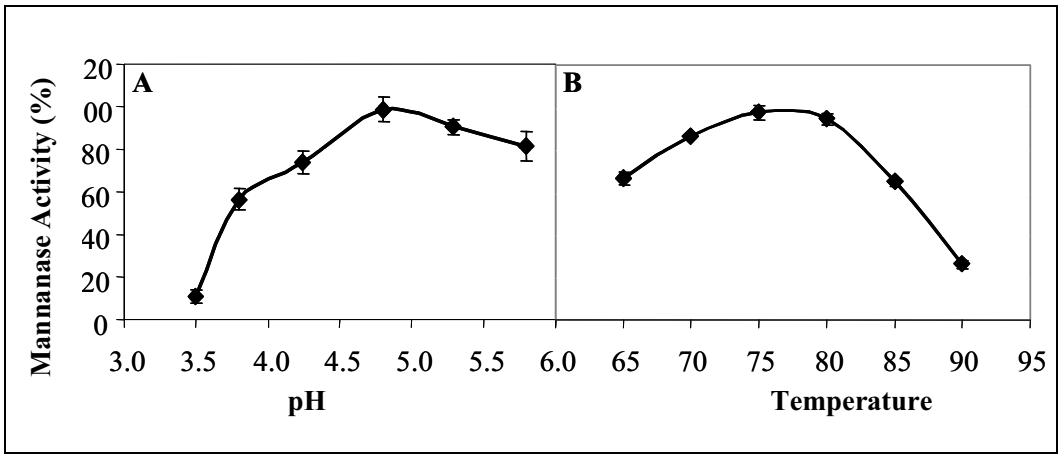

Figure 6. The effect of (**A**) pH and (**B**) temperature on the enzymatic activity of Man1 produced by *A. niger* D15[*man1*]. The highest activity for Man1 was measured at pH 4.8 using sodium citrate buffer and at 75 - 80°C. Optimal conditions were determined as described in La Grange et al. (1996).

The production of the heterologous Man1 by *A. niger* D15[*man1*] was followed over 10 days in 2xMM (Figure 7). The highest level of Man1 activity by *A. niger* D15[*man1*] was produced on day 8 (6,800 nkat.ml^{-1}), although the levels started to stabilise from day 6 at above 6,000 nkat.ml^{-1}. These levels of activity exceeded the best levels of activity

obtained with expression of *man1* in *S. cerevisiae* (521 nkat.ml^{-1}) by approximately 13-fold (Setati et al. 2002).

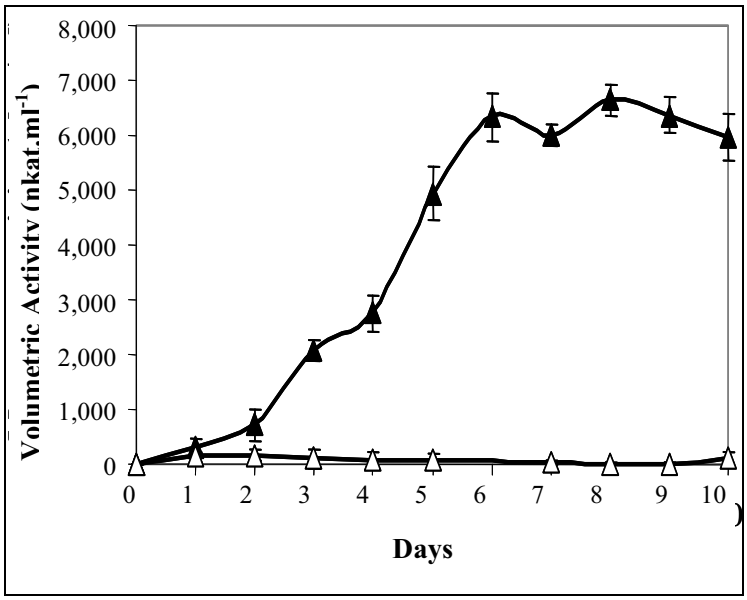

Figure 7. The heterologous production of Man1 (▲) by *A. niger* D15[*man1*] was monitored over a period of 10 days in 2xMM medium and compared to the activity produced by *A. niger* D15[pGT] (Δ, representing the level of β-mannanase expressed by the reference strain). The pH of the media did not change significantly. The pH dropped from pH 5.5 to 4.5 after 3 days of cultivation and remained at pH 4.5 for the duration of the experiment. Enzyme activity assays were determined in triplicate using 6 parallel cultures. Enzyme activity was determined as described in La Grange et al. (1996).

SDS-PAGE Analysis of Man1

The supernatant of *A. niger* D15[*man1*], cultivated on CSLM, was separated by 10% SDS-PAGE (Figure 8). The recombinant Man1 protein was present as a slightly diffuse band with a molecular size varying from 45 - 50 kDa. It is evident that the recombinant Man1 protein constitutes a significant portion of the total amount of protein produced extracellularly, whereas the rest of the natively secreted proteins are barely visible.

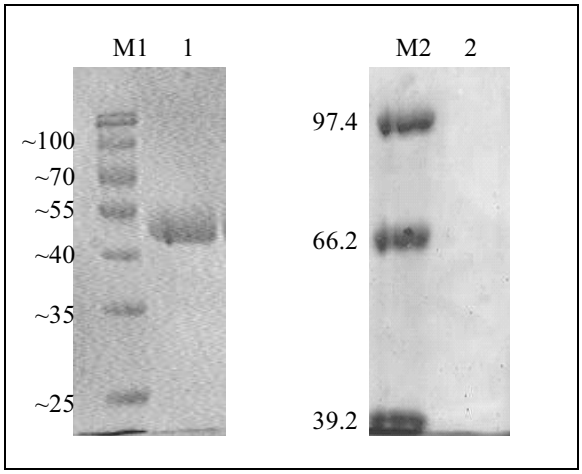

Figure 8. Separation of the total extracellular protein fractions (10 µl) of *A. niger* D15[*man1*] (lane 1) and *A. niger* D15[pGT] (lane 2) on 10% SDS-PAGE. Lanes M1 and M2 contain the molecular weight markers with the sizes depicted on the left hand side in kDa. The recombinant Man1 is present at approximately 45 -50 kDa, similar to the Man1 expressed in *S. cerevisiae* (Setati et al. 2001). *A. niger*[pGT] had no equivalent protein band.

Media composition

1. The effect of CSL and yeast extract on Man1 production

The effect of inorganic nitrogen supplementation on the production of Man1 by *A. niger* D15[*man1*] was evaluated in shake flasks with two different types of CSL, viz, Roquette CSL^{PT} (spray dried, phytase-treated and centrifuged) and Roquette CSL^{SD} (spray-dried) and in yeast extract-containing medium (YEM). Stationary growth phase was reached after one day irrespective of the growth medium tested (Figure 9). The highest biomass of 35 $g.l^{-1}$ was obtained in YEM medium. This was 6% higher than the maximum biomass of 33 $g.l^{-1}$ obtained in medium supplemented with CSL^{SD} (CSLM) and 16% higher than the maximum biomass obtained with medium containing phytase-treated CSL (PCSLM) (Table 9). The limiting nutrient in all cases was not glucose as this was only depleted by day 4 in all flasks. The biomass concentrations decreased steadily after the initial peak in concentration, most probably due to autolysis.

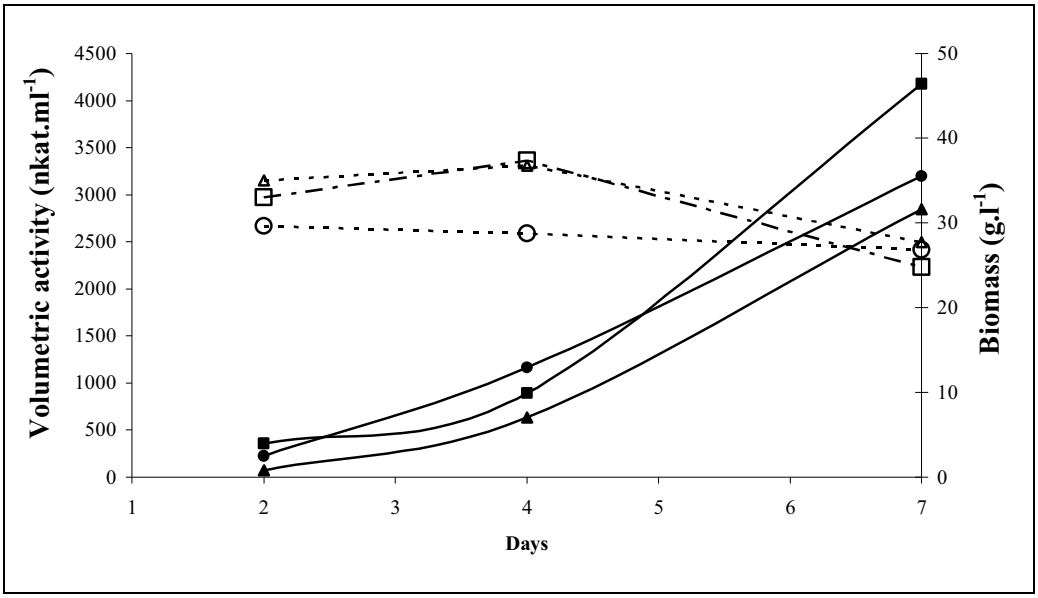

Figure 9. The heterologous production of Man1 by *A. niger* D15[*man1*] monitored over a period of 7 days in different media to determine the effect of CSL and yeast extract on Man1 production. The volumetric activity (nkat.ml^{-1}) is represented by the solid icons and line, and the biomass (g.l^{-1} dry cell weight) by the open icons and dotted lines. ▲ YEM, ■ CSLM, ● PCLM.

Table 9. The effect of nitrogen supplementation heterologous production of Man1 by *A. niger* D15[*man1*]

Medium	Nitrogen supplementation	Biomass (g.l^{-1} dcw)	Volumetric enzyme activity (nkat.ml^{-1})	Specific enzyme activity (nkat.mg^{-1})
YEM	Yeast extract	35 (±2.6)	2848 (±164)	103 (±8)
CSLM	Roquette CSL^{SD}	33 (±5.2)	4175 (±323)	159 (±17)
PCSLM	Roquette CSL^{SP}	30 (±0.9)	3199 (±529)	119 (±28)

Extracellular enzyme activity was observed after day 2 and increased until day 7. It is unclear whether the production of the enzyme only occurs after day 2 or if the residual high glucose concentration in the medium masks the measurable β-mannanase activity. The maximum β-mannanase activity of 4,175 nkat.ml^{-1} was obtained in CSLM. This was 23% higher than the maximum activity of 3,199 nkat.ml^{-1} in PCSLM and 46% higher than the 2,848 nkat.ml^{-1} with YEM.

The increase in enzyme activity during stationary growth phase, as shown in Figure 9, was not expected as the *gpd* promoter is constitutive (Punt et al. 1998) and part of the EM pathway. However, secretion of heterologous proteins in fungi is known to be

problematic and therefore the prolonged production may be due to slow secretion of intracellular enzyme (Punt et al. 1991 and Ásgeirsdóttir et al. 1998).

Due to the small differences in biomass obtained in the different medium compositions, the differences in volumetric enzyme activity were due to differences in the specific enzyme production. The highest specific enzyme activity of 159 nkat.(mg DCW)$^{-1}$ was obtained with *A. niger* D15[*man1*] cultivated in CSLM, 35% higher than that achieved in YEM, which had the lowest specific activity of 103 nkat.(mg DCW)$^{-1}$. The specific enzyme activity increased during the stationary growth phase, indicating a metabolic shift away from biomass production towards enzyme production. This is in contrast with results published by Talabardon and Yang (2005) who reported that the production of a glucoamylase-green fluorescent protein fusion by *A. niger* is growth-dependent. However, it should be noted that the strong promoter from the *A. niger* glucoamylase *glaA* gene was used, compared to the *gpd* promoter used in this study. Based on this data, CSL^{SD} was chosen as the organic nitrogen supplementation of choice for further media evaluation.

The effect of AspA+N salts and glucose concentrations on Man1 production

The effect of glucose and inorganic nitrogen concentration in CSLM was subsequently tested. Decreasing the glucose concentration resulted in a concomitant decrease in biomass (Table 10). When the glucose concentration was reduced from 100 g.l^{-1} to 25 g.l^{-1}, the yield of biomass on glucose fed increased from 0.33 g.g^{-1} to 0.40 g.g^{-1}, indicating that some metabolic overflow of carbon was present at the higher glucose concentration. The yield of biomass on glucose was very high in Medium 3 (2 g.l^{-1} glucose), but it assumed that at this low glucose concentration the organism will start using protein for carbon and energy requirements. The maximum yield of 0.44 g.g^{-1} was obtained in Medium 4, at a glucose concentration of 5 g.l^{-1} and 0.5 x AspA+N salts.

Table 10. Yield of biomass per glucose fed ($g.g^{-1}$) for *A. niger* D15[*man1*] in shake flasks with different glucose and AspA+N salt concentrations

Medium	Glucose ($g.l^{-1}$)	AspA+N salts (final conc.)	Biomass (g)	Yield of biomass per glucose fed ($g.g^{-1}$)	Volumetric Activity ($nkat.ml^{-1}$)	Specific Activity ($nkat.mg^{-1}$)
1	100	2 X	33	0.33	16,596	574
2	25	2 X	10	0.40	3,542	515
3	2	2 X	4	2	684	342
4	50	0.5 X	22	0.44	3,274	197
5	50	0.1 X	15	0.30	597	36

Decreasing the glucose concentration by 75% from 100 $g.l^{-1}$ (Medium 1) to 25 $g.l^{-1}$ (Medium 2) resulted in a 78% decrease in the volumetric enzyme activity from 16,596 $nkat.ml^{-1}$ to 3,542 $nkat.ml^{-1}$ at Day 7, when the maximum activity was detected (Figure 10). Maintaining a fixed glucose concentration of 50 $g.l^{-1}$ but decreasing the AspA+N stock solution from 0.5 x to 0.1 x (an 80% decease in concentration) resulted in an 81% decrease in the volumetric Man1 activity from 3,274 $nkat.ml^{-1}$ to 597 $nkat.ml^{-1}$ (Medium 4 vs Medium 5).

The decrease in the glucose concentration from 100 $g.l^{-1}$ to 25 $g.l^{-1}$ (Medium 1 to Medium 2) had very little effect on the specific activity of Man1 by *A. niger* D15[*man1*], with maximum specific enzyme activities were 574 $nkat.(mg\ DCW)^{-1}$ and 515 $nkat.(mg\ DCW)^{-1}$ respectively. The concentration of the AspA+N salts in the medium had a major effect on the specific production of the enzyme and decreasing the AspA+N salts 4-fold from 2 x to 0.5 x resulted in a 3-fold decrease in the specific enzyme activity (Medium 1 vs Medium 4). It is assumed that this response in the specific production of Man1 is due to the decrease in AspA+N salt concentration and not the decrease in the glucose concentration from 100 $g.l^{-1}$ to 50 $g.l^{-1}$, as it was shown that a decrease from 100 $g.l^{-1}$ to 25 $g.l^{-1}$ glucose did not have a significant impact. A further 5-fold decrease in AspA+N salt concentration to 0.1 x resulted in the specific Man1 activity decreasing 5-fold from 197 $nkat.(mg\ DCW)^{-1}$ to 36 $nkat.(mg\ DCW)^{-1}$ (Medium 4 vs Medium 5). The response to the changes in the AspA+N salt concentration does not seem to be due to changes in the morphology of the fungal growth as similar morphologies were deserved irrespective of the salt concentrations. The smallest bead

sizes were observed in Medium 1, which consistently gave the best results. The fungal bead size was bigger in all the other media.

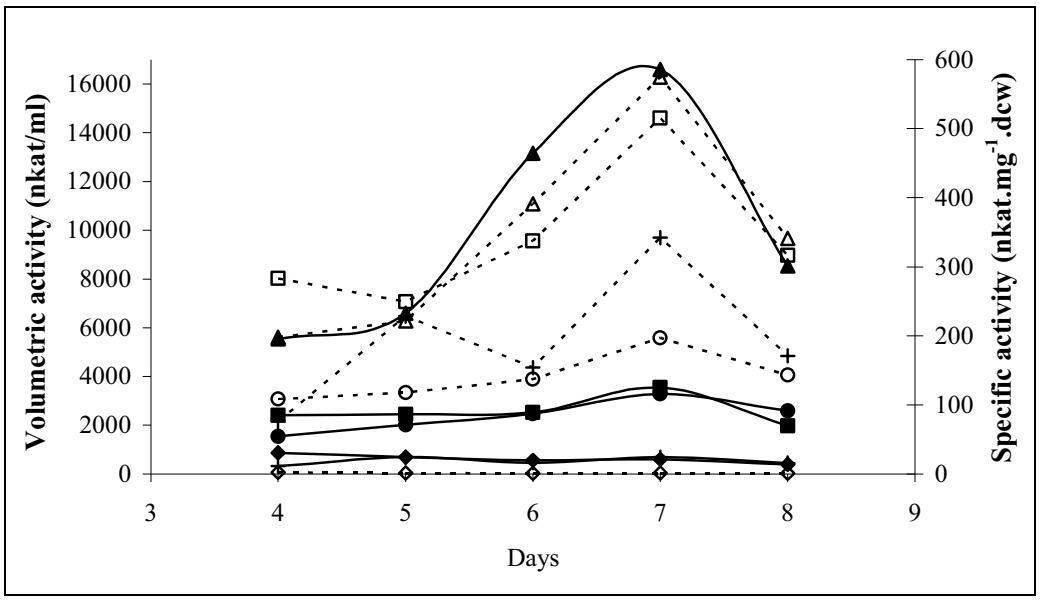

Figure 10. The heterologous production of Man1 by *A. niger* D15[*man1*] was monitored over a period of 7 days in different media. Medium 1 is supplemented with 100 g.l^{-1} glucose, 2 x AspA+N salts, Medium 2 with 25 g.l^{-1} glucose, 1 x AspA+N salts, Medium 3 with 2 g.l^{-1} glucose, 2 x AspA+N salts, Medium 4 with 50 g.l^{-1} glucose, 0.5 x AspA+N salts and Medium 5 with 50 g.l^{-1} glucose, 0.1 x AspA+N salts. The volumetric activity (nkat.ml^{-1}) is represented by: ▲ Medium 1, ■ Medium 2, + Medium 3, ● Medium 4 and ♦ Medium 5. The specific activity (nkat/g.dcw) is shown by: Δ Medium 1, □ Medium 2, + Medium 3, ○ Medium 4 and ◊ Medium 5.

The effect of shaker speed on the production of Man1 by *A. niger* D15[*man1*] was evaluated on an orbital shaker at 220 rpm, 100 rpm and with no shaking (Figure 11), using Medium 1. It was found that decreasing the shaker speed from 220 to 100 rpm resulted in a decrease of 46% in the maximum volumetric enzyme activity from 12,984 nkat.ml^{-1} to 7,012 nkat.ml^{-1} at Day 7. An absence of shaking did not result in a significant further decrease in the maximum volumetric enzyme activity, but instead decreased the rate of production. The maximum volumetric enzyme activity was reached after 9 days compared to 7 days with either shaking speed. In the absence of shaking, *A. niger* D15[*man1*] formed a pellicle on the surface of the medium. This would have resulted in only a small part of the fungal population being in contact with the substrate and therefore the slower rate of enzyme production.

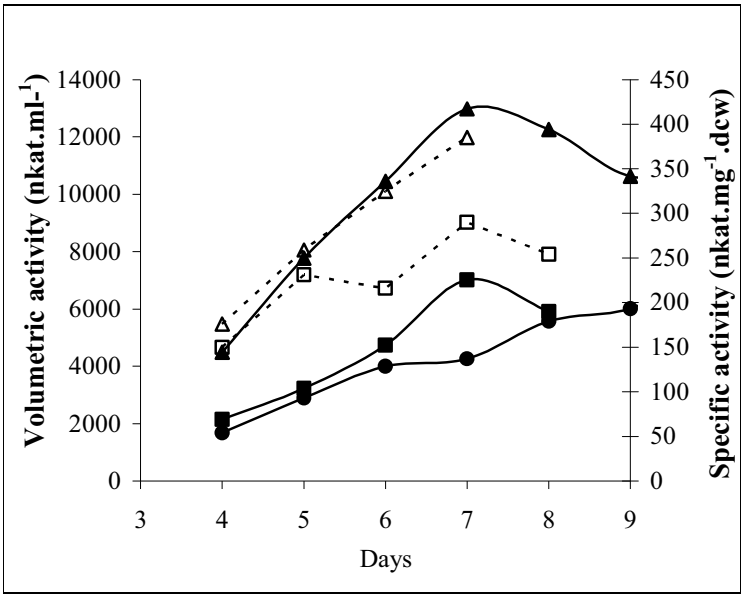

Figure 11. The heterologous production Man1 by *A. niger* D15[*man1*] was monitored over a period of 9 days in Medium 1 at different rates of agitation. ▲ represents the volumetric activity (nkat.ml^{-1}) at 200 rpm, ■ 100 rpm, and ● at 0 rpm. The specific activity at 200 rpm is shown by Δ, and at 100 rpm is indicated by □.

Decreasing the shaker speed affected the biomass production, and as a result also the specific enzyme production. At day 5 the specific enzyme production was only 11% lower at 100 rpm compared to 220 rpm. However, the effect of lower shaker speed became more pronounced during the later stages of the production of the enzyme. At day 7, the specific enzyme activities were 385 nkat.(mg DCW)$^{-1}$ at 220 rpm and 290 nkat.(mg DCW)$^{-1}$ at 100 rpm, representing a 24% decrease in specific enzyme activity. This decrease in specific enzyme activity during the later stages of production is most probably due the increased viscosity of the medium at the lower shaker speed, resulting in the mixing becoming a problem. This observation is confirmed by results of other researchers (McIntyre et al. 2001; Wang et al. 2003).

2. The combined effect of CSL^{SD} and yeast extract on the production of Man1

A. niger D15[*man1*] was subsequently grown in media containing different concentrations of CSL^{SD} and yeast extract to evaluate the impact of organic nitrogen composition and concentration on the production of Man1. The organic nitrogen composition had an effect on the maximum volumetric and specific enzyme activities.

The highest volumetric and specific enzyme activities of 11,634 nkat.ml and 300 nkat.mg^{-1} respectively, were obtained in Medium 6, containing casamino-acids, CSL and no yeast extract (Table 11).

Table 11. The effect of organic nitrogen composition on the production of Man1 by *A. niger* D15[*man1*]

Medium	Organic nitrogen composition	Concentration (g.l^{-1})	Volumetric enzyme activity (nkat.ml^{-1})	Specific enzyme activity (nkat.mg^{-1})
6	CSL	4	11,636	300
7	CSL Yeast extract	4 20	10,498	257
8	CSL Yeast extract	2 20	9,315	230

The addition of yeast extract had a negative effect on the production of Man1 (Medium 6 vs Medium 7). Although the previous results showed that the addition of yeast extract to the medium resulted in lower enzyme activities compared to media containing CSL, this result was still unexpected as it was thought that the combination of yeast extract and CSL would have a positive effect. It has, however, been reported that the addition of yeast extract to the medium resulted in a more viscous broth, due to changes in the morphology of the fungal growth (Wang et al. 2003). This decreased the mixing efficiency of the broth and resulted in a decrease in the production of the green fluorescent protein (GFP) by *A niger*. The addition of yeast extract to D15[*man1*] resulted in a slightly higher biomass concentration at the point of maximum enzyme activity, as could be expected from the extra protein available in the medium, but the extra biomass did not increase the production of Man1. The specific enzyme activity decreased by 14% to 257 nkat.(mg DCW)$^{-1}$. Decreasing the CSL concentration in the medium by 50% from 4 g.l^{-1} (Medium 7) to 2 g.l^{-1} (Medium 8) resulted in only an 11% decrease in both the volumetric and specific enzyme activities. The relatively small change in both would suggest that CSL at 4 g.l^{-1} was in excess.

3. The effect of inorganic nitrogen concentration on the production of Man1

AspA+N salts (1 x: 0.05% KCl, 0.15% KH_2PO_4, 0.6% $NaNO_3$) were added to all previous media. Previous results showed that decreasing the AspA+N salt concentration from 2 x to 0.5 x did not have an effect on the volumetric β-mannanase activity but

resulted in a 3-fold decrease in specific enzyme activity (Medium 3 vs Medium 4). A further decrease in the AspA+N salts concentration from 0.5 x to 0.1 x (an 80% decease in concentration) resulted in an 81% decrease in the volumetric β-mannanase activity from 3,274 nkat.ml^{-1} to 597 nkat.ml^{-1} (Medium 5). To test an assumption that it was the $NaNO_3$ concentration within the AspA+N salts which was responsible for the change in activity levels, different $NaNO_3$ concentrations were used in Media 9, 10 and 11. Table 12 shows the effect of $NaNO_3$ concentration on Man1 production. The highest volumetric (10,498 nkat.ml^{-1}) as well as specific (257 nkat.mg^{-1}) Man1 production was obtained in Medium 9, with 12 g.l^{-1} $NaNO_3$, the amount in 2 x AspA+N salts.

Table 12. The effect of inorganic nitrogen composition on the production of Man1 by *A. niger* D15[*man1*]

Medium	$NaN0_3$ (g.l^{-1})	Volumetric enzyme activity (nkat.ml^{-1})	Specific enzyme activity (nkat.mg^{-1})
9	12	10,498	257
10	20	8,983	208
11	30	9,415	197

A negative correlation was found between the total nitrogen concentrations and the specific enzyme production when evaluating the effect of different medium compositions on enzyme production by *A niger* at a fixed CSL concentration of 4 g.l^{-1} and a glucose concentration of 100 g.l^{-1} (Figure 12).

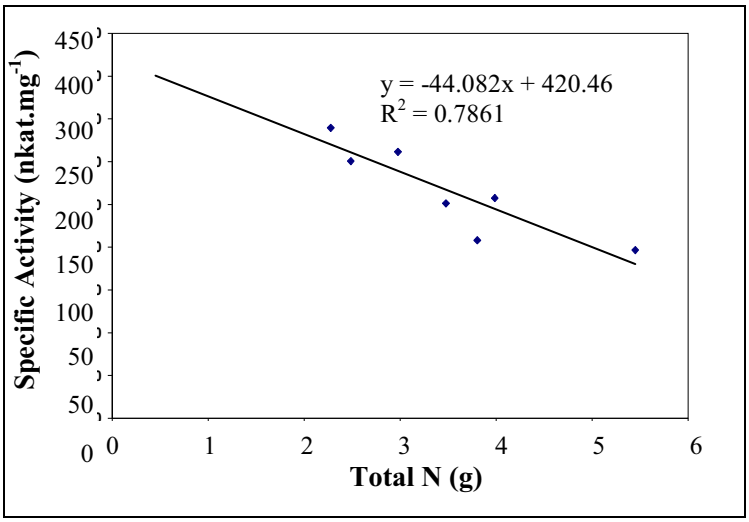

Figure 12. The effect of total nitrogen concentration on heterologous production of Man1 by *A. niger* D15[*man1*].

The higher the total nitrogen concentration, the lower the specific enzyme production, with a 1.7-fold decrease in enzyme activity for a 2.4-fold increase in the total nitrogen concentration. The ratio of glucose to nitrogen in the medium was also found to be a major contributor to the production of β-mannanase by *A. niger* (D15) (Figure 13).

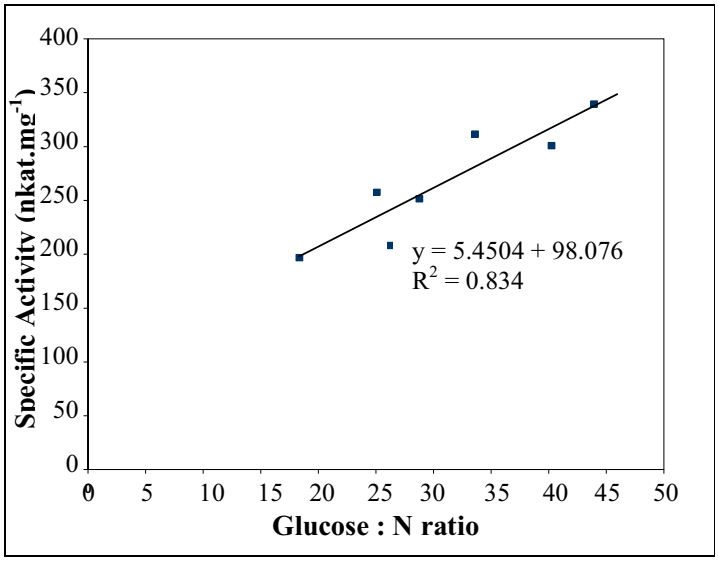

Figure 13. The effect of glucose to total nitrogen ratio on heterologous production of Man1 by *A. niger* D15[*man1*].

No collaboration could be found between the volumetric enzyme production and the total nitrogen concentration or the glucose to nitrogen ratio. The ratio of inorganic to organic nitrogen did not have an effect on either the specific or the volumetric production of Man1, although the concentration of $NaNO_3$ had an impact on enzyme production when all other parameters were kept constant, as discussed above.

Discussion

Yarrowia lipolytica low copy transformants were constructed using pINA1313 (Nicaud et al. 2002) for the expression of *Aspergillus aculeatus Man1* under the control of the inducible hp4d promoter, fused in-frame with the *LIP2* secretion signal. These low copy *Y. lipolytica* [*man1*low] transformants resulted in very low levels of β-mannanase expression. It has previously been reported that multi-copy vectors in *Y. lipolytica* can give a 10-20 fold increase in the expression of proteins (Madzak et al. 2004) and

therefore multi-copy transformants were constructed. These were based on pINA1293 and pINA1291 (Nicaud et al. 2002), using the *LIP2* or the native *Man1* secretion signal, respectively. The use of these recombinant strains, *Y. lipolytica* [*man1-LIP2*] and *Y. lipolytica* [*man1*], resulted in higher levels of β-mannanase production. The production of β-mannanase using its natural secretion signal was more than twenty-fold higher than the expression using the homologous *LIP2* secretion signal. In shake flasks, *Y. lipolytica* [*man1*] (13) produced maximum volumetric activity of 13,073 nkat.ml^{-1} and a specific activity of 1,020 nkat.(mg DCW)$^{-1}$. Both homologous and heterologous secretion signals have successfully been used in *Y. lipolytica* (Madzak et al 2004). The homologous secretion signals most commonly used are the *XPR2* prepro-region (Hamsa and Chattoo 1994), the *XPR2* pre region alone (Swennen et al. 2002), the prepro-region of the *Y. lipolytica LIP2* gene (Pignède et al. 2000) and a hybrid between *XPR2* and *LIP2* prepro regions (Nicaud et al. 2002). Enzymes successfully produced under the direction of their native secretion signals include *A. aculeatus*'s cellulase and galactanase (Müller et al. 1998), *Humicola insolens*'s xylanase (Müller et al. 1998) and *Trichoderma reesei*'s endogluconase I (Park et al. 2000).

Both *Y. lipolytica* [*man1*] (3) and (13) gave similar relative copy numbers. This result implies that it is not the absolute copy number of the *man1* gene that causes *Y. lipolytica* [*man1*] (13) to be a better performer than *Y. lipolytica* [*man1*] (3). The site of integration may also have a large affect on Man1 activity. This has also been observed in *A. niger* transformants expressing glucoamylase (Verdoes et al 1993), as well as in *S. cerevisiae* and *Lactococcus lactis* (Thompson and Gasson 2001). In *L. lactis,* there was a three-fold difference in expression of randomly integrated β-glucuronidase (GUS) genes, whereas in *S. cerevisiae*, a 14-fold variation was found in the expression of the *lacZ* reporter gene. This difference may reflect the greater complexity of the yeast genome. This underlines the need to screen all transformants for activity, as genetic screening is not able to be directly extrapolated into function. An interesting result was the low copy number determined for *Y. lipolytica* [*man1-LIP2*] (4). This may be due to undesired recombination effects. However, this transformant (4) was selected after activity screening of all *Y. lipolytica* [*man1-LIP2*] transformants, implying the other

transformants were inferior Man1 producers. A possible explanation is that the expression cassette integrated at a site in the genome which is highly active, and the expression of the *ura3d4* selection marker, as well as the *man1* gene, was influenced by regulatory elements in the vicinity of the site of integration. In the shake-flask analysis of *Y. lipolytica* [*man1*-low] transformants, activity also varied greatly, indicating a difference in region of integration. These transformants are likely to contain only one or two copies of the integrated cassette, as the expression cassette contains the unmutated *ura3d1* selection marker, so a single copy is enough to alleviate the auxotrophy of the Po1h host. The relative copy number determined for the best Man1 producer, *Y. lipolytica* [*man1*-low] (3), by RT-PCR confirmed the *man1* gene is only present in two copies. So, its improved *man1* activity is potentially entirely due to the region of integration, not copy number. The secreted Man1 protein appears to be glycosylated, which has been observed in *Y. lipolytica* (Madzak et al. 2004). No hyperglycosylation has been reported, but moderate glycosylation (an additional 10 kDa) of heterologous products is known, which do not appear to impair activity (Nicaud et al. 1989, Park et al. 2000)

The volumetric enzyme activity achieved by *Y. lipolytica* [*man1*-low] (3) in batch fermentation was 5.5-fold higher than in shake flask production. This compares with the 5.75-fold increase in batch fermentations reported by Nicaud et al. (2002). The integration of multi-copy gene inserts into the host organism resulted in a metabolic load on the *Y. lipolytica* [*man1*] transformants compared to the *Y. lipolytica* [*man1*-low] constructs as indicated by the 50% lower biomass yield. A similar decrease in biomass and growth rate as a result of multi-copy integrations of the *Aspergillus oryzae* leucine amino peptidase II gene into *Y lipolytica* was observed by Nicaud et al. (2002).

Assuming no enzyme was produced during the first 6 hours of the 2-L batch fermentations, it was observed *Y. lipolytica* [*man1*] (3) growing at maximum growth rate of 0.09 h^{-1} produced β-mannanase at a specific rate of 2.7 $nkat.mg^{-1}.h^{-1}$ whereas *Y. lipolytica* [*man1*] (13), growing at a μ of 0.06 h^{-1} produced at a specific rate of 8 $nkat.mg^{-1}.h^{-1}$. Similar to previous results obtained when comparing *Y. lipolytica* [*man1*-

low] versus *Y. lipolytica* [*man1*], where the higher volumetric enzyme activities are attained at the expense of biomass production, indicating a metabolic burden on the cells.

However, the volumetric enzyme activity obtained in the batch fermentation of *Y. lipolytica* [*man1*] (13) decreased by almost 2-fold when compared to activities obtained in shake flasks (6,719 nkat.ml^{-1} versus 13,073 nkat.ml^{-1}). It is hypothesised that the conditions in fermenters are more suited for growth than in flasks and therefore metabolic flux is directed towards growth at the cost of enzyme production. The specific enzyme activity of *Y. lipolytica* [*man1*] (13) in fed-batch fermentation was also 1.3-fold lower than the specific enzyme activity in batch fermentation and a 4.3-fold lower compared to the shake flask activity. This suggests that not all of the biomass that was produced during the length of the fermentation contributed to the production of the enzyme. This could be explained by a shift in metabolic flux towards an increase in biomass and not enzyme expression, suggesting that media and growth condition optimisation may have a further positive effect on the Man1 production, as was seen in the *A. niger* optimisations.

Initial shake flask Man1 production by *A. niger* D15[*man1*] Man1 in 2xMM with 100 g.l^{-1} glucose resulted in a volumetric activity of ~6,800 nkat.ml^{-1}. Further media composition optimisation increased the maximal levels obtained to 16,596 nkat.ml^{-1} and 574 nkat.ml^{-1}, with supplementation by 100 g.l^{-1} glucose and 2x AspA+N salts. Decreasing the glucose and AspA+N salts concentrations resulted in a concomitant decrease in both biomass and volumetric and specific activity. Further efforts to optimise the organic and inorganic nitrogen composition and concentration did not increase these values. The results indicate that a high glucose concentration is essential for both high volumetric enzyme activity and biomass production, whereas a high AspA+N salt concentration is essential for high specific enzyme activity.

The main parameter influencing the specific production of β-mannanase by *A. niger* D15[*man1*] is the total nitrogen concentration, as well as ratio of glucose to nitrogen. A negative correlation was found between the total nitrogen concentration and the specific

enzyme production when evaluating the effect of different medium compositions on enzyme production by *A niger* at a fixed CSL and glucose concentration. A positive correlation was noted between the glucose: nitrogen ratio and the specific enzyme production.

Decreasing the CSL concentration had a negative effect on production of the enzyme, but not by the level that was expected. This indicates that the higher concentrations of CSL used are possibly too high when in combination with the other nitrogen sources. The use of yeast extract rather than either of the two types of CSL tested had a negative effect on the volumetric and specific activities of *A. niger* D15[*man1*]. Even when present in combination with CSL, it reduced the Man1 activity levels. A decrease in agitation also had a negative effect on the production of β-mannanase during the later stages of production, most probably due to increased viscosity of the medium, resulting in oxygen limitation. The temperature and pH optima determined for the Man1 produced by *A. niger* D15[*man1*], pH 4.8 and 75 to 80°C, differ from pH 3 and 60°C observed with the native enzyme and that expressed in *S. cerevisiae*.

Our results indicate that *Y. lipolytica* is also a promising heterologous producer of hydrolases, such as *A. aculeatus* β-mannanase. Multi-copy transformants with the *man1* gene directed by its own secretion signal give good extracellular yields of β-mannanase. The increase in size of the enzyme produced, presumably due to some glycosylation effects, does not appear to affect the activity of the enzyme. Recombinant *S. cerevisiae* expressing *man1,* under the control of the *ADH2* and *PGK1* promoters, produced 521 and 379 nkat.ml^{-1}, respectively. In contrast, *Y. lipolytica*[*man1*] (13) produced 13,073 nkat.ml^{-1} in shake flasks, which increased to 26,140 nkat.ml^{-1} in fed-batch fermentations. *A. niger* D15[*man1*] produced maximum levels of 16,596 nkat.ml^{-1} and 574 nkat.ml^{-1} in shake flasks, after media optimisations.

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Chapter 6.

Heterologous Expression of *Trametes versicolor* Laccases in *Pichia pastoris* and *Aspergillus niger*

by

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The expression of *T. versicolor* laccases in *A. niger*, and the purification of the wild-type *T. versicolor* laccase was done by the author.
The expression in *P. pastoris* was done by Christina Bohlin of Karlstad University.

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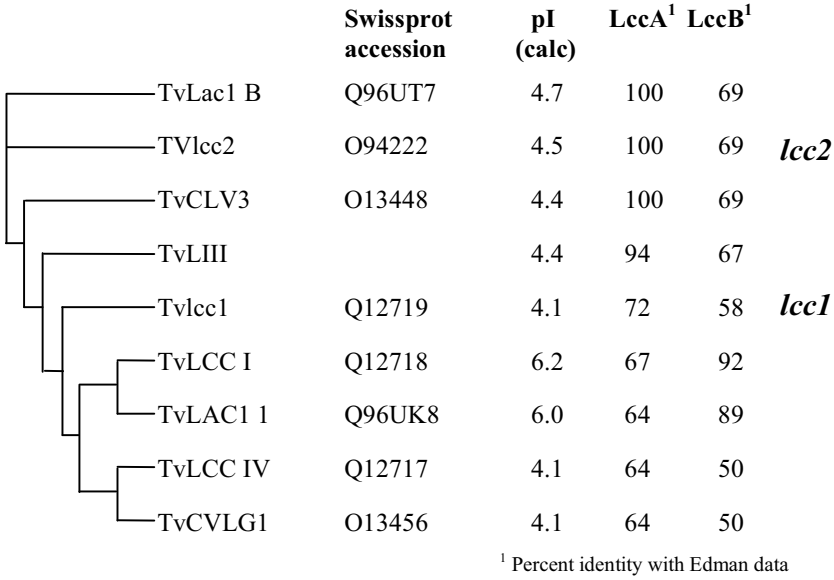
Introduction

The following publication, "Heterologous expression of *Trametes versicolor* laccase in *Pichia pastoris* and *Aspergillus niger*" by C. Bohlin, L. J. Jönsson, R. Roth, and W. H. Van Zyl, appeared in Applied Biochemistry and Biotechnology, Vol 129–132 (2006), pg 195-214.

Laccases are important enzymes for bioremediation, and the best characterised enzymes are from the fungus *Trametes versicolor.* Figure 1 shows the family of laccase genes identified from *T. versicolor*. The objective of the research was to optimise expression of *T. versicolor* laccases (*lcc1* and *lcc2*) in *Aspergillus niger* and *Pichia pastoris*[1]. The recombinant laccase Lcc2, produced by *A. niger,* was characterised and its properties compared to those of the native laccases, LccA and LccB, produced by *T. versicolor*.



	Swissprot accession	pI (calc)	LccA[1]	LccB[1]	
TvLac1 B	Q96UT7	4.7	100	69	
TVlcc2	O94222	4.5	100	69	*lcc2*
TvCLV3	O13448	4.4	100	69	
TvLIII		4.4	94	67	
Tvlcc1	Q12719	4.1	72	58	*lcc1*
TvLCC I	Q12718	6.2	67	92	
TvLAC1 1	Q96UK8	6.0	64	89	
TvLCC IV	Q12717	4.1	64	50	
TvCVLG1	O13456	4.1	64	50	

[1] Percent identity with Edman data

Figure 1. Dendogram of *T. versicolor* laccases. Tvlcc2 and TVlcc1 were used for this research.

[1] The expression of *T. versicolor* laccases in *P. pastoris* was done by Christina Bohlin of Karlstad University. The expression in *A. niger* was done by Robyn Roth of CSIR.

Expression of *lcc2* in both expression systems was superior to *lcc1*, and optimisation was conducted using the best Lcc2 producer from each host. A summary of the results obtained for the heterologous expression of Lcc2 in the different expression systems is shown in Table 1.

Table 1. Summary of results of heterologous expression of Lcc2 in *P. pastoris* and *A. niger*

	P. pastoris [1]	***A. niger*** [2]	***T. versicolor***
Promoter	glyceraldehyde-3-phosphate dehydrogenase (GAP)	glyceraldehyde-3-phosphate dehydrogenase (GPD)	
Carbon source	Glucose	Sucrose	
Fermentation time	5-7 days	10 days	
Glycosylation (%)	~15[3]	~16	10-14[4], ~11[5]
Activity ($U.L^{-1}$)	2.8	2,700	

[1] SMD1168 [2] D15 [3] AOX1 system [4] Reinhammer 1984 [5] This study

It was found that *A. niger* D15 was a superior Lcc2 producer under the conditions tested in this research.

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Heterologous Expression of *Trametes versicolor* Laccase in *Pichia pastoris* and *Aspergillus niger*

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Abstract

Convenient expression systems for efficient heterologous production of different laccases are needed for their characterization and application. The laccase cDNAs *lcc1* and *lcc2* from *Trametes versicolor* were expressed in *Pichia pastoris* and *Aspergillus niger* under control of their respective glyceraldehyde-3-phosphate dehydrogenase promoters and with the native secretion signal directing catalytically active laccase to the medium. *P. pastoris* batch cultures in shake-flasks gave higher volumetric activity (1.3 U/L) and a better activity to biomass ratio with glucose than with glycerol or maltose as carbon source. Preliminary experiments with fed-batch cultures of *P. pastoris* in bioreactors yielded higher activity (2.8 U/L) than the shake-flask experiments, although the levels remained moderate and useful primarily for screening purposes. With *A. niger*, high levels of laccase (2700 U/L) were produced using a minimal medium containing sucrose and yeast extract. Recombinant laccase from *A. niger* harboring the *lcc2* cDNA was purified to homogeneity and it was found to be a 70-kDa homogeneous enzyme with biochemical and catalytic properties similar to those of native *T. versicolor* laccase A.

Index Entries: Laccase; heterologous expression; *Pichia pastoris*; *Aspergillus niger*.

Introduction

Laccases are phenol-oxidizing enzymes that are of interest in several different applications (reviewed in ref. *[1]*). Possible applications include textile processing, detoxification of industrial effluents and pollutants, detoxification of lignocellulose hydrolysates in fuel ethanol production, utilization as an environmentally benign oxidant in the production of chemicals, delignification of pulp for paper manufacture, catalysis of grafting

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processes in the development of novel polymers, production of fiberboards, use in fuel cells, and utilization in biosensors for monitoring phenolic pollutants and drugs. The properties of different laccases show a great deal of divergence. Laccases with high-redox potential, such as the laccase from the white-rot fungus *Trametes (Polyporus, Coriolus) versicolor (2)*, are required for oxidation of recalcitrant substrates. Considering the wide range of applications for laccases *(1)*, there is a need for heterologous expression systems to screen mutated laccases for novel properties or for large-scale production of selected laccases.

Previous studies suggest that yeasts such as *Pichia pastoris* and *Saccharomyces cerevisiae* are convenient systems for rapid expression of laccase genes. However, production levels in yeast have been quite low (up to approx 5 mg/L), whereas filamentous fungi in general have given 2–30 times higher levels (10–135 mg/L) *(3)*. *P. pastoris* is easy to manipulate genetically, easy to use in conventional fed-batch fermentations, secretes low levels of native proteins, and capable of adding both *O*- and *N*-linked glycans to secreted proteins *(4)*. Filamentous fungi, such as *A. niger*, have the ability to produce and secrete exceptionally large amounts of properly folded proteins with the correct cofactors incorporated and can produce proteins that contain *O*- and *N*-linked glycans without extensive hyperglycosylation *(5–7)*.

It has been shown previously that production of laccase in *P. pastoris* using the *AOX1* promoter system is negatively affected by increasing methanol concentration *(8)*. The *AOX1* promoter requires methanol for induction, which makes it difficult to use lower concentrations because that would negatively affect the mRNA expression level. Therefore, it should be of interest to consider the glyceraldehyde-3-phosphate dehydrogenase promoter system as an option for expression in *P. pastoris*, as well as in *A. niger*.

In this study, we have explored the potential in using *P. pastoris* for screening purposes and *A. niger* for production of selected laccases. The cDNAs *lcc1* and *lcc2* from *T. versicolor* were expressed in *P. pastoris* and *A. niger*, and the effects of different media and cultivation conditions on the laccase production levels were investigated. The recombinant laccase expressed in *A. niger* was purified to homogeneity and its biochemical and catalytic properties were compared to the well-characterized native *T. versicolor* laccases A (LccA) and B (LccB) *(2)*.

Methods

Microbial Strains and Recombinant DNA

The *lcc1* and *lcc2* cDNA genes from *T.* (*Coriolus, Polyporus*) *versicolor (9–11)* were used in the construction of plasmids for expression of laccases in *P. pastoris* and *A. niger*.

For the expression in *P. pastoris*, the *lcc1* and *lcc2* cDNA genes were inserted into the vectors pGAPZ A and pGAPZ B, respectively. The expression cassette of the pGAPZ vectors includes the glyceraldehyde-3-phosphate

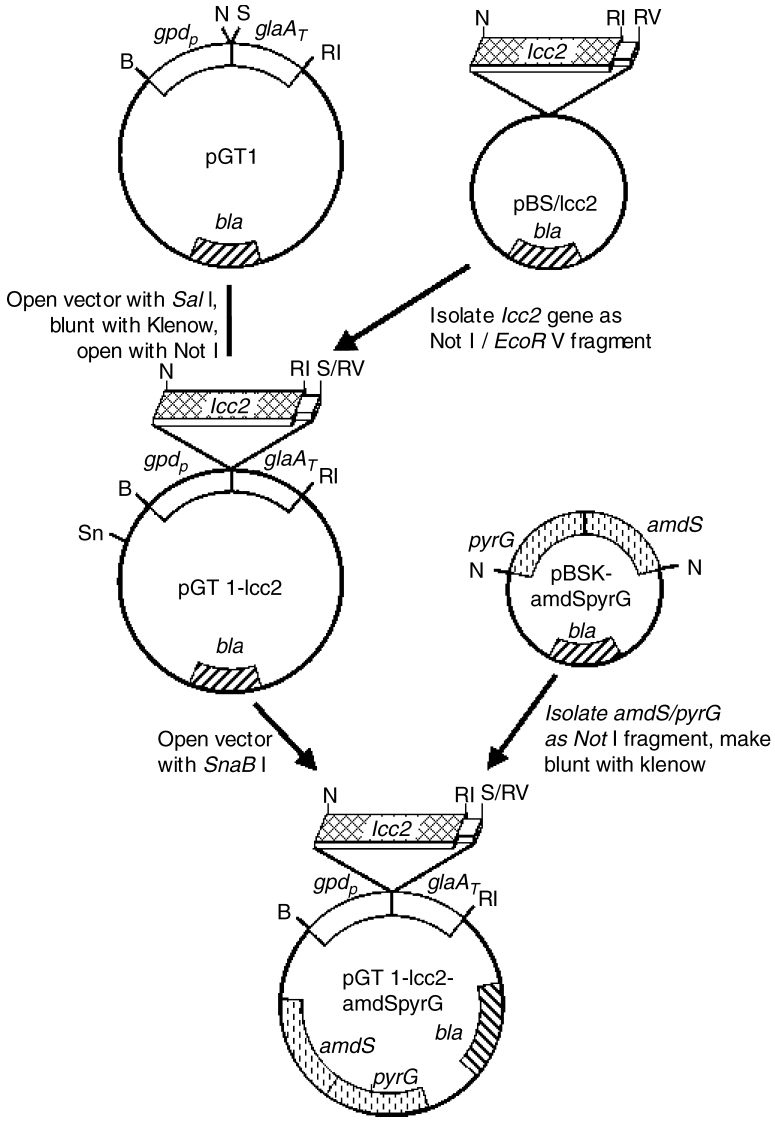

Fig. 1. Construction of plasmids for expression of laccase in *A. niger*. B, *BamH I*; N, *Not I*; RI, *EcoR I*; RV, *EcoR V*; S, *Sal I*; Sn, *SnaB I*. pGT1-*lcc1-amdSpyrG* was made similarly.

dehydrogenase gene (*GAP*) promoter region and the transcriptional terminator of the alcohol oxidase 1 gene (*AOX1*). The pGAPZ vectors are designed for constitutive expression in *P. pastoris* (Invitrogen, Carlsbad, CA). *P. pastoris* SMD1168, a *his4 pep4* strain, was transformed by electroporation.

Figure 1 summarizes the construction of plasmids for the expression in *A. niger* D15 (obtained from TNO, Zeist, The Netherlands). Insertion of the *lcc1* and *lcc2* cDNA genes into the plasmid pGT1, which harbors the *Aspergillus* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter and glucoamylase (*gla*) terminator from pSPORT *(12)*, generated pGT1-*lcc1*

and pGT1-*lcc2*, respectively. Insertion of an *amdSpyrG* fragment taken from pBSKII(+)-*amdSpyrG* generated pGT1-*lcc1-amdSpyrG* and pGT1-*lcc2-amdSpyrG*, respectively (Fig. 1). *A. niger* spheroplasts were formed using Novozyme 234 (Sigma, St. Louis, MO) and transformed in accordance with the method of Punt and van den Hondel *(13)*.

Selection of Laccase-Expressing Transformants

P. pastoris transformants that survived a concentration of 0.1 mg/mL zeocin were spread on BMG agar plates (Invitrogen) containing glucose instead of glycerol, 0.1 m*M* $CuSO_4$, and 0.2 m*M* ABTS [2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid)]. The color development was followed at room temperature (approx 22°C). Laccase-expressing transformants were selected and incubated in 50 mL buffered minimal glycerol (BMG) medium with 0.1 m*M* $CuSO_4$. An addition of 0.3 mL of a 0.6 *M* solution of potassium phosphate (pH 6.0) and 0.2 mL of a 0.4 *M* solution of NaOH was made daily. For selection of top laccase producers, samples were taken daily to determine the laccase activity (*see* Analyses of Samples).

A. niger transformants containing the *lcc1* and *lcc2* cDNA constructs as well as a negative control strain obtained from a transformation of *A. niger* D15 with the pBSKII(+)-*amdSpyrG* plasmid (Fig. 1) were plated onto agar plates containing 5% glucose, 0.2% amino acid pool without tyrosine or phenylalanine (200 mg each of adenine, uracil, tryptophan, arginine, methionine, and histidine; 300 mg of lysine; 600 mg of leucine; 2000 mg of threonine; and 65 mg of inositol), 0.5% yeast extract, 0.1 m*M* $CuSO_4$, 2 m*M* $MgSO_4$, 0.1% ABTS, 0.1% 1000× trace elements [the 1000X trace element solution contained (per 100 mL deionized water) 2.2 g $ZnSO_4 \cdot 7H_2O$, 1.1 g H_3BO_3, 0.5 g $MnCl_2 \cdot 4H_2O$, 0.17 g $CoCl_2 \cdot 6H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, 0.15 g $Na_2MoO_4 \cdot 2H_2O$, 5 g ethylene diamine tetraacetic acid (EDTA), and the pH was 6.5], and 1× AspA with nitrate [50X AspA with nitrate contained (per 500 mL deionized water): 150 g $NaNO_3$, 13 g KCl, 38 g KH_2PO_4]. The plates were incubated at 30°C for 48 h and the color development was followed. Laccase-expressing *A. niger* transformants were transferred to 50 mL medium containing 0.5% yeast extract, 1% glucose, 0.1% casamino acids (Difco, BD, Franklin Lakes, NJ), 2% $MgSO_4 \cdot 7H_2O$, 1X trace element solution, and 1X AspA with nitrate by inoculation with spores to a concentration of 1×10^6 spores/mL (experimental series 1). The cultures were then grown for 48, 72, or 144 h. The mycelia were harvested by filtering through Miracloth (Calbiochem, San Diego, CA) and the culture supernatants were collected to determine the laccase activity.

Shake-Flask Cultivations of P. pastoris

A selected laccase-producing transformant (designated *P. pastoris/lcc2*) was incubated in BMG medium and also in the same medium but with glucose or maltose instead of glycerol. The initial concentration of carbon source was 0.5–3%. The concentration of YNB (Yeast Nitrogen Base w/o amino acids, a component of BMG medium), was varied in the range 1–3%. In addition, the medium contained 0.4% histidine, 1 m*M* $CuSO_4$, and

0.8% alanine. The cultivation was allowed to continue for 7 d at 20°C and samples were taken daily to determine the laccase activity.

Parallel Fed-Batch Cultivations of P. pastoris *in Multibioreactor*

Preparation of Inoculum

A colony of *P. pastoris*/*lcc2* containing pGAPZ/*lcc2* was transferred from a yeast peptone dextrose (YPD) agar plate into a baffled flask containing 200 mL of yeast peptone dextrose (YPD) (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) and incubated at 30°C with shaking (200 rpm in a G25 orbital shaker, New Brunswick Scientific, Edison, NJ) for 20 h until an OD_{600} (optical density at 600 nm) of greater than 3 was reached.

Batch Phase

A Sixfors multibioreactor system (Infors, Bottmingen-Basel, Switzerland) equipped with three parallel temperature-controlled 500 mL bioreactors with sensors for pH and pO_2 was used. Each bioreactor contained 180-mL basal salt medium (Invitrogen) not including glycerol but supplemented with 4% glucose, 2 g/L histidine, 4.35 mL/L PTM trace salt solution (Invitrogen), and 0.2 m*M* $CuSO_4$. The pH of the medium was adjusted to 5.0 with 28% ammonium hydroxide before the inoculum (3 mL) was added to a final OD_{600} of 1. The bioreactors were maintained at a temperature of 20°C and at an agitation rate of 1000 rpm. Air was introduced into the bioreactor at a rate of 30 L/h and the pH was automatically maintained at 5.0 by the addition of a 2.8% ammonium hydroxide solution. The batch phase was allowed to continue until the glucose was depleted, which occurred after approx 40 h. Thereafter, the fed-batch phase was initiated.

Fed-Batch Phase

The medium that was fed into the bioreactor was the same as in the batch phase, except that different concentrations of glucose were used. Bioreactors A–C were fed with glucose at a rate of 0.06, 0.17, and 0.43 g/h, respectively. Samples were taken daily and centrifuged in a Mini Spin microcentrifuge (Eppendorf, Hamburg, Germany) at maximum speed for 15 min. The supernatants were transferred to fresh tubes, which were snap-frozen in liquid nitrogen. Samples were analyzed with respect to laccase activity, protein concentration, protease activity, OD_{600}, wet cell weight (WCW), and glucose concentration (*see* Analyses of Samples).

Media Optimization for A. niger *Transformants Using Shake-Flask Cultures*

Experimental Series 2 and 3

To determine optimal medium composition for laccase production, spores of the *A. niger* transformant expressing the highest level of laccase (designated *A. niger*/*lcc2*) were used to inoculate different media (Table 1)

Table 1
Media Used for Optimization of Laccase Production by *A. niger*

	Media composition						
Ingredient (g/L)	A	B	C	D	E	F	G
$(NH_4)_2SO_4$	45						3[a]
KH_2PO_4	23	1.5[b]	1.5[b]		1.5[b]		
$FeSO_4 \cdot H_2O$	0.1						
$MgSO_4 \cdot 7H_2O$	7	0.49[c]	0.49[c]	0.49[c]	0.49[c]	0.49[c]	0.98[d]
Sucrose	50		50				100
Urea	11						
Yeast extract	5		5		5	5	
Glucose		10		100	10		
1000X trace elements		1 mL	1 mL	1 mL	1 mL	1 mL	2 mL
$NaNO_3$		6[b]	6[b]	5.95	6[b]	6[b]	12[a]
KCl		0.52[b]	0.52[b]	0.52	0.52[b]	0.52[b]	1.04[a]
NaH_2PO_4				35.60			
Initial pH	4, 5, 6	4, 5, 6	4, 5, 6	5	5	5	5

[a]As 40 mL 50X AspA+N salts.
[b]As 20 mL 50X AspA+N salts.
[c]As 2 mL of 1 *M*.
[d]As 4 mL of 1 *M*.

to a final concentration of 1×10^6 spores/mL. The inoculum was generated by resuspending spores in a sterile 0.9% NaCl solution. The spore suspension was stored at 4°C until use. The cultures were incubated in a volume of 100 mL at 30°C with shaking (200 rpm). The performance of the transformant in media A–D (experimental series 2) (Table 1) was studied for 4 d. In the experiments with media E–G (experimental series 3) (Table 1), the cultivation was allowed to continue for 10 d.

Experimental Series 4

The media optimization was continued by investigating the effect of the addition of 0.5% yeast extract to the medium, as well as the use of a preculture. The 20-mL precultures were all inoculated to a final concentration of 5×10^6 spores/mL using the *A. niger/lcc2* transformant. The precultures were incubated at 30°C for 36 h and then transferred to 80-mL fresh media.

Experimental Series 5

Additional experiments were carried out at pH 4.0–6.0 and at 25°C and 30°C. The laccase activity was measured daily. The results of these and earlier experiments were used to plan the following experiment.

Experimental Series 6

The production of laccase by the transformant *A. niger/lcc2* was followed over a time period of 10 d in cultures with three different media in a volume of 250 mL. The first medium consisted of a minimal salt medium

(0.05% $MgSO_4 \cdot 7H_2O$, 0.6% $NaNO_3$, 0.05% KCl, 0.15% KH_2PO_4, 1X trace elements) supplemented with 1% glucose and 0.5% yeast extract. The second medium was a double-strength minimal salt medium (0.1% $MgSO_4 \cdot 7H_2O$, 1.2% $NaNO_3$, 0.1% KCl, 0.3% KH_2PO_4, 2X trace elements) with 10% sucrose. The third medium was the same as the second but supplemented with 0.5% yeast extract. The pH of the first two media at the time of inoculation was 5.0, whereas the pH of the third was 6.0.

Purification of Heterologously Expressed Laccase From A. niger

The transformant *A. niger*/*lcc2* was cultivated in 250 mL of previously optimized medium (0.1% $MgSO_4 \cdot 7\ H_2O$, 1.2% $NaNO_3$, 0.1% KCl, 0.3% KH_2PO_4, 2X trace elements) with 10% sucrose and 0.5% yeast extract, and with the pH adjusted to 6.0. The culture was inoculated to 1×10^6 spores/mL and incubated at 30°C and 200 rpm for 11 d. The extracellular fraction was harvested by filtration. Ammonium sulfate precipitation was carried out at 70% saturation (at pH 4.2). The harvested protein precipitate was resuspended in 20 m*M* imidazole buffer, pH 7.0, and then snap-frozen in liquid N_2 in aliquots. The aliquots were freeze-dried and stored at –70°C. They were then resuspended in a 20 m*M* solution of potassium phosphate (pH 6.0) and dialyzed against the same buffer before purification.

The first purification step was done by loading the dialyzed sample onto a DEAE Sepharose Fast Flow column (Amersham Biosciences, Uppsala, Sweden), pre-equilibrated with 100 m*M* phosphate buffer (pH 6.0). A NaCl gradient from 0 to 500 m*M* was applied to the column, and the laccase started eluting at 40 m*M* NaCl. Fractions collected between 40 and 125 m*M* NaCl were pooled and dialyzed against 20 m*M* 3-(*N*-morpholino propanesulfonic acid) MOPS buffer, pH 7.2, overnight.

The second purification step was done by loading the dialyzed DEAE fractions onto a HiTrap Q FF column (Amersham Biosciences) equilibrated with 20 m*M* MOPS, pH 7.2. The protein was eluted with a 0–500 m*M* NaCl gradient. The laccase eluted from 150 m*M* NaCl. Fractions collected between 150 and 250 m*M* NaCl were pooled and dialyzed overnight against 10 m*M* MOPS, pH 6.5.

The dialyzed protein solution was concentrated 15 times, using 5-kDa cutoff spin columns (Millipore, Bedford, MA). This preparation was used for kinetic analysis and comparison with two different forms of the native protein, laccase A (LccA) and laccase B (LccB), isolated from *T. versicolor (2,14)*.

A further purification step was carried out by loading the HiTrap Q-purified protein onto a MonoP HR 5/20 column (Amersham Biosciences). The column was equilibrated with 20 m*M* MOPS buffer, pH 7.2, and a pH gradient from 7.2 to 3.0 was applied using 10% PolyBuffer 74 (Amersham Biosciences), pH 3.0, as eluent. A peak was visible for fractions eluting at pH 3.0–4.0 and the relevant fractions were neutralized by adding 100 µL of 1 *M* Tris-HCl per 2 mL fraction and pooled. The protein solution was dialyzed

against 10 m*M* phosphate buffer, pH 6.0, and concentrated using the 5-kDa spin columns.

Analyses of Samples

Laccase Activity

Assays were performed as described previously *(9)*. One unit was defined as the amount of laccase that forms 1 µmol of ABTS radical cation ($\varepsilon = 3.6 \times 10^4/M/\text{cm}$ at 414 nm *[15]*). To avoid assay interference by medium components, the laccase activity in samples from *P. pastoris* cultures was measured after purification of the samples using Microspin G-25 columns (Amersham Biosciences, Sweden) equilibrated with 10 m*M* phosphate buffer (pH 6.0).

Protein Concentration

The protein concentrations in samples from *P. pastoris* cultures were determined using Coomassie protein assay reagent (Pierce, Rockford, IL) with bovine-serum albumin as the standard. The protein concentrations in samples from *A. niger* cultures were estimated using the Lowry method *(16)*. During the purification of laccase from *A. niger*, the protein concentration was determined using the BCA protein assay reagent kit (Pierce, IL).

Optical Density

The OD of the samples was measured against distilled water at 600 nm with a ultraviolet-1601PC spectrophotometer (Shimadzu, Kyoto, Japan) after appropriate dilution.

Wet Cell Weight

The WCW was determined by centrifugation of the samples at maximum speed with a Mini Spin centrifuge (Eppendorf, Germany) for 15 min in preweighed test tubes and subsequent removal of the supernatant.

Glucose Concentration

The glucose concentration was determined using a Glucometer Elite XL (Bayer, Leverkusen, Germany) after appropriate dilution in artificial liquor containing (per liter of deionized water) 8.65 mg NaCl, 176.4 mg $CaCl_2{\cdot}2H_2O$, 182.9 mg $MgCl_2{\cdot}6H_2O$, 201.3 mg KCl, and NaOH to a pH of 7.4.

Electrophoresis

Estimation of the purity, size, and pI of the enzyme was obtained by using the PhastGel system (Amersham Biosciences), with a 4–15% gradient gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and an isoelectric focusing (IEF) 3–9 gel for IEF. Two identical IEF gels were run at the same time and one was stained with Coomassie brilliant blue. The other was used for zymogram analysis and was immersed for 5 min in a solution of 0.4 m*M* ABTS in 50 m*M* NaAc, pH 5.0.

Glycosylation

To determine the level of glycosylation of the recombinant laccase and the native LccA sample, deglycosylation was carried out using *N*-glycosidase F (Roche, Mannheim, Germany). First, 8 µg of laccase (in 5 µL of a solution of 1% SDS, 1% β-mercaptoethanol, and 20 m*M* sodium phosphate [pH 8.6]) was denatured by boiling for 3 min. The denatured sample was subsequently deglycosylated in a final volume of 20 µL containing 1% Nonidet P-40, 0.5% EDTA, 1% β-mercaptoethanol, 25 m*M* sodium phosphate buffer (pH 7.2), 0.25% SDS, and 5 U of the deglycosylation enzyme. The reactions were incubated at 37°C for 2 h, and 4 µL was loaded onto a 12% homogeneous SDS-PAGE gel using the PhastGel system. Control reactions without *N*-glycosidase F addition were done in parallel.

Kinetic Analysis

The K_M values of LccA, LccB, and the recombinant laccase purified from *A. niger* were determined using the substrate 2,4,5-trimethoxylbenzyl alcohol and were performed as described elsewhere *(32)*.

Protease Activity

The protease activity was determined using the QuantiCleave fluorescent protease assay kit (Pierce, IL), which is based on proteolytic digestion of a fluorescein thiocarbamoyl-casein conjugate and measurement of the fluorescence at 538 nm. The proteolytic activity at pH 5.0 was assayed using an LS 55 luminescence spectrometer (PerkinElmer, Wellesley, MA).

Results and Discussion

The expression of two isoenzymes of laccase from *T. versicolor* in *P. pastoris* and *A. niger* was investigated. In both cases, the expression was under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter, the *GAP* promoter of *P. pastoris (17)* and the *gpd* promoter of *Aspergillus (18)*. The *GAP* promoter gives constitutive expression, although its strength varies depending on the carbon source *(17)*. This is the first report of a laccase expressed in *P. pastoris* with the *GAP* promoter, as previous attempts have been made using the *AOX1* promoter.

Analysis of shake-flask cultures revealed that *P. pastoris* transformants with the *lcc2* gene gave approx three to four times higher activity than transformants with the *lcc1* gene (Fig. 2A). The transformant giving the highest activity (*P. pastoris*/*lcc2*) (approx 0.6 U/L) (Fig. 2A) was chosen for further studies. The choice of laccase gene for expression appears to have a great impact on the levels of activity reached.

Shake-flask cultures of *P. pastoris*/*lcc2*, in which the type and concentration of carbon source were varied, gave higher laccase activity (1.3 U/L) with glucose than with glycerol (0.7 U/L) or maltose (0.6 U/L) (Fig. 2B). Cells grown on glycerol showed higher growth rate than cells grown on glucose, which in turn showed higher growth rate than cells grown on

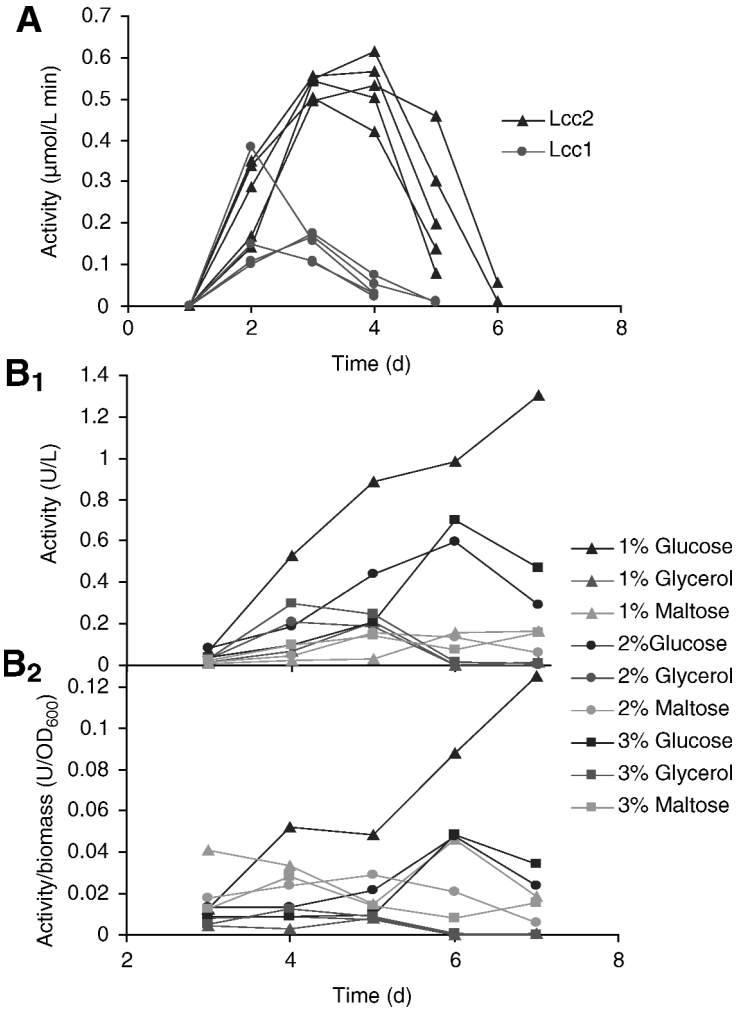

Fig. 2. Shake-flask cultures of *P. pastoris*. **(A)** Comparison of *lcc1* and *lcc2* transformants, **(B_1)** volumetric activity with *P. pastoris*/*lcc2*, **(B_2)** ratio of activity to biomass with *P. pastoris*/*lcc2*.

maltose. The cultures reached an OD_{600} of 27 (glycerol), 20 (glucose), and 10 (maltose). Previous results suggest that a slow growth rate is usually better for reaching high-laccase activity *(8)*, but the maltose cultures did not follow that trend. The ratio of activity to biomass was higher with glucose than with maltose or glycerol (Fig. 2B). This result shows that the laccase activity depends on more than just the growth rate. Growth on different carbon sources results in distinct patterns of intracellular proteins *(19)*, which in turn may affect the production of heterologous proteins. For laccase production, a glucose concentration of 1% was better than 2–3% (Fig. 2B). When even lower glucose concentrations were studied, 0.5% glucose gave about the same activity to biomass ratio as 1% glucose (not shown). The activities reached with the *GAP* promoter can be compared

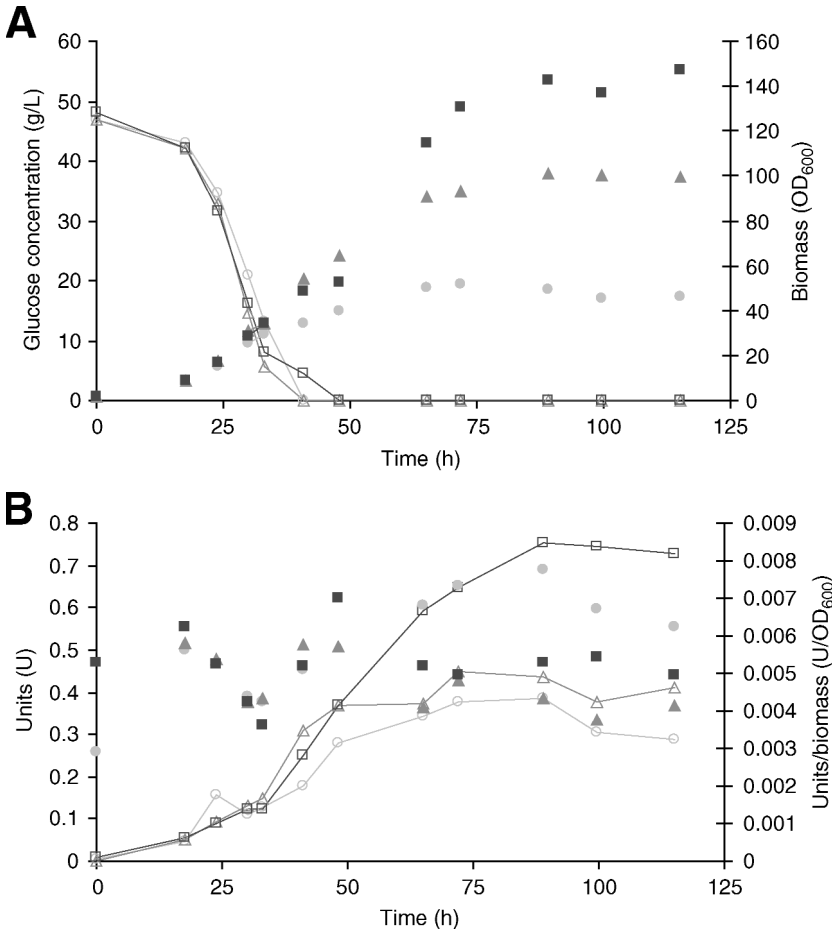

Fig. 3. Bioreactor cultures of *P. pastoris/lcc2*. **(A)** Glucose concentration and biomass, **(B)** units (µmol/min) and units/biomass. Filled symbols show biomass and units/biomass [fermentor A (●), fermentor B (▲), and fermentor C (■)]. Open symbols show glucose concentration and units.

with the expression of the same gene but with the *AOX1* promoter, which gave an activity of 0.35 U/L in the X-33 strain.

The influence of different feeding rates of glucose on the selected *P. pastoris/lcc2* transformant was investigated in parallel fed-batch cultures using a multibioreactor system (Fig. 3). The batch phase lasted for about 40 h (Fig. 3A) and resulted in a biomass concentration of 40–60 g/L. In the fed-batch phase, glucose was added at different rates for 82 h and the biomass concentrations in the bioreactors started to vary (Fig. 3A). The biomass concentration in fermentor A did not increase that much in the fed-batch phase, whereas fermentors B and C reached biomass concentrations of 80–90 g/L and 130–140 g/L, respectively. Fermentor C reached the highest volumetric activity (2.8 U/L), whereas fermentors B and A reached 1.8 and 1.3 U/L, respectively. The activity increased until approx 90 h, and

thereafter the level was constant or decreased slightly (Fig. 3B). The ratio of activity to biomass was slightly higher in fermentor A (Fig. 3B), but the relatively low values compared to the shake-flask cultures (Fig. 2B) indicate that further improvements should be possible. The specific activity (not shown) increased for about 50 h and subsequently leveled off.

Compared to a number of reports documenting high-level expression of foreign genes in *P. pastoris (4,20)*, the concentration of secreted protein in this study (0.1 g/L) was satisfactory. When laccases from *Pleurotus sajor-caju (21)* and *Pycnoporus cinnabarinus (22)* were expressed in *P. pastoris* using the *AOX1* system, the protein concentrations were 0.11 and 0.008 g/L, respectively. Although the total protein concentration in our study was at the same level or higher, the specific activity was much lower than in those studies *(21,22)*. The use of controlled conditions in a bioreactor instead of using shake-flasks generally improves heterologous protein production *(20)*. The laccase production reported in the current study could probably be enhanced further by optimization of medium and cultivation conditions.

When *P. pastoris* was used to express and determine the size of laccases from *T. versicolor (8)*, *P. cinnabarinus (22)*, *Fome lignosus (23)*, and *P. sajor-caju (21)*, the recombinant laccases were found to be between 5% and 36% larger then the native enzymes, strongly indicating hyperglycosylation. However, no reports show that hyperglycosylation affects the activity of the enzyme produced.

The use of a proteinase A mutant (*pep4*) strain (SMD1168) has been reported to be beneficial for the production of secreted recombinant proteins *(24)*, and that has also been observed for laccase *(9)*. In this study, the protease level in the fermentation medium was around 0.6 µg/mL (with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) trypsin as the standard) after 17 h and was fairly constant throughout the whole fermentation, never reaching a level greater than 1 µg/mL. It has been shown that cells grown on glycerol have lower levels of total protease activity compared with cells grown on methanol as the sole carbon source *(25)*. This could be a problem when laccase is expressed with the *AOX1* promoter and is one of the reasons why alternative carbon sources are of interest to study.

Very few transformants were obtained for *A. niger*. The transformation with pGT1-*lcc1-amdSpyrG* and pGT1-*lcc2-amdSpyrG* gave rise to nine and seven transformants, respectively. Of these 16 transformants, 11 showed color development on ABTS plates. No development of color was observed with the control transformed with pBSKII(+)-*amdSpyrG*.

To select a top laccase producer, transformants were grown in shake-flasks (experimental series 1). The results obtained showed that most of the activity was lost by 144 h, with the relative activities at 48 and 72 h being very similar (data not shown). Again, the control transformant showed no activity. Specific activities of laccase in the supernatants were calculated indicating that *lcc2* gave higher specific activity than *lcc1*, although the

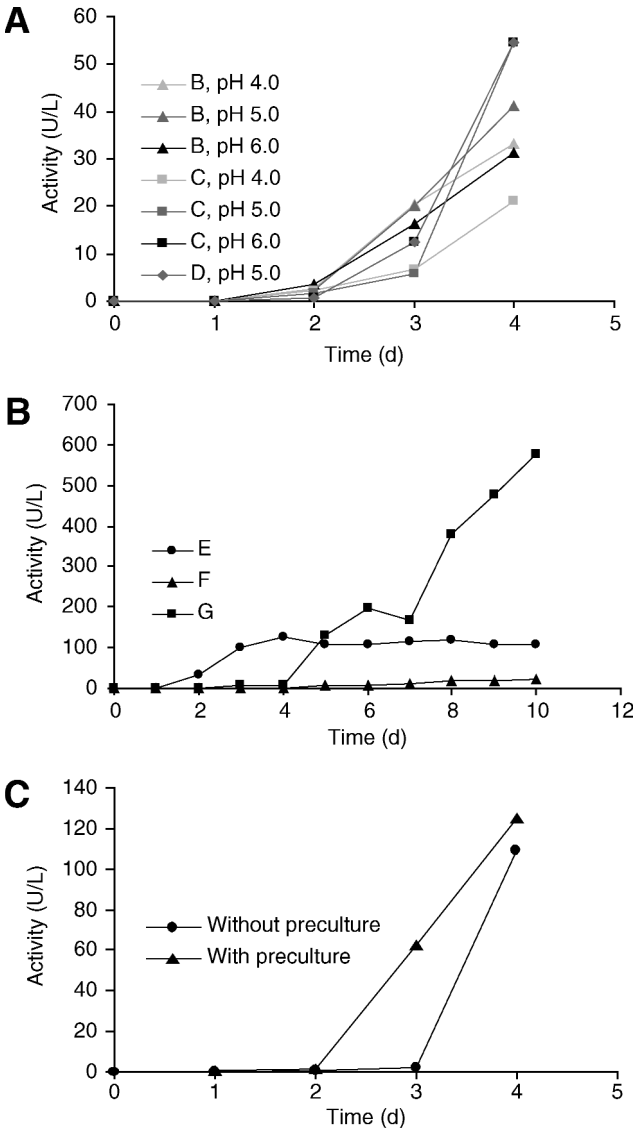

Fig. 4. Media optimization for *A. niger*/*lcc2* in **(A)** experimental series 2 with media A–D, **(B)** experimental series 3 with media E–G, and **(C)** experimental series 4 with and without preculture.

activity also varied between *lcc2* transformants (data not shown). The average volumetric and specific activity was almost 10 times higher for the *lcc2* transformants in comparison to the *lcc1* transformants. An *lcc2* transformant (*A. niger*/*lcc2*) was selected to be used in shake-flask analysis for media optimization.

Laccase production in media A–D was studied during 4 d of cultivation (Fig. 4A). Medium A (high salts) was used by Balasubramaniem et al. *(26)* for the production of β-fructofuranosidase in *A. niger* NRRL 330.

Growth in medium A gave no extracellular laccase activity, and very low biomass concentration (4–5 g/L after 4 d) was obtained. Medium B gave extracellular laccase activity levels of 31–41 U/L depending on the pH. The biomass production was good (19 g/L at pH 6.0). At low pH, the biomass concentration was lower (only approx 10 g/L at pH 4.0). Medium C also gave extracellular laccase activity (21–55 U/L), with the activity levels increasing as the pH increased. The biomass concentration was 14–15 g/L regardless of pH. Medium D was used by Record et al. *(27)* for expression of the *P. cinnabarinus* laccase in *A. niger* D15 No. 26, similar to the host strain being used in our experiments. A maximum activity of approx 50 U/L was obtained (Fig. 4A), which is in agreement with the reported levels of *P. cinnabarinus* laccase activity of 50 U/L using the native secretion signal. The biomass production was low, only approx 6 g/L. As the laccase activity was still increasing at the end of the experiment, a longer time period was used in further studies (experimental series 3–6).

Laccase production in media E–G was studied over 10 d (Fig. 4B). Medium E showed excellent laccase activity. By d 4 of the experiment (to compare with media A–D), the activity levels were 128 U/L, which was the maximum level obtained in this medium. The glucose was spent by 48 h, which was the point at which the biomass peaked (6.1 g/L). The cells may have lyzed during the next 2 d, as there was a small increase in extracellular laccase activity until 96 h (128 U/L), after which the activity dipped slightly and remained constant at approx 110–120 U/L until d 10. This may indicate that there is very little protease activity in the medium, as the laccase is not disappearing. *A. niger* D15 (the host organism) is a pH mutant which does not naturally acidify the medium, unlike like wild-type *Aspergillus*, and the acid proteases are not activated *(28)*. Medium F showed very little activity, as well as very little biomass, which was to be expected as the only carbon source added was yeast extract. Medium G combined sucrose as a carbon source with double strength minimal medium. This medium gave by far the best laccase activity, which reached 576 U/L after 10 d (Fig. 4B). The results show that yeast extract has a positive effect on laccase activity when added to the minimal medium (medium B vs medium E). The lag phase before activity was seen could be reduced from 3–4 to 2 d. This phenomenon was investigated further in experimental series 4 using 2× minimal medium with 10% sucrose (medium G) with and without yeast extract, as well as using glucose in place of sucrose in this medium. The presence of yeast extract resulted in laccase production, with low levels seen after 48 h (data not shown). An attempt to reduce the lag phase of laccase activity even further was done by using precultures for inoculation of the shake-flasks. The lag phase was reduced from 3 to 2 d (Fig. 4C).

In experimental series 5, the effects of pH and temperature were determined through the cultivation of *A. niger/lcc2* in sucrose 2× minimal medium supplemented with yeast extract. Spores were inoculated into 50-mL medium with the pH adjusted to 4.0, 5.0, or 6.0 and the cultures were

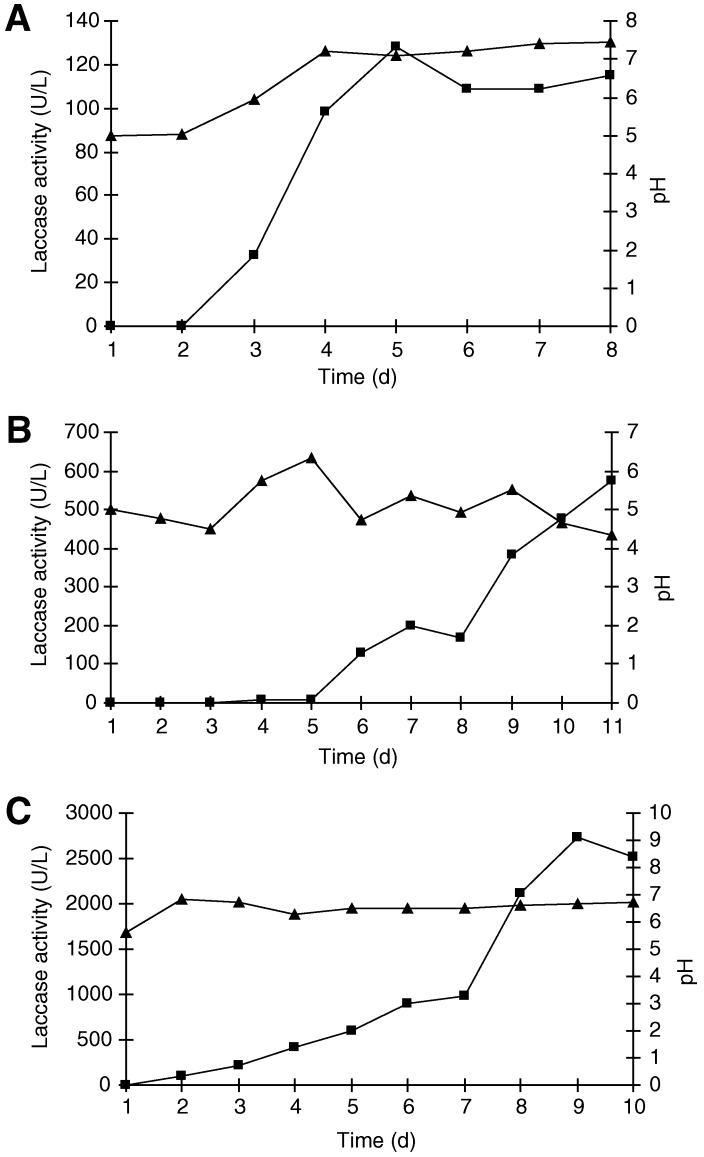

Fig. 5. The production of laccase by the transformant *A. niger/lcc2* in **(A)** glucose minimal medium supplemented with yeast extract, **(B)** double-strength sucrose minimal medium, and **(C)** double-strength sucrose minimal medium supplemented with yeast extract. Activity (■) and pH (▲) are indicated.

grown at 25°C or 30°C. The best results were obtained with the pH 6 medium (data not shown).

Media composition and growth conditions for further studies were selected on basis of results from experimental series 1–5. In experimental series 6, the cultivation of *A. niger/lcc2* was carried out over a period of 10 d in glucose minimal medium supplemented with yeast extract. A rise in pH from 5.0 to 7.0 was seen during the cultivation (Fig. 5A). This should not

Table 2
Purification of Recombinant Laccase From *A. niger/lcc2*

Purification step	Total volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Resuspended freeze-dried sample	5	56.8	15.55	3.65	100	1
DEAE fractions	2.5	28.4	1.53	18.6	50	5.1
HiTrap fractions	1.5	10.7			19	
Mono P fractions	0.125	1	0.04	25	2	6.9

be a problem as it has been previously shown that laccase from *T. versicolor* has best stability between pH 6.0 and 7.0 *(29)*. The activity increased rapidly between the second and the third day after inoculation to approx 100 U/L and then stayed at that level (Fig. 5A). A higher activity was reached with sucrose 2× minimal medium (Fig. 5B). In that case the activity appeared much later (d 6) and the highest activity was observed after 11 d. The pH dropped to 4.0, which is suboptimal for native laccase, with 70% of the activity remaining in comparison to pH 6.0–7.0 (*29*). In sucrose 2× minimal medium with yeast extract, the pH was stable at pH 6.0–7.0 during the whole period. The highest activity was reached after 9 d and reached approx 2700 U/L (Fig. 5C). In comparison to sucrose 2× minimal medium, the activity appeared sooner (d 2–3) in sucrose 2× minimal medium with yeast extract. The activity was similar to that determined in the work of Record et al. *(27)*, who expressed a *P. cinnabarinus* laccase in *A. niger* using a similar strain but replaced the native laccase signal peptide with the 24-amino-acid-residue glucoamylase (*gla*) preprosequence from *A. niger*, as the production of laccase with the native signal peptide was quite low. In this study, we demonstrate that it is possible to express high levels of laccase with the native secretion signal. Recombinant Lcc2 protein was purified from the culture medium of *A. niger/lcc2* in three steps (Table 2). The purification was not optimized further regarding the yield, as enough enzyme was obtained to characterize the recombinant protein.

Recombinant Lcc2 from different stages of purification were run on an SDS-PAGE gel, with LccA and LccB preparations from *T. versicolor* as controls. Molecular weight determination indicated that the recombinant Lcc2 was similar to LccA rather than to LccB (Fig. 6A). This was to be expected, as the *lcc2* gene has been associated with the LccA form of the protein in the native host (*10*). The calibration indicated that the recombinant Lcc2 is 74 kDa vs 68 kDa of LccA. IEF was also carried out, using LccA and LccB as controls (Fig. 6B). The pI of the recombinant Lcc2 protein and LccA appeared identical and very acidic, at about 3.5. LccB showed a different pattern indicating a higher pI and the presence of several forms in the preparation (Fig. 6B). The expected pI ranges of preparations of

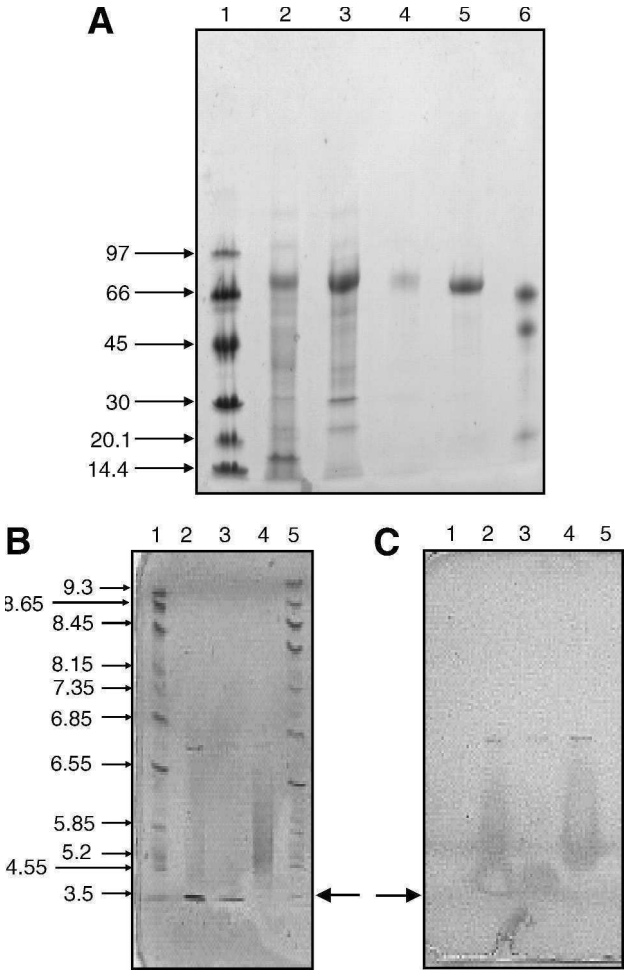

Fig. 6. (A) SDS-PAGE analysis: lane 1, marker proteins (sizes in kDa indicated to the left); lanes 2–4, recombinant Lcc2 protein from *A. niger/lcc2* after $(NH_4)_2SO_4$ precipitatation (lane 2), purification on HiTrap Q (lane 3), and purification on MonoP (lane 4); lanes 5 and 6, native LccA (lane 5) and LccB (lane 6). **(B)** IEF analysis: lanes 1 and 5, marker proteins (pI indicated to the left); lane 2, recombinant Lcc2 protein from *A. niger/lcc2*; lane 3, native LccA; lane 4, native LccB. **(C)** Zymogram analysis: lanes 1 and 5, marker proteins; lane 2, native LccA; lane 3, recombinant Lcc2 protein from *A. niger/lcc2* (position indicated by arrow); lane 4, native LccB.

LccA and LccB are 3.07–3.27 and 4.64–6.76, respectively *(2)*. LccB may contain at least ten components. Zymogram analysis confirmed that the recombinant laccase was similar to LccA, whereas most of the LccB activity was found around pH 5.0 (Fig. 6C).

As can be seen in Fig. 7, both recombinant Lcc2 protein and native LccA have similar carbohydrate contents. The calculated carbohydrate content of recombinant Lcc2 protein is 16%, whereas LccA shows a carbohydrate content of 11%. Previous estimates of the carbohydrate content

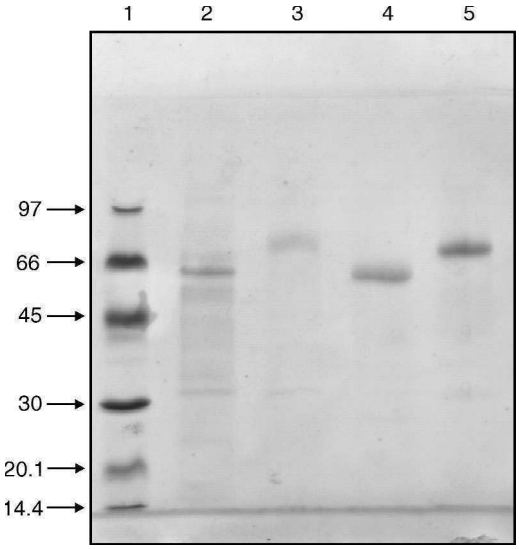

Fig. 7. Deglycosylation of recombinant Lcc2 protein from *A. niger*/*lcc2* and native LccA. Lane 1, marker proteins (sizes in kDa indicated on left); lane 2, deglycosylated recombinant Lcc2; lane 3, untreated recombinant Lcc2; lane 4, deglycosylated LccA; lane 5, untreated LccA.

of LccA range from 10% to 14% *(2)*. The molecular weight of the unglycosylated laccase as deduced from the amino-acid sequence is 53 kDa *(10)*. The recombinant laccase appeared to be homogeneous. Laccase from *P. cinnabarinus* expressed in *A. niger* gave rise to a 70-kDa enzyme, which was the same as for the native protein *(27)*. Laccase from *Trametes villosa* expressed in *Aspergillus oryzae* showed 0.5% and 10% glycosylation for the native and the recombinant enzyme, respectively *(30)*. Recombinant laccase from *Myceliophthora thermophila* expressed in *A. oryzae* also showed a higher degree of glycosylation *(31)*.

The K_M values were determined using 2,4,5-trimethoxybenzyl alcohol (Hong et al., in this volume) as the substrate. For LccA and the recombinant Lcc2 protein, substrate concentrations of 1–60 m*M* were tested, whereas for LccB the maximum concentration was increased to 90 m*M*, as the rate continued to increase at 60 m*M*. The results suggest that the K_M of LccA and recombinant laccase are similar, whereas the K_M of LccB is considerably higher.

Conclusions

Production of catalytically active laccase from the *T. versicolor* cDNAs *lcc1* and *lcc2* under control of glyceraldehyde-3-phosphate dehydrogenase promoters was achieved in both *P. pastoris* and *A. niger*. Expression of laccase in *P. pastoris* using the *GAP* system gave better results with glucose than with glycerol or maltose as carbon source. The activity obtained with *P. pastoris* was considerably lower than for *A. niger*, but the *P. pastoris* system

may still be of interest for screening studies owing to convenience and speed. With *A. niger*, high laccase activity levels (2700 U/L using ABTS as reducing substrate) were obtained with the native secretion signal by using a medium containing sucrose and yeast extract.

Recombinant laccase produced by *A. niger* D15 transformed with the *lcc2* cDNA was purified to homogeneity, and its biochemical and catalytical properties were found to be similar to those of native laccase A from *T. versicolor*. This is an important finding for the applicability of *A. niger* for heterologous production of enzymes, as it is an indication that the activity and characteristics of enzymes produced in this manner can be correlated to the native enzymes.

Acknowledgments

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Discussion

Laccases are important enzymes for bioremediation, and the best characterised enzymes are from *T. versicolor*. The enormous potential for fungal laccases for various environmental and industrial laccases has long been recognised, in fields from biolpulping and fermentation of lignocellulosic hydrolysates, to degradation of environmental pollutants and dyes (Wong and Mansfield 1999; Larsson et al. 2001; Fahr et al. 1999; Abadulla et al. 2000).

Previous attempts at expressing *T. versicolor* laccases in different expression systems have been widely reported. An extract from Table 10 (Chapter 2) summarising these results, is reproduced here for ease, and the results reported in this chapter for the expression of Lcc1 and Lcc2 in *A. niger* and *P. pastoris* are now included (in bold).

Extract from Table 10 (Chapter 2). Examples of heterologously-expressed *T. versicolor* laccase genes

Laccase	Host	Comments	References
Lcc1	*P. pastoris*	Secreted, 24 $U.L^{-1}$ (ABTS [1])	Jönsson et al. 1997; O'Callaghan et al. 2002
	P. pastoris	Secreted, 39 $mU.L^{-1}$	Gelo-Pujic et al. 1999
	Z. mays *High II*	50 ppm dry weight aqueously extractable laccase (up to 2% TSP [2]), remainder of laccase imobilised in seed in active form	Bailey et al. 2004
	P. methanolica	Secreted, 12,600 $U.L^{-1}$	Guo et al. 2006
	P. pastoris	**Secreted, 0.4 $U.L^{-1}$ (unoptimised)**	**This work**
	A. niger	**Secreted, 6 $U.L^{-1}$ (unoptimised)**	**This work**
Lcc2	***P. pastoris***	**Secreted, 0.6 $U.L^{-1}$ (unoptimised)**	**This work**
		Secreted, 2.8 $U.L^{-1}$ (optimised)	
	A. niger	**Secreted, 30 $U.L^{-1}$ (unoptimised)**	**This work**
		Secreted 2,700 $U.L^{-1}$ (optimised)	
	S. cerevisiae	Secreted, 0.0022 $nkat.ml^{-1}$ (ABTS) = 0.13 $U.L^{-1}$	Cassland and Jönsson 1999; Larsson et al. 2001
lccα	*S. cerevisiae*	Secreted, 35 $mU.L^{-1}$ (ABTS)	Necochea et al. 2005
LacIIIb	*Y. lipolytica*	Laccase secreted activity of 230 $U.L^{-1}$ (ABTS) 2.5 $mg.L^{-1}$	Jolivalt et al. 2004
LccIII	*Nicotiana*	Secreted, in cell-free extracts of roots	Sonoki et al. 2004

Laccase	Host	Comments	References
(*cvl3*)	*tabacum*		
	T. versicolor	Secreted, 1 $U.L^{-1}$ (Guaiacol)	Kajita et al. 2004
LccIV	*P. pastoris*	Secreted, 1,500 $U.L^{-1}$ (ABTS).	Brown et al. 2002
	A. niger	Solid-state fermentation: 592 $U.L^{-1}$ Submerged fermentation: 13 $U.L^{-1}$ (ABTS)	Téllez-Jurado et al. 2006
Lac2	*S. cerevisiae*	Undetectable laccase activity in the medium.	Klonowska et al. 2005
Lac3	*S. cerevisiae*	Secreted, 0.5 $U.L^{-1}$ (SGZ [3]), 2 $mg.L^{-1}$	Klonowska et al. 2005

[1] 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid

[2] Total soluble protein

[3] Syringaldazine

In Chapter 2, it was postulated that *A. niger* gives the best expression of fungal laccases, based on the results reported in literature. This theory was tested in the research presented in this chapter. The *lcc1* and *lcc2* genes from *T. versicolor* were expressed in *A. niger* D15 and *P. pastoris* SMD1168, both of which have native protease levels reduced, and levels of activity were compared. The average volumetric and specific activities of *A. niger/lcc2* transformants were significantly higher than the *A. niger/lcc1* transformants. Similarly, the *P. pastoris/lcc2* transformants displayed activities approximately three – four-fold higher than the *P. pastoris/lcc1* transformants. *A. niger/lcc2* and *P. pastoris/lcc2* were subsequently chosen for optimisation. The Lcc2 enzyme from the recombinant *A. niger* strain was subsequently purified and characterised in terms of molecular weight and glycosylation, and compared to enzymes purified from *T. versicolor*. A maximum activity of ~2,700 U.L-1 was obtained, confirming the supposition that *A. niger* is a superior laccase producer, compared to *P. pastoris*.

Another *T. versicolor* laccase gene, laccase IIIb, was also recently expressed in *Y. lipolytica* (Jolivalt et al. 2005; Madzak et al. 2006), the other heterologous expression system in focus in this thesis. The native lacIIIb signal peptide was used for extracellular production, as well as the *XPR2* pre-sequence. The transformants with the native lacIIIb signal peptide were selected for further work based on their better zone formation on ABTS plates. A slight increase in glycosylation compared to the native laccase was determined in the recombinant lacIIIb. Extracellular activity was measured at 230 $U.L^{-1}$ after 6 days cultivation.

Although there are numerous differences in the heterologous expression of laccases described above, certain deductions can be made. For *T. versicolor* laccases, a precise comparison is impossible, given the difference in laccase genes chosen. Differences also occur in the copy number of the laccase gene in the heterologous host, control mechanisms utilised (e.g. promoters and different secretion techniques), and possibly different cultivation protocols. However, the overwhelming conclusion that can be drawn from currently available data is that *A. niger* is a better expression host for overall maximal production. The levels obtained by *A. niger* of ~2,700 $U.L^{-1}$ were almost 1000-fold higher than in *P. pastoris* (2.8 $U.L^{-1}$). The *Y. lipolytica* expression of lacIIIb (Jolivalt et al. 2005) was 16 times less than the *A. niger* /*lcc2* expression levels. Even given the differences in laccase gene chosen and the copy number of the transformants, this is a significant difference. *Y. lipolytica* expression may be improved through the use of a multi-copy system, unlike the one used by Jolivalt et al. (2005). *Y. lipolytica* expression also resulted in unexpected slight hyperglycosylation, while the *A. niger* recombinant Lcc2 was of a similar molecular weight and glycosylation as the native LccA.

A. niger D15 was also used to express the laccase gene *lac1* from *Pycnoporus cinnabrinus,* under control of the glyceraldehyde-3-phosphate dehydrogenase promoter (Record et al. 2002). Similarly superior yields of ~7,000 $U.L^{-1}$ were reported when fused to the GLA prepro-sequence. When expressed with *lac1*'s own secretion signal, as was done in our research with *lcc2*, the production decreased 80-fold. The purified heterologous laccase was found to be 70 kDa, the same as that produced by *P. cinnabarinus*. This suggests an absence of hyperglycosylation, as was also found with our expression of *lcc2* in *A. niger*. The same *P. cinnabarinus lac1* gene was expressed in *Y. lipolytica* (Madzak et al. 2005). This was also achieved using the native *lac1* signal peptide, as well as heterologous secretion signals, the *XPR2* pre and prepro sequences. In contrast to the expression of *T. versicolor* lacIIIb in *Y. lipolytica* (Jolivalt et al. 2005), in this case the *XPR2* constructs were superior to the native *lac1* signal peptide construct. In comparison to the *A. niger*-expressed laccases, the laccase expressed by *Y. lipolytica* again appeared to be hyperglycosylated, with a molecular weight of 90 kDa.

A precise comparison may be made using the data for *P. cinnabarinus lac1* expression in the different systems, as was analysed by Madzak et al. (2005). The same gene was expressed in *Y. lipolytica*, *P. pastoris* (Otterbein et al 2000) and *A. niger* (Record et al. 2002). As was found with the expression of *T. versicolor* laccases, *P. pastoris* was not competitive for the expression of *P. cinnabarinus* laccase, with a highly hyperglycosylated heterologous protein produced, and at levels three-fold lower than the *Y. lipolytica* system. In contrast, *A. niger* produced higher levels of enzyme. The *A. niger*-produced protein was the only one to produce a protein with glycosylation levels similar to the native enzyme.

These results are an indication that the choice of an expression system cannot be made entirely based on theory. What works for one enzyme is not necessarily the best host for another enzyme. Within the same system, different control mechanisms can have different effects on the production. An example is the presence or absence of heterologous or native secretion peptides. For the expression of *T. versicolor* lacIIIb, the native secretion signal was better than the *XPR2* pre sequence, whereas the expression of *P. cinnabarinus lac1* was significantly enhanced when the *XPR2* pre and prepro sequences were added in place of the native signal.

The most commonly used heterologous production system, *P. pastoris*, is generally not a suitable host for the expression of fungal laccases. The enzymes are expressed at much lower levels than other hosts, and in a hyperglycosylated state. However, the recent article by Guo et al. (2006) reported the use of *P. methanolitica* for expression of *lcc1*. They obtained vastly improved expression levels compared to the *P. pastoris/lcc1* expression system. A laccase from a novel *Trametes* was recently also produced in *P. pastoris* at levels of 8.3×10^4 $U.L^{-1}$ (Hong et al. 2007). *Y. lipolytica* shows promise, though the systems currently reported do not match *A. niger*. However, there is broad scope for maximising the expression from *Y. lipolytica*, including working with a multi-copy system. *A. niger* is currently the most promising heterologous expression system for fungal laccases.

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Chapter 7.

Discussion

Saccharomyces cerevisiae had traditionally been used for the production of foreign proteins in a yeast host. When Müller et al. (1998) compared *Saccharomyces cerevisiae, Hansenula polymorpha, Kluyveromyces lactis, Schizosaccharomyces pombe* and *Yarrowia lipolytica* as expression hosts, they found that *Y. lipolytica* was one of the most attractive alternative host organisms. This 'non-conventional' yeast has many advantages over the traditional yeast expression systems, including glycosylation patterns more similar to mammalian cells (reviewed in Madzak et al. 2004), which is important for products to be used in the pharmaceutical industry. It has been used successfully for the expression of a number of different enzymes and shows great secretion capacity (Titorenko et al. 1997). Filamentous fungi such as *Aspergillus niger* have the ability to produce and secrete exceptionally large amounts of properly folded proteins and can produce proteins that contain *O*- and *N*-linked glycans without extensive hyperglycosylation (Hombergh et al. 1997; Maras et al. 1999; Punt et al. 1994). The main attraction of filamentous fungi is their natural ability to secrete large amounts of proteins into the growth medium. The use of both these highly valuable heterologous expression systems was reviewed in this study, for the production of a number of different biocatalytic enzymes. Both were found to be useful host strains, and their comparisons with the more traditional yeasts *S. cerevisiae* or *Pichia pastoris* have highlighted their utility and value.

Epoxide hydrolases (EHs) are hydrolytic enzymes that convert epoxides to vicinal diols by ring-opening. They are present in mammals, vertebrates, invertebrates, plants, insects and micro-organisms, and have been studied extensively in recent years for their potential application as biocatalysts for the production of optically active epoxides and vicinal diols. The use of EHs for the production of these enantiopure building blocks represents an alternative to asymmetric organic syntheses and may surpass the chemical processes in yield, enantiomeric purity of the product and environmental friendliness.

The development of a single highly efficient expression system for producing functional heterologous EHs from any source, including animal, insect, plant and microbial origins, would be a significant advantage given the limitations and constraints of the current

options available. A microbial whole-cell biocatalyst that is able to functionally express a broad range of epoxide hydrolases from diverse origins as active intracellular proteins is commercially sought after. Furthermore, an expression system that is capable of producing high-levels of high quality heterologous proteins from diverse origins without, for example, loss of the desired characteristics identified in the original wild-type source, is highly desired. This is particularly useful for the development of an array of chirally specific EH biocatalysts to provide a range of chiral epoxide substrates useful as building blocks in chiral pharmaceutical syntheses. High-level functional expression of EHs from diverse origins is also required to produce high-quality proteins in sufficient quantities to allow structure-function studies.

The applicability of *Yarrowia lipolytica* as an expression system for EHs was explored. Two new fungal EHs from *Rhodosporidium toruloides* NCYC 3181 and *Cryptococcus curvatus* NCYC 3158 were identified and cloned. A number of additional EHs from different sources, including bacteria, yeasts, fungi and plants, were chosen for expression in *Y. lipolytica*, in order to determine its suitability as the expression system of choice for the production of EHs. The soluble sEH from *Solanum tuberosum* (potato) displayed selectivity for styrene oxide that is opposite to those of yeast EHs. The EH from *Agrobacterium radiobacter* was selected as an example of a sEH from bacterial origin, due to the good selectivity of this enzyme reported for styrene oxide (Lutje Spelberg et al. 1998). However, this enzyme reportedly became unstable if epoxide concentrations exceeded the solubility limit. The kinetic characteristics of this enzyme was thus only reported at very low concentrations (5 mM) by Lutjie Spelberg et al. (1998), while it was shown in this study that *A. radiobacter* sEH produced by the *Y. lipolytica* could perform the biotransformation at substrate concentration of 100 mM. Evaluation of the use of a secretion signal for extracellular expression was done using selected EHs, and was found to negatively affect the activity of the expressed EHs. The broad applicability of *Y. lipolytica* as an effective expression system for EHs is shown, with a number of different EHs from various sources, with highly different selectivities and activities being successfully expressed and used for biotransformations.

Halohydrins can be considered direct precursors of epoxides. Halohydrin dehalogenases (HHdHs) catalyse the nucleophilic displacement of a halogen ion in halohydrins by a vicinal hydroxyl group, yielding an epoxide, a proton and a halide ion. They can also efficiently catalyse the reverse reaction, the halogenation of epoxides. The interest in HHdHs increased when it was found that the dehalogenation of halohydrins may proceed with high enantioselectivity, making these enzymes useful catalysts to prepare various optically active epoxides.

The HHdH-encoding *HheC* gene of *Agrobacterium radiobacter* AD1 (van Hylckama Vlieg et al. 2001) was codon-optimised to match the codon usage of *Y. lipolytica,* and over-expressed in this host. Codon-optimisation was carried out to ensure maximal levels of activity, although it was shown in Chapter 3 that the epoxide hydrolase from the same *A. radiobacter* strain was functionally expressed in *Y. .lipolytica.* Codon-optimisation is also an option in that instance, as the epoxide hydrolase activity may be further improved. Expression levels were optimised by increasing the number of expression cassettes (consisting of hp4d promoter – s*HheC – LIP2* terminator) integrated into the genome of *Y. lipolytica*. To increase the copy number, vectors were constructed which contained up to five expression cassettes per single *ura3d4* selection marker, compared to the usual single expression cassette per *ura3d4* gene. Copy number determination estimated that up to 40 copies of the s*HheC* gene was integrated into the genome of *Y. lipolytica*. The ring-closure reactions of 2-chloro-1-phenylethanol displayed a broadly dose-dependent response, with the highest levels of activity demonstrated in the transformants with the most copies of the s*HheC* gene

The successful expression of a tetrameric protein such as *A. radiobacter* AD1 HHeC in *Y. lipolytica* is important. The recombinant activity in these strains is evidence that the heterologous HheC is successfully folded and aggregates into a functional tetramer. This is further confirmation of the utility of *Y. lipolytica* as an expression host for a variety of different biocatalysts.

The *Aspergillus aculeatus man1* gene, encoding endo-1,4-β-mannanase, was expressed in *Y. lipolytica* and *Aspergillus niger*. Man1 was successfully expressed in *A. niger*, under control of the *A. niger* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter[1]. The enzyme was efficiently secreted by *Y. lipolytica* using its own secretion signal, under control of the growth-phase dependent synthetic hybrid promoter hp4d (Nicaud et al. 2002). The effect of supplementation by organic nitrogen on *A. niger* Man1 production, as well as glucose and inorganic nitrogen concentration, was investigated. The results indicate that both *Y. lipolytica* and *A. niger* are promising heterologous producers of *A. aculeatus* mannanase. Multi-copy *Y. lipolytica* transformants with the *man1* gene directed by its own secretion signal give good extracellular yields of mannanase. The increase in size of the enzyme produced, presumably due to some glycosylation effects, does not appear to affect the activity of the enzyme. *Y. lipolytica*[*man1*] produced 13,073 nkat.ml^{-1} in shake flasks, which increased to 26,140 nkat.ml^{-1} in fed-batch fermentations. *A. niger* D15[*man1*] produced maximum levels of 16,596 nkat.ml^{-1} and 574 nkat.ml^{-1} in shake flasks, after media optimisations. In contrast, recombinant *S. cerevisiae* expressing *man1* produced up to 521 nkat.ml^{-1} (Setati et al. 2001).

The volumetric enzyme activity achieved by low-copy *Y. lipolytica* transformants in batch fermentation was 5.5-fold higher than in shake flask production. The integration of multi-copy gene inserts into the host organism resulted in a metabolic load on the transformants, as indicated by a 50% lower biomass yield than that for the low copy transformants, indicating an increased metabolic burden on the cells. However, the volumetric enzyme activity obtained in batch fermentations decreased by almost 2-fold when compared to activities obtained in shake flasks, possibly due to the conditions in fermenters being more suited for growth, with metabolic flux is directed towards growth at the cost of enzyme production. The specific enzyme activity was also 1.3-fold lower in fed-batch fermentation than in batch fermentation and a 4.3-fold lower compared to the shake flask activity. This suggests that not all of the biomass that was produced during

[1] The construction of the Man1-producing *A. niger* strain was done by Dr Shaunita H. Rose of the University of Stellenbosch. The construction of the *Y. lipolytica* Man1 production was done by Robyn L. Roth of CSIR Biosciences. Further optimisation of the Man1 production by these strains was done by the team at CSIR

the length of the fermentation contributed to the production of the enzyme. This could again be explained by a shift in metabolic flux towards an increase in biomass and not enzyme expression. Media and growth condition optimisation may have a further positive effect on the Man1 production by *Y. lipolytica*, as was seen in the *A. niger* optimisations.

Laccases are important enzymes for bioremediation, and the best characterised enzymes are from the fungus *Trametes versicolor*. The objective of this research was to optimise expression of *T. versicolor* laccases (*lcc1* and *lcc2*) in *Aspergillus niger* D15 and *Pichia pastoris*[2]. The *lcc2* gene from *T. versicolor* was expressed in *A. niger* and *P. pastoris*, and levels of activity compared. The Lcc1 enzyme was less active than Lcc2, in both hosts. *P. pastoris* secreted 0.4 $U.L^{-1}$ Lcc1 compared to 6 $U.L^{-1}$ secreted by *A. niger*. *P. pastoris* secreted 0.6 $U.L^{-1}$ Lcc2, which improved to 2.8 $U.L^{-1}$ following optimisation. This can be compared to 30 $U.L^{-1}$ produced by *A. niger,* under initial screening conditions and which was increased to 2,700 $U.L^{-1}$ by media manipulation. The Lcc2 enzyme from the recombinant *A. niger* was subsequently purified and characterised in terms of molecular weight and glycosylation, and compared to the wild-type enzyme purified from *T. versicolor*.

For *T. versicolor* laccases, a precise comparison of reported heterologous expression is impossible, given the difference in laccase genes chosen. Differences also occur in the copy number of the laccase gene in the heterologous host, control mechanisms utilised (e.g. promoters and different secretion techniques), and possibly different cultivation protocols. However, the overwhelming conclusion that can be drawn from currently available data is that *A. niger* is a better expression host for overall maximal production. The levels obtained by *A. niger* of ~2,700 $U.L^{-1}$ were almost 1000-fold higher than in *P. pastoris* (2.8 $U.L^{-1}$). The expression of lacIIIb in *Y. lipolytica*, the other heterologous expression system in focus in this study, was 16 times less than the *A. niger* /*lcc2* expression levels (Jolivalt et al. 2005; Madzak et al. 2006). Even given the differences in

[2] The expression of *T. versicolor* laccases in *P. pastoris* was done by Christina Bohlin of Karlstad University.

laccase gene chosen and the copy number of the transformants, this is a significant difference. *Y. lipolytica* expression may be improved through the use of a multi-copy system, unlike the one used by Jolivalt et al. (2005). *Y. lipolytica* expression also resulted in unexpected slight hyperglycosylation, while the *A. niger* recombinant Lcc2 was of a similar molecular weight and glycosylation as the native LccA.

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different effects on the production. An example is the presence or absence of heterologous or native secretion peptides. For the expression of *T. versicolor* lacIIIb, the native secretion signal was better than the *XPR2* pre sequence (Jolivalt et al. 2005), whereas the expression of *P. cinnabarinus lac1* was significantly enhanced when the *XPR2* pre and prepro sequences were added in place of the native signal (Madzak et al. 2005).

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The work presented here underscores the requirement for experimentation before finalising the choice of an appropriate expression system for the optimal production of the desired protein. Every system available has both advantages and disadvantages, and when considering which system to use for producing a recombinant protein, the following factors could be considered initially: (1) origin of gene, *i.e.* prokaryotic or eukaryotic; (2) secretion versus intracellular expression; (3) post-translational modifications for optimal activity; (4) ease of use of expression system; (5) cost; (6) recovery of product; and (7) scalability of the process. This would direct the selection towards a group of systems, *e.g.* bacterial, yeast, or mammalian cell line. However, within these groups, the choice is broad and still needs to be made empirically.

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Appendix 1

Further results from Chapter 3: Isolation of epoxide hydrolases, functional expression in *Yarrowia lipolytica*, evaluation of recombinant strains as whole-cell biocatalysts and comparison to recombinant *Saccharomyces cerevisiae* strains.

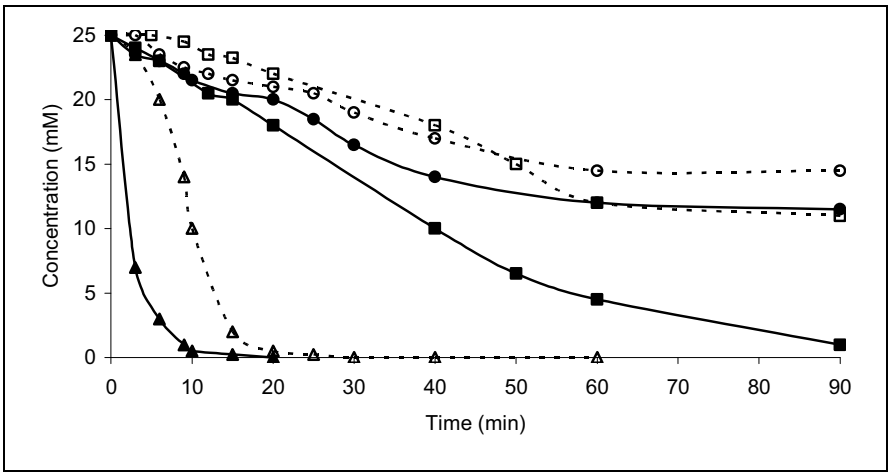

Figure A1. Comparison of the kinetic resolution of 50 mM 1,2-epoxyoctane using *R. araucariae* 25 (WT-25), and the recombinant EH enzyme expressed in *Y. lipolytica* with the *LIP2* signal peptide (YL25HmL). Solid icons indicate (*R*)-1,2-epoxyoctane, open icons indicate (*S*)-1,2-epoxyoctane. -▲- = WT-25, -■- = YL25HmL cell-bound activity, -●- = YL25HmL extracellular activity. (PCT WO 2007/010403).

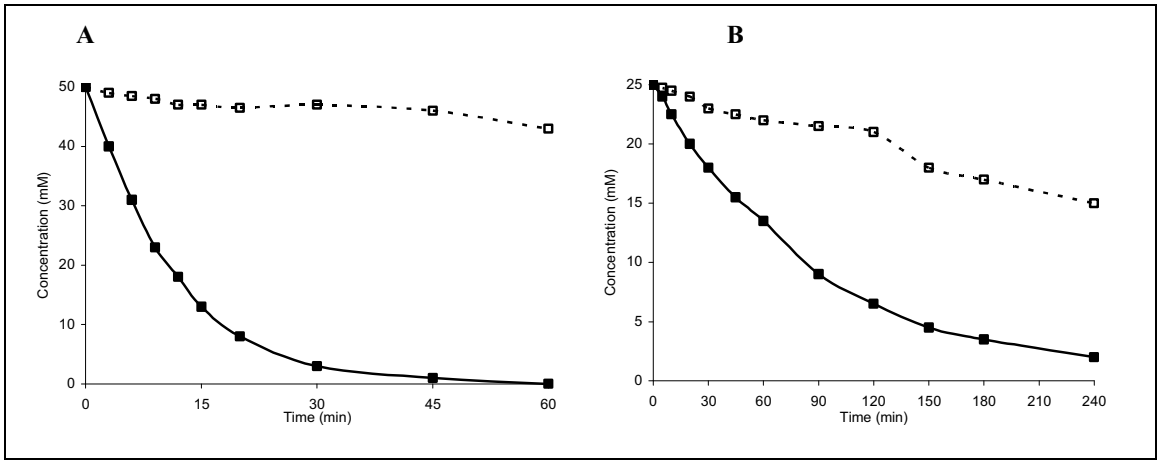

Figure A2. A comparison of the kinetic resolution of racemic Type II oxide, styrene oxide, by the recombinant *Y. lipolytica* strains containing the *R. paludigenum eph* gene. **(A)** YL692HmA and **(B)** YL692HmL. YL692HmA was tested using 10% (w/v) cell loading with a 100mM substrate concentration, whereas YL692HmL was done using 50% (w/v) cell loading and 50 mM styrene oxide. Closed icons indicate (*R*)-styrene oxide, open icons represent (*S*)-styrene oxide. *Note the differences in time (x-axis) and the difference in initial substrate concentration (y-axis)* (PCT WO 2007/010403). YL25HmA/YL25HmL and YL46HmA/YL46HmL were similarly tested and gave similar results (not shown).

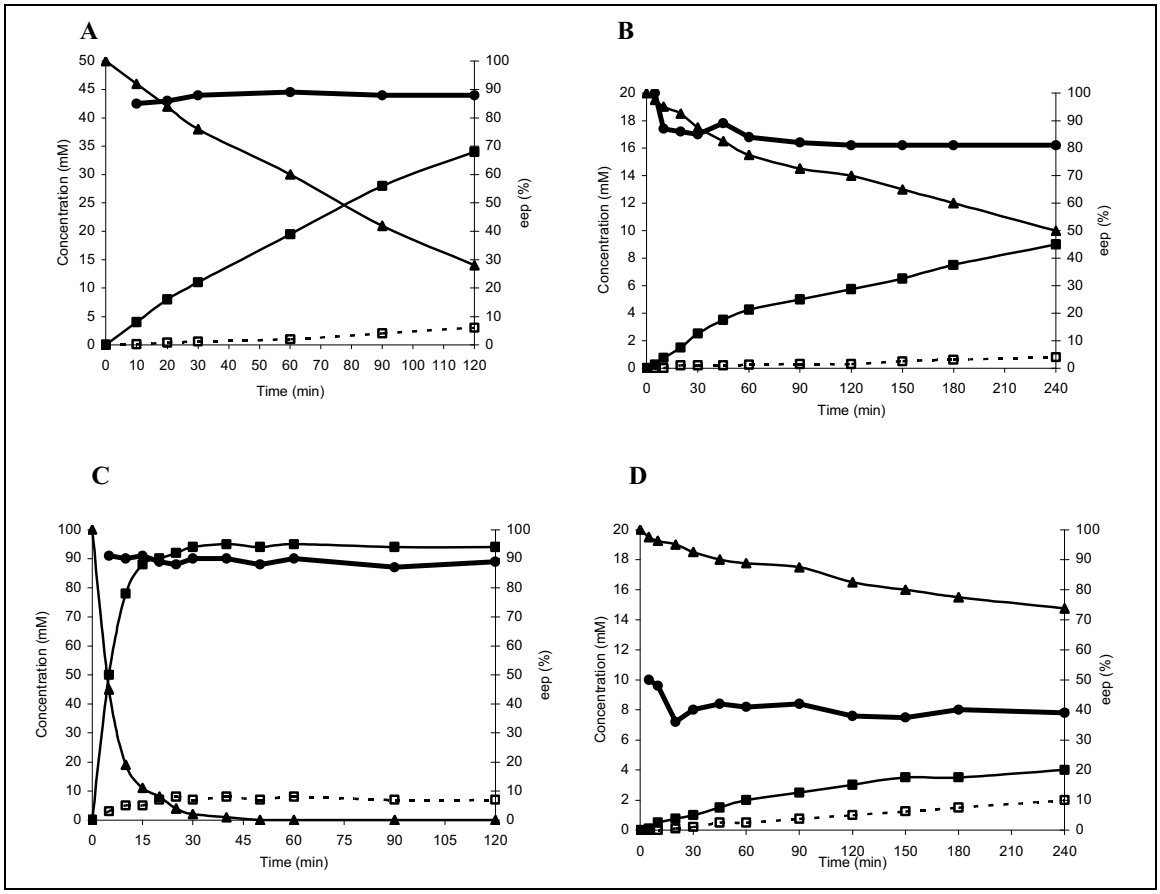

Figure A3. A comparison of the kinetic resolution of *meso*-epoxide cyclohexene oxide by the recombinant *Y. lipolytica* **(A)** YL25HmA, **(B)** YL25HmL, both with *R. araucariae eph,* and **(C)** YL46HmA and **(D)** YL46HmL, both with *R. toruloides eph*, all using a 50% (w/v) cell loading. YL-HmL strains **(B)** and **(D)** were tested with a 20 mM substrate concentration, whereas YL25HmA **(A)** and YL46HmA **(C)** were done using 50 mM and 100 mM CO, respectively. -▲- = CHO, -●- = ee_p. -■- indicates the produced (*R.R*)-cyclohexanediol, -□- indicates the produced (*S.S*)-cyclohexanediol. *Note the differences in time (x-axis) and the difference in initial substrate concentration (y-axis*) (PCT WO 2007/010403). YL692HmA/YL692HmL gave results similar to YL46HmA/YL46HmL. Activity and enantioselectivity (in this case indicated in the graphs by the ee_p traces, were far superior in YL-HmA transformants in both cases. The ee_p is a measure for how much of one enantiomer is present compared to the other, in the product.

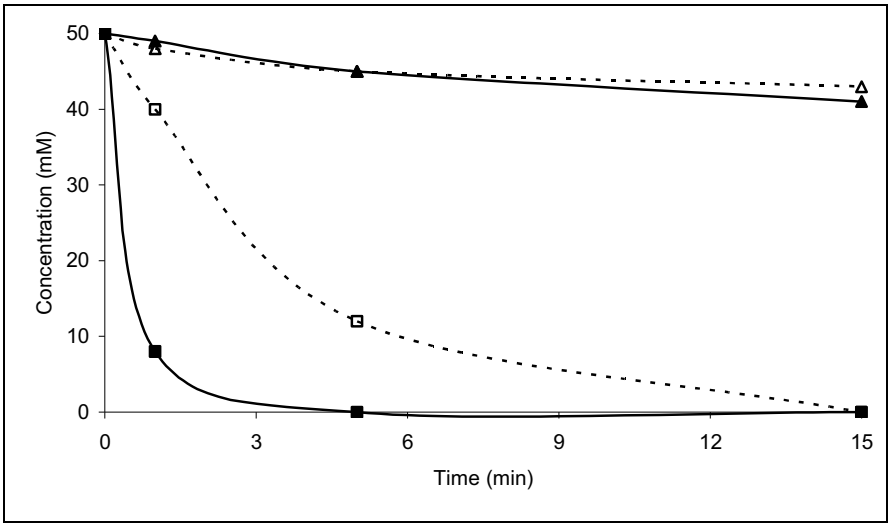

Figure A4. Comparison of kinetic resolution of the *cis*-2,3-disubstituted epoxide, indene oxide (100 mM), by YL692HmL versus YL692HmA (*R. paludigenum eph*). Both were tested using 10% (w/v) cell loading. Solid icons indicate (*1S,2R*)-indene oxide, open icons indicate (*1R,2S*)-indene oxide. -■- = YL692HmA, -▲- = YL692HmL (PCT WO 2007/010403). Activity and enantioselectivity were far superior in YL-HmA transformant.

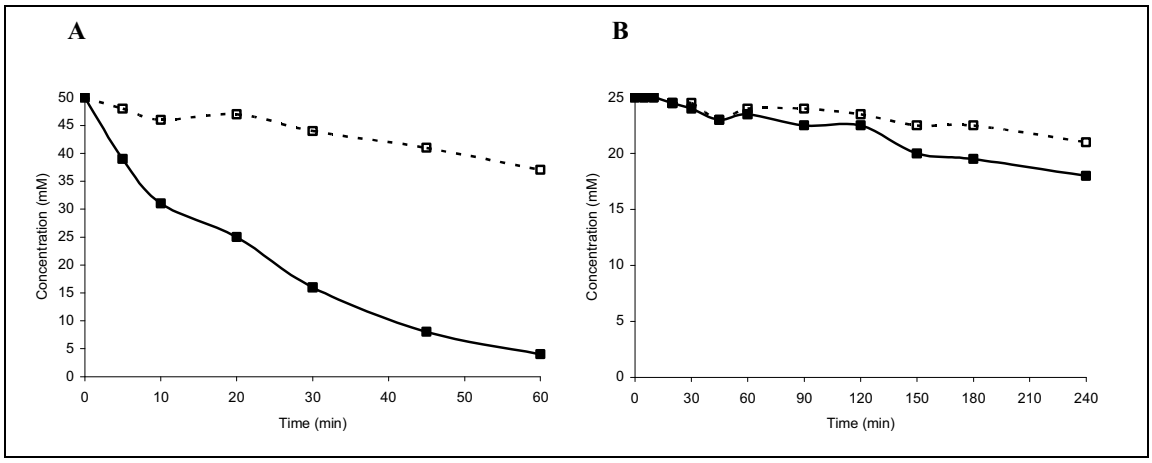

Figure A5. A comparison of the kinetic resolution of the 2,2-disubstituted (Type III) epoxide, 2-methyl-3-phenyl-1,2-epoxypropane (MPEP), by the recombinant *Y. lipolytica* containing *R. paludigenum eph:* **(A)** YL692HmA and **(B)** YL692HmL, using 10% cell loading (w/v) for YL692HmA and 50% for YL692HmL. YL692HmA was tested with a 100 mM substrate concentration, whereas YL692HmL was done using 50 mM. Closed icons indicate the (*R*)-enantiomer and open icons represent the (*S*)-enantiomer of MPEP. *Note the differences in time (x-axis) and the difference in initial substrate concentration (y-axis).* YL46HmA/YL46HmL gave similar patterns with this substrate (data not shown).

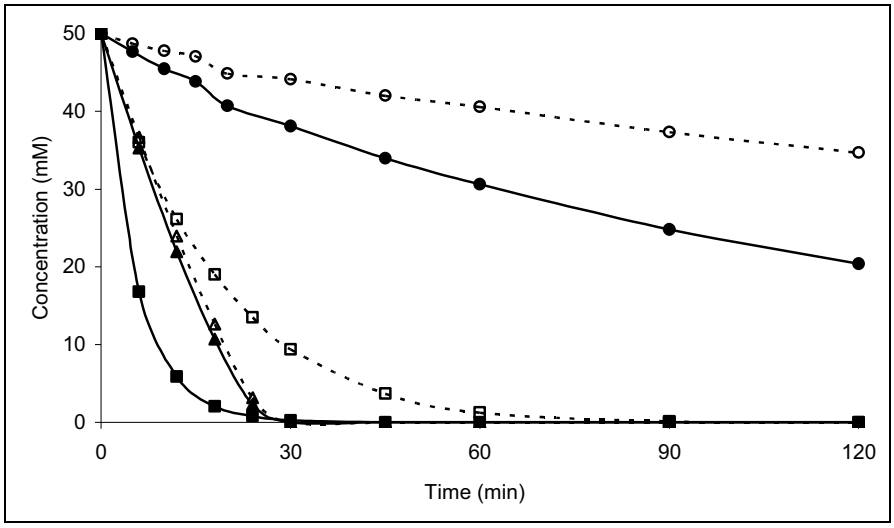

Figure A6. Kinetic resolution of 100 mM epichlorohydrin by the recombinant EHs expressed in *Y. lipolytica*, using 10% (w/v) cell loading. Solid icons indicate (*R,R*)-epoxide, open icons indicate (*S,S*)-epoxide. -■- = YL1HmA (*R. toruloides* UOFX Y-0517), -▲- = YL23HmA (*R. mucilaginosa eph*), -●- = YL25HmA (*R. araucariae eph)*. The Type 1 epoxide, epichlorohydrin, was effectively hydrolysed by all strains tested. YL23HmA (-▲-) and YL777HmA (data not shown) were too efficient at the concentrations tested to determine enantioselectivity. The other strains depicted all initially selectively hydrolysed one enantiomer. In all strains, the less preferred enantiomer is also hydrolysed, though at a slower rate.

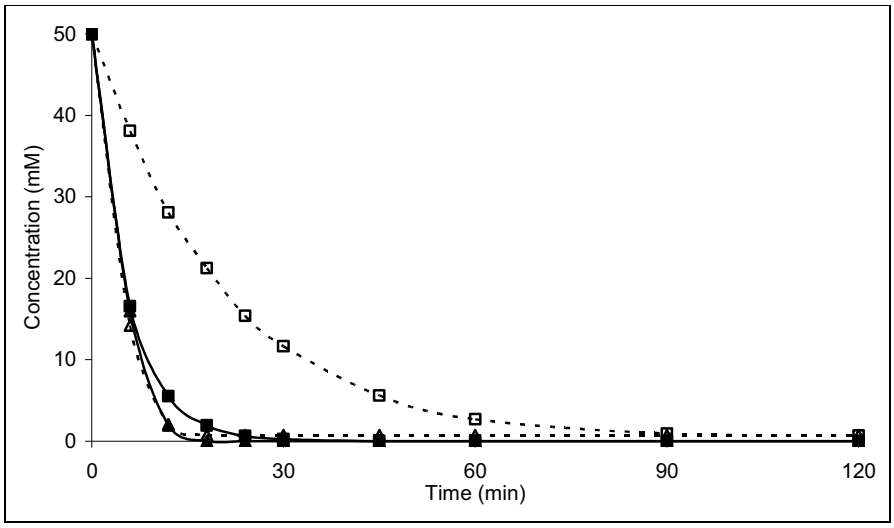

Figure A7. Kinetic resolution of 100 mM epibromohydrin (EBH) by the recombinant EHs expressed in *Y. lipolytica*, using 10% (w/v) cell loading. Solid icons indicate (1)-EBH, open icons indicate (2)-EBH. -■- = YL1HmA (*R. toruloides* UPFS Y-0157), -▲- = YL777HmA. (*C. neoformans eph)* The Type 1 epoxide, epibromohydrin, was effectively hydrolysed. YL1HmA displays better enantioselectivity than both YL777HmA and YL23HmA (*R. mucilaginosa* eph) (data not shown), which show very little enantioselectivity, possibly due to the extreme efficiency of the reaction.

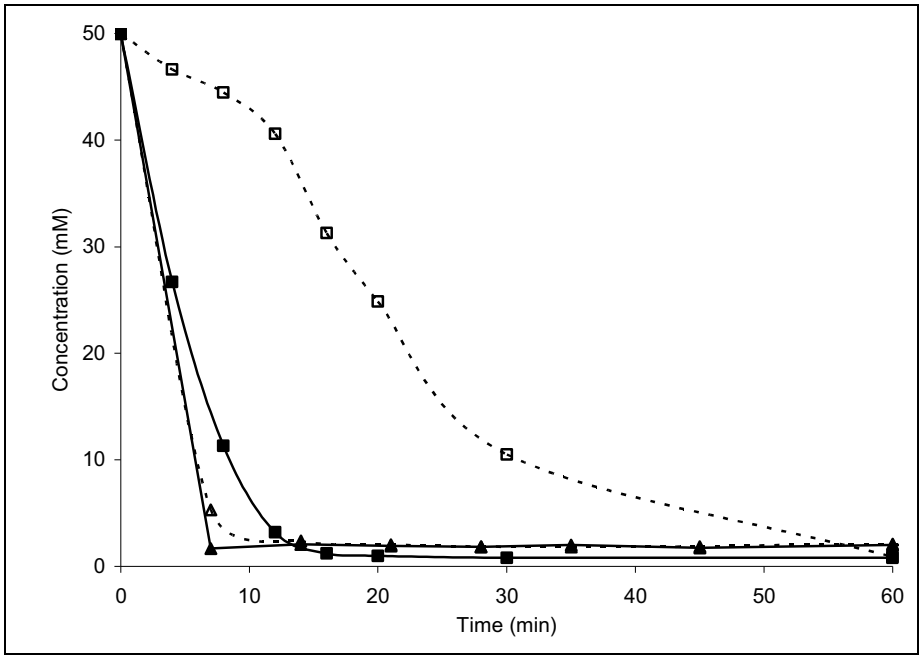

Figure A8. Kinetic resolution of the glycidyl ether, benzyl glycidyl ether (100 mM) by recombinant EHs expressed in *Y. lipolytica*. Solid icons indicate (*R*)-benzyl glycidyl ether, open icons indicate (*S*)-benzyl glycidyl ether. -■- = YL23HmA (*R. mucilaginosa eph)* with 5% (w/v) cell loading, -▲- = YL777HmA (*C. neoformans eph)* , using 10% (w/v) cell loading. The (*S*)-enantiomer is preferentially utilised, though not with the same absolute preference as for the (*S*)-enantiomer of phenyl glycidyl ether. YL23HmA (*R. mucilaginosa eph)* shows exceptionally high activity levels, and may also show better enantioselectivity if the cell loading were further reduced.

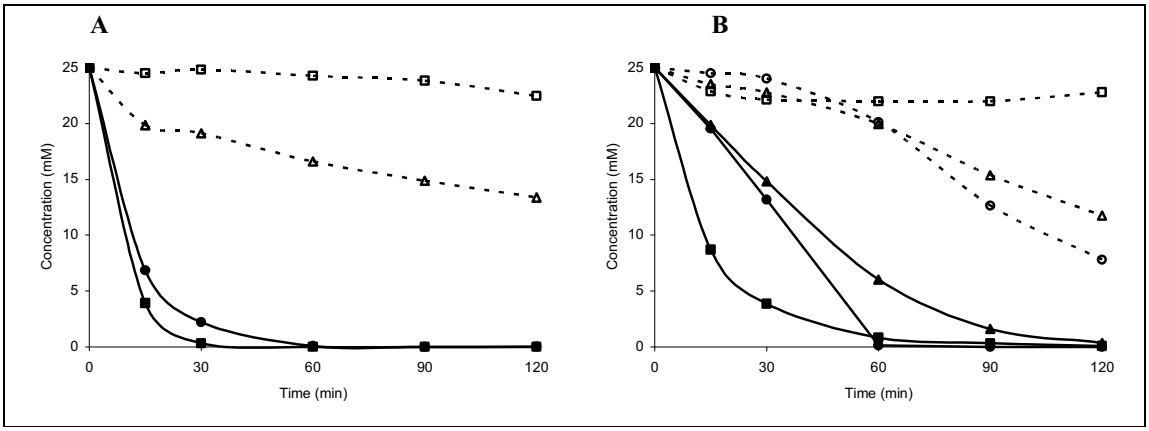

Figure A9. Comparison of the kinetic resolution of 50 mM 4-nitrostyrene oxide (4-NSO) by recombinant EHs expressed in *Y. lipolytica* with a 10% (w/v) cell loading. Solid icons indicate (*R*)-4-NSO, open icons indicate (*S*)-4-NSO. A. -■- = YL1HmA (*R. toruloides* UOFS Y-0157), -●- = YL692HmA (*R. paludigenum eph)*. B. -■- = YL-Car054-HmA, -▲- = YL-Tn1-HmA (*T. ni* TNU73680), -■- = YL-Tn2-HmA (*T. ni* AF035482). YL1HmA shows remarkable activity and enantioselectivity for 4-nitrostyrene oxide. YL46HmA shows a similar profile (not shown). YL-Car054-HmA also displays similar good enantioselectivity, though its overall activity is lower. YL692HmA indicates good activity for the preferred substrate, though its enantioselectivity is poorer over the 120 min. The insect EHs from *T. ni* (Tn1 and Tn2) display poorer enantioselectivity than the represented fungal EHs.

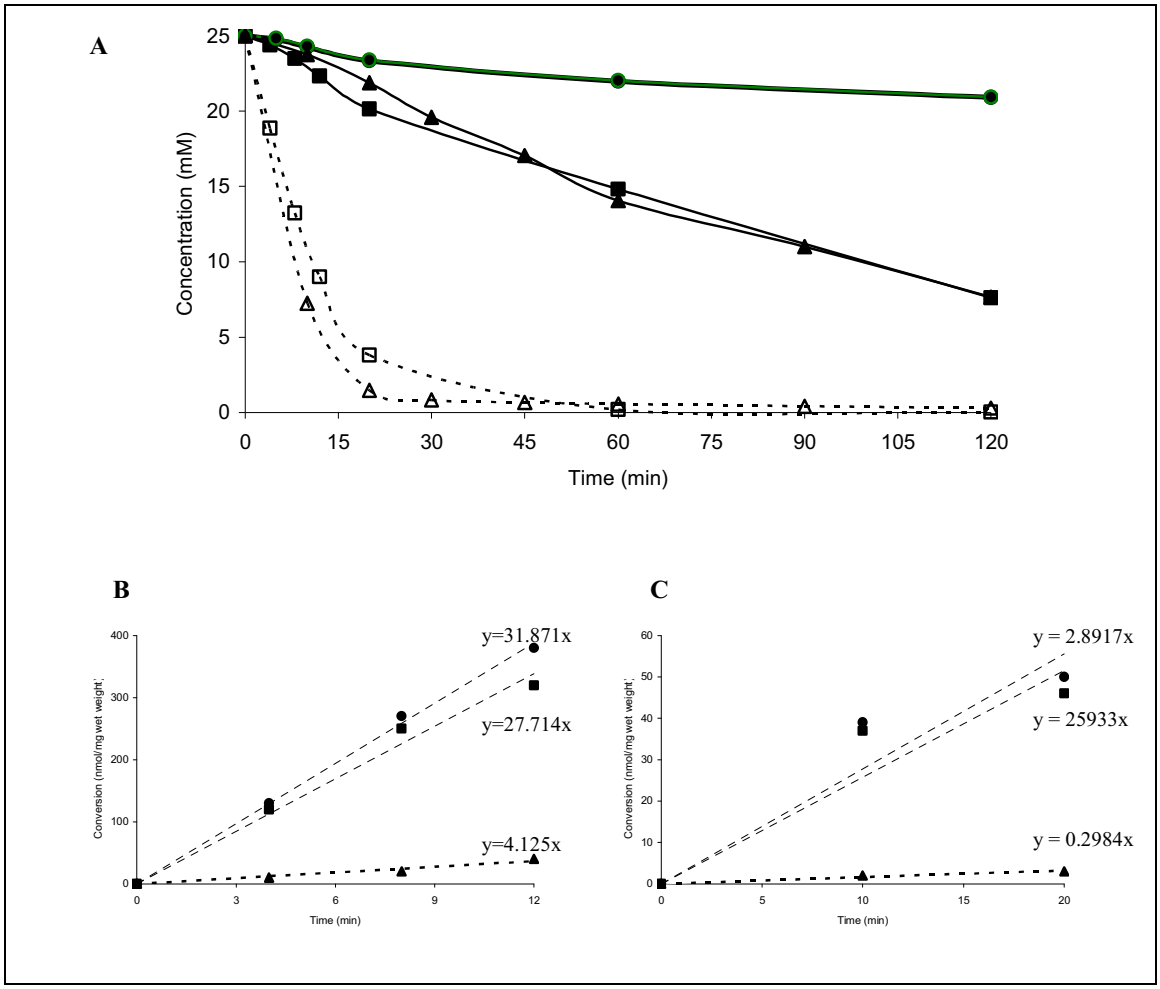

Figure A10. **(A)** Kinetic resolution of 3-chlorostyrene oxide by recombinant EHs expressed in *Y. lipolytica*. -■- = YL-An-HmA, -▲- =YL25HmA, -●- = *Y. lipolytica* Po1h**.** Solid icons indicate (*S*)-3-chlorostyrene oxide, open icons indicate (*R*)-3-chlorostyrene oxide. **(B)** Initial rate of hydrolysis of 3-chlorostyrene oxide by YL-An-HmA and **(C)** Initial rate of hydrolysis by YL25HmA. ▲- = (*R*)-3-chlorostyrene oxide, -■- = (*S*)- 3-chlorostyrene oxide, -●- = total conversion. These strains are initially very enantioselective, as shown in B) and (C), though over time the second enantiomer is also hydrolysed, as can be been in (A).

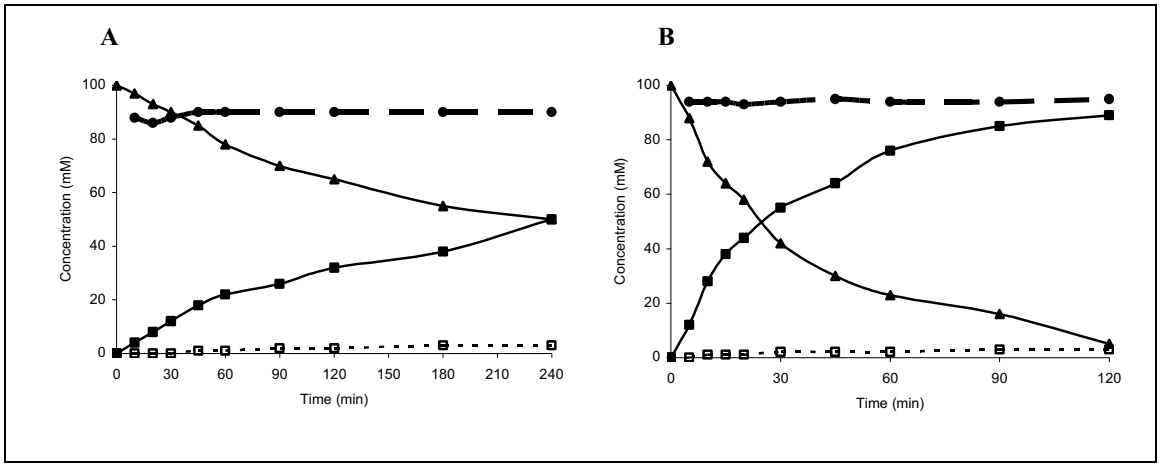

Figure A11. Kinetic resolution of the *meso*-epoxide, cyclopentene oxide (100 mM), by **(A)** YL25HmA (*R. araucariae eph)* and **(B)** YL46HmA (*R. toruloides* UOFS Y-0471), using 50% (w/v) cell loading. -▲- = cyclopentene oxide, -■- indicates (*R,R*)-cyclopentanediol, -□- indicates (*S,S*)- cyclopentanediol, -●- = ee_p. All strains shown display an ee_p of approximately 90%, indicating high enantioselctivity. However, YL25HmA (and YL692HmA, data not shown) converts only approximately half of the supplied 100 mM substrate to the (*R,R*)-diol in the time analysed, whereas YL46HmA converts all the substrate to the (*R,R*)-diol.

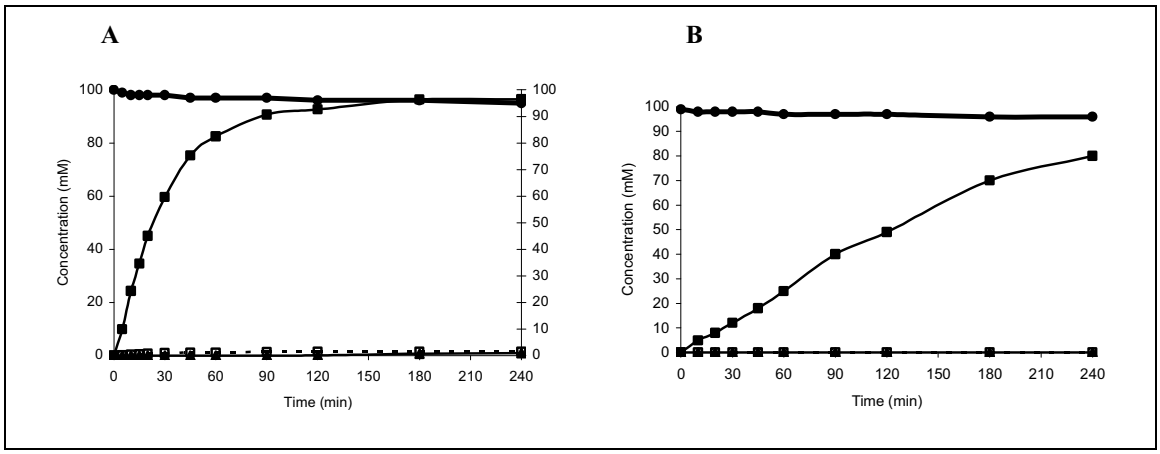

Figure A12. Kinetic resolution of 100 mM *cis*-2,3-epoxybutane by **(A)** YL46HmA and **(B)** YL25HmA, using 50% (w/v) cell loading. -■- indicates (*R,R*)-diol, -□- = (*S,S*)-diol, -▲- = *meso*-diol, -●- = ee_p (PCT WO 2007/069079). The (*R,R*)-diol of *cis*-2-3-epoxybutane is absolutely selectively produced by the selected recombinant strains. The 100 mM substrate added was completely hydrolysed in a number of the other strains tested (YL1HmA, YL23HmA, YL777HmA).

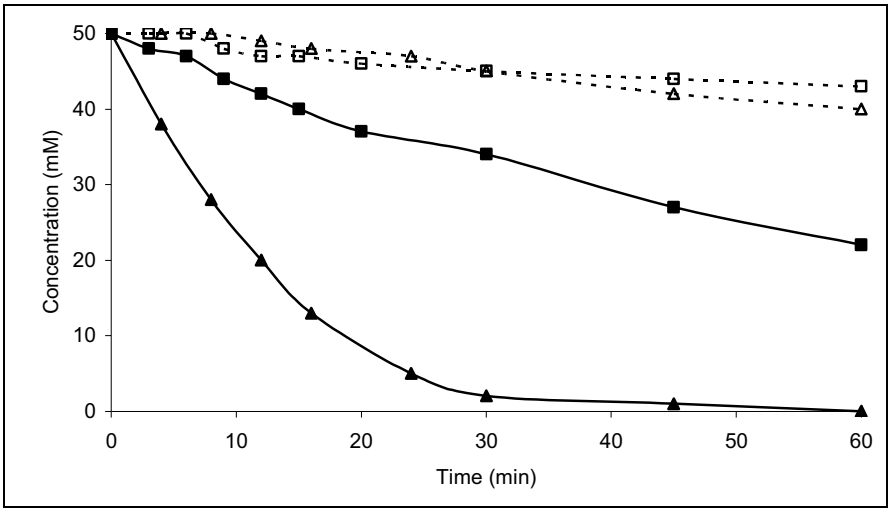

Figure A13. Kinetic resolution of 100 mM styrene oxide by YL-HmA transformants harbouring the sEH *echA* gene from *A. radiobacter* and the EH gene from *R. paludigenum* 692, using 10% (w/v) cell loading. Solid icons indicate (*R*)-SO, open icons indicate (*S*)-SO. -■- = YL-Ar-HmA -▲- = YL692HmA. (PCT WO 2007/010403)

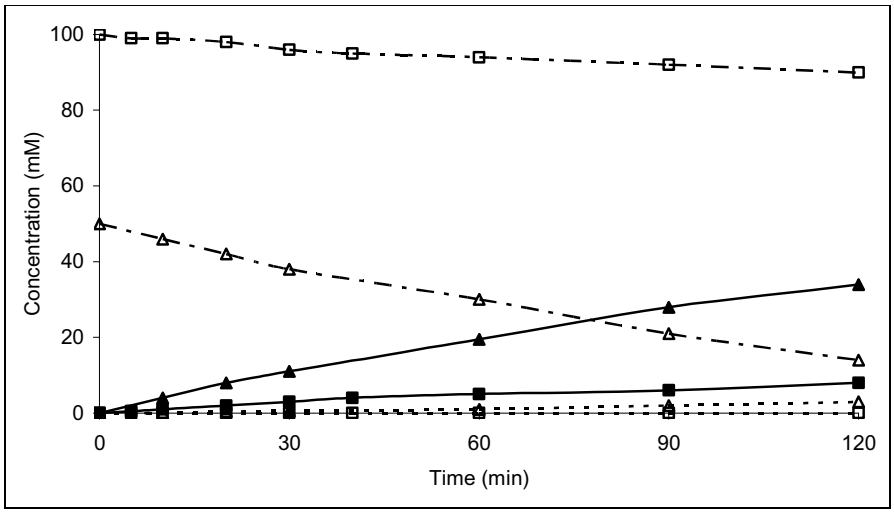

Figure A14. Comparison of the kinetic resolution of cyclohexene oxide by the recombinant enzyme from *R. araucariae* 25 expressed in *S. cerevisiae* (SC-25), and *Y. lipolytica* (YL25HmA). Solid icons indicate production of (*R,R*)-diol, open icons indicate production of (*S,S*)-diol, - — - — = consumption of CHO. -■- = SC-25, -▲- = YL25HmA (PCT WO 2007/010403).

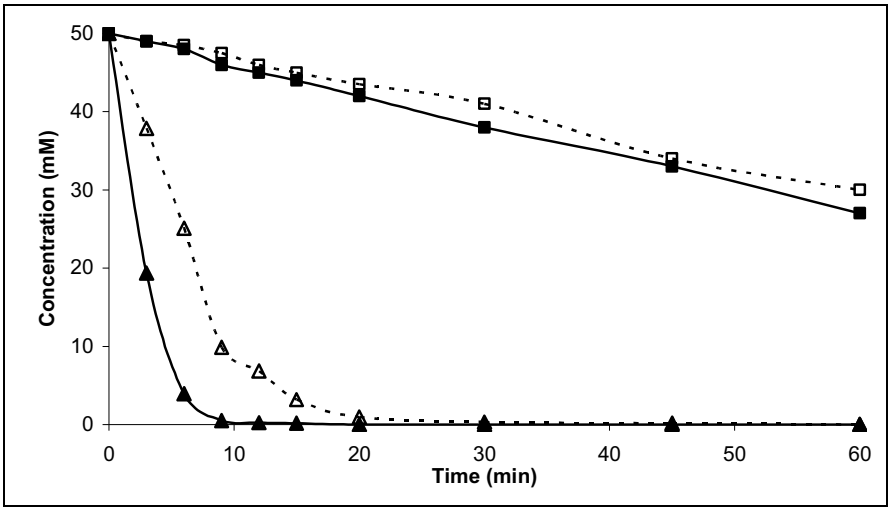

Figure A15. Kinetic resolution of 100 mM indene oxide by the recombinant enzyme from *R. araucariae* 25 expressed in *S. cerevisiae* (SC-25) and *Y. lipolytica* (YL25HmA). -■- = SC-25, -▲- = YL25HmA. Closed icons indicate (*1S,2R*)-IO, open icons = (*1R,2S*)-IO.