Development of *Yarrowia lipolytica* for enhanced production of heterologous proteins

by

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Date: March 2011

“You don’t have to be great to start dreaming you start by dreaming to be great” - Unknown

Table of contents

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The thesis is dedicated to my daughter and my family, particularly my late father.

List of abbreviations used

ACT = Actin

AEP = Alkaline extracellular protease

AIDS = Acquired immune deficiency syndrome

ARS = autonomous replicating sequences

BLAST = Basic Local Alignment Search Tools

CCL5 = Chemokine (C-C motif) ligand 5

CCR5 = Chemokine (C-C motif) receptor 5

CD4 = cluster of differentiation 4

CXCR4 = Chemokine (C-X-C motif) receptor 4

CW = Calcofluor white

CWP = Cell wall protein

cDNA = complementary DNA

CR = Congo red

DES = diethylsulfate

DNA = Deoxyribonucleic acid

EH = Epoxide hydrolase

EK = Bovine enterokinase

ER = endoplasmic reticulum

EMS = ethyl methane sulfonate

EtN = ethanolamine

FDA = Food and Drug Administration

GPI = Glycosylphosphatidylinositol

GPI7 = GPI biosynthesis gene 7 encoding the putative GPI:protein transamidase

GRAS = Generally Regarded As Safe

GEFs = guanine nucleotide exchange factors

HIV = Human immunodeficiency virus

HygR = Hygromycin-B

Ins = inositol

leu = leucine

LS = locus specific

LTR = long terminal repeats

mRNA = messenger RNA

MMS = methyl methane sulfonate

MNNG = N-Methyl-N'-Nitro-N-Nitrosoguanidine

MTC = mutagenesis cassette

NCAM = neural cell adhesion molecule

Ni-NTA = Ni (II)-nitrilotriacetic acid

PM = Plasma membrane

pNPP = *p*-nitrophenyl palmitate

pNP = *p*-nitrophenol

NG = Nitrosoguanidine

NTG = N-methyl-N′-nitro-N-nitrosoguanidine

OD_{600}/A600 = Optical density at wavelength 600 nm

OD_{595} = Optical density at wavelength 595 nm

OD_{450} = Optical density at wavelength 450 nm

ORFs = open reading frames

PHT = Promoter-Hygomycin-Terminator

P = phosphate

PKC = Protein kinase C

PT = Promoter-Terminator

qRT-PCR = Quantitative real time PCR

RANTES = **R**egulated upon **A**ctivation, **N**ormal **T** cell **E**xpressed and presumably **S**ecreted

RNA = Ribonucleic acid

SCP = Single-cell protein

SRP = signal recognition particle

SSAPCR = single-stranded amplification PCR

SDS–PAGE = Sodium dodecyl sulphate polyacrylamide

SPPS = Solid phase peptide synthesis

SDS = Sodium dodecyl sulfate

START-dependent signals

TEs = transposable elements

tRNA = transfer RNA

UAS = upstream activating sequences

Ura = Uracil

VSV-V = Vesicular Stomatitis Virus-V

www = world wide web

List of figures

Yarrowia lipolytica, a host for heterologous protein expression, a review

1.1. General introduction

In the last three decades a wide range of recombinant proteins have been produced based on heterologous gene expression in bacteria, mammalian cells and several yeasts and fungi (Gellissen, 2005; Melmer, 2005, Böer *et al*., 2005, Yin *et al*., 2007). Prokaryotic cells are usually preferred as they allow production of target proteins at a relatively cheaper cost (Dominguez *et al*., 1998). The main drawback of heterologous production of proteins in bacteria is that the proteins are sometimes expressed in bacteria as inclusion bodies requiring solubilization and reconstitution to attain activity. This complicates downstream processing (Gellisen *et al*., 2005). Bacteria also very often possess endotoxins which affect biosafety of the expressed bioproducts (Gellisen *et al*., 2005). The application of bacteria as hosts for production of therapeutic proteins is also affected by the failure of prokaryotes to perform certain post translational modifications which are critical for protein function. This is typified by the inability of prokaryotes to glycosylate heterologous proteins which has limited their application particularly as host in the production of therapeutic proteins (Dominguez *et al*., 1998). Mammalian cells have become preferred hosts for production of therapeutic proteins. However, mammalian cell culture and maintenance result in high costs for production in addition to the risks of cells possessing oncogenic or viral molecules (Morton and Potter, 2000).

With regard to protein production, yeasts offer considerable advantages over alternative prokaryotic and mammalian cell systems (Dominguez *et al*., 1998). They provide low-cost screening and production systems for efficiently processed and modified proteins (Gellisen, 2005). In most cases, yeasts meet safety prerequisites in that they do not harbour pyrogens, pathogens or viral inclusions (Gellissen *et al*., 2005). Certain yeasts have also been recently genetically engineered to enable them to add either humanized *N*-glycans of the intermediate mannose type (Kim *et al*., 2006; Jolivet *et al*., 2007) or complex type glycans (Hamilton *et al*., 2006). This provides yeasts with the option to produce biopharmaceuticals suitable for application in humans. The recognition of yeasts as attractive expression platforms for recombinant proteins is also met by genome analysis of an increasing number of yeast species, among others that of *Saccharomyces cerevisiae* (Goffeau *et al*., 1996), *Hansenula polymorpha* (Ramezani-Rad *et al*., 2003), *Kluyveromyces lactis* and *Yarrowia lipolytica* (Dujon *et al*., 2004).

The extensively studied *S. cerevisiae* has been applied to produce biopharmaceuticals for human application such as insulin (Melmer, 2005), hepatitis B vaccines (Brocke *et al*., 2005), granulocyte-macrophage colony stimulating factor (GMCSF) (Marini *et al*., 2007) and glucagons (Wu *et al*., 2009, Zhang *et al*., 2010). However, certain limitations and drawbacks

are encountered when using this yeast. *Sacharomyces cerevisiae* tends to hyperglycosylate recombinant proteins and this can make the target protein allergenic (Jolivet *et al*., 2007). The majority of proteins are retained in the periplasmic space or associated with the cell wall, resulting in low production and increased costs for production and downstream processing (Romanos *et al*., 1992; Buckholz and Gleeson, 1997). In addition *S. cerevisiae* has limited carbon source utilization. This imposes restrictions on the design of fermentation processes. In addition, preferential use of epitome vectors generates unstable recombinant strains and as a result inconsistencies with fermentation runs are of major concern (Böer *et al*., 2007).

These limitations have been circumvented by the development of expression systems for several other alternative yeasts generally referred to as non-conventional yeasts. These yeasts include *Hansenula polymorpha (Pichia angusta)*, *Kluyveromyces lactis*, *Pichia pastoris*, *Schizosaccharomyces pombe*, *Schwanniomyces occidentalis* and *Y. lipolytica* (Buckholz and Gleeson, 1997; Dominguez *et al*., 1998). In recent years, production of proteins for pharmaceutical and industrial applications has been done using these yeasts. Initially, this was to complement some of the drawbacks associated with *S. cerevisiae* but it was later established that some of these yeasts are better producers of heterologous proteins than the conventional *S. cerevisiae* yeast (Dominguez *et al*., 1998, Müller *et al*., 1998). However, high-level production of proteins was still found to be hampered by several intrinsic and extrinsic factors. Some of the causes of poor production in both conventional and non-conventional yeasts include low level expression, insufficient heterologous protein processing for secretion and degradation caused by host yeast cell proteases (Gellisen *et al*., 2005; Müller *et al*., 1998). General strategies aimed to achieve efficient heterologous protein production included the optimization of the coding sequence, maximisation of gene dosage and the use of strong promoters amenable with controlled expression of the target protein (Klabunde *et al*., 2007).

Yarrowia lipolytica is a yeast that has over the years been considered a potential candidate for production of bioproducts and concentrated efforts to understand its physiology, genetics and molecular biology appear in literature (Barth and Gaillardin, 1997, Nicaud *et al*., 2002, Madzak *et al*., 2004). This yeast is considered to be non-pathogenic and has GRAS (Generally Regarded As Safe) status. It has been used in the production of citric acid at industrial scale (Rywińska and Rymowicz, 2010). The pathways leading to the assimilation of hydrophobic substrates such as *n*-alkanes, fatty acids, fats and oils by the *Y. lipolytica* yeast have been the subject of extensive studies with the aim of enhancing citric acid production (Mauersberger *et al*., 2001; Fickers *et al*., 2005, Thevenieau *et al*., 2007). *Yarrowia lipolytica* is also known to be a prolific secretor of extracellular enzymes, notably proteases, lipases,

esterases and RNase (Barth and Gaillardin, 1996). The yeast was reported to produce to these extracellular proteins to levels as high as 2 $g.L^{-1}$ (Ogrydziak, 1988, 1993). Post-translational protein modification by *Y. lipolytica* is reported to be co-translational, a pattern resembling that in mammalian cells (Beckerich *et al*., 1998; Boisramé *et al*., 1998). In a study done by Müller and co-workers (1998), *Y. lipolytica* was found to be a more superior host for heterologous protein production than *S. cerevisiae* as it produced most active enzymes.

This chapter reviews current understanding of the *Y. lipolytica* physiology focusing on fundamental and applied researches which enable the development of strains with increased capacity to produce heterologous proteins for biotechnological applications. In an effort to obtain mutant yeast strains with increased capacity to express proteins, several physiological properties associated with protein production have been studied. These include transcription, translation, translocation and secretion of proteins. The capacity of *Y. lipolytica* to grow on hydrophobic substrates has facilitated isolation of several promoters with potential application in expression systems (Nicaud *et al*., 2002). In attempts to achieve optimal protein translations the codons of genes encoding target open reading frames (ORF) have been optimised in accordance with the preferred codon usage for particular hosts (Xuan *et al*., 1990). This has resulted in synthesis of ORF to be expressed in *Y. lipolytica* to resemble codons of endogenous proteins to improve their expression. In addition, chaperone proteins have been found to play an essential role in protein folding, maturity of nascent polypeptides and translocation (Boisramé *et al*., 2002). Protein secretory pathways involve translocation of proteins across the cell wall. The composition of the cell wall and cell wall permeability may become a barrier for selective uptake and secretion of macromolecules. This rigid but dynamic structure surrounding cells is primarily composed of an interconnected array of mannoproteins (mannan) and glucans linked to small amounts of chitin (Duran and Nombela, 2004). Disruption of mannoprotein encoding genes have been observed to enhance the secretion of proteins through enhancing cell permeability (Bartkeviciūte and Sasnauskas, 2004; Zhang *et al*., 2008).

1.2. Yarrowia lipolytica expression systems

The manipulation of *Yarrowia lipolytica* as a host for heterologous protein production is made possible by the large number of genetic markers and molecular tools available for this yeast (Müller *et al*., 1998; Barth and Gaillardin, 1996; Pignede *et al*., 2000). *Y. lipolytica* is typically transformed with shuttle vectors comprising of a bacterial DNA moiety for propagation in *E. coli* and the expression cassette for *Y. lipolytica* (Madzak *et al*., 2004). Typically, the expression cassette comprises a selection marker, promoter, secretion signal, gene of

interest, transcription terminator and with some systems the fragment responsible to target the expression cassette to a defined locus in the host genome (Figure 1.1.).

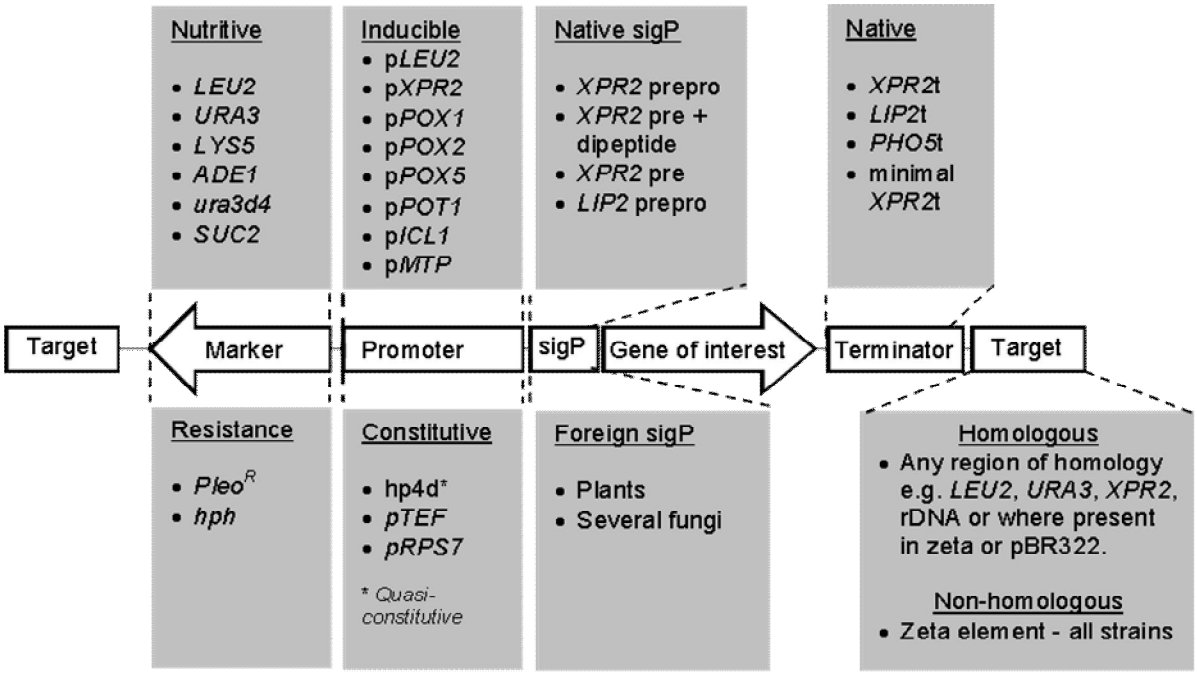

Figure 1.1. A schematic diagram showing the molecular and cellular tools available to manipulate *Y. lipolytica*. The diagram is based on an integrative vector which can be targeted to several available target sites through either homologous or non-homologous recombination. The promoters that have been used are either inducible or constitutive; and several native and foreign secretion signals have been used to produce heterologous proteins. Auxotrophic selection markers have been predominantly used with hygromycin and phleomycin occasionally applied as antibiotic selective markers. A number of transcription terminators have also been applied in the expression of heterologous proteins. Target sites include several homology based regions such as the LEU2, URA3, XPR2 and rDNA genes as well as the pBR322 sequence and in cases where the genome is fitted with this docking platform. There is also homologous integration into the zeta elements where these are present or random non-homologous intergration based on the zeta *Ylt1* retrotransposon (Taken from Albertyn *et al*., 2009).

1.2.1. Vectors

There are two major classes of expression systems used in the transformation of *Y. lipolytica*, differing by their mode of maintenance, namely episomaly replicating (autonomously replicating ARS/CEN vectors) and integrative vectors (Madzak *et al*., 1999). These vectors are introduced in *Y. lipolytica* host strains using either the lithium acetate method (Barth and Gaillardin, 1996) or electroporation (Fournier *et al*., 1993) for transformation. The lithium acetate method yields very high transformation efficiencies, but regular recombination events between short repeated sequences are observed when replicative vectors are used (Barth and Gaillardin, 1996). The lithium acetate method is usually recommended for integrative vectors, whilst electroporation is preferred when *Y. lipolytica* is transformed with autonomously replicating vectors (Fournier, 1991).

1.2.1.1. Episomal vectors

Unlike *S. cerevisiae* containing an autonomously replicating vector such as 2µ*, Y. lipolytica* is naturally devoid of any episomal DNA in its genome (Juretzek *et al*., 2001), although replicative plasmids using chromosomal replicating origins can be designed (Madzak *et al*., 2004). The autonomously replicating vectors are usually limited to 1-3 copies/cell as they are only stable as ARS/CEN plasmids (Juretzek *et al*., 2000; Vernis *et al*., 1997, 1999). Consequently, these vectors allowed limited gene amplification and increases of gene expression (Nicaud *et al*., 1991).

1.2.1.2. Integrative vectors

Integrative vectors carry sequences homologous to regions of the *Y. lipolytica* chromosome that direct insertion of the vector into the genome by either homologous or non-homologous recombination (Juretzek *et al*., 2001). The single cross-over event occurs by linearization of the vector within the homology region, and this usually results in very high transformation frequencies (Madzak *et al*., 2004). In most cases, a single complete copy of the vector integrates at the selected site (Barth and Gaillardin, 1996), while remaining events will favour either multiple tandem integration, gene conversion or out of site integration. With integrative vectors, transformants are obtained with 12-60 plasmid copies/cell, and this correlates with increase in gene expression and results in a 10 fold increase in protein production (Le Dall *et al*., 1994). In addition, these vectors exhibit high stability, as they can be maintained without rearrangements for 100 generations under non-selective conditions (Hamsa and Chattoo, 1994).

Several sites in the genome of *Y. lipolytica* have been used to target integrative vectors (Juretzek *et al*., 2001). These include the LEU2, URA3, and XPR2 genes as well as rDNA sequences. For multicopy integration, the rDNA clusters are used to facilitate homologous cross-over of the vector to clusters. In addition, the *Y. lipolytica* retrotransposon *Ylt1* long terminal repeats (LTR) named *zeta* (Schmid-Berger *et al*., 1994) is present in some *Y. lipolytica* strains in at least 35 copies/genome in a dispersed manner. LTR often exists as solo elements with up to 50-60 copies in several strains and function as sites of homologous recombination for multiple integration (Juretzek *et al*., 2001). Transformation of *zeta* containing vectors into *Y. lipolytica* strains devoid of the LTR results in non-homologous integration (Nicaud *et al*., 1998). In other instances, the genome may be fitted with a target site, such as the pBR322-docking platform for directed monocopy integration of the vector (Jeretzek *et al*., 2001)

1.2.2. Promoters

The ability of *Y. lipolytica* to assimilate several hydrophobic substrates was used as a tool to isolate several promoters of key enzymes from these pathways. Promoters from isocitrate lyase (*ICL1*), 3-oxo-acyl-CoA thiolase (*POT1*), and acyl-CoA oxidases (*POX1*, *POX2* and *POX5*) have been evaluated, and compared to those of glycerol-3-phosphate dehydrogenase (*G3P*), *XPR2*, and hp4d, regarding their regulation and activity during growth on various carbon sources (Figure 1.1.) (Juretzek *et al*., 2001). The study showed that p*ICL1*, p*POT1* and p*POX2* were the strongest inducible promoters; with the latter two showing high induction by fatty acids although they were repressed by glucose and glycerol. Two other promoters isolated from *Y. lipolytica* were also described by Müller *et al*. (1998). These promoters, namely those of the *TEF* and *RPS7* genes are relatively strong and the genes are constitutively expressed. They were intended to be used for isolation of enzyme genes through expression-cloning and not for heterologous production of proteins. This is because fully constitutive promoters are not recommended for that purpose since early expression of the heterologous protein can affect the culture growth if the protein particularly if the protein is toxic to the cell.

1.2.3. Secretion signals

Heterologous proteins destined for secretion into the culture medium have a signal peptide to target the protein to the secretion pathway. Several endogenous and heterologous secretion signals have been described in *Y. lipolytica* (Nicaud *et al*., 2002; Madzak *et al*., 2004). To date the *XPR2* prepro region has obtained most attention as it has been shown to target the early steps of protein secretion to the co-translational pathway of translocation (He *et al*., 1992; Yaver *et al*., 1992). Naturally, the *XPR2* pro region itself is required for AEP transit, acting as an internal chaperone allowing the mature part of AEP to adopt a conformation compatible with secretion (Fabre *et al*., 1991, 1992). More recently, alternative secretion signals were used: (i) the *XPR2* pre region alone (with or without the dipeptide stretch) which was shown to be sufficient to drive an efficient heterologous secretion (Swennen *et al*., 2002); (ii) the prepro region of the *Y. lipolytica LIP2* gene (Pignède *et al*., 2000); (iii) an hybrid between *XPR2* and *LIP2* prepro regions (Wang *et al*., 2002).

1.2.4. Selection markers

Yarrowia lipolytica is resistant to most of the commonly used antibiotics. However, it is sensitive to hygromycin-B and the antibiotics in the bleomycin/phleomycin group (Barth and Gaillardin, 1997). Expression of heterologous genes using vectors containing genes conferring resistance to these antibiotics has been applied with success. However, their use

for selection was hampered by a high frequency of spontaneous resistance (Cordero Otero and Gaillardin, 1996)

Yarrowia lipolytica is unable to assimilate sucrose as a sole carbon source (Barth and Gaillardin, 1997), and this property was exploited to use heterologous *ScSUC2* expression as a dominant selective marker (Nicaud *et al*., 1989, Mauersberger *et al*., 2001, Förster *et al*., 2007). However, the drawback of this strategy was the residual growth of wild-type *Y. lipolytica* strains on sucrose plate impurities (Barth and Gaillardin, 1996).

The availability of non-leaky non-reverting *LEU2* and *URA3* recipient strains has made auxotrophic markers, particularly *LEU2* and *URA3*, the best choice for selection in *Y. lipolytica*. To facilitate the selection of multiple vector integrations, defective versions of the *URA3* marker were developed (Le Dall *et al*., 1994). Sequential truncations of the *URA3* promoter were evaluated for their ability to allow *Y. lipolytica* transformation by multiple integrations. Thus, the *ura3d4* allele (Juretzek *et al*., 2001), which retained only 6 bp upstream from the *URA3* ATG sequence, was no longer able to confer a Ura+ phenotype as a single copy, but could promote the amplification of the vector copy number in multiple integrations (Le Dall *et al*., 1994; Pignède *et al*., 2000; Nicaud *et al*., 2002).

1.3. Strain enhancement

Over the past 15 years, significant progress has been made in developing techniques for the modification of *Y. lipolytica* strains to enhance heterologous protein production. This has further been made possible by the completion of the *Y. lipolytica* genome sequencing project and annotation of the finished sequence (Dujon *et al*., 2004). This has paved ways for researchers to rapidly modify the *Y. lipolytica* genome to construct novel strains with properties beneficial to heterologous protein production. Improvements to protein production have been explored at many major points of protein synthesis, folding and secretion.

1.3.1. Mutagenesis

Heterologous protein production strains are often improved for their ability to secrete a desired protein by various mutagenesis techniques (van Ooyen *et al*., 2006). It has been shown that chemical and insertional mutagenesis in *Y. lipolytica* enhances protein production (Fickers *et al*., 2003; Mauersberger *et al*., 2001).

1.3.1.1. Chemical mutagenesis

Several chemical mutagens modify the genetic make-up of yeasts by mispairing the nucleotides (Brockman *et al*., 1984). These chemicals react directly with certain nucleotides and do not require active DNA synthesis in order to act but still do require DNA synthesis in order to be "fixed" (Beranek, 1990). Examples of alkylator mutagens include ethyl methane sulfonate (EMS), methyl methane sulfonate (MMS), diethylsulfate (DES), and nitrosoguanidine (NTG, NG, MNNG). These mutagens tend to prefer G-rich regions, reacting to form a variety of modified G residues (Ahmed and Hadi, 1988).

Fickers *et al*, (2005) constructed non-genetically modified mutants with increased capacities to produce extracellular lipase by employing chemical mutagenesis. *Y. lipolytica* cells were treated with NTG and the mutants were screened for lipase hyperproduction phenotype. A mutant was selected that showed a 10-fold increase in lipase productivity upon addition of oleic acid and exhibited lipase production uncoupled from catabolite repression by glucose. Furthermore, treatment of a lipase producing fungal strain *Aspergillus japonicas* by the chemical mutagenic agents HNO_2 and NTG generated strains that could accumulate up to 139% and 156% lipase when compared to the parent strain (Karanam and Medicherla, 2006).

1.3.1.2. Insertional mutagenesis

The *Y. lipolytica* LTR direct random integration of the transforming DNA into the genome of strains devoid of *Ylt1* (Juretzek *et al*., 2001). This property has been used to identify strains with enhanced abilities to produce enzymes that assimilate hydrophobic substrates (Mauersberger *et al*., 2001; Thevenieau *et al*., 2007). Tagged mutants that were affected in the degradation of hydrophobic compounds (HC) were generated by insertion of a *zeta-URA3* mutagenesis cassette (MTC) into the genome of a *zeta*-free ura3 deleted strain of *Y. lipolytica*. More than 200 mutants were obtained, and about 70 were affected in HC degradation, representing different types of non-alkane-utilizing mutants and triacylglycerol degradation mutants (Mauersberger *et al*., 2001). The regions flanking the integrated MTC were sequenced using genome walking PCR techniques to identify the disrupted genes. Sequence analysis revealed known and novel genes required for HC utilization, e.g. for AlkD/E mutants MTC insertion had occurred in genes of thioredoxin reductase, peroxines PEX14 and PEX20, succinate-fumarate carrier SFC1, and isocitrate lyase ICL1 (Thevenieau *et al*., 2007).

1.3.2. Modification of the glycosylation pathway

Several proteins of therapeutic interest are glycosylated, and the nature of this modification affects their activity and immunogenicity (Hamilton *et al*., 2003; Madzak *et al*., 2004). There are differences in the glycosylation pathway of yeasts and mammalian cells (Cereghino and Cregg, 2000). Mammals generate three types of oligosaccharide residues (high-mannose, complex and hybrid types), while lower eukaryotes such as yeasts perform only the addition of mannose outer chains. Fungi including yeasts and mammals share initial steps of protein *N*-glycosylation, which involves the site-specific transfer of $(Glc)_3$-$(Man)_9$-$(GlcNAc)_2$ from the luminal side of the endoplasmic reticulum (ER) to the *de novo* synthesized protein by an oligosaccharyltransferase complex (Choi *et al*., 2003). Following the export of predominantly $(Man)_8$-$(GlcNAc)_2$ containing glycoproteins to the Golgi, the pathways diverge notably between mammals and yeast (Hubbard and Ivatt, 1981). In the human Golgi α-1,2-mannosidases (IA–IC) remove Man to yield the $(Man)_5$-$(GlcNAc)_2$ structure, which forms the precursor for complex N-glycans. These mannosidases are typically type II membrane proteins with an *N*-terminal cytosolic tail, a transmembrane domain, a stem region, and a C-terminal catalytic domain.

N-glycosylation has been studied extensively in *S. cerevisiae*, and in contrast to the mammalian *N*-glycan processing, it involves the addition of numerous Man sugars throughout the entire Golgi apparatus, often leading to hypermannosylated *N*-glycan structures with >100 Man residues (Figure 1.2.). This process is initiated in the early Golgi by an α-1,6-mannosyltransferase (OCH1) that prefers $(Man)_8$-$(GlcNAc)_2$ as a substrate but is able to recognize various other Man oligomers with the notable exception of the human $(Man)_5$-$(GlcNAc)_2$ intermediate, which is not a substrate (Nakayama *et al*., 1997). After addition of the first α-1,6-Man by OCH1, additional α-1,6-mannosyltransferases extend the α-1,6 chain, which then becomes the substrate for medial- and trans-Golgi-residing α-1,2- and α-1,3-mannosyltransferases as well as phosphomannosyltransferases that add yet more Man sugars to the growing *N*-glycan structure (Dean, 1999). In *Y. lipolytica* a similar process occurs; however, hypermannosylation occurs less frequently and to a lesser extent (Song *et al*., 2007).

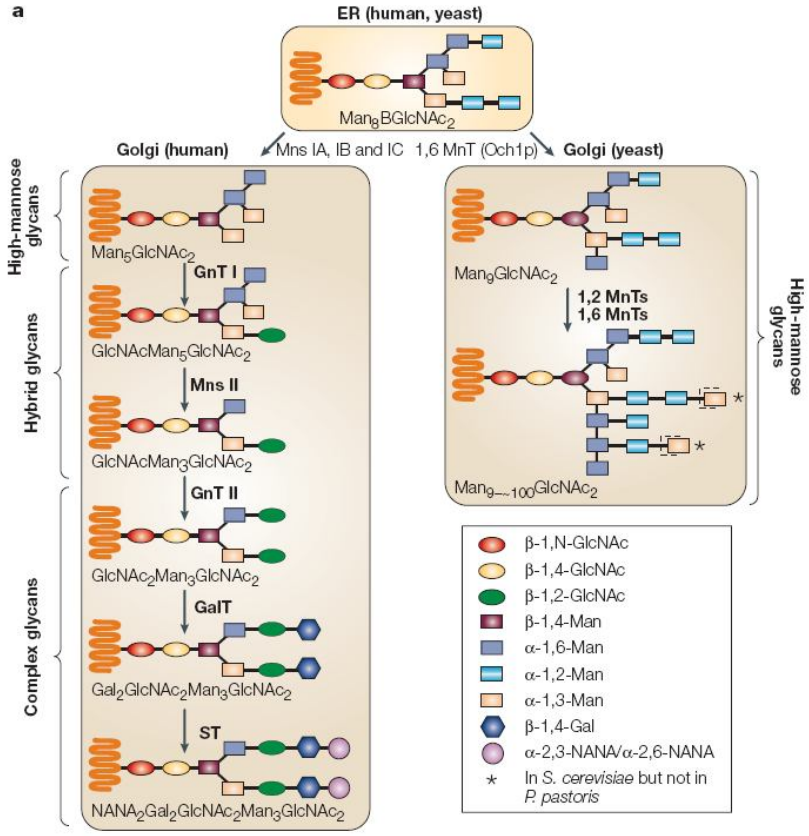

Figure 1.2. Major N glycosylation pathways in humans and yeast. Representative pathway of N-glycosylation pathway in humans (left) provides a template for humanizing N-glycosylation pathways in yeast (right). Early oligosaccharide assembly mutants can be best used to recreate synthetic glycosylation pathways that lead to complex *N*-glycosylation in yeast. ER, Endoplasmic reticulum; GalT, galactosyltransferase; GlcNac, N-acetylglucosamine; GnTI, N-acetylglucosaminyl transferase I; GnTII, N-acetylglucosaminyltransferase II; Man, Mannose; MnsII, Mannosidase II, MnTs Mannosyl transferase; ST, Sialtransferase. (Taken from Wildt and Gerngross, 2005).

There have been attempts to engineer yeast to enable biosynthesis of mammal-compatible glycans (Wildt and Gerngross, 2005). Humanizing the glycosylation machinery of a *Pichia pastoris* yeast strain required the (*i*) elimination of some endogenous glycosylation reactions and (*ii*) the recreation of the sequential nature of human glycosylation in the ER and Golgi (Hamilton *et al*., 2003). The first step involves the generation of a gene knockout of the α-1,6-mannosyltransferases encoding gene, *OCH1* (Wildt and Gerngross, 2005). OCH1 is responsible for the elongation of the outer chain to result in hyperglycosylation (Hamilton *et al*., 2003). In *Y. lipolytica*, disruption of the *YlOCH1* gene resulted in a homogenous glycosylated reporter protein containing $(Man)_8$-$(GlcNAc)_2$ in contrast to the $(Man)_9$-$(GlcNAc)_2$ glycan structure (Song *et al*., 2007). The subsequent steps required the proper localization of active mannosidases, glycosyltransferases, and possibly nucleotide sugar transporters to specific organelles (Choi *et al*., 2003).

1.3.3. Construction of protease deficient strains

Although *Y. lipolytica* has advantages for production of heterologous proteins, product yield can be reduced due to proteolysis. Consequently, genetic manipulation of the host proteases can reduce host-specific degradation; therefore, it has been used to develop many protease-deficient yeast strains. In *Y. lipolytica*, two major extracellular proteases have been studied in great detail; the acid extracellular protease (AXP) (Young *et al*., 1996) and an alkaline extracellular protease (AEP) (Davidow *et al*., 1987). Protease deficient strains have been used to improve the yield of expressed proteins. *Y. lipolytica* extracellular protease null mutant strains have been constructed. For example, *Y. lipolytica* Po1d, was deleted of the AEP (Le Dall *et al*., 1994). In addition, other *Y. lipolytica* strains Po1g and Po1h have been deleted for both extracellular proteases (Madzak *et al*., 2000).

1.3.4. Hyper-protein secreting mutants

Mutations in genes involved in the construction and in the maintenance of the cell wall, such as *PMR*1, *SEC*14, *ERD*1, *MNN*9 and *MNN*10, have in some cases been demonstrated to lead to supersecretive mutants in *S. cerevisiae* and other yeasts (Bartkeviciūte and Sasnauskas, 2004). The results also substantiate a tight correlation between glycosylation processes and protein secretion (Bankaitis *et al*., 1990). It has been hypothesized that this correlation is not directly linked to the heterologous secreted proteins, but rather to an altered structure of the glycosidic residues added to the cell wall (glycol) proteins (Bartkeviciūte and Sasnauskas, 2004), which result in altered permeability of the cell wall. The inactivation of the *GAS*1 gene, whose product is directly involved in the synthesis of the cell wall, has also led to a hypersecretive phenotype in *S. cerevisiae* (Vai *et al*., 2000). The *GAS*1 gene disruption resulted in morphological and physiological phenotype changes due to an altered cell wall structure and composition. As a result of these cell wall structure modifications, the *gas1* mutant shows higher protein secretion levels when compared to the wild type, both for total and for the heterologous recombinant proteins, as the human insuline-like growth factor (rhIGF1) (Vai *et al*., 2000). Additionally, the disruption of the *P. pastoris GAS*1 was found to enhance secretion of a heterologous lipase protein (Marx *et al*., 2006).

1.3.5. Other protein production enhancement strategies

Many proteins are still secreted only at comparatively low levels even though the transcription, translation or secretion level of the target protein is optimized sufficiently for overexpression in the most suitable host system (Punt *et al*., 2002; Macauley-Patrick *et al*., 2005; Porro *et al*., 2005; Schröder 2007). Thus, improvement strategies for heterelogous protein production included codon usage and utilisation of chaperones.

1.3.5.1. Codon usage

For efficient translation, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage. A study conducted by Gasmi *et al*., (2011) investigated the effect of codon bias and consensus sequence (CACA) at the translation initiation site on the expression level of heterologous proteins in *Y. lipolytica*; using human interferon alpha 2b (hIFN-alpha2b) as a model. A codon optimized hIFN-alpha2b gene was synthesized according to the frequency of codon usage in *Y. lipolytica*. Both wild-type (IFN-wt) and optimized hIFN-alpha2b (IFN-op) genes were expressed under the control of a strong inducible promoter acyl-coA oxidase (POX2). Codon optimization increased protein production by 11-fold, whereas the insertion of CACA sequence upstream of the initiation codon of IFN-op construct resulted in 16.5-fold increase of the expression level; indicating that translational efficiency played an important role in the increase of hIFN-alpha2b production level.

1.3.5.2. Co-expression with chaperones

In *Y. lipolytica*, secretory proteins start their journey on the intracellular secretory pathway by co-translational translocation, through the Sec61 translocon into the crowded environment of the ER lumen where they are folded into their native structure via the ER-resident protein-folding machinery (Ellgaard and Helenius, 2003). After translocation into the ER, nascent polypeptides are bound by the ER-resident chaperone protein binding protein (BiP; encoded by Kar2) for folding to native structures, whereas the nascent glycoproteins are bound by the ER chaperone calnexin (encoded by CNE1) to undergo their correct folding and *N*-glycan processing. The ER sustains a set of covalent modifications, which include signal sequence processing, disulfide bond formation, *N*-glycosylation, glycosyl-phosphatidyl-inositol addition, degradation, and sorting.

After a substantial folding and modification process in the ER, only properly folded and assembled proteins can be exported from the ER to the Golgi apparatus, where they are further modified, before being transported to the extracellular space, vacuoles or other organelles (Klausner, 1989). At the same time, misfolded or aggregated proteins in the ER are recognized by the cell, which leads to binding of the proteins by the BiP complex and eventual redirection to the cytosol for degradation (Yoshida, 2007). Prolonged binding of BiP to partially misfolded proteins leads to the induction of unfolded protein response (UPR), which also stimulates proteolysis and inhibits the transcription and translocation of the target protein.

To enhance translocation of heterologous proteins, overexpression of multiple chaperones and other folding helpers seem to be an effective approach. Some studies have suggested that overexpression of the chaperone BiP, a member of the Hsp70 family of ATPases, stimulates protein secretion in *S. cerevisiae*, for example, a 5-fold increase in secretion of human erythropoietin (Robinson *et al*., 1994) and a 26-fold increase in bovine prochymosin (Harmsen *et al*. 1996). Moreover, in some cases, reduction of BiP levels leads to decreased secretion of foreign proteins (Robinson *et al*., 1996). In addition to BiP, overexpression of PDI also resulted in increased secretion of some heterologous proteins (Butz *et al*., 2003; Damasceno *et al*., 2006). When coupled with Ero1p the secretion of recombinant human albumin in *K. lactis* was accelerated (Gross *et al*. 2004; Lodi *et al*., 2005).

1.4. Conclusion

The properties of *Y. lipolytica* as a prolific secretor of endogenous proteins, accompanied by several genetic tools that can be used for its manipulations make it attractive as a host for recombinant protein production. In addition, the other feature that has resulted in *Y. lipolytica* gaining widespread usage are the co-translational pathway of protein synthesis resembling that in mammalian cells and lack of protein hypermannosylation. Extensive data on *Y. lipolytica* in large bioreactors has been accumulated. However, several systematic approaches can be followed. Heterologous protein production can be enhanced by modifying the expression vector. Different classes of promoters and secretion signals as well as target sites are available to suit expression of a particular protein. *Y. lipolytica* mutants with enhanced capacity to produce heterologous proteins can also be constructed following random mutagenesis. Mutagenic strategies such as chemical and insertional mutagenesis have proven that *Y. lipolytica* can be improved for heterologous protein accumulation with up to more than a 10 fold increased protein production being reported.

With this background, the objective of the study was to develop a *Y. lipolytica* strain with enhanced abilities to produce industrial and therapeutic proteins. A desirable property would be a hyperproducing strain that secretes the protein of interest into the surrounding medium. In the first research chapter (Chapter 2) of the study, the use of LIP2 as a carrier protein to express therapeutic peptides in *Y. lipolytica* is described. In Chapter 3 of the study, the construction of a *ylGPI7* null mutant strain with a lipase hyperproduction phenotype is described, as well as the effect of the disruption on other physiological factors. Chapter 4 describes the deletion of *YlOCH1* in the *ylGPI7* null mutant strain for preliminary assessment as a candidate yeast amenable to glycoengineering.

1.5. References

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Development of an expression system for production of therapeutic peptides in the *Yarrowia lipolytica* yeast

2.1. Introduction

Peptides are attracting increasing attention as therapeutics. The therapeutic value of peptides was once dominated by their use in oncology, but has now broadened to encompass cardiovascular metabolic and nervous systems diseases (Song *et al*., 2004, Alvares *et al*., 2010, Torres and Miranda, 2010**,** Behme *et al*., 2003, Carlson and Cummings, 2006, Zhou *et al*., 2004). The other pathological conditions where peptides have huge potential application is in the treatment of diseases caused by microbial and viral infections (Shah *et al*., 2010). Peptide therapeutics are attractive as therapeutic agents because they show high biological activity, they are associated with low toxicity and high specificity (Watt, 2006; Oyston *et al*., 2009). The pharmaceutical and biotechnology industries are increasingly turning towards peptides in their search for drug discovery targets (Watt, 2006; Hu, 2010). The study conducted by the Tufts Center for the Study of Drug Development revealed that the annual number of therapeutic peptides entering clinical study worldwide in the 2000 to 2007 period nearly doubled to 16.9 from 9.7 during the 1990s (http://csdd.tufts.edu). This is a further indication that the peptide therapeutics market is providing new commercial opportunities to biotechnology and pharmaceutical industries. To exploit these markets, biotechnology and pharmaceutical companies are actively pursuing the development of a variety of peptide-based technologies, peptide manufacturing technologies and drug delivery methods.

Natural peptides such as insulin, vancomycin (Song *et al*., 2004), oxytocin (Alvares *et al*., 2010), and cyclosporine (Prignano *et al*., 2010) and synthetically produced ones such as enfuvirtide (Torres and Miranda, 2010) and integrilin (eptifibatide) (Shah *et al*., 2010) are among the approved peptide-based drugs for treatment of various pathological conditions. Several other therapeutic peptides that serve as potent agonists and antagonists against a number of receptors involved in disease progression have been identified**.** Notable among the different classes of therapeutic peptides are the agonists such as glucagon-like peptide-1 (GLP-1) for the control of diabetes (Behme *et al*., 2003), ghrelin to treat obesity (Carlson and Cummings, 2006), gastrin-releasing peptide used in cancer treatments (Zhou *et al*., 2004), and defensin (Simon *et al*., 2008), which has found use as an antimicrobial agent. These peptides account for almost 50 per cent of the market (Bionest, 2005).

Peptides are generally unstable in the body and have short *in vivo* half lives owing to rapid renal clearance and degradation by proteases (Sood and Panchagnula, 2001).

This is particularly challenging when designing agents for oral administration, often the preferred administration route. It also requires large or frequent dosages, which challenges the large scale manufacturing of long peptides in a cost-effective manner. Although novel technologies to create peptides with extended half lives are being developed (Sato *et al.*, 2006), problems with delivery technologies and peptide bioavailability necessitate the requirement for high dosage amounts. Because high doses of these products are needed per patient, relatively large amounts of protein must be produced presenting a challenge for cost-effective manufacturing.

There are many technological challenges associated with the production and therapeutic applications of peptides (Parmar, 2004). Historically, therapeutic peptides were obtained from their original sources making such peptides limited in availability and expensive (Watt, 2006). As an example, human growth hormone (hGH) was obtained from human corpses, and insulin for treating diabetes was collected from slaughtered pigs (Hu, 2010). Currently, peptides for therapeutic use are manufactured through chemical synthesis, transgenic, or recombinant methods (Brandsma *et al*., 2009). Three distinct chemically based techniques are currently the biggest sources of peptides: solid phase synthesis, solution phase synthesis, and a combination of both (Verlander, 2007). Although chemical synthesis provides an ideal solution to early stage manufacturing, it encounters serious potential problems when used for producing materials for late stage clinical trials and commercialisation. The process is inefficient for peptides longer than 50 amino acids and anything over 35 amino acids is generally not economically feasible (Verlander, 2007). In addition, chemical syntheses involve the use of chemicals that are fairly toxic, presenting potential ecological and purity problems when manufacturing in bulk. The processes are step-intensive, and do not scale well for mass production. For example, chemical synthesis of the enfuvirtide peptide used in the treatment against HIV/AIDS required at least 106 steps to produce (Torres and Miranda, 2010).

The alternative approach for therapeutic peptide manufacturing is through recombinant DNA techniques (Marv, 2005). The option is particularly attractive for peptides longer than 35 amino acids where chemical synthesis is technically difficult and economically not feasible. The expression of recombinant peptides in bacteria is by far the simplest and most inexpensive means to produce large amounts of products of interest. However, it is difficult for small peptides to be expressed in bacteria at levels that are sufficiently high to enable economic recovery from the production media (Valore *et al*., 1998). To alleviate the challenge, several biological

expression systems have been developed by fusing the peptide with a partner protein. The presence of the endogenous gene is considered essential in the fusion partner to result in the efficient expression of the peptide and this has an added advantage that it avoids probable toxicity of the peptide to the host cells. Fusion partner proteins that have been used to produce peptides include bovine prochymosin (Haught *et al*., 1998), F4 of *Escherichia coli* intracellular protein PurF (Lee *et al*., 2000), and green fluorescent protein (EGFP) (Skosyrev *et al*., 2003). Although these studies showed that certain carrier molecules could greatly improve the stability of passenger proteins in the expression host, difficulties are often connected with the relative low yield and the subsequent isolation of the target products (Skosyrev *et al*., 2003).

The past decade saw tremendous advances in cell culture technology producing higher protein and peptide yields. As a result, peptide purification and recovery has become a bottleneck, or at least a major cost driver with a high potential for cost cutting. The outcome is a gap in which upstream cell culture advances have outpaced downstream separation and purification advances. The challenge is to devise downstream purification technologies that are well integrated with upstream efforts to purify and recover therapeutic peptides. The design of efficient, economic and safe purification processes for proteins and peptides produced using recombinant DNA technology is a major challenge in bringing new biopharmaceuticals to the market. The pharmaceutical and biotechnology industry needs to continue to evolve towards technology integration and market expansions.

This study investigated the potential of the *Yarrowia lipolytica* as a robust alternative platform for expression of peptides and proteins for application in the therapeutic fields. *Yarrowia lipolytica*, with a Generally Regarded As Safe (GRAS) status, is a prolific producer of heterologous proteins (Madzak *et al*., 2004), and is currently a subject for glycoengineering studies to enable it to produce proteins with human like N-linked glycans (Song *et al*., 2007). The successful engineering of *Y. lipolytica* to produce peptides and proteins that have human like posttranslational modifications (Jolivet *et al*., 2007) could result in the generation of peptides with improved therapeutic properties such as serum half lives (Zalevsky *et al*., 2010) alleviating some of the problems identified to be drawbacks during patient treatments with peptide and protein based therapeutic drugs.

Yarrowia lipolytica is notable for production to high levels and to the extracellular the lipase protein, LIP2 (Fickers *et al*., 2005; Pignede *et al*., 2000a, 200b). The fermentation processes for industrial scale production of the LIP2 for application in food industry are documented in literature (Pignede *et al*., 2000a, 2000b). In addition, genetically modified strains capable of enhanced production of Lip2p have also been described in literature (Mauersberger *et al*., 2001; Fickers *et al*., 2005) suggesting that optimised fermentation processes of genetically modified *Y. lipolytica* strains could result in even higher yields of the Lip2p in the extracellular medium. It could be hypothesized that Lip2p could be used as a carrier protein for production and release to the extracellular therapeutic peptides. To demonstrate this concept, the LIP2-based expression and secretion system was used with the RANTES (**R**egulated upon **A**ctivation, **N**ormal **T** cell **E**xpressed and presumably **S**ecreted) peptide as a model therapeutic peptide.

The RANTES peptide belongs to the family of chemokines and plays a primary role in the inflammatory immune response via its ability to chemoattract leucocytes and modulates their function (Calderon and Berman, 2005). Human RANTES cDNA encodes a 91 amino acid residue precursor polypeptide with a 23 amino acid signal peptide that is cleaved to generate the 68 amino acid mature protein (Schall *et al*., 1988; Schall *et al*., 1991). RANTES, is known to interact with four identified seven transmembrane G-protein coupled receptors: CCR1, CCR3, CCR4 and CCR5 (Neote *et al*, 1993; Ponath *et al*, 1996, Power *et al*, 1995; Combadiere *et al*, 1996). The RANTES receptor CCR5 is also the primary co-receptor for R5 (M-tropic) variants of HIV-1 (Deng *et al*., 1996; Alkhatib *et al*., 1996). It has been demonstrated that RANTES can competitively inhibit CCR5/HIV-1 interaction and suppress viral infection in vitro (Cocchi *et al*., 1995; Trkola *et al*., 1998). These effects apparently do not require fully intact signalling from the CCR5 receptor (Simmons *et al*., 1997). Consequently, modified forms of RANTES that block the interaction of HIV-1 with CCR5 are being considered as therapies against HIV infection (Simmons *et al*., 1997; Baba *et al*, 1999; Scozzafava *et al*., 2002; Pastore *et al*., 2003). It has been demonstrated that small synthetic peptides are able to mimic large proteins (Kawai *et al*., 1991; Hayashi *et al*., 1995). In concert with these findings, several studies have demonstrated that the entire RANTES sequence is not required to block infection; peptide fragments are able to mimic the inhibitory effect of the entire protein (Nishiyama *et al*., 1997). Both the Ac [Ala^{10}] RANTES-(1-10)NH_2 {RANTES I} and Ac[Ala^{10}, ^{11}] RANTES-(5-14)NH_2 {RANTES II} have been shown to display significant

antiviral activity with the latter fragment being significantly less potent (Nishiyama Y *et al*., 1999). The RANTES I peptide was selected as a model to be expressed in *Y. lipolytica*. RANTES has been produced previously by fusion to the Maltose Binding protein (MBP) in soluble form in *E. coli* (Cho *et al*., 2008). However, given the possibility of co-producing pyrogens, *E. coli* is not an ideal system. To facilitate downstream processing of the RANTES peptide, the enterokinase cleavage site was introduced between the peptide sequence and the LIP2 carrier protein together with the sequence encoding 6XHis for protein purification using Nickel affinity chromatography.

2.2. Materials and Methods

2.2.1. Three dimensional modelling of LIP2 *and* RANTES

The three-dimensional structure of the LIP2-RANTES fusion protein was predicted by SWISS-MODEL (Arnold *et al*., 2006). The amino acid sequences coding for *Y. lipolytica* LIP2 and RANTES were submitted for a search against the Protein Data Bank using the PSI-BLAST algorithm (Bordes *et al*., 2009). Three lipases with known 3D structures were identified as having a significant homology with LIP2. The identified lipases belong to the fungal lipase family: lipases from *Rhizomucor miehei* (sequence homology 46 %), *Rhizopus niveus* (sequence homology 47 %) and *Thermomyces lanuginose* (sequence homology 47%). Similarly, the RANTES amino acid sequence was submitted for a search against the Protein Data Bank using the PSI-BLAST algorithm. It displayed 44% sequence homology with the anti-HIV chemokine variant P2-RANTES (Jin *et al*., 2010). The identified 3D-structures were used as templates to model LIP2 and RANTES. The two 3D-structures were used to predict the overall LIP2-RANTES 3D-structure.

2.2.2. Strains, plasmids, reagents, and growth conditions

Escherichia coli XL10-Gold® strain (Stratagene, La Jolla, USA) was used for transformation and amplification of recombinant plasmid DNA. Cells were grown in Luria-Bertani medium (Sambrook *et al*., 1989) supplemented with ampicillin (100 $\mu g.mL^{-1}$, Roche, Mannheim, Germany) or kanamycin (40 $\mu g.mL^{-1}$, Sigma-Aldrich, St. Louis, MO) for plasmid selection. *Yarrowia lipolytica* strains used in this study are listed in Table 3.1. They were grown at 28°C in YPD (Yeast extract, Peptone and Glucose (1% w/v) of each), YPG (Glucose was replaced with glycerol (1% v/v) and YNBcasa (1.7 $g.L^{-1}$ of YNB without $(NH_4)_2SO_4$ and amino acids (Difco, Detroit, MI), 4 $g.L^{-1}$ NH_4Cl, and 0.2% (w/v) Casamino acids). For solid media, 15 g of agar per liter

was added. Unless otherwise stated, all the chemicals were from Merck (Wadeville, South Africa). Restriction enzymes and T4 DNA ligase were from Promega (Madison, USA) and New England Biolabs (Beverly, MA). The oligonucleotides used were from Integrated DNA Technologies (Coralville, USA) and are listed in Table 2.1 written in the 5′-3′ orientation. PCR products were subcloned into pGemT-Easy (Promega) and p410 (Labuschagne and Albertyn, 2007).

Table 2.1 Plasmids, strains and oligonucleotide primers used in the study

Plasmids, strains, primers	Genotype, primer (5´- 3´)	Reference, source, purpose
Plasmids		
pGemlip2	pGemT containing *Y. lipolytica LIP2* and 6X His tag.	This study
pGemlip2-R	pGemlip2 containing the enterokinase cleavage and RANTES coding sequences	This study
p410	Yeast expression vector carrying the 26S rDNA fragments for targeting to the rDNA clusters, selection marker *Ura3d4*, Kan^R, hp4d promoter and *LIP2* terminator	This study
p410*LIP2*	*LIP2* cloned under hp4d	This study
p410*LIP2*-R	p410 containing the LIP2-RANTES expression cassette	This study
Yarrowia lipolytica strains		
Y. lipolytica W29	*MatA (ATCC 20460, CLIB89)*	Madzak *et al.*, 2000 2004
Y. lipolytica Po1f	*MatA*, *leu2-270*, *ura3-302::URA3*, *xpr2-322*, *axp1-2*	Madzak et al., 2000 2004
Yl410	*Y. lipolytica* Po1f strain transformed with p410	This study
Yl414	*Y. lipolytica* Po1f strain transformed with p410*LIP2*	This study
Yl415	*Y. lipolytica* Po1f strain transformed with p410*LIP2*-R	This study
Primers		
Lip2-3F	ATG*AAGCTT*TCCACCATCCTTTTCAC	*Hind*III
Lip2-1018 HisR	*CCTAGG*TCATTA*ATCGAT* GTGGTGGTGG TGGTGGTGGATACCACAGACACCCTCGG TGACGAAGTA	*Avr*II/*Cla*I
RANTESR	TTAAGACATCTCCAGGGAGTTGATGTAC	Screening

2.2.3. Nucleic acid isolation and manipulation

Escherichia coli transformation and other Recombinant DNA techniques were performed as described by Sambrook *et al.* (1989). *Yarrowia lipolytica* genomic DNA was isolated from 5 ml YPD cultures grown for 48 h with shaking as described

previously (Labuschagne *et al*., 2007). PCR amplifications were performed on an MJ Mini Gradient Thermal Cycler (Biorad, Hercules, USA) with Expand High Fidelity^{PLUS} (Roche) or Phusion DNA polymerase (Finnzymes, Finland). PCR and gel band purifications were performed using the Biospin PCR DNA and Gel Band Purification Kits (Bioer, Tokyo, Japan). Plasmid purifications were done using the Biospin plasmid Purification Kit (Bioer). Clones were sequenced at Inqaba Biotech (Pretoria, South Africa).

Nucleotide sequences were analysed using Chromas 2.33 (http:www.technelysium .com.au.chromas.html) and compared with sequences in the database at NCBI using BLAST (http://www.ncbi.nlm. nih.gov/blast/BLAST.cgi) (Altschul *et al*., 1997) against the *Y. lipolytica* RST and genome databases at the genolevures sites (http://cbi.labri.fr./Genolevures and at http://www.ncbi.nlm.nih.gov/BLAST/). Amino acid sequence alignments were performed using DNAssist Version 3.0. The plasmid diagram was constructed using Clone Manager (http://www.scied.com).

2.2.4. Construction of the LIP2-RANTES expression vector

The DNA fragment encoding *LIP2* was amplified excluding its stop codon from the *Y. lipolytica* W29 genomic DNA with primer pair Lip2-3F and Lip2-1018HisR (Table 3.1). The primers introduced *Hind*III and *Cla*I/*Avr*II restriction sites at the 5´ and 3´ ends of the amplicon, respectively. The thermal cycling reaction was performed as follows; initial denaturation at 94°C for 2 min, 30 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 2 min), and a final extension step (72°C for 10 min). The amplicon was purified with the PCR purification kit and subcloned into pGemT-Easy. A RANTES encoding DNA fragment was synthesized based on *Y. lipolytica* preferred codons (Geneart, Regensburg, Germany). Recognition sequences for *Cla*I and *Avr*II were incorporated in the 5´and 3´ ends, respectively. The fragment was fused to *LIP2* as a *Cla*I-*Avr*II fragment. The LIP2-RANTES open reading frame was released from pGemT-Easy as a *Hind*III-*Avr*II fragment and subcloned into pKOV410 for expression (Figure 3.1).

2.2.5. DNA transformation

Yarrowia lipolytica was transformed by the lithium acetate method as described previously by Labuschagne and Albertyn (2007). The expression vector (5 ng) was digested with *NotI* and subjected to electrophoresis. The bands corresponding to the expression cassette was extracted from the gel and used for transformation. Typically, 0.05 or 2 ng of the expression cassettes was used to transform the *Y.*

lipolytica Po1f strain and transformants were selected on YNBcasa agar. Genomic DNA was used to perform PCR using Lip2-3F and RANTESR (Table 2.1) to confirm integration of the expression cassette.

2.2.6. Expression of LIP2-RANTES in shake flask cultures

Shake flask cultivations were performed in 500 ml Erlenmeyer flasks using *Y. lipolytica* Yl410 and Yl415 (Table 2.1) that were maintained as cryopreserved cultures at -70°C. Single cryovials containing the clones (1.5 ml) were used to inoculate triplicate 500 ml Erlenmeyer flasks each containing 100 ml of YPD or YPG medium. The pH of the medium was adjusted to 6.8 prior to autoclaving at 121°C for 15 min. Erlenmeyer flasks inoculated with the strains were subsequently incubated at 28°C on a rotary shaker at 210 rpm for a period of 144 h. The growth profile was monitored over the cultivation period and the supernatants were either analysed immediately or stored at ± 20°C for future analyses.

2.2.7. Purification of histidine-tagged LIP2 gene products from Y. lipolytica

Three milliliters of nickel resin, NI-NTA, was added to 30 ml of yeast extracellular media obtained from *Yarrowia lipolytica* Yl414. The mixture was agitated for 3 h at 4°C. The mixture was poured into an empty syringe cartridge with a filter at the bottom. This separates the resin from the extracellular media. Any histidine tagged protein in the media i.e. lipase will bind to the resin through the nickel. Non histidine tagged proteins will wash away as part of the flow though fraction. Histidine tagged lipase or Lip2-RANTES bound to the nickel resin was eluted using different concentrations of imidazole (10 mM, 20 mM, 50 mM, 80mM, and 100 mM) dissolved in 5ml of buffer made up of Tris/HCl pH 8 and 20mM NaCl. Non-specific proteins bound to the resin were washed away from the resin with 10ml of buffer without any imidazole.

2.2.8. SDS–PAGE analysis of proteins

Supernatant and purified fractions of the cultures were harvested and subjected to acetone precipitation. Acetone was added to 300 µl of the supernatants in a ratio of 3:1 (v/v). Samples were centrifuged at 12,000 *g* for 20 min in a microcentrifuge to pellet the precipitated proteins. Aliquots of total protein in 10 µl of deionized water were mixed with 10 µl of 2X Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 10% SDS and 5% β-mercaptoethanol. The mixture was boiled for 10 min prior to SDS-PAGE analysis. Prestained molecular weight markers (Page ruler™

prestained protein ladder, Fermentas, Canada) were loaded on 12.5% (v/v) polyacrylamide gels and separated at 100 V for 1 h. The gels were stained with Bio-Safe Coomassie G-250 dye (Bio-Rad).

2.2.9. Bradford Assay for protein quantification

To estimate the total amounts of protein in the supernatants of *Y. lipolytica* Yl410 and Y1415, the dye-binding assay described previously by Bradford *et al*, (1976) was used. Briefly, Coomassie G-250 dye (100 mg) was dissolved in 50 ml of methanol. The solution was added to 100 ml of 85% (v/v) H_3PO_4, and diluted with water. The assay reagent was prepared by diluting 1 volume of the dye stock with 4 volumes of distilled H_2O, filtered through Whatman 540 paper. Bovine serum albumin (BSA) was selected as a protein standard and a standard curve was generated using concentrations 0, 10, 20, 30, 40, 50 $\mu g.mL^{-1}$ for the microassay. The Beckman DU 800 spectrophotometer was set to collect the spectra over a wavelength range from 400 to 700 nm and over an absorbance range of 0 to 2 Absorbance units, and to overlay the collected spectra. The absorbance spectra of the samples were recorded from 400 to 700 nm, particularly noting absorbance at 595 nm wavelength. A graph of Absorbance at 595 nm vs protein concentration was prepared for the protein standards. Optical density values obtained from the samples (supernatants from Yl410 and Yl415) were used to estimate the protein concentration values from the standard curve.

2.2.10. ELISA to detect RANTES in the supernatant

Levels of CCL5/RANTES were determined using a mouse CCL5/RANTES ELISA development system (R & D Systems®, Minneapolis, Minnesota). Briefly, monoclonal antibody MAB678 (R & D Systems®) was used to coat 200 ng anti-RANTES to a 96-well microtiter plate (Dynex Technologies, Chantilly, VA) for 16 h at 4°C. The plate was washed three times with ELISA wash buffer (1XPBS + 0.1% (v/v) Tween 20). The non-specific sites were blocked with 200 µl blocking buffer (phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 and 5% (w/v) dry milk), followed by agitation on a rotary shaker for an hour. This was followed by three more washes with the ELISA wash buffer. The RANTES protein standard (278RN-rhCCL5/RANTES) was prepared by serial dilutions. The test samples (supernatants from Yl410 and Yl415) were also subjected to a series of dilutions in triplicate. The diluents were washed with the ELISA wash buffer three times. N-RANTES IgG biotinylated anti-RANTES antibody BAF278 (R & D Systems®) at a concentration of 1

µg.mL^{-1} in blocking buffer was added and incubated for an hour on a shaker. Another set of washes was done on the plate. Peroxidase-conjugated goat anti-rabbit Ig: Streptavidin-HRP (R & D Systems ®) (125 ng.mL^{-1} in blocking buffer) was added and the mixture was incubated for 30 min. Three more washes were done on the plate. The 3,3,5,5-tetramethylbenzidine (TMB) liquid substrate used to detect the amount of bound Ig was added. The plate was incubated at 22°C for colour development. The reaction was stopped by the addition of 1 M H_2SO_4. Spectrophotometer readings were done at 450 nm, and the absorbance values were compared with a standard curve generated with serial concentrations. The values were used to calculate the concentrations of RANTES present in the supernatant of *Y. lipolytica* Yl410 and Yl415.

2.2.11. Separation of RANTES from LIP2-RANTES

The Ni-NTA chromatography purified LIP2-RANTES was loaded onto a protein concentrating column (MWCO 10 kDa) (Separations, Johannesburg, South Africa) to remove the salts and imidazole that inhibit enterokinase activity. The sample was washed with the enterokinase capture buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 2 mM $CaCl_2$, 50% (v/v) glycerol) to exchange the buffer used to elute the protein during Ni-NTA chromatography purification. After buffer exchange, the sample was resuspended in enterokinase cleavage buffer (200 mM Tris-HCl pH 7.4, 500 mM NaCl, 20 mM $CaCl_2$). The sample was treated with enterokinase at 16°C for 16 h. The cleavage products were loaded onto another protein concentrating column (MWCO 10 kDa) to separate the peptide from LIP2, enterokinase and undigested LIP2-RANTES. The retentate and eluate were analysed on 12.5% (v/v) SDS gel.

2.2.12. Peptide mass finger-printing

The peptides containing segment of the SDS gel were excised and cut into small chips. The gel chips were destained by washing in 20 µl aliquots of 50 mM $(NH_4)_2CO_3$ in 30% (v/v) acetonitrile for 1 h repeatedly until they were colourless. The samples were dried in a centrifugal evaporator and treated as described by Yan *et al*., (2000). Modified (methylated) porcine trypsin (Promega, Madison, USA) was prepared as a stock solution in water (0.1 µg.µL^{-1}). For digestion, 4 µl of trypsin solution were added to 21 µl of Tris buffer (5 mM pH 8.8, prepared fresh for each use) and added to the gel pieces before incubating overnight at room temperature. Digestion was stopped by addition of 15 µl of 50% acetonitrile, 0.1% TFA. MALDI-MS was performed using a QSTAR® Elite mass spectrometer (Applied Biosystems Inc., Ontario, Canada)

operated in the positive ion reflection mode at 20 kV accelerating voltage with 'time-lag focusing' enabled. Peptide mass maps were searched against SWISS-PROT/TrEMBL release 35, using Protein Probe (Micromass), or against a non-redundant database maintained by the National Center for Biotechnology Information (NCBI) using the Mascot (Matrix Science Inc., Boston, MA, USA) search engine (Helsens *et al*., 2007).

2.2.13. RANTES activity assays

The neutralization assay was performed as described previously by Montefiori (2004). This assay measures neutralization of viral particles as a function of reductions in Tat-regulated Luc reporter gene expression after a single round of infection in TZM-bl cells. Briefly, a 96-well plate (NUNC, Roskilde, Denmark) was seeded with 10,000 actively growing cells and incubated overnight at 37°C. Different concentrations of RANTES were prepared on another plate. The media was removed from the cells, and the prepared RANTES was transferred to the plate containing TZM-bl cells. The mixture was incubated for an hour and the dextran containing the virus was added. The mixture was incubated at 37°C in 5% CO_2 for 48 h. The media was removed from each well and discarded. Bright Glo^{TM} Reagent (Promega, Madison, USA) was added to each well, and the plate was incubated at room temperature for 2 min to allow complete lysis. The mixture was mixed by pipet action and transferred to a corresponding 96-well plate. The samples were analysed immediately in a Luminometer. The ID_{50}/IC_{50} (the reciprocal concentration where 50% of the virus is neutralized by the test sample) was calculated.

2.2.14. RANTES toxicity assays

Cell viability studies were also conducted to confirm if the activity obtained in the efficacy tests was due to the peptide. A 96-well plate (NUNC) was seeded with 10,000 actively growing TZM-bl cells and incubated overnight at 37°C. The media was removed from the cells. Maraviroc, which served as a control, and RANTES I were added to the plate containing the cells, and the mixture was incubated further for 48 h at 37°C. A CellTiter 96$^{®AQueous}$ Non-Radioactive Cell proliferation Assay kit (Promega) was used to determine toxicity effects of the peptide and the controls on the cells.

2.3. Results

2.3.1. Three dimensional modelling of LIP2 and RANTES

A search of the Protein Data Bank with the PSI-BLAST algorithm using the *Y. lipolytica* Lip2p sequence as query yielded the structures of the lipases from *R. miehei* and *Rh. nieveus* with 29 and 33% sequence identity, respectively (Bordes *et al*., 2009). In a similar fashion, the RANTES sequence showed a 37% sequence identity with the anti-HIV chemokine variant P2-RANTES (Jin *et al*., 2010). These proteins were selected and used as templates to design the 3-D models of Lip2p and RANTES using the SWISS-MODEL (http://swissmodel.expasy.org/). The models revealed protein structures were the C and N terminus of Lip2p and RANTES are characterised by free loops (Figure 2.1). The freely hanging loops show that joining the two proteins generated a structure with the junction exposed.

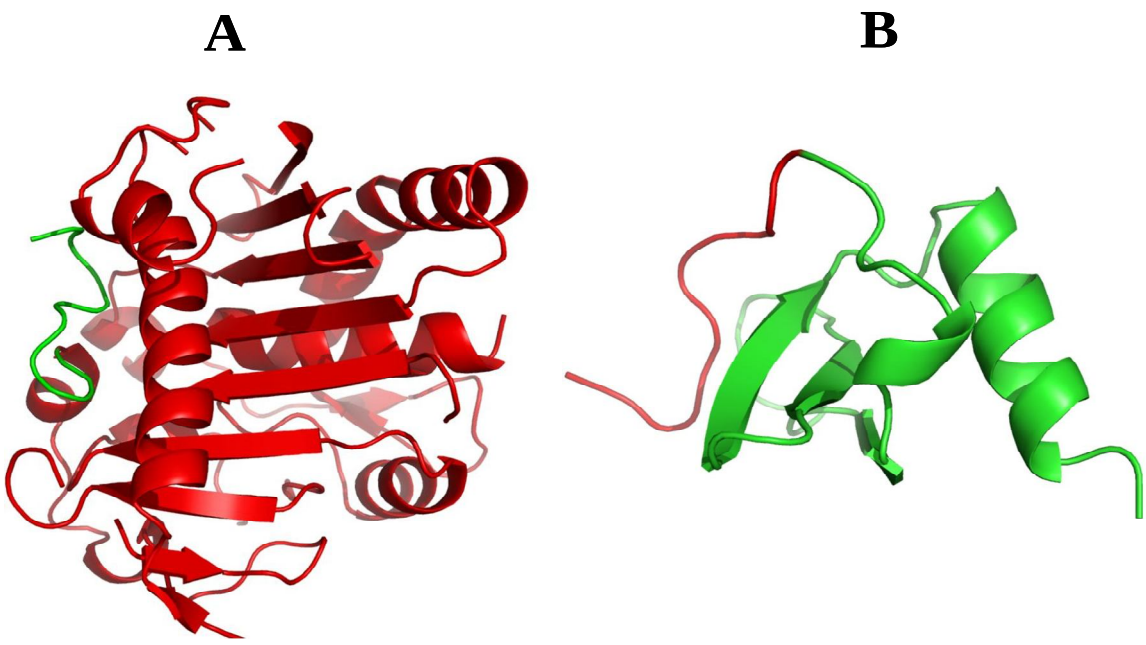

Figure 2.1. Computer models of the likely three-dimensional structures of (A) Lip2p and (B) RANTES. The interest was to obtain models illustrating the position of the C and N terminus of Lip2p and RANTES, respectively. The structures were used to predict the probable folding mechanism of Lip2-RANTES. Both the C and N terminus of the Lip2 and RANTES proteins are characterised by free loops, shown in green and red colours respectively. The 3D-structures were modelled using the SWISS-MODEL and the structures were analysed viewed with the program DeepView (Swiss Pdb-Viewer).

2.3.2. Cloning, sequence analysis and expression of the LIP2 gene

The amplification by PCR using *LIP2* specific primers and *Y. lipolytica* genomic DNA resulted in the generation of a 1000 bp DNA fragment that corresponded to the expected size of the *LIP2* gene. The PCR fragment was subcloned into pGemT-Easy vector to yield pGemT-LipHis gene in which the LIP2His open reading frame is flanked by *Hind*III and *Cla*I/*Avr*II restriction sites. Nucleotide sequence analyses revealed a 100% match at nucleotide level to the reference sequence containing the artificially introduced hexa histidine tag before the stop codon (Pignede *et al*, 2000). This Lip2His DNA fragment was sub-cloned into p410 to eventually yield the rDNA based multi-copy integrative vector p410*LIP2* (Figure 2.2). The *Y. lipolytica* Po1f strain transformed with p410*LIP2* (denoted Yl414) resulted in several clones exhibiting increased lipolytic activity towards the tributyrin substrate in the solid media, judged by the sizes of the clearing zones compared to a control strain harboring only the wild-type Lip2p activity (data not shown). The *Y. lipolytica* Y414 strain was grown in shake flasks in culture media containing glucose or glycerol as source of carbon. The production of lipase was observed in cultures grown in both the glucose and the glycerol medium. SDS-PAGE analysis of precipitated supernatant samples from *Y. lipolytica* Yl414 revealed the presence of a strong band in the expected size range of 38.5 kDa (Figure 2.3). The strain cultured in the growth medium containing glycerol resulted in predominantly a single protein band as judged by SDS-PAGE analysis while the growth on glucose medium resulted in several additional protein bands (Figure 2.3). The culture supernatant obtained from the *Y. lipolytica* Yl414 culture grown on YPG was subjected to Nickel affinity chromatography purification. A single protein band of about 38 kDa corresponding to the size of Lip2HIS protein was obtained on SDS-PAGE (Figure 2.4)

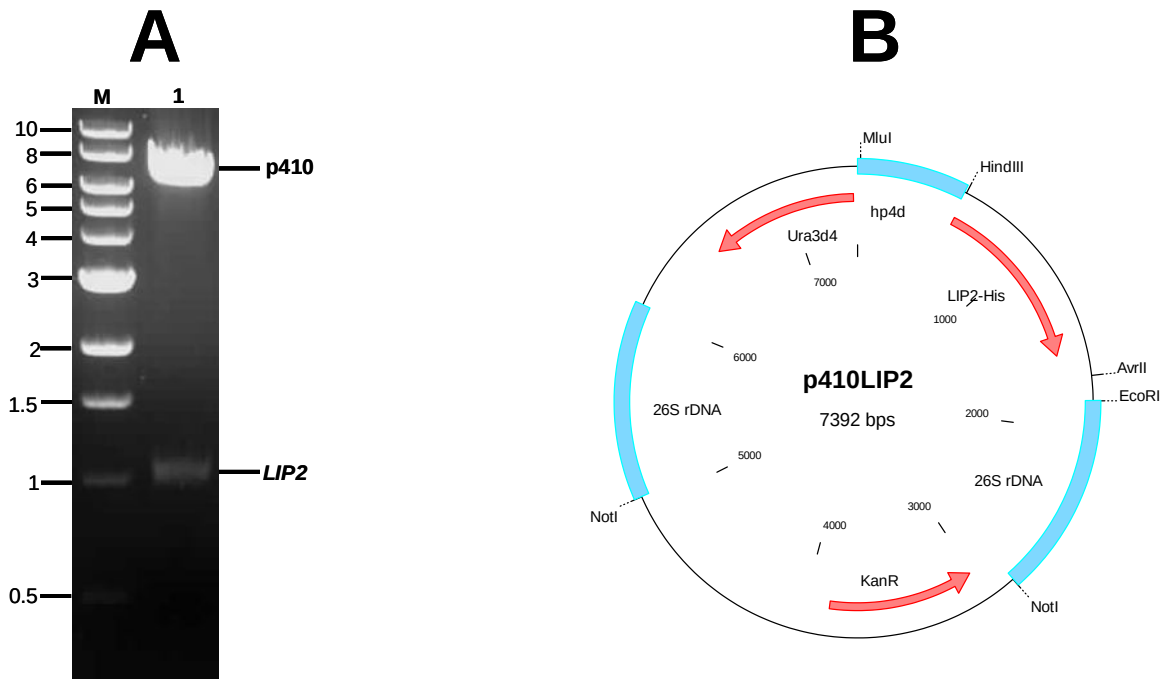

Figure 2.2. Construction of the Lip2p expression cassette. (**A**) A 1% (w/v) agarose gel to show the p410LIP2 construct digested with *Hind*III and *Avr*II in a single reaction. Digestion with the two enzymes resulted in the release of the lipase encoding fragment of 1 kbp from the p410 plasmid of 6.392 kbp. M = GeneRuler^{TM} (NEB) 1 kb DNA Ladder. (**B**) A schematic diagram indicating the p410 vector containing the *LIP2* gene under the hp4d promoter. The vector targets the expression cassette to the rDNA clusters upon transformation.

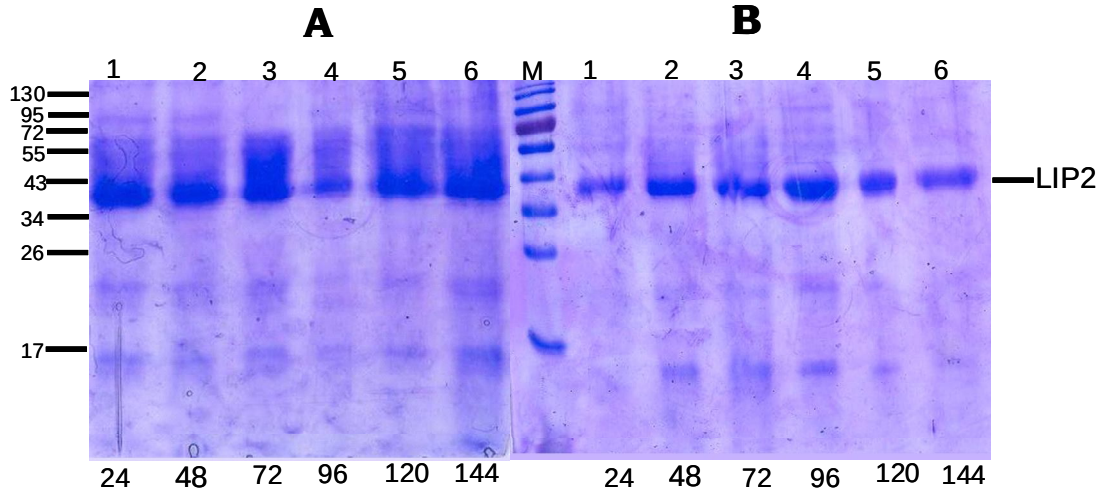

Figure 2.3. SDS-PAGE analysis of the supernatants from cultures of *LIP2* transformed *Y. lipolytica* YI414. The strain was cultured in (**A**) YPD (Glucose) and (**B**) YPG (Glycerol) media for six days. The supernatant was harvested and precipitated with acetone in a 3:1 ratio. Sampling times were 24, 48, 72, 96, 120, and 144 h (lanes 1 to 6, respectively). The gel was stained with Coomassie brilliant blue R-250. The precipitates were resolved on 12.5% SDS-PAGE gel. The band indicated by the arrow is representing the 38.5 KDa LIP2. M is the Pageruler^{TM} page ruler protein ladder (Fermentas) molecular weight standard in KDa.

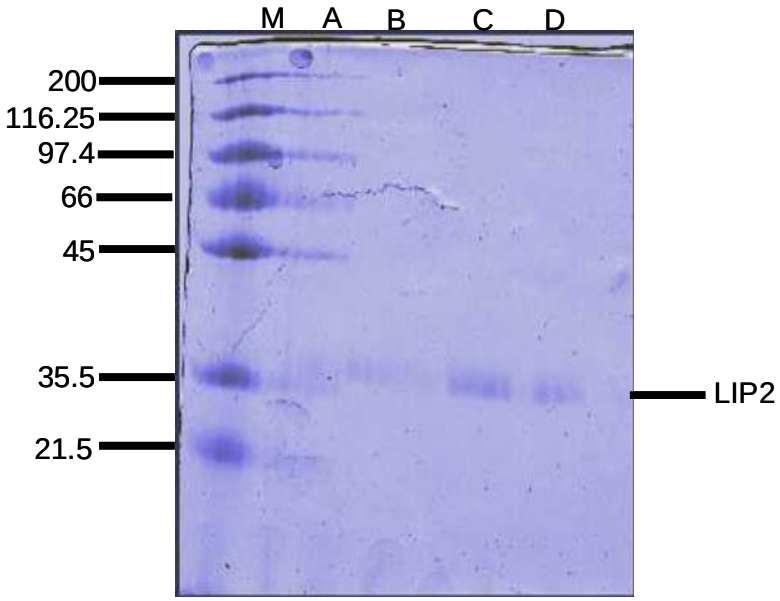

Figure 2.4. SDS-PAGE analysis of different fractions eluted from the nickel resin with different concentration of imidazole. M is the broad range molecular weight standards in kDa (Bio-rad). A is the 10 mM immidazole fraction, B the 20 mM fraction, C the 50 mM fraction and D the 80mM fraction.

2.3.3. Construction of LIP2-RANTES expression system

The *LIP2*-RANTES expression system was constructed by introducing to the 3´-end of the *LIP2* gene synthetic sequences encoding the 6XHis tag, the enterokinase cleavage site and the RANTES I in a single open reading frame. To enhance the possibility of the RANTES encoding gene to be efficiently transcribed, the coding regions were codon-optimized to mimic *Y. lipolytica* preferred codons. This was because one of the limitations facing the development of yeasts into successful recombinant production hosts is their inability to overcome differences in codon-usage. The fusion open reading frame was subcloned into p410 to create p410*LIP2*-R which placed the fused open reading frame under the hp4d promoter (Figure 2.5).

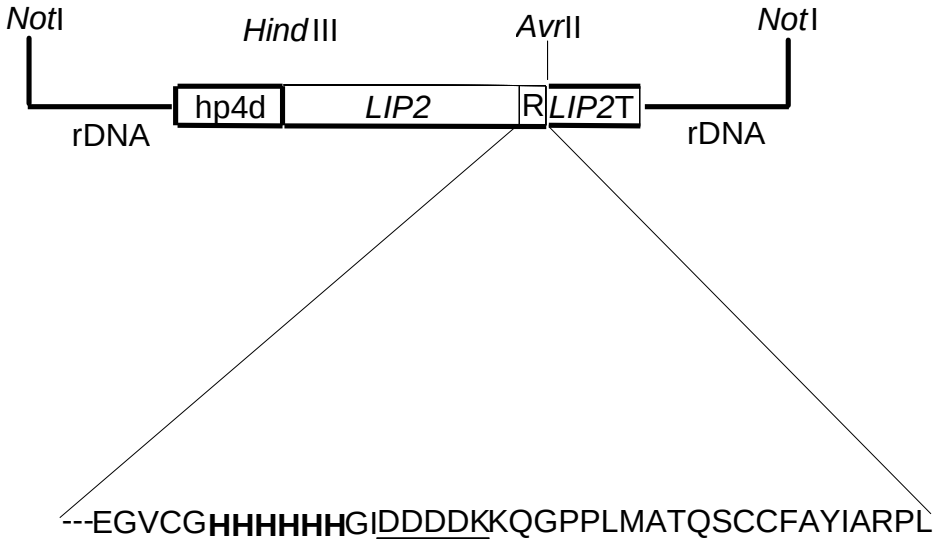

Figure 2.5. Schematic diagram of the Lip2-RANTES expression cassette. The expression cassette was constructed with the LIP2-RANTES fusion in frame with the hp4d promoter. The C-Terminus sequence of LIP2 is represented by (-) and the amino acids before the bolded 6XH residues representing the Histidine tagging sequence. The enterokinase cleavage site is indicated by the underlined DDDDK sequence. The sequence downstream represents part of the RANTES I encoding sequence.

The construction of the expression cassette was confirmed by restriction analysis using *Hind*III, *Cla*I and *Avr*II restriction enzymes resulting in the expected DNA fragments of 0.6, 1.1, 1.8 and 6 kbp (Figure 2.6).

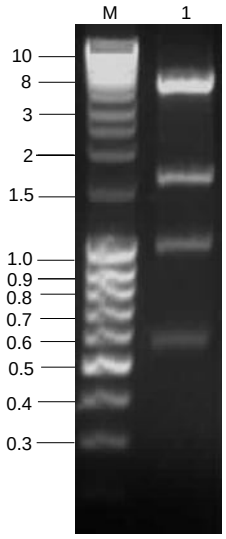

Figure 2.6. A 1% (w/v) agarose gel showing the DNA fragments generated by digestion of p410*LIP2*-R. The plasmid was digested with *Hind*III, *Cla*I, and *Avr*II. The expected four bands of 0.6, 1.1, 1.8 and 6 kbp were obtained in lane 1. M = GeneRuler^{TM} (NEB) 1 kbp DNA Ladder.

The p410*LIP2*-R expression cassette was transformed into *Y. lipolytica* Po1f targeting the *Y. lipolytica* rDNA through the rDNA sequences of the expression cassette. The presence of the p410*LIP2*-R within the *Y. lipolytica* genome sequence was confirmed by PCR using primers derived from the hp4d promoter and the LipT terminator sequences and the expected DNA fragment of ~1.57 kbp was obtained (data not shown). The PCR fragment was cloned into pGemT-Easy vector and sequenced to confirm the in-frame arrangement of the fused open reading frame with the correct integrant denoted *Y. lipolytica* Yl415. The *Y. lipolytica* Po1f strain was transformed with p410 to serve as the negative control and the strain was denoted *Y. lipolytica* Yl410. *Yarrowia lipolytica* Yl410 produced low levels of extracellular lipase, and this in consensus with the findings of Pignede and co-workers (2000a) were Po1d produced low levels of lipolytic activity when compared to the transformant when they were grown on rich media.

2.3.4. Production of LIP2-RANTES *in Y. lipolytica*

The *Y. lipolytica* Yl415 and Yl410 strains grew to comparable maximum optical densities when cultured in YPG medium. The production of extracellular lipase activity was observed in *Y. lipolytica* Yl415 where maximum activity of ~10 $U.mL^{-1}$ was detected after 66 h of incubation (Figure 2.7). The total extracellular protein concentrations determined by the Bradford assay using BSA as standard were 10.03±0.015 and 2.89±0.032 $\mu g.mL^{-1}$ for the *Y. lipolytica* Yl415 and Yl410 strains, respectively. The amount of Lip2-RANTES fusion protein was thus estimated to be 7 $\mu g.mL^{-1}$. The amount of RANTES contained in the growth culture supernatant was determined to be 274 $pg.mL^{-1}$ using the human CCL5/RANTES immunoassay.

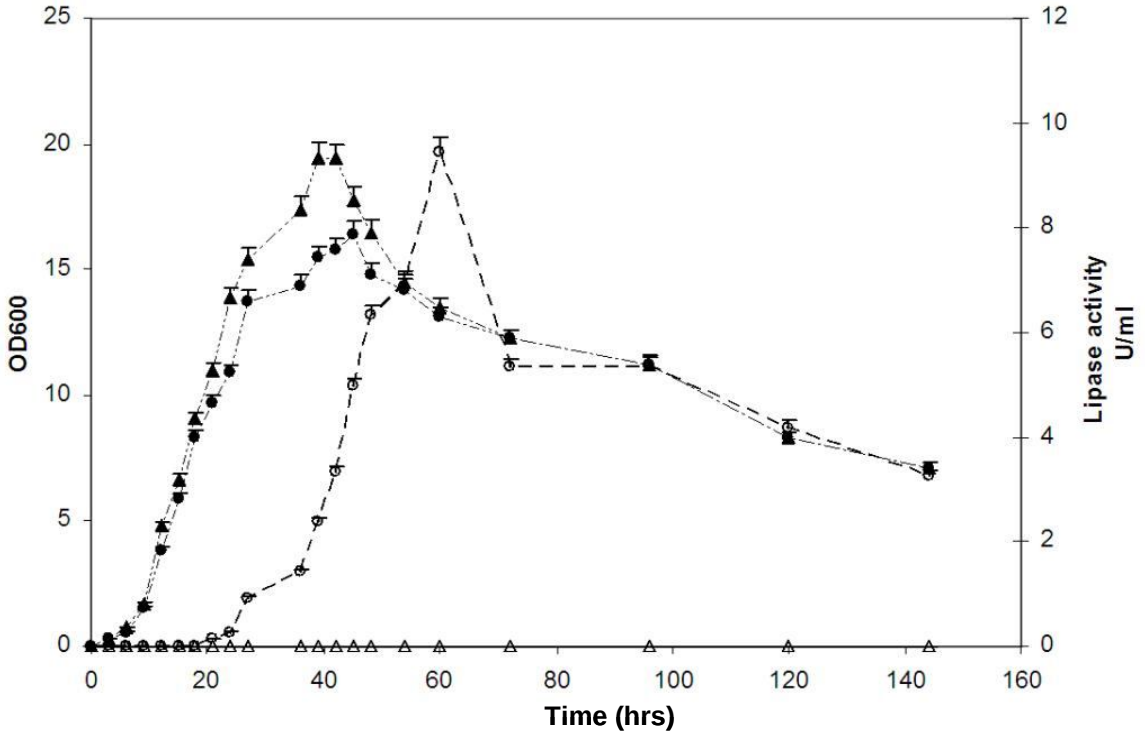

Figure 2.7. Growth and production of extracellular lipase activity by Yl410 and Yl415 [YlLIP2-RANTES] strains grown in YPG medium. The growth of *Y. lipolytica* Yl410 is represented by solid triangle (▲) while lipase production of Yl410 is denoted by the open triangle (∆). The growth of *Y. lipolytica* Yl415 is represented by the closed circle (●) and the production of the lipase-RANTES fusion by theYl415 strain is represented by the open circle (○).

2.3.5. Purification and peptide finger printing of the RANTES I

The cell free culture from *Y. lipolytica* Yl415 was harvested and treated with enterokinase to release the RANTES peptide fragment. The expected sizes of 38 and 4.5 kDa corresponding to the Lip2His and the RANTES peptide were observed on SDS-PAGE gel although the relative amount of the RANTES I peptide was proportionally higher than the Lip2His protein (Figure 2.8A). The RANTES peptide was separated from the Lip2His by passing the mixture through the column with a 10kDa molecular size cut off membrane. The flow through which contained RANTES peptide was concentrated by passage through a 7 kDa molecular weight cut off membrane and a protein fragment of about 4.5 kDa could be observed on SDS-PAGE gel (Figure 2.8B).

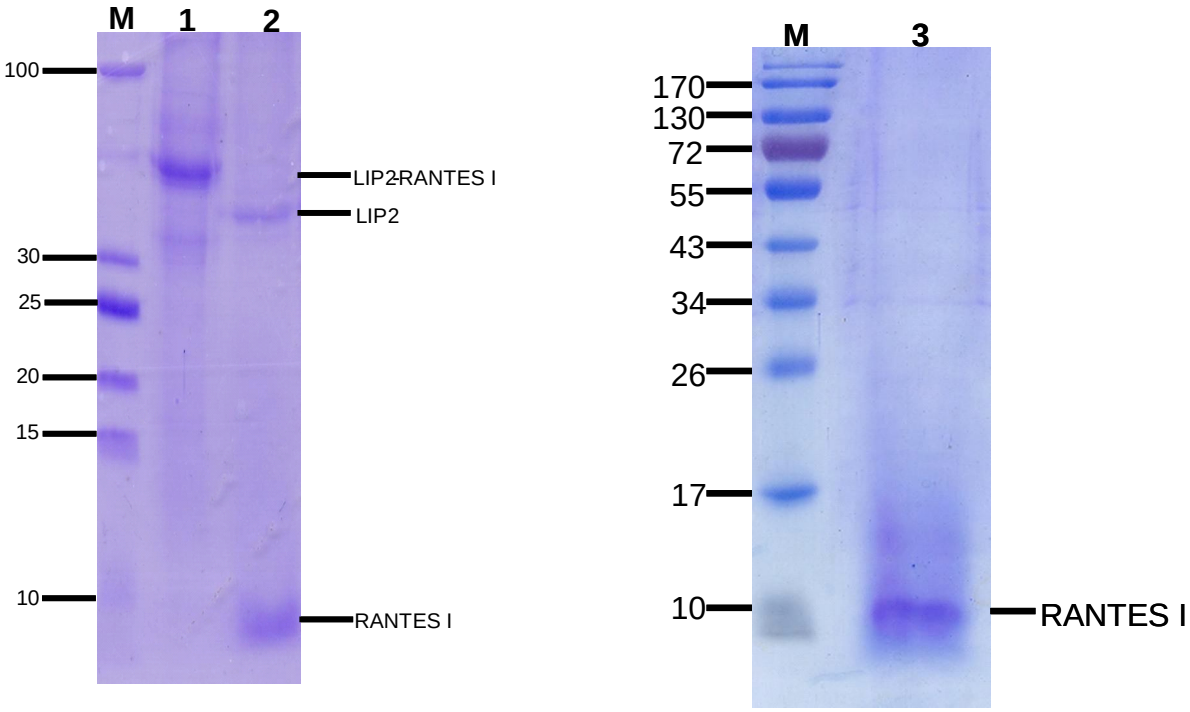

Figure 2.8. SDS-PAGE analysis of the supernatants from *Y. lipolytica* YI415. The cell free culture from YI415 was harvested and treated with EK as described. The cleavage mixture was loaded onto a 10 kDa MWCO column to separate RANTES from LIP2. RANTES was concentrated by loading the 10 kDa MWCO eluent onto a 7 kDa MWCO column. M = Pageruler^{TM} protein ladder (Fermentas) molecular weight standards in KDa, lane 1 the supernatant fraction from *Y. lipolytica* YI415, lane 2 is the enterokinase cleavage products showing the separation of the RANTES peptide from Lip2p, lane 3 is the purified RANTES.

The cleaved peptides were subjected to peptide fingerprinting using MALDI-TOF-MS. Analysis of the data through Mascot BLAST searches revealed that the peptide belonged to the family of RANTES as it aligned with synthetic RANTES (Swiss-Prot. accession number 1B3AA) giving a 60% sequence identity match. Database searches were conducted on Mascot using the following parameters: all species in the NCBI database, trypsin digestion allowing for up to two missed cleavages and 0.1 Da tolerance for MS and MS/MS, peptide charges of +1, +2 and +3 and monoisotopic masses. The profiles of unknown peptides were compared with theoretical peptide libraries generated from sequences in the different databases (Barrett *et al*., 2005). Table 2.2 lists the hits obtained from the Mascot BLAST search. High-score identifications were obtained from the Swiss-Prot database for chemokines and Lip2p. Two different fingerprinting profiles were observed (Figure 2.9). The profile patterns illustrate a difference in the arrangement of the amino acids. The two are presumably representing the peptides corresponding to RANTES and LIP2. RANTES was identified in the group of chemokine proteins listed in Table 2.3.

Table 2.2. Proteins detected from the NCBI database by Mascot search. RANTES was classified under the proteins in the group of accession number CAD32620.

Accession number	Protein name	Species	Peptides (#)
Q9P8F7	Triacylglycerol lipase precursor	Y. l	4
TRPGTR	Trypsin	Pig	3
Q6KFR8	Putative ubiquitin	A. t	2
CAD32620	Sequence 23 from patent		
	WO0220615 (putative cytokine A27)	Mus sp	1
KRHU2	Keratin 1, type II, cytoskeletal	Human	1
Q29M10	Acylphosphatase	D.p	1
WNIPA	Protein W	Nipah virus	1

Y. l = *Yarrowia lipolytica*; A. t = *Arabidopsis thaliana*, D. p = *Drosophila pseudoobscura*. # = Number of peptide fragments identified.

Table 2.3. Protein group of chemokines identified. (One of the excised peptides displayed a 60% sequence similarity with the peptide from accession number 1B3AA).

Accession number	Protein name	Species
AAP36897	Homosapiens chemokines (C-C motif)	Synthetic
AAN76984	Chemokine (C-C motif) ligand 5	M.m
AAF73070	C-C motif chemokine 5	Human
A28815	Monocyte chemoattract RANTES	Human
1HRJA	RANTES, Chain A	Human
1B3AA	RANTES, Chain A	Synthetic

M.m = *Macatta mulatta*

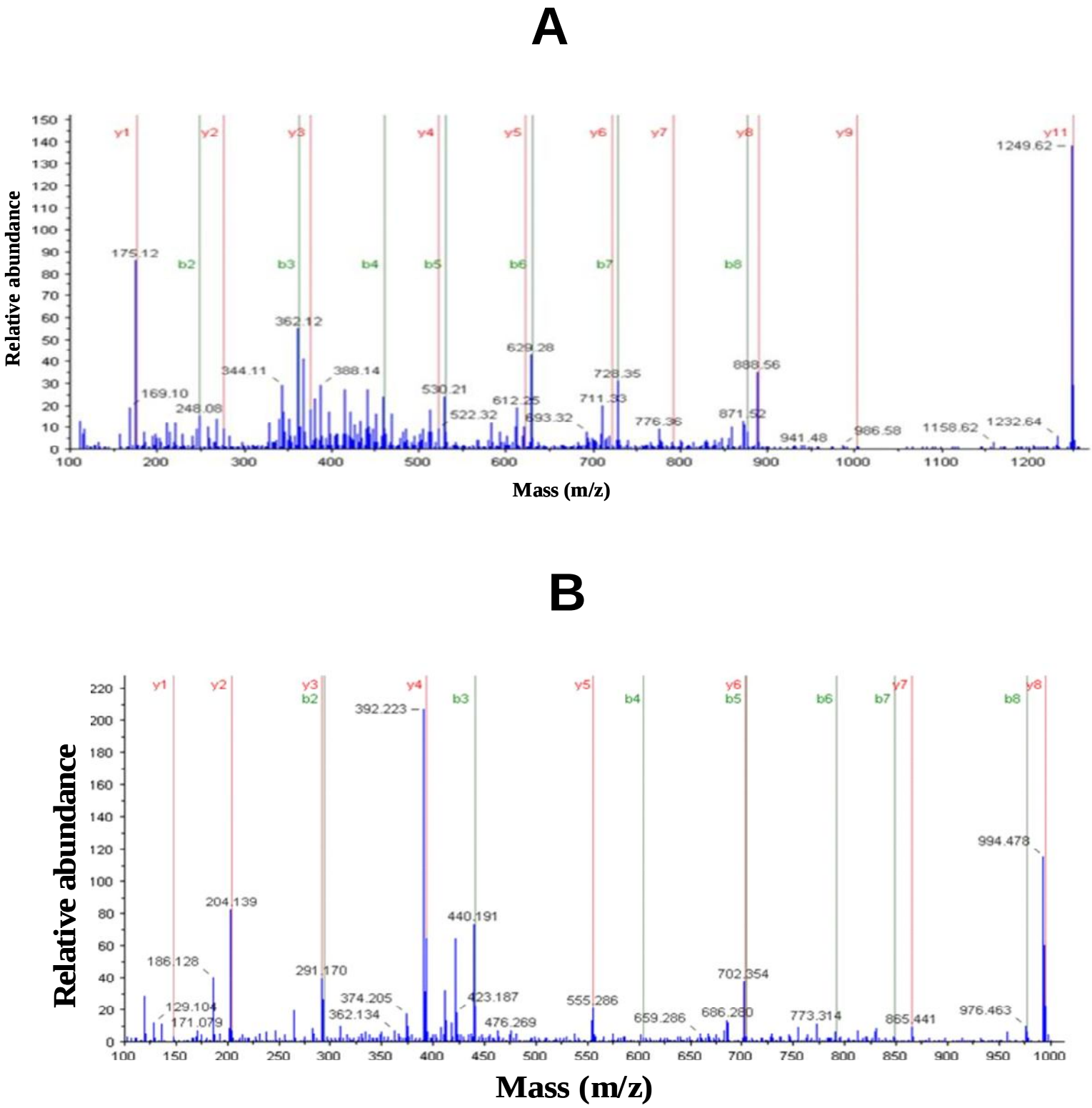

Figure 2.9. MALDI-TOF mass spectra of peptide samples collected from *Y. lipolytica* YI415**.** The profiles were generated by the peptides excised from the SDS gel and digested with trypsin before analysis by mass spectrometry. Two different mass profiles were observed and they presumably correspond to RANTES and Lip2p peptides as revealed by Mascot BLAST searches alignments. The blue coloured peaks are representing the identified amino acids.

2.3.6. RANTES I functionality assay

RANTES was assessed for its ability to neutralise the infection of TZM-bl cells by two model viruses. The antiviral activity was tested against Vesicular Stomatitis Virus-V (VSV-V) and the HIV-type 1 subtype B viral strain, QHO692.42. The inhibition of entry of viral particles into TZM-bl cells was measured by reduction in luciferase activity. TZM-bl cells are CXCR4-positive HeLa cell clones that were engineered to express CD4 and CCR5 (Platt *et al*., 1998). They were also engineered to contain the integrated reporter gene for firefly luciferase under control of an HIV long-terminal repeat sequence (Wei *et al*., 2002). After infection, the expression of the reporter luciferase gene is induced in *trans* by viral Tat protein. Luciferase activity is quantified as relative luminescence units (RLU) and is directly proportional to the number of infectious virus particles present in the initial inoculum over a wide range of values (Montefiori *et al*., 2004). Thus, luciferase activity was measured after incubating a mixture of RANTES, TZM-bl cells and viral particles for 48 h. It was revealed that in the presence of RANTES, luciferase activity was reduced (Figure 2.10). This showed that RANTES blocked the binding of the viral particles to the CD4 and CCR5 receptors on the TZM-bl cells.

2.3.7. RANTES I toxicity assay

Neutralization assays measured the reduction of luciferase activity expressed from the TZM-bl cells. The reduction could have been either as a result of inhibition by RANTES I or cell death. Consequently, the toxicity of RANTES I towards TZM-bl was tested. A well known viral entry inhibitor, Maraviroc (Selzentry) was used as a control. Maraviroc specifically blocks gp120 (V3 loop), which normally associates with the chemokine receptor CCR5. When TZM-bl cells were treated with both RANTES I and Maraviroc, the cells remained viable in the presence of both RANTES I and Maraviroc (Figure 2.11A and B). This shows that the purified RANTES I peptide derived from the LIP2-RANTES fusion inhibits viral entry but does not kill the cells.

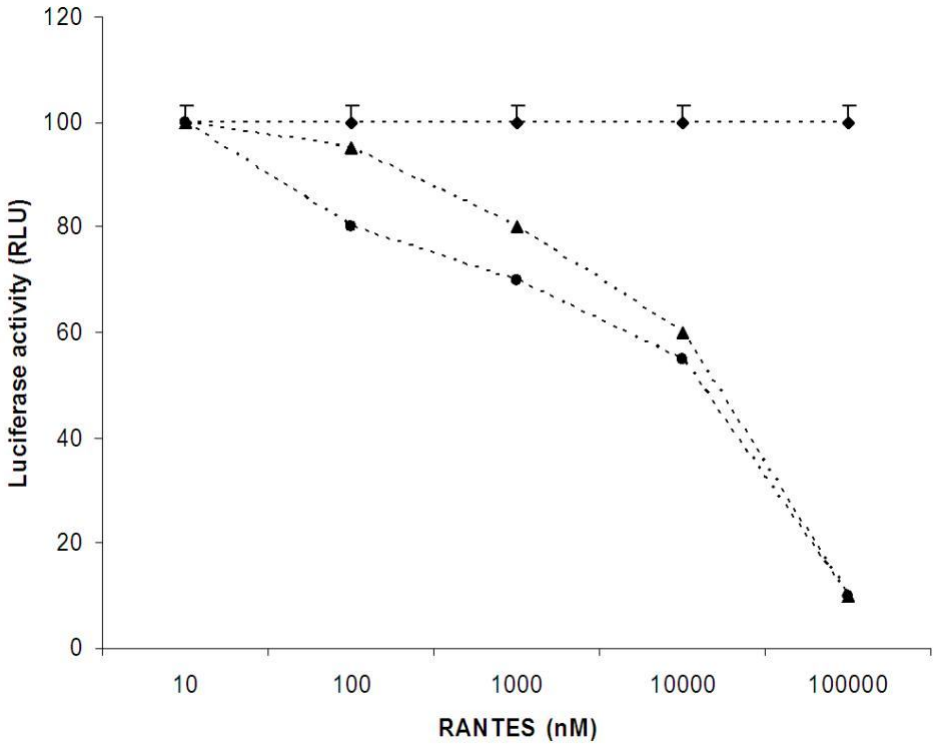

Figure 2.10. Inhibition of VSV-G (▲) and the subtype QHO692.42 (●) infection of TZM-bl cells. Wells containing TZM-bl cells were infected with VSV-G and QHO692.42 viral particles. A mixture of TZM-bl cells and viral particles was used as a control (♦). The cells were then treated with different dilutions of RANTES for 1 h. Luciferase activity of the mixture was assessed by luminescence. The results represent mean ± SD of three independent experiments, each done in duplicate wells.

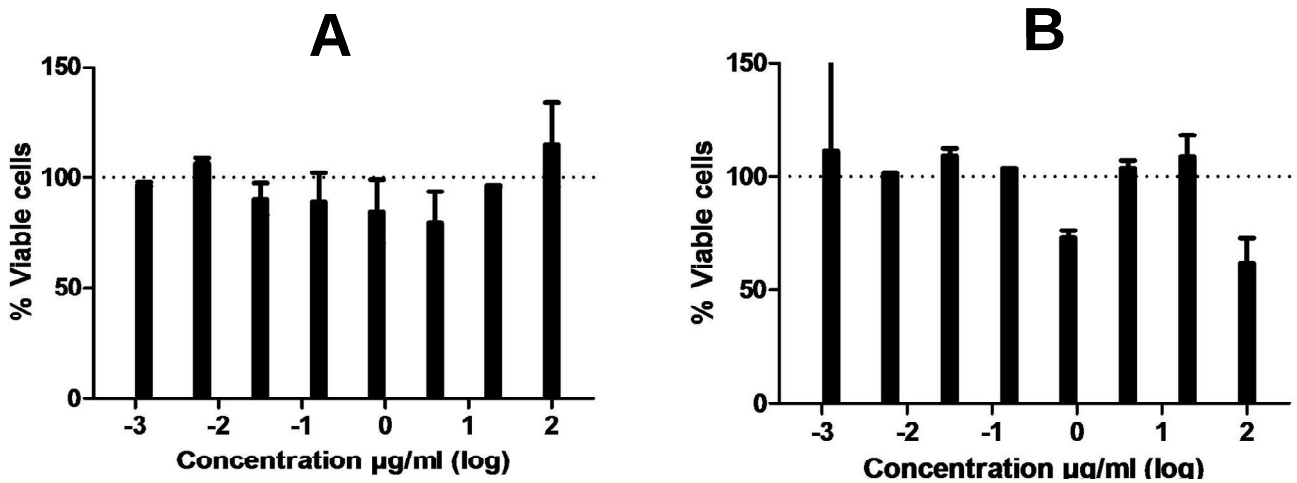

Figure 2.11. Toxicity assays of (**A**) RANTES I and **(B)** Maraviroc towards TZM-bl cells. The bar charts are showing the percentage of viable cells at different concentrations of both RANTES I and Maraviroc.

2.4. Discussion

Chemical synthesis is currently mainly used as the method of choice for the production of therapeutic peptides (Hey *et al*., 2006). However, both solid or liquid phase chemical synthesis have several limitations including low yields and high cost, prohibiting industrial scale use (Torres and Miranda, 2010). Production through recombinant prokaryotic and eukaryotic cells is a viable alternative but it is not always pursued due to perceived problems with efficiency. *Escherichia coli* have been extensively used to produce a number of therapeutic peptides. These include insulin, human growth hormones (hGH) and interferons which according to chemical and pharmacological studies have proven to be indistinguishable from their native counterparts (Schmidt *et al*., 1999). However, some of the major obstacles encountered in the production of peptides by recombinant *E. coli* is the rapid intracellular degradation of the recombinant protein (Shen, 1984). Since they are produced in inclusion bodies, it is often necessary to reconstitute the protein after expression. Alternatively, Chinese hamster ovary (CHO) cells are the most widely used mammalian cells for transfection, expression, and large-scale recombinant protein production. Since CHO cells provide stable and accurate glycosylation, they offer a post-translationally modified product and thus a more accurate *in vitro* rendition of the natural protein (Werner *et al*., 1998).

Maintenance of mammalian cell lines requires the addition of complex and expensive additives such as serum to the medium. Cell death is another major barrier to maintaining high cell densities at high viability and often leads to lower protein yields and quality (Arden and Betenbaugh, 2004). Consequently, yeasts become the most attractive host for recombinant therapeutic peptide production. This is mainly because they produce a complete therapeutic molecule with proper post-translation modifications under relatively cheaper culturing conditions. As an example, high-level expression and efficient assembly of Hepatitis B surface Antigen (HBsAg) particles have been reported in *Pichia pastoris* by integrating a single copy of the HBsAg gene under the control of the alcohol oxidase (AOX1) promoter and the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter (Vassileva *et al*., 2001).

A fundamental requirement for an ideal expression platform would be the quick availability of an active protein, i.e. secretion into the culture medium of a properly folded protein. There have been attempts to produce RANTES in *E. coli* (Kuna *et al*., 1992), and they were typically expressed in poorly soluble or insoluble aggregate in

inclusion bodies. However, an *E. coli* based MBP fusion protein system was used and the findings showed that the incorporation of a fusion partner results in high-yield expression of functional RANTES (Cho *et al*., 2008). Secchi and co-workers (2009) performed a study to compare the efficiency of *Lactobacillus jensenii* as an alternative system for the production of recombinant RANTES. Although the level of production was lower, it was possible to release properly folded RANTES directly into the culture supernatant. However, like *E. coli*, *L. jensenii* lacks post-translational pathways required for eukaryotic protein modification. This might be a bottleneck when expressing peptides that require stringent modifications such as glycosylation. Efforts to develop a mammalian cell-based recombinant RANTES production system using CHO cells was hampered by the low quantity of protein secreted and the unwanted covalent dimerization event (Secchi *et al*., 2009). This is hypothesized to be as a result of a highly-specialized secretion system in CHO cells that could induce RANTES to undergo extensive ER retention and intracellular degradation.

The *Y. lipolytica* yeast is known for producing to high levels the extracellular Lip2p. The protein is secreted to the extracellular medium in almost pure form which greatly simplifies the downstream processing of the protein (Pignede *et al.*, 2000a, 2000b; Nicaud *et al.*, 2002). The production of recombinant Lip2p in almost a pure form was also observed in this study when the recombinant *Y. lipolytica* 414 strain was grown in media containing glycerol as a carbon and energy source. In the medium which contained glucose multiple contaminating proteins were observed on SDS-PAGE gel. The passage of the crude supernatant through a Nickel chromatography column resulted in the purification of the Lip2His tagged protein as judged by the presence of a single protein band on SDS-PAGE gel. It was hypothesized that fusing proteins to the LIP2 protein could result in high level production and secretion to the extracellular of relatively pure forms of the LIP2-fusion proteins. To this end, RANTES I peptide was selected as a model peptide for co-expression with the Lip2p. The model structures for both Lip2p and RANTES I peptide revealed no secondary structures on the C and N termini, suggesting that the junction of the two fusion proteins would result in the fused protein being exposed to the surface of the folded protein structure. The His tag and the enterokinase cleavage sites were incorporated between the Lip2p and the RANTES I to facilitate downstream processing of the peptide.

The developed *Y. lipolytica* host strain containing the Lip2His-RANTES I was designated *Y. lipolytica* Yl415. The expression cassette contained the multicopy Ura3d4 marker gene and the hp4d promoter with Lip2p as a carrier protein. The Ura3d4 sequence has been reported to result in multiple copies of the expression cassette integrating within the *Y. lipolytica* genome (Nicaud *et al*., 2002; Madzak *et al*., 2004). The expression cassette was targeted to the rDNA which in *Y. lipolytica* genome could give as many as 12-60 plasmid copies/cell (Juretzek *et al*., 2001). The developed Lip2p based expression system for peptides allows indirect monitoring of the production of the peptide during the growth of the yeast which is achieved by monitoring lipase activity using standard lipase activity assay methods. This could be vital during optimization of processes for the production of the peptides. *Yarrowia lipolytica* based protein expression systems capable of producing to the extracellular medium 0.5 g per liter of highly purified Lip2p have been described in literature (Pignede *et al.*, 2000a, 2000b, Nicaud *et al.*, 2002). The development of fed-batch processes for the production of lipase in bioreactors optimizing different extracellular factors such as growth medium composition, air pressure, pH fluctuations and nutrient feeding rates have significantly improved production levels of the enzyme (Fickers *et al*., 2004; Kar *et al*., 2008; Lopes *et al*., 2009; Turki *et al*., 2010).

Mutant strains of *Y. lipolytica* developed by chemical mutagenesis with enhanced capabilities to produce and secrete to the extracellular high levels of the lipase enzyme have also been described in literature (Fickers *et al*., 2004, 2005a). Extracellular lipase production was used to monitor the production of RANTES. It was observed that the wild type Yl410 strain produced very low levels of extracellular lipase, and this was in agreement with the results obtained by Pignede and co-workers (2000a) with Po1d. Although there are other genes, LIP7 and LIP8, that code for extracellular lipases (Fickers *et al*., 2005b), low levels of lipase accumulated in the media. In this study, Lip2-RANTES I was produced in shake flask cultures under non-optimised cultivation conditions to 0.01 $g.L^{-1}$. This suggests that there was significant production of Lip2-RANTES. While this study demonstrated application with peptides, the system has also been developed to co-express human therapeutic Erythropoietin (EPO) which is 72 kDa in size (unpublished data). More importantly, without co-expression, EPO expression could not be detected in extracellular medium, suggesting that the system could serve both as a carrier and a facilitator for expression of proteins that are otherwise difficult to express in *Y. lipolytica*.

The amount of RANTES I produced by the *Y. lipolytica* production system was estimated to be 0.274 µg.L^{-1} as determined by the CCL5/RANTES immunoassay method. It is worthy to note that this assay is based on enzyme-linked polyclonal antibody raised against the synthetic human RANTES which is at least 68 amino acids long. The RANTES I is a 36 amino acid long variant of the human RANTES peptide. The relative mass level of 274 pg.mL^{-1} could therefore be an under estimation of the actual amount of RANTES produced by the *Yarrowia lipolytica* system due to the differences in peptide mass size and therefore unavailability of all polyclonal antibody epitopes. This can result in non-conformation to the linear curve for standard of measurements.

The RANTES I peptide fragment was released from Lip2-RANTES using recombinant bovine enterokinase. The recognition site for the enterokinase proteolytic enzyme was positioned in such a way that cleavage with the enzyme results in the peptide of interest without an additional amino acid residue. The relative proportion of the RANTES I peptide released from the LIP2-RANTES protein was visually higher than the Lip2-His protein suggesting unspecific cleavage of the Lip2-RANTES by the enterokinase enzyme, which complicates peptide purification. The MALDI-TOF MS analysis of the tryptic digests of the released peptide fragment matched synthetic RANTES as well as Lip2p confirming unspecific cleavage of the Lip2-RANTES. It has been reported in literature that enterokinase does not exhibit high stringency in its specificity for cleavage of the DDDDK (Jenny *et al*., 2003; Hillar *et al*., 2007; Liew *et al*., 2005)**.** Ley and co-workers (2005) identified enterokinase recognition sequences that result in improved specificity and cleavage rate by the enterokinase as compared to the DDDDK sequence. The introduction of the SRLLR residues downstream of the DDDDK recognition sequence has also been reported in literature to enhance enterokinase cleavage efficiency (Liew *et al*., 2007).

Production of the Lip2-RANTES in glycerol containing medium resulted in the secretion to the extracellular of the Lip2-RANTES I as predominantly a single protein band with few impurity problems and significantly facilitating the downstream processing of the RANTES I peptide. The protein was cleaved with enterokinase without further purification to release the RANTES I peptide from the Lip2His carrier protein. The theoretical sizes of Lip2His, enterokinase and RANTES I are 38, 20, and 5 kDa as determined using EXPASY (Altschul *et al*., 1997). This enables easy purification of the protein mixture by gel exclusion chromatography. In this study,

membrane filtration was used to separate the RANTES I from the Lip2His protein resulting in a concentrated RANTES I peptide fragment. Alternative methods for the purification of the RANTES I protein from the Lip2His-RANTES I include treatment with enterokinase and the removal of the Lip2His by Nickel affinity chromatography and collecting the RANTES I as the eluant. This study demonstrated this possibility by describing a one step purification of the Lip2His using Nickel Affinity chromatography. In addition, Ley *et al*., 2005 described one step affinity chromatography procedures for purification of proteins involving the incubation of the enterokinase enzyme with the fusion protein immobilized on a solid support.

The RANTES I peptide produced in this study was reactive to polyclonal antibodies raised against human RANTES and functionality assays showed that the peptide inhibited the binding of the pseudoviral particles to TZM-bl cells through the CD4/CCR5 receptors. The TZM-bl cells are CXCR4-positive HeLa clones engineered to express CD4 and CCR5 and the genes for firefly luciferase and β-galactosidase under control of an HIV long-terminal repeat sequence that has been integrated within the genome (Platt *et al.*, 1998, Wei *et al.*, 2002). RANTES I was able to neutralise both VSV-V and the HIV- type 1 subtype B viral strain, QHO692.42 (Li *et al*., 2006) as demonstrated by the reduction in luciferase activity by the cells in the presence of RANTES (Montefiori, 2004). The lack of expression was due to complete neutralisation of the viral particles by the RANTES, prohibiting interaction with the receptors contained within the TZM-bl cell membranes. β-Chemokines such as RANTES compete with viral particles for the CCR5 binding site, thereby preventing viral fusion with the host cell (Cocchi *et al*., 1995). In certain instances, they cause receptor internalization and subsequent down-regulation which in turn eliminates chemokine receptor-mediated viral entry (Amara *et al*., 1997; Alkhatib *et al*., 1997). It was observed that RANTES inhibited viral entry into TZM-bl cells, rather than killing the cells.

In conclusion we described in this study a lipase based expression system for production of peptides. The system offers great potential for an economic production of therapeutic peptides, integrating upstream efforts for high level production of proteins with various options for downstream purification and recovery of the peptide. Further developments are aimed at the optimization of the production of fused extracellular proteins, cleavage with the enterokinase proteolytic enzyme and assessment of peptide quality by N-terminal peptide –sequencing.

2.5. References

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Development and characterisation of extracellular lipase hyperproducing mutants of *Yarrowia lipolytica*

3.1. Introduction

Yarrowia lipolytica is notable for the biosynthesis of lipolytic proteins (Ota *et al*., 1982; Barth and Gaillardin, 1997). The extracellular LIP2 enzyme has in particular received attention over the decade due to its commercial prospects. The enzyme was recently suggested as a good candidate for pancreatic exocrine insufficiency treatment (Turki *et al*., 2010). The accumulation of extracellular lipase *from Y. lipolytica* is induced by the presence of long chain fatty acids, triacylglycerols and organic nitrogen sources such as peptone and urea (Pignede *et al*., 2000a, 2000b). In a medium containing glucose, extracellular lipase activity is detected at relatively low levels and only after glucose has been depleted (Fickers *et al*., 2003). There is extensive information on the biochemical, catalytic properties and biotechnological application of lipases in general (Hasan *et al*., 2006) and little information on the factors and mechanisms that regulate lipase biosynthesis. The lack of extensive knowledge on the biosynthesis of extracellular proteins is hampering development of host strains with increased capabilities to produce these proteins.

In order to increase extracellular lipase production by *Y. lipolytica*, several methods including chemical, ultraviolet and insertional mutagenesis techniques have been employed (Fickers *et al*., 2003, Nga *et al*., 1989, Destain *et al*., 1997, Mauersberger *et al.*, 2001). A *Y. lipolytica* mutant, obtained through chemical mutagenesis, yielded 10-fold increase in extracellular lipase production compared to wild type upon optimisation of the cultivation medium (Fickers *et al*., 2003). This lipase hyperproduction phenotype was suggested to have resulted from the uncoupling of catabolite repression by glucose through involvement of the hexokinase gene (Fickers *et al.*, 2003; Fickers *et al.*, 2005a). Mauersberger *et al.*, (2001) reported on the generation of lipase hyper producing mutants of *Y. lipolytica* based on the halo based assay for lipase activity. The genes involved in the hypersecretion phenotype of the *Y. lipolytica* mutants have not been characterised.

In this study the *Y. lipolytica* extracellular *LIP2* gene was used in the identification of mutants with enhanced extracellular protein production. The ultimate objective was to identify genetic elements that could be targeted for the development of *Y. lipolytica* strains with improved capacity to produce and secrete to the extracellular heterologous proteins. This is important for the non-conventional yeast *Y. lipolytica* given that it is proving to be a competent host in the production of heterologous proteins (Müller *et al*., 1998). The versatility of *Y. lipolytica* as a host for production of

industrially and therapeutically relevant proteins is further enhanced by recent progress in genetically engineering the yeast to produce proteins with human glycans (Song *et al*., 2007). In addition the Generally Regarded As Safe (GRAS) status of *Y. lipolytica* makes it suited for production of bioactive protein products for human applications (Madzak *et al*., 2004).

3.2. Materials and Methods

3.2.1. Plasmid, strains and media

The integrative zeta based mutagenesis vector pFR1 (Figure 3.3) used in this study is a monocopy derivative of JMP5 (Mauersberger *et al*., 2001). The plasmid diagram was constructed using Vector NTI (http://www.invitrogen.com/site/us/en/home/productServices/Applications/Cloning/Vector-DesignSoftware/Vector-NTI-Software.html).

Yarrowia lipolytica strains used in this study are listed in Table 2.1. *Escherichia coli* XL10-Gold® strain (Stratagene, La Jolla, USA), used for transformation and propagation of recombinant plasmid DNA, was grown at 37ºC in Luria–Bertani (10 $g.L^{-1}$ tryptone, 5 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ NaCl) medium supplemented with 100 $\mu g.mL^{-1}$ of ampicillin (Roche diagnostics, Mannheim, Germany) or 40 $\mu g.mL^{-1}$ kanamycin sulphate (Sigma-Aldrich, St. Louis, MO). Unless otherwise stated, all the chemicals were from Merck (Wadeville, South Africa). The media, growth conditions and techniques used for *Y. lipolytica* have been described elsewhere (Barth and Gaillardin, 1997). Yeasts were grown at 28ºC on YPD (20 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ glucose and 10 $g.L^{-1}$ yeast extract) or yeast nitrogen base (YNB) supplemented for auxotrophic requirements (1.7 $g.L^{-1}$ of YNB without $(NH_4)_2SO_4$ and amino acids (Difco, Detroit, MI), 4 $g.L^{-1}$ NH_4Cl, 5 $g.L^{-1}$ casamino acids (Difco). For hygromycin-B (HygR) selection, transformants were plated on YPD containing 600 $\mu g.mL^{-1}$ hygromycin-B (Roche diagnostics), whereas for selection of $Uracil^+$ (Ura^+) and $Leucine^+$ (Leu^+) clones, transformants were plated on YNBcasa (1.7 $g.L^{-1}$ YNB without amino acids and $(NH_4)_2SO_4$ (Difco), 0.2% w/v casamino acids, 4 $g.L^{-1}$ NH_4Cl, 20 $g.L^{-1}$ glucose) and YNB_{5000} (1.7 $g.L^{-1}$ YNB without amino acids and $(NH_4)_2SO_4$, 4 $g.L^{-1}$ NH_4Cl, 20 $g.L^{-1}$ glucose), respectively. YNB-tributyrin (YNBT) (1.7 $g.L^{-1}$ of YNB without $(NH_4)_2SO_4$ and amino acids, 4 $g.L^{-1}$ NH_4Cl, 10 $g.L^{-1}$ of tributyrin) plates were used for extracellular lipase activity detection as previously described (Pignède *et al*., 2000a), and YPDO (20 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ glucose, 10 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ of olive oil) was used to grow *Y. lipolytica* strains for lipase assays. Agar (2% (w/v); Merck) was added for solid media. Media were buffered with 50 mM phosphate buffer, pH 6.8. Stock solutions of tributyrin (20% tributyrin, 0.5% Tween 80) and olive

oil (10% olive oil, 0.5% Tween 40) were emulsified on ice by sonication three times for 1 min.

Table 3.1 *Yarrowia lipolytica* strains and oligonucleotide primers used in this study

Strains and Primers	**Genotype, primer sequence (5´- 3´)**	**Reference/purpose**
***Y. lipolytica* strains**		
Po1d	*MatA*, *leu2-270*, *ura3-302*, *xpr2-322*	Le Dall *et al.*, 2004
Yl5	Po1d derivative, extracellular lipase overproducer	This study
Yl6	Po1d derivative, extracellular lipase overproducer	This study
Yl10	Po1d derivative, extracellular lipase overproducer	This study
Yl12	*MatA*, *leu2-270*, *ura3-302*, *xpr2-322, ΔGPI7*	This study
Yl25HmA	*MatA*, *ura3-302*, *xpr2-322*, *axp1-2, EH*$^+$	Maharajh *et al., (2008)*
Yl25HmAΔGPI7	*MatA*, *ura3-302*, *xpr2-322*, *axp1-2, EH*$^+$*, ΔGPI7*	This study
Oligonucleotides		
Yl3485356F	GAGTAGTGGCAAGCTTGTCCTCCACCGTTTG	Confirm integration
Yl3487261R	GTTGACGTTTGTGTCCAACTGGATTGGCCCTCTG	Confirm integration
ZetaF	GGCCGCTGTCGGGAACCGCGTTCAGC	LSP[2]
ZetaR	GCACTGAGGGCTTTGTGAGGAGGTAAT	LSP
Zeta1F	GGCCGCTGTCGGGAACCGCGTTCAGG	LSP
Zeta2F	GGGAAAGCGATACTGCCTCGGACAC	LSP
Zeta1R	GCCGCACTGAAGGGCTTTGTGAGAG	LSP
Zeta2R	CCCCACTATGAGCTACATCAGCCGATAC	LSP
CSP-1	GGCGGGATTGGCCTGTACCAGGTAGTAG	CSP[3]
CSP-2	GAAAGGGTTTGATGACCAGACCCGCG	CSP
PTF	GTGCGCCACGCACCTTCACAGTCAGTGTCTAGC	Deletion
PTR	GATTACCCTGTTATCCCTAGGAGACACACACTCTCTCCAC	Deletion
PPF	CTAGGGATAACAGGGTAATCCTTCCTACCGAGGACGTTGG	Deletion
PPR	CCGGCATGTCCAGGCCGATGGAACCCTTAAAAAG	Deletion
GTF	GTGGCAAGCTTGTCCTCCACCGTTTGACACATTATC	Deletion
GTR	GATTACCCTGTTATCCCTTAGCGTCAGGTTGCAGGGTCTG	Deletion
GPF	CTAGGGATAACAGGGTAATGATTGGCCTTTACGAGAATGT	Deletion
GPR	GAGTTGTACCAGGTAGTAGGATAGGCGGTGGAGG	Deletion
GPI7F	CACGGATCCATGCTCTGGAAAAGGTC	Deletion screening
GPI7R	CATCATCATCATCATTTACACCGATCTAT	Deletion screening
PKURTF	GGCCCGCTTCGTCGTT	RT-PCR
PKURTR	GGACAAGCTCAAACT	RT-PCR
PKDRTF	CGGTCTGAGGTCCGCGT	RT-PCR
PKDRTR	GAAGGAATTGGAGGAA	RT-PCR
GPI7URTF	GCTTGGGAGAAAAGACTG	RT-PCR
GPI7URTR	CTTCTCAATGGGTAACT	RT-PCR
GPI7DRTF	GTCTTATGTCCAATATGAT	RT-PCR
GP77DRTR	CAGGCATGTTTGACGAC	RT-PCR
ActF	TATTGCCGAGCGAATGC	RT-PCR
ActR	CTTGGAGATCCACATCTGC	RT-PCR

Note: EH = epoxide hydrolase [1]Mutagenesis cassette**,** [2]Locus specific primer**,** [3]Cassette specific primer**,** Underlined segments indicate the *I-Sce-I* restriction sites

3.2.2. Transformation, screening and confirmation of integration

3.2.2.1. Transformation

Yarrowia lipolytica strains were transformed according to the method described by Xuan *et al.* (1988). Five nanograms of the PCR amplified integration cassettes were used to transform *Y. lipolytica* cells. This was done to facilitate genomic integration to the target sites. Plates were incubated at 28ºC for a minimum of 48 h.

3.2.2.2. Screening for lipase hyperproducing transformants

To detect lipolytic activity on solid media, *Y. lipolytica* cells were streaked out on YNBT plates containing emulsions of tributyrin (Sigma-Aldrich, Steinheim, Germany). To prepare the substrate, the solution was emulsified in a sonicator (Bandelic electronics, Berlin, Germany) (three pulses of 2 min each at maximum speed).

3.2.2.3. Southern blot analysis

Samples of genomic DNA (25 µg) were digested with *Eco*RI, *Hind*III and *Bam*HI; and the resulting fragments were separated by electrophoresis using 0.8% (w/v) agarose gels in TAE buffer (40 mM Tris/HCl pH 7.6, 5 mM CH_3COONa, 1 mM EDTA). The agarose gels were submerged in 0.25 M HCl for 15 min, followed by immersion in a 0.5 M NaOH/1.5 M NaCl solution for 30 min. Finally, the gel was submerged in 0.5M Tris/HCl (pH 7) /1.5 M NaCl solution for a further 30 min. The DNA was transferred onto a positively charged nylon membrane (AEC-Amersham, Claremont, Cape Town) by capillarity action, and the membrane was baked at 120°C for 30 min to ensure DNA immobilization. Pre-hybridization was performed in 5x SSC, 0.1% (w/v) *N*-laurylsarcosine, 0.02% (w/v) SDS, 1% (v/v) Blocking Reagent (Roche Prehybridization Solution) for 1 h at 42°C. DNA fragments amplified using the *ylURA* and *ylGPI7* reading frames were used to prepare digoxigenin (DIG)-labelled DNA probes according to the protocols provided by the manufacturer (Roche diagnostics), at a concentration of 20 $ng.mL^{-1}$ in Prehybridization Solution for at least 16 h at 42°C. The membrane was hybridized with the probe overnight and washed twice in 2x SSC, 0.1% (w/v) SDS for 5 min at room temperature, and twice more in 0.1x SSC, 0.1% (w/v) SDS at 68°C. Detection of the hybridized probe was carried out according to the manufacturer's instructions for the DIG-DNA labelling and detection kit (Roche Diagnostics).

3.2.3. Amplification of the MTC borders

The upstream and downstream borders were amplified using cassette ligation mediated PCR described previously (Nthangeni *et al*., 2005) as illustrated in Figure 3.1. Locus specific oligonucleotide primers (LSPs) were constructed according to the known MTC DNA fragment. For the amplification of the downstream flanking region, the *Hind*III sticky end-containing ligation cassette was ligated with *Hind*III-digested genomic DNA from *Y. lipolytica* Po1d, Yl5, Yl6 and Yl10. ZetaF (LSP-1) was used as a LSP-1 primer in single-stranded amplification PCR (SSA-PCR) while Zeta1F (LSP-2) and CSP-1 were used in the second PCR. Finally, Zeta2F (LSP-3) and CSP-2 were used in the third PCR.

The upstream border was amplified with the *Hind*III ligation mixture of cassette-genomic DNA as the template using ZetaR as a LSP-1 in SSA-PCR. The second and third rounds were performed with Zeta1R/CSP-1 and Zeta2R/CSP-2 as LSP-2 and cassette-specific primers, respectively. PCR amplification was done according to standard conditions with general purpose Taq DNA polymerase. In the first PCR, the genomic DNA-ligation cassette mixture was used as a template. To perform the second and third PCRs, the amplicons obtained in the first PCR were used as templates and similarly the amplicons from PCR 2 used as templates for PCR 3. DNA fragments obtained by PCR amplifications were ligated into pGemT-Easy and sequenced. To confirm that the vector integrated at the identified region, PCR was performed using Yl3485356F and Yl3487261R.

Nucleotide sequences were analysed using Chromas 2.33 (http://www.technelysium.com.au/chromas.html). Nucleotide sequences were compared with sequences in the database at NCBI using BLAST (http://www.ncbi.nlm.nih.gov/blast/BLAST.cgi) (Altschul *et al*., 1997) against the *Y. lipolytica* RST and genome databases at the genolevures sites (http://cbi.labri.fr/Genolevures and http://www.ncbi.nlm.nih.gov/BLAST/). Amino acid sequence alignments were performed using DNAssist Version 3.0.

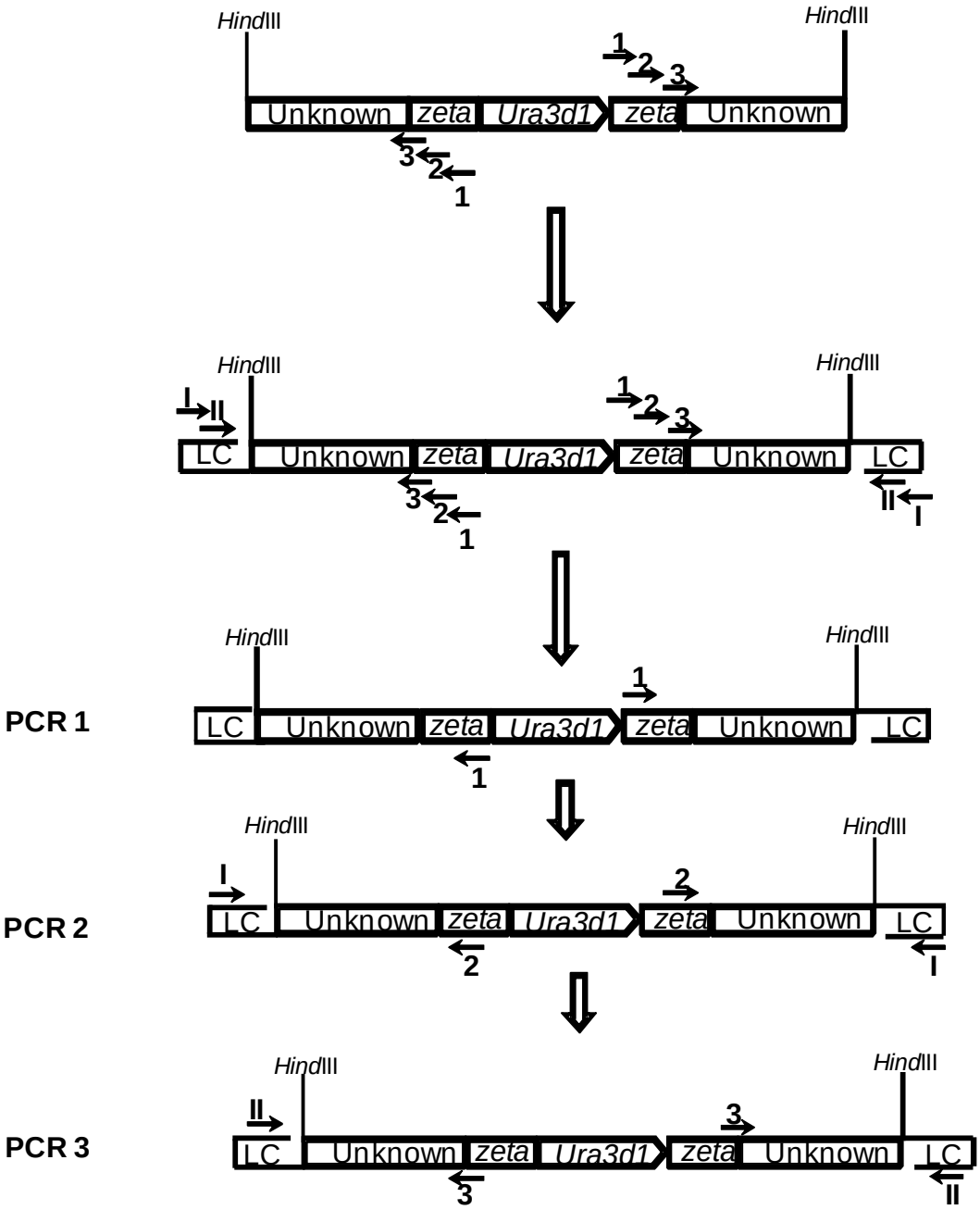

Figure 3.1. Graphical representation of the cassette ligation mediated PCR. The genomic DNAs from the selected strains were digested with *Hind*III and ligated to a ligation cassette compatibly digested. The Ligation mixture was used as a template in the first PCR to perform Locus specific SS-PCR using LSP-1 (1 representing ZetaF and ZetaR) for the upstream and downstream regions. PCR 2 was done with PCR 1 products by pairing 2 (representing Zeta1F and Zeta1R) and I (representing CSP-1). The last amplification, PCR 3 was performed with PCR 2 amplicons as a template, pairing 3 (representing Zeta2F and Zeta2R) and II (representing CSP-2). LC is the ligation cassette and the locus specific and cassette specific primers are represented by arrows. The gaps at the junctions are indicating the nicks between the cassette and DNA fragments upon ligation.

3.2.4. Quantitative Real Time PCR (qRT-PCR)

3.2.4.1. Purification of total RNA from Y. lipolytica

Total RNA was isolated from the *Y. lipolytica* Po1d, Yl10 and Yl12 cultured under similar growth conditions. The RNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) by mechanical disruption of cells. Briefly, cells were harvested by centrifugation at 3000 *g* for 5 min at 4°C. The supernatant was decanted and the pellet was resuspended in 600 µl of resuspension buffer and vortexed. Approximately 600 µl of acid washed beads were added to the suspension. The sample was agitated and vortexed in the tissuelyser with regular cooling. The lysate was transferred to a new microcentrifuge tube and centrifuged for 2 min at 14000 *g*. One volume of 70% (v/v) ethanol was added to the homogenised lysate, and mixed by pipeting. The sample was transferred to an RNeasy spin column and centrifuged for 15 s at 10000 *g*. The flow through was discarded and 700 µl of RW1 buffer was added to the spin column. The sample was centrifuged for 15 s at 10000 *g*. The flow through was decanted and two consecutive washes were done with 500 µl of buffer RPE. The sample was centrifuged for 15 s at 10000 *g*. The flow through was discarded and the sample was centrifuged for 1 minute at 14000 *g* to remove excess RPE buffer. Pure total RNA was eluted from the spin column with 30-50 µl of RNase-free water. The RNA was either stored at -20ºC or used immediately to prepare cDNA.

3.2.4.2. Complementary DNA (cDNA) synthesis for RT-PCR

Complementary DNA (cDNA) was prepared with the Superscript ^{TM}III First-Strand Synthesis System for RT-PCR (Invitrogen, Basel, Switzerland). Microcentrifuge tubes were used to mix 3 µg of total RNA from each sample with 50 µM oligo $(dT)_{20}$, 10 mM dNTP mix, and DEPC-treated water. The mixtures were incubated at 65 ºC for 5 min and placed on ice for at least one minute. A cDNA synthesis mix was prepared for all the samples and it comprised the following: 10X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNaseOUT^{TM}, Superscript^{TM}III RT (200 U.µL^{-1}). Twenty microlitres of the cDNA mixtures were added to the RNA/oligonucleotide mixtures, and the samples were incubated at 50ºC for 50 min. The reactions were terminated by incubating the samples at 85ºC for 5 min followed by chilling them on ice. RNase H was added to each tube and the samples were incubated at 37ºC for 20 min. Complementary DNA was diluted into a 10 ng.µL^{-1}, 2 ng.µL^{-1}, and 0.4 ng.µL^{-1}concentration series. The prepared cDNA were used immediately to perform RT-PCR or stored at -20ºC for later use.

3.2.4.3. Quantitative Real Time PCR (qRT-PCR)

Real time PCR was performed using the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit (Roche). Briefly, the required number of LightCycler® capillaries was placed in a LightCycler® Sample Carousel in a precooled LC Carousel Centrifuge Bucket. To prepare for RT-PCR, a 10X stock of the oligonucleotides was prepared. Fifteen microlitres of the PCR master mix was pipeted into the capillary and 5 µl of the DNA template was added. The capillaries were sealed with a stopper, and centrifuged with an LC Carousel Centrifuge 5000 *g* for 30 s. The LightCycler® Sample Carousel was placed in the LightCycler® 2.0 instrument to run the RT-PCR programmed as follows: 95°C for 3 min, 45 cycles of 95°C for 30 s, 48°C for 30 s, 72°C for 30 s and a final extension step of 5 min at 72°C. Melting curves ranging between 45 and 95°C were analysed after the amplification. Reactions were performed in triplicates and repeated three times. The threshold was set to a standard deviation of 10 over the cycle range for each run and increasing fluorescence was monitored during repetitive cycling of the amplification reaction. Results from the RT-PCR assay were expressed as relative expression by measuring the maximum amount of the PCR product generated by the primers after 25 cycles as measured by fluorescence. The relative mRNA levels were determined by the difference between the β-actin, *PK* and *GPI7* PCR products from *Y. lipolytica* Po1d and Yl10.

3.2.5. Construction of the disruption cassettes

3.2.5.1. Promoter-terminator (PT) cassette construction

The promoter-terminator (PT) cassette was obtained in a two-step PCR reaction in which first the promoter (P) and terminator (T) regions of *PK* and *GPI7* were separately amplified. Then the combined P and T PCR products were used as a template in a second PCR reaction to obtain the full *PK* and *GPI7* PT PCR products (Figure 3.2), which were purified and cloned into pGemT–Easy vector. Verification of the right constructs was carried out by restriction digest(s) as well as sequence analysis.

3.2.5.2. Construction of the promoter-hph-terminator (PHT) cassette

The deletion cassette loxR-*hph*-loxP was rescued from plasmid JMP115 by *I-Sce* I digestion and cloned into pGemT-Easy PT at the corresponding *I-Sce* I site. Thus the correct pGemT-Easy-PHT carried the full *ylPK* and *ylGPI7* deletion cassettes containing the *hph* marker gene. The linear disruption PHT cassettes for *PK* and

GPI7 were generated by PCR amplification from pGemT-Easy-PHT using oligonucleotide primer pair PTF/PPR and GTF/GPR (Figure 3.2).

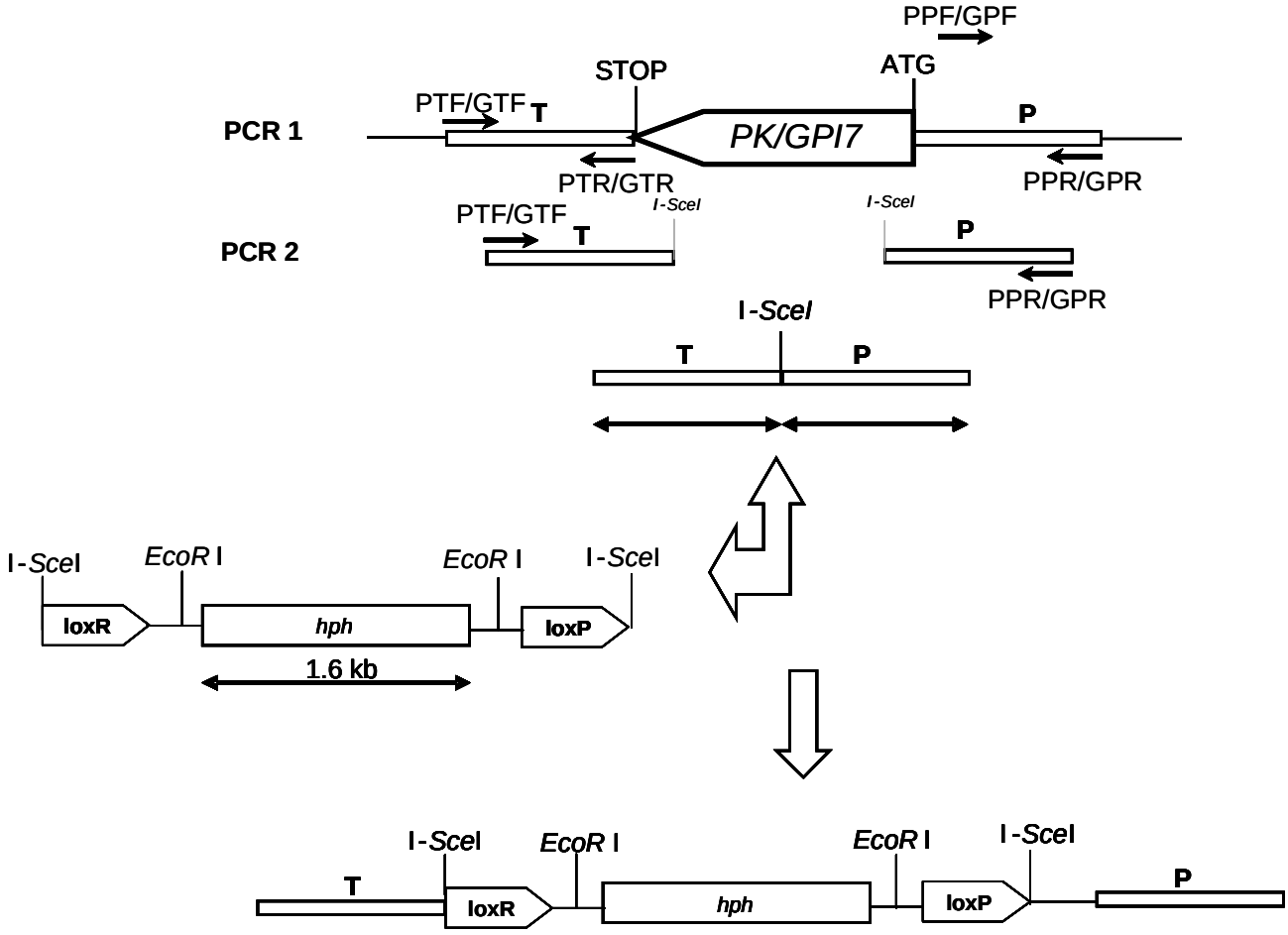

Figure 3.2. Graphical representation of the strategy used to delete the protein kinase and GPI7 encoding genes. The promoter and terminator regions of the two genes were used to construct primers with the rare meganuclease *I-SceI* incorporated as indicated. *hph* is the DNA fragment inclusively containing the hygromycin resistance gene and the loxR/P fragments for marker recycling.

3.2.5.3. Deletion of PK/GPI7

Yeast cells were transformed by the lithium acetate method using approximately 1 mg of purified PHT PCR product of the *ylPK* and *ylGPI7* genes. Ura+ transformants, which appeared after approximately 48 h, were selected on YNBcasa plates. Verification of disruption was done by PCR on genomic DNA of transformants using primer pairs GPI7F/GPI7R and GPS/HygR. The primer pair GPI7F/GPI7R amplifies the *ylGPI7* ORF.

3.2.5.4. Hph marker rescue by expression of Cre recombinase

To allow excision of the selectable *hph* marker gene between the two *lox*P sites, the *GPI7::hph* strain was transformed with the *cre*-expressing plasmid, pRRQ2 and selected on YNB_{5000} plates. Loss of the marker followed by loss of the *cre* plasmid was achieved by growing the transformants in non-selective YPD broth supplemented with 0.5% (w/v) uracil and 1% (w/v) leucine in two successive 24 h cultures.

3.2.6. Phenotype analysis of the Δylgpi7 strain

The sensitivity of *Y. lipolytica* Yl12 to substrates that interfere with the cell wall of yeast was tested by growing the strain on YPD supplemented with Calcofluor white (7.5 and 10 $\mu g.mL^{-1}$) and Congo red (10 $\mu g.mL^{-1}$ and 15 $\mu g.mL^{-1}$). Single colonies were selected from YPD plates and suspended in YPD broth. Serial dilutions of the suspension, $10^{-1,}$ $10^{-2,}$ $10^{-}3$, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} were made. Aliquots (5 µl) from each dilution were streaked on YPD agar plates containing Calcofluor white and Congo red. The plates were incubated for 48 h at 28ºC and 37 ºC for temperature sensitivity assay on YPD.

3.2.7. Assay for zymolyase sensitivity

To test *Y. lipolytica* Yl12 sensitivity to zymolyase, cells from exponentially growing cultures were adjusted to an OD_{600} ~1 in 10 mM Tris–HCl (pH 7.5) containing 10 µg zymolyase 20 T, and the decrease in OD_{600} was monitored over a 2 h period. In brief, cells were grown in YPD media until the mid-log phase (OD_{600} 0.3). Cells (5×10^8) were collected by filtration and resuspended in CE buffer (0.1 M sodium citrate, 10 mM EDTA, adjusted to pH 7.2 with HCl) in glass tubes. After 10 min of treatment with 3% 2-mercaptoethanol, zymolyase was added at a concentration of 5 $U.\mu L^{-1}$ (Sigma Aldrich, Switzerland) was added to each tube and the cells were incubated at 30°C under gentle agitation. Spheroplast lysis after dilution in water was determined by measurements of OD_{600}, monitored after every 15 min using a DU800 Beckman spectrophotometer (Beckman Coulter Inc, Brea, United States). The experiment was done in triplicate and the decrease in OD readings was taken as cell lysis.

3.2.8. The effect of GPI7 deletion on cell separation

3.2.8.1. Slide preparation with agarose cushion

To prepare the agarose solution, 1.2% (w/v) of agarose was prepared in YPD media. The agarose solution was completely melted in a microwave. Approximately 200 µl of

melted agarose was added to a slide prewarmed to 60°C. The agarose-coated slide was covered with a regular microscopic cover slide by placing it directly onto the agarose, and pressed evenly against the bottom slide to allow agarose to harden. Pure petroleum jelly was applied to the extreme edge of the coverglass.

3.2.8.2. Cell mounting

Aliquots of both *Y. lipolytica* Po1d and Yl12 cells growing at 28 and 37°C were withdrawn at the late exponential phase for microscopic observations. The cells were sedimented by transferring 1 ml of the log-phase yeast culture to a microcentrifuge tube and centrifuged at low speed (4000 *g*) for 1 min. The supernatant was decanted and the cells resuspended in 0.5 ml of fresh YPD medium. The cover slide was removed to expose the agarose pad gently pushing the top slide along the length of the other slide. The cell culture resuspensions (2.2 µl) was transferred to the smooth agarose and the cell culture was covered with a petroleum jelly soaked coverslide. The slide was subjected to live imaging using a Zeiss microscope Axioskop 40 (Carl Zeiss, Inc., North America).

3.2.9. Expression of LIP2 and epoxide hydrolase (EH) in shake flask cultures

Shake flask cultivations were performed in 500 ml Erlenmeyer flasks using *Y. lipolytica* Po1d, Yl12, Yl25HmA and Yl25HmAΔGPI7 strains (Table 3.1) that were maintained as cryopreserved cultures at -70^{o}C. Cryovials containing 1.5 ml of each strain was used to inoculate triplicate 500 ml Erlenmeyer flasks each containing 100 ml of YPDO medium for *Y. lipolytica* Po1d and Yl12 and YPD for Yl25HmA and Yl25HmAΔGPI7. The pH of the medium was adjusted to 6.8 before it was autoclaved at 121°C for 15 min. Erlenmeyer flasks inoculated with the strains were incubated at 28°C on a rotary shaker at 210 *g* for a period of 144 h. The growth profile was monitored over the cultivation period and the supernatants were stored at -20°C for further analyses.

3.2.10. The effect of ylGPI7 deletion on protein production in shake cultures

3.2.10.1. Lipase activity assay

Lipase activity was measured spectrophotometrically using supernatant fractions with *p*-nitrophenyl palmitate (pNPP) as the substrate according to the method of Winkler and Stuckman (1979). The supernatant of the cell cultures (50 µl), was added to 600 µl of substrate emulsion prepared in a mixture containing 50 mM phosphate buffer, pH 6.8 (Na_2HPO_4-KH_2PO_4), 0.2% (w/v) sodium deoxycholate, 0.1 % (w/v) gum arabic

and *p*NPP (0.30 mM final concentration). Lipase activity was determined by the rate of *p*-nitrophenol (*p*NP) production, measured at 410 nm in a model DU600 spectrophotometer (Beckman Coulter, Fullerton, CA) at 37°C. Lipase activity was assayed using enzyme free substrate blanks as controls. All lipase activity assays were performed in triplicates. One unit of enzyme activity was defined as the amount of enzyme forming 1 µmol of *p*NP min^{-1} under the mentioned conditions.

3.2.10.2. Determination of epoxide hydrolase (EH) activity

EH activity was determined as described previously by Maharajh *et al*. (2008), using 1,2-epoxyhexane as a substrate. Briefly, epoxyhexane was added to a final concentration of 200 mM to 500 µl of the supernatant (2.5% w/v) in KH_2PO_4 buffer [50 mM, pH 7.5 containing 20% (v/v) glycerol]. The reactions were incubated (25 °C) on an Eppendorf shaker with gentle shaking. After 10 min the reactions were stopped with the addition of 500 µl of ethyl acetate for extraction. The samples were vortexed for 30 s and centrifuged in a bench top centrifuge at 13000 *g*. The organic fractions were dried over anhydrous $MgSO_4$ and analyzed for non-racemic 1,2-epoxyhexane by gas chromatography (GC). Quantitative analysis of 1,2-epoxyhexane bioconversion was performed on a Hewlett Packard 5890 series II gas chromatograph (GC; GMI, MN,USA) equipped with flame ionization detector (FID) and Agilent 6890 series auto sampler injector (GMI), using hydrogen as a carrier gas at a constant column head pressure of 10 psi. The analysis of 1,2-epoxyhexane was achieved using a capillary GC column MDN 5S (Supelco, MS, USA) 30 m length×0.25 mm internal diameter×0.25 µm film thickness, and 10 mM 1-heptanol (Sigma-Aldrich, MS, USA) as the internal standard. Total extracellular proteins were using the Pierce BCA assay using bovine serum albumin (BSA) as the calibrating standard.

3.2.12. General protein techniques

Proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with a 12.5% (v/v) polyacrylamide resolving gel and a 4% (v/v) polyacrylamide stacking gel using prestained broad-range protein markers (Fermentas, Brazil) as molecular mass standards (Laemmli, 1970). Proteins gels were stained with Coomassie brilliant blue.

3.3. Results

3.3.1. Construction of the MTC

A monocopy derivative of JMP5 (Mauesrberger *et al*., 2001) denoted pFR1 (Figure 3.3A), was constructed. Digestion of the vector with *Not*I to release the MTC resulted in the expected two DNA bands of about 2.3 and 2.5 kbp respectively corresponding to the MTC and the DNA sequence for plasmid selection and propagation in *E. coli* (Figure 3.3B).

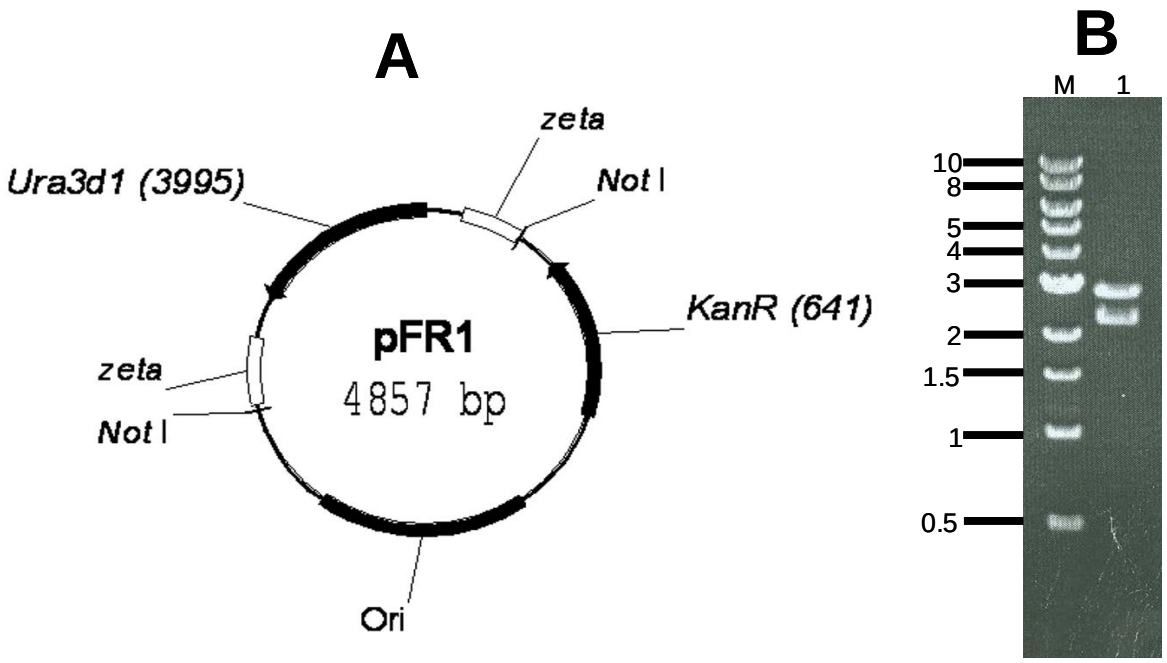

Figure 3.3. Construction of the MTC. (**A**) A schematic diagram indicating the pFR1 vector containing the MTC. For insertion mutagenesis, the plasmid was digested with *Not*I prior to transformation to eliminate the bacterial moiety and to liberate the MTC containing only the non-defective *Y. lipolytica ura3d1* allele, flanked by two inverted partial *zeta* regions of 401 and 312 bp. (**B**) A 1% (w/v) agarose gel to show the pFR1 construct digested with *Not*I. Digestion with this enzyme resulted in the release of the (lane 1) 2.3 kbp MTC from the 2.557 kbp bacterial moiety. M is the Generuler^{TM} (NEB) molecular weight marker in kbp.

3.3.2. Random mutagenesis of Y. lipolytica Po1d

The MTC was used to transform the *Y. lipolytica* Po1d strain. The integration of the MTC within the *Y. lipolytica* Po1d genome resulted in up to 10^3 transformants per µg cassette DNA after incubation at 28°C for 72 hours on YNBcasa agar medium. The colonies transformed with the MTC were transferred to the YNBT agar medium to screen for enhanced extracellular lipase activity. Three colonies denoted *Y. lipolytica* Yl5, Yl6 and Yl10 displayed enhanced extracellular lipase activity as judged by the ratio of the size of the colony to the surrounding zone of clearance. The *Y. lipolytica* Yl5, Yl6 and Yl10 strains displayed zones of clearance around the colony with

hydrolysis/colony diameter ratios of 4.0, 3.66 and 3.07 respectively in comparison to 1.09 from Po1d wild type (Figure 3.4) an indication of enhanced extracellular lipase production by the mutants.

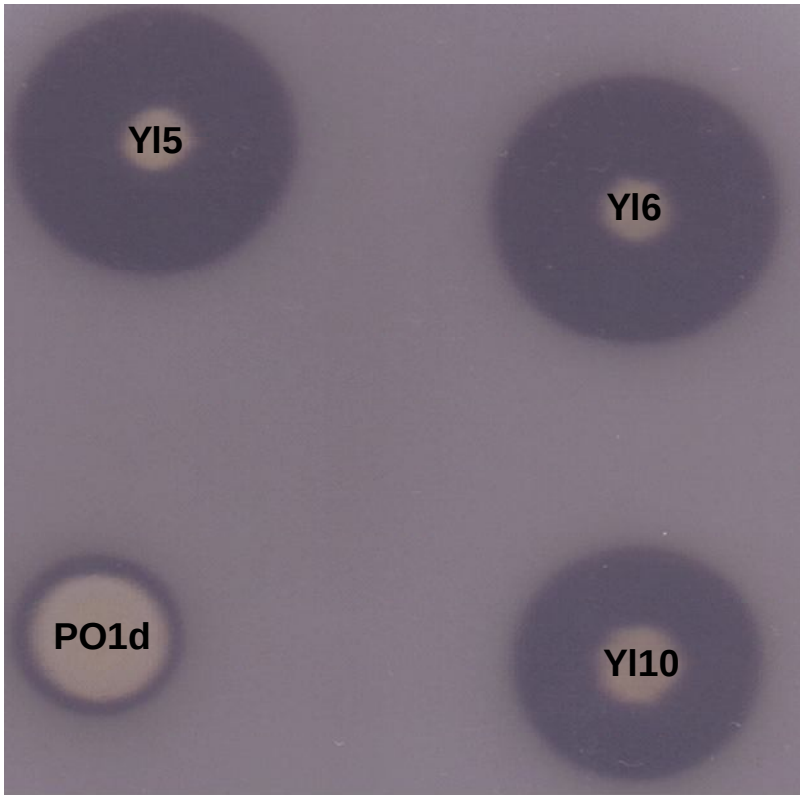

Figure 3.4. Extracellular lipase production by *Y. lipolytica* Po1d and its derivatives denoted Yl5, Yl6 and Yl10 on YNBT agar medium after incubation at 28°C for 5 days. The H/C ratios (diameter of hydrolysis halo/diameter of cell colony) were measured and compared.

The *Y. lipolytica* Yl5, Yl6 and Yl10 transformants were subjected to Southern blot using as a probe the *YlURA* DNA fragment derived by PCR from the *Y. lipolytica* genome. The MTC which integrates randomly within the genome contains the *URA* gene as a transformation selection marker. The random integration in *Y. lipolytica* Po1d of the MTC derived from pFR1 results in at least two copies of the *URA* gene fragment as the *Y. lipolytica* Po1d contains one copy of the dysfunctional *URA* gene. The hybridization of the *YlURA* probe to the genomic DNA restricted with *Eco*RI, *Bam*HI or *Hind*III revealed predominantly two bands with each of the *Y. lipolytica* Yl5, Yl6 and Yl10 clones; and single hybridisation bands with the *Y. lipolytica* Po1d control strain (Figure 3.5). This data indicated that the MTC integrated in single copies per genome. The different hybridisation patterns in the three different strains

demonstrated that the integration of the mutation cassette occurred at different loci within the *Y. lipolytica* Po1d genome.

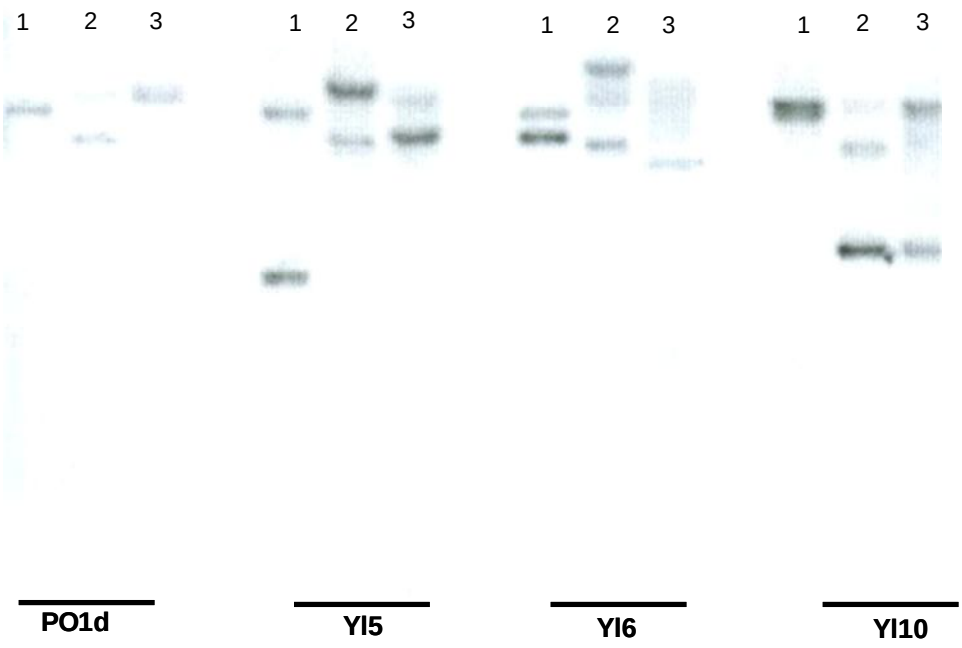

Figure 3.5. Southern blot analysis of *URA*$^{+}$ transformants revealing random integration of the MTC within the *Y. lipolytica* Po1d genome. Genomic DNA from *Y. lipolytica* Po1d (lanes Po1d) and 3 MTC transformants; *Y. lipolytica* Yl5, Yl6 and Yl10 (lanes Yl5, Yl6 and Yl10) from one transformation plate were digested with (**1**) *Eco*RI, (**2**) *Hind*III and (**3**) *Bam*HI and probed with the entire *URA* open reading frame.

3.3.3. Identification of MTC integration locus

SSA-PCR based genome walking technique (Nthangeni *et al*., 2005) was used to clone the DNA fragments flanking the site of MTC insertion. SSA-PCR was only successful with the genomic DNA derived from *Y. lipolytica* Yl10 where a PCR fragment of about 0.9 kbp was obtained (Figure 3.6).

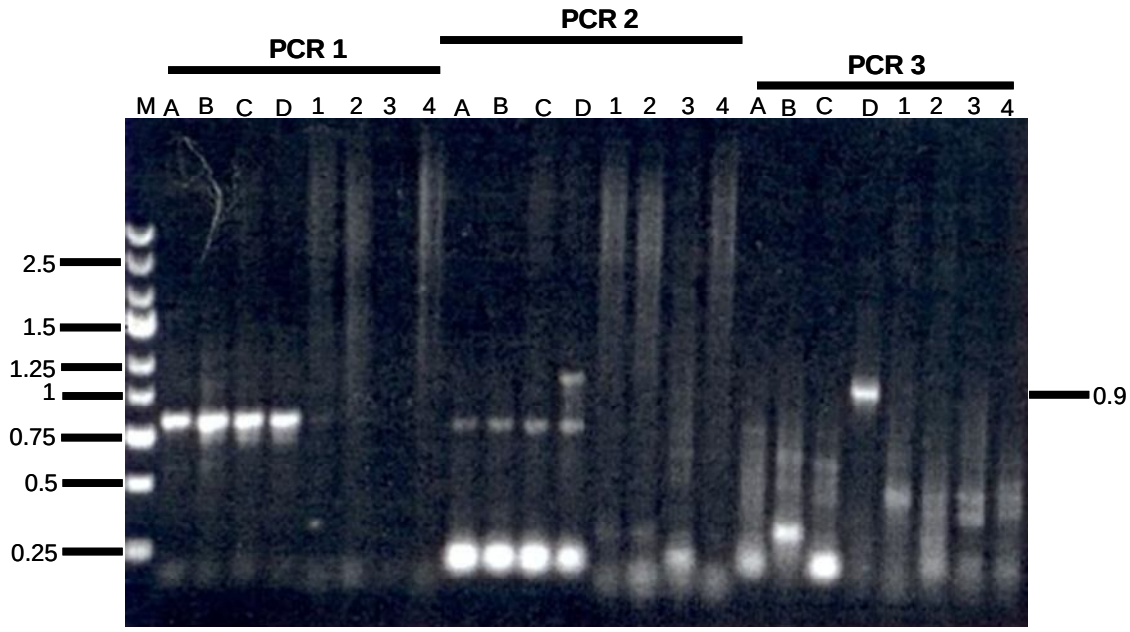

Figure 3.6. SSA-PCR-based genome walking amplicons run on 1% (w/v) agarose gel. The letter numbered lanes represent the upstream region while the numerically numbered lanes represent the downstream border. (Lane A and 1 represent *Y. lipolytica* Po1d; lane B and 2 represent *Y. lipolytica* YI5; lane C and 3 represent *Y. lipolytica* YI6 and lane D and 4 represent *Y. lipolytica* YI10). M is the Generuler^{TM} (NEB) molecular weight marker in kbp.

The final SSA-PCR amplified DNA fragment was cloned into pGemT-Easy and sequenced. Nucleotide sequence analysis and BLAST searches against *Y. lipolytica* genome sequences available in Genelovures nucleotide database *(*www.genolevures.org/blast) revealed that the MTC integrated between the open reading frames encoding the protein kinase (*PK*) and glycosylphosphatidylinositol-anchor biosynthesis protein 7 (*GPI7*) genes between two guanosine residues at position 3486191 and 3486192 of the *Y. lipolytica* CLIB122 chromosome D sequence and surprisingly without affecting any known ORF (Figure 3.7). The integration within this locus was confirmed by PCR using sense and antisense primers designed based on the nucleotide sequence flanking this locus as published in the nucleotide database. The primers were designed to flank the site of MTC insertion as illustrated in Figure 3.6. The primers specified the amplification of a DNA fragment of ~1.9 kbp in size from the genomic DNA of the *Y. lipolytica* Po1d control strain (Figure 3.8). The genomic DNA of the *Y. lipolytica* YI10 strain resulted in a DNA fragment of approximately 4.3 kbp (Figure 3.7), indicating the presence of an additional DNA fragment of about 2.3 kbp, the expected size of the MTC (Figure 3.3).

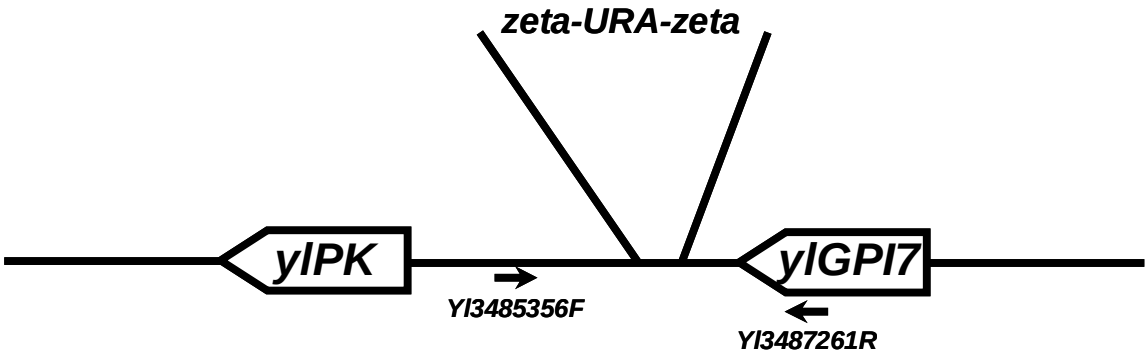

Figure 3.7. Schematic diagram showing the integration locus of the MTC in the genome of *Y. lipolytica* Yl10. The MTC integrated as a sandwich between adjacent genes encoding *PK* and *GPI7* on the complementary strand. The black arrows are indicating the sense and antisense primers to amplify the locus for integration.

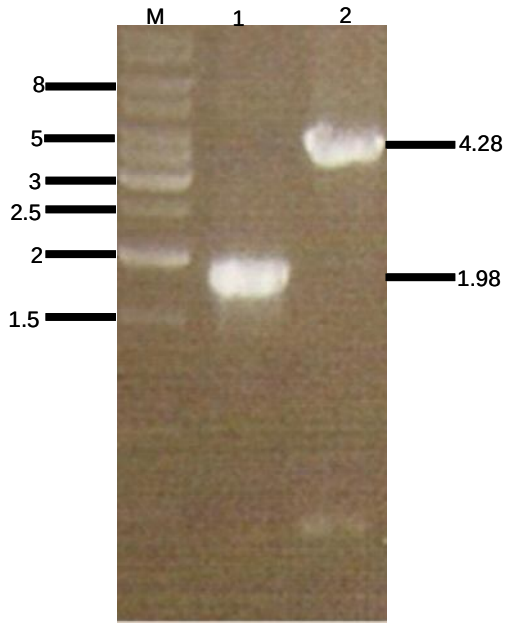

Figure 3.8. A 1% (w/v) agarose gel showing the PCR products to confirm the integration of the MTC in the genome of *Y. lipolytica* Yl10. M is the Generuler^{TM} (NEB) molecular weight marker in kbp. Lanes 1 and 2 are amplicons obtained from the genomic DNA of *Y. lipolytica* Po1d and Yl10 strains, respectively.

3.3.4. Quantitative Real Time PCR (qRT-PCR)

Quantitative RT-PCR was carried out to establish which of the two genes, *ylPK* or *ylGPI7* genes is affected by the MTC integrated within the *Y. lipolytica* Po1d genome. The amounts of the mRNA transcripts accumulated by *Y. lipolytica* Po1d and Yl10 strains during the exponential phase of the yeast growth were determined with β-actin mRNA transcripts as the reference. Complementary DNA was synthesised from the mRNA isolated from Po1d and Yl10. RT-PCR was performed using serially

diluted cDNA. The amount of PCR products that accumulated were monitored by fluorescence. The fluorescence curves of the target *PK* and *GPI7* PCR products were compared to the standard curve of the β-actin product (Figure 3.9A). The relative expression of the *PK* gene was found to be similar in *Y. lipolytica* Po1d and Yl10 strains, judging by the amount of DNA that accumulated in both strains (Figure 3.9A and B). However, low amounts of *ylGPI7* PCR products were observed in *Y. lipolytica* Yl10 (Figure 3.9B) in comparison to the *Y. lipolytica* Po1d control strain. The complete sequence of *YlGPI7* encodes a predicted protein of 860 amino acids with 33% identity with GPI7 (830 amino acids) of *S. cerevisiae.* The protein is predicted to encode an integral membrane protein.

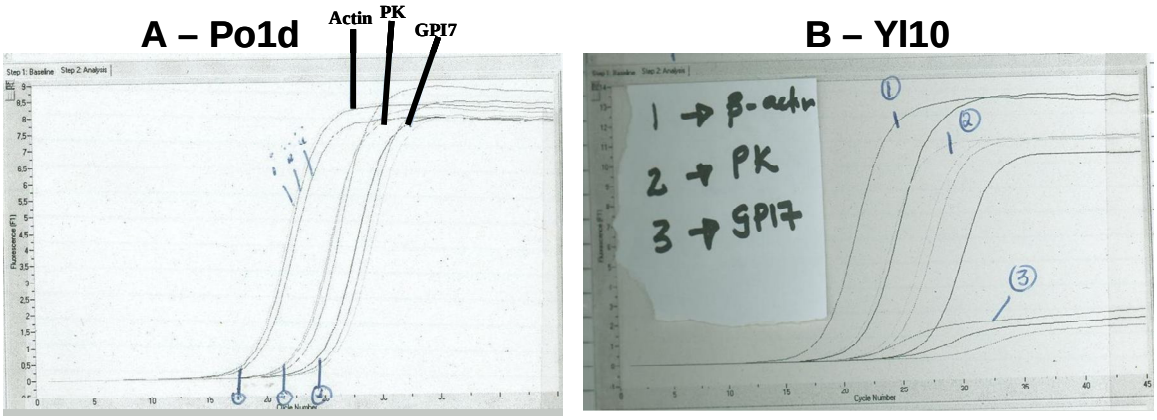

Figure 3.9. Quantitative RT-PCR analysis of *PK, GPI7* and β-actin PCR products in *Y. lipolytica* Po1d and Yl10 strains grown in YPD. The peaks are indicating fluorescence values generated by the β-actin (Actin), protein kinase (PK) and GPI7 primers, respectively.

3.3.5. Disruption of the ylGPI7 and ylPK encoding genes

The disruption of *ylPK* and *ylGPI7* genes was carried out in *Y. lipolytica* Po1d to create strains deleted for the respective genes and without the MTC using the two-step 'pop in/pop out' method (Rothstein, 1991) and hygromycin-B was used as the selection marker. The upstream and downstream border regions inclusively containing the promoter and terminator of the *ylGPI7* region were amplified as fragments of 1200 and 1150 bp, respectively (Figure 3.10A). Similarly, the promoter and terminator regions bordering the *ylPK* gene were amplified as 1110 and 1050 bp PCR fragments (Figure 3.10B), respectively. The respective promoter and terminator fragments were pooled together and subjected to PCR to create promoter-terminator (PT) fusions of 2170 and 2352 bp corresponding to *ylGP17* and *ylPK* flanking regions (Figure 3.10C).

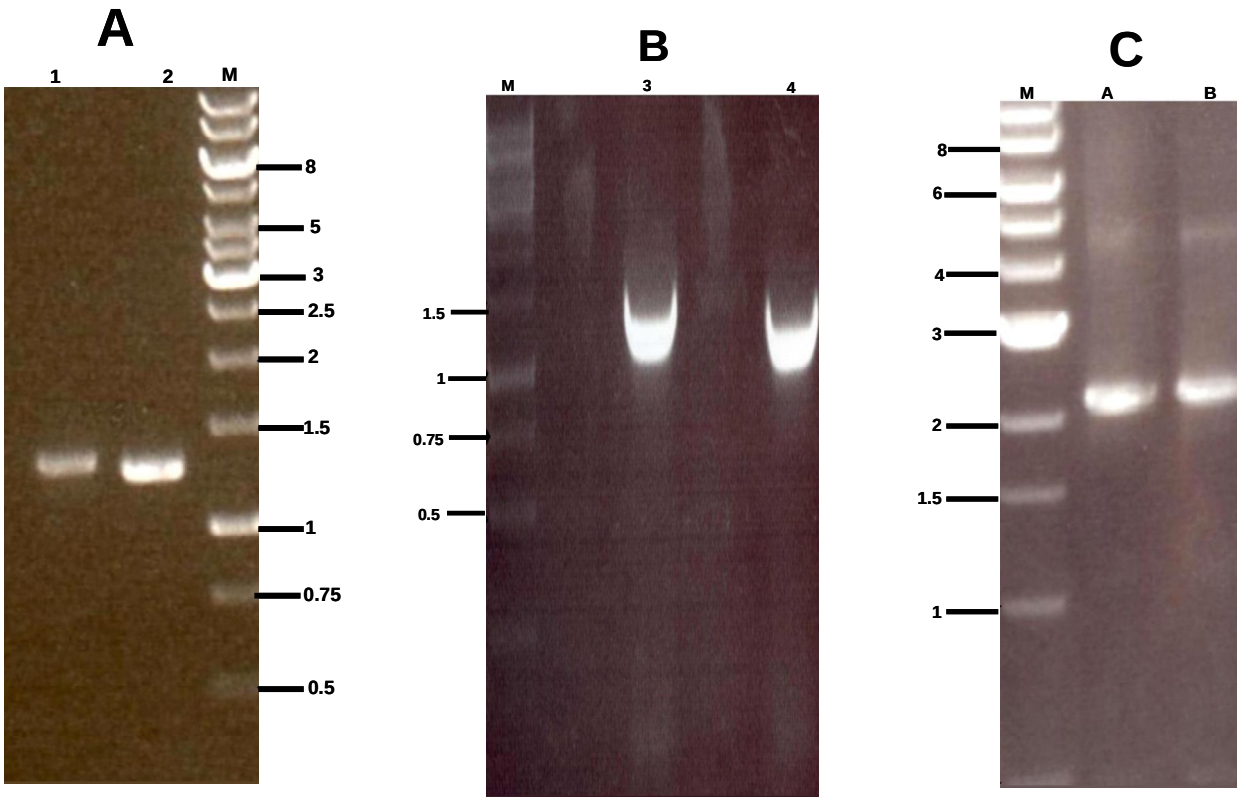

Figure 3.10. Ethidium bromide stained agarose gels (1% w/v) showing the amplicons generated by the promoter and terminator primers of *ylPK* and *ylGPI7*. Lanes M indicate the Generuler^{TM} (NEB) standard molecular weight markers in kbp. Products of the first PCR in which the promoter (lanes 1 and 3) and terminator (lanes 2 and 4) regions of (**A**) the *GPI7* (1.2 and 1.152 kbp respectively) and (**B**) *ylPK* (1.112 and 1.058 kbp) were separately amplified. (**C**) Products of the second PCR reaction in which the promoter and terminator PCR products were used as templates to obtain the combined promoter/terminator (PT) product (A = GPI7 and B = PK).

A DNA fragment containing the loxR-hph-loxP was ligated to the PT fragments to generate the final PHT cassette for deletion. The PCR amplified deletion cassettes were used to transform *Y. lipolytica* Po1d. Transformants capable of growing on HygR-agar medium were selected and the transformants tested for disruption of the *ylGP17* and *ylPK* gene by PCR. The PCR aimed at the amplification of the region within the *GPI7* locus yielded a DNA fragment of about 2700 bp with the *Y. lipolytica* Po1d strain and there was no amplification using genomic DNA isolated from one of the transformants denoted *Y. lipolytica* Yl12 (Figure 3.11). The genomic DNA from the *Y. lipolytica* Po1d and Yl12 strains were also subjected to Southern blot analysis using a PCR amplified fragment of the *ylGPI7* gene as a probe. A hydridization band was obtained in the *Y. lipolytica* Po1d control strain and was absent in *Y. lipolytica* Yl12 (Figure 3.12A). A similar procedure was followed to delete the *ylGPI7* gene in *Y. lipolytica* YlHmA25 strain over-producing epoxide hydrolase (EH) (Maharajh *et al*., 2008). The successful deletion of the *ylGPI7* in *Y. lipolytica* YlHmA25 was confirmed

by the absence of hybridisation in the *ylGPI7* deleted strain denoted YlHmA25ΔGPI7 strain while present within the original YlHmA25 strain (Figure 3.12B). Numerous transformants were obtained when both *Y. lipolytica* Po1d and YlHmA25 were transformed with the *GPI7* deletion cassette. However, a large number of these transformants had to be screened to identify the *GPI7* deleted strains. Several attempts to delete the ORF encoding *ylPK* were unsuccessful.

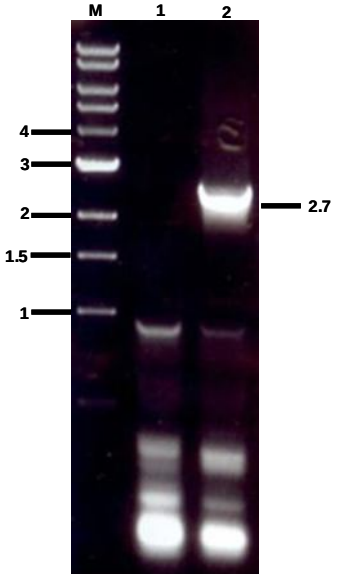

Figure 3.11. A 1% (w/v) agarose gel showing the PCR products obtained for verification of correct disruption of *GPI7* using primers GPI7F/GPI7R. The primers amplify the *GPI7* reading frame which is 2.7 kbp. Lane 1 represents the amplicons from the genomic DNA of *Y. lipolytica* YI12 and lane 2 are the amplicons of the genomic DNA from *Y. lipolytica* Po1d. M = GeneRuler™ (NEB) 1 kbp DNA Ladder.

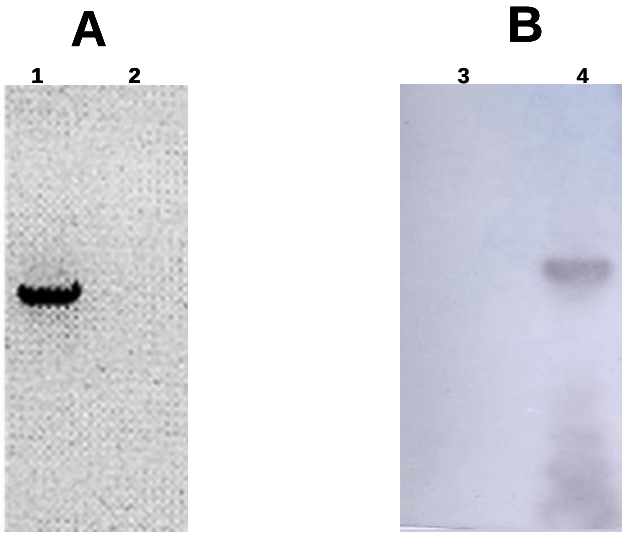

Figure 3.12. Southern blot hybrids of the genomic DNA from *Y. lipolytica* Po1d, YI12, YlHmA25 and YlHmA25ΔGPI7 probed with the *GPI7* ORF. The genomic DNA was digested with *Eco*RI. Lane 1 is representing the genomic DNA from *Y. lipolytica* Po1d, 2 is representing the genomic DNA from *Y. lipolytica* YI12, 3 is representing the genomic DNA from *Y. lipolytica* YlHmA25ΔGPI7 and 4 is representing the genomic DNA from *Y. lipolytica* YlHmA25.

3.3.6. Phenotypic properties of GPI7 deleted Y. lipolytica strain

The *Y. lipolytica* Po1d and Yl12 strains were plated on YNBT agar medium to reassess the production of extracellular lipase activity. The release of extracellular lipase activity as judged by the zones of clearance around the yeast colony was higher with *Y. lipolytica* Yl12 displaying a hydrolysis/colony diameter of 2.56 while that of *Y. lipolytica* Po1d was 1.3 (Figure 3.13). The obtained diameter ratio of *Y. lipolytica* Yl12 (2.56) was comparable with the diameter previously observed with the *Y. lipolytica* Yl10 strain (3.03). The data indicated that *ylGPI7* is indeed implicated in the enhanced release of extracellular lipase activity by the *Y. lipolytica* Yl12 strain.

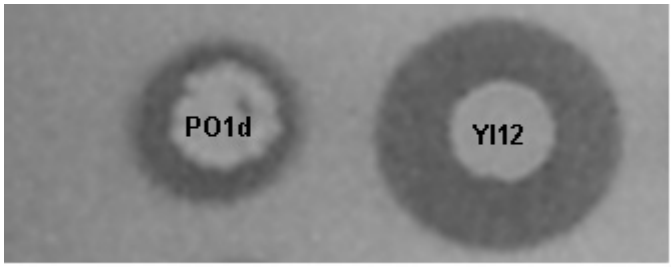

Figure 3.13. Hyperproduction of LIP2 on YNBT by *Y. lipolytica* Yl12. Lipase detection was done on YNBT plates and the hydrolysis/colony diameter was measured after 48 h.

The ability of *Y. lipolytica* Yl12 to grow on YPD supplemented with Calcofluor white and Congo red was assessed on YPD agar plates. Calcofluor white and Congo red are known to interfere with yeast cell wall construction by inhibiting chitin microfibril assembly (Zhang *et al*., 2008). While the *Y. lipolytica* Yl12 and Po1d strains grew similarly on YPD agar medium, the *Y. lipolytica* Yl12 exhibited hypersensitivity to Calcofluor white and Congo red as the cells showed reduced ability to grow (Figure 3.14).

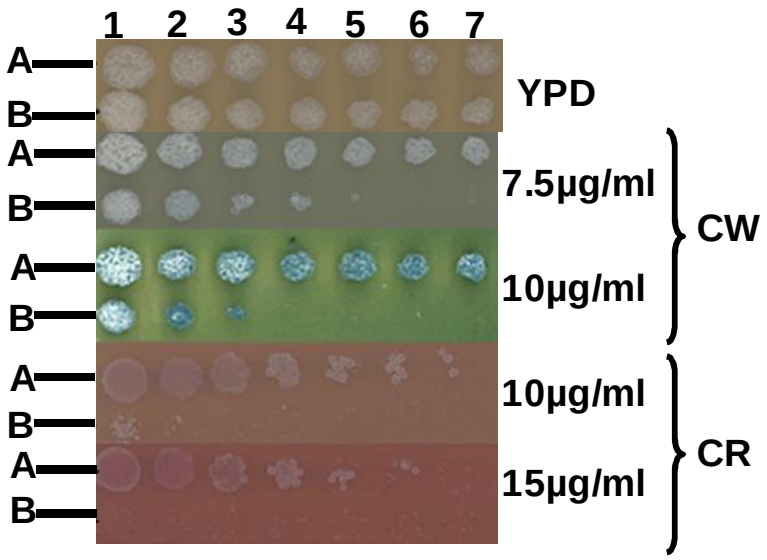

Figure 3.14. *Yarrowia lipolytica* Po1d (**A**) and Yl12 (**B**) were grown on YPD media containing Calcofluor white (CW) and Congo red (CR). 1 to 7 are representing the serial dilutions of the cell suspensions starting with an initial concentration of 7.5 µg.mL^{-1}. The plates were incubated at 28°C for 48 hours.

The *Y. lipolytica* Yl12 strain was also treated with zymolyase which is a mixture of cell wall hydrolyzing enzymes. The sensitivity of yeast cells to zymolyase has been used to monitor changes in cell wall composition and arrangement (van der Vaart *et al.*, 1995). It was observed that *Y. lipolytica* Yl12 demonstrated marked sensitivity to the lytic action of zymolyase as demonstrated by the higher rate of change in absorbance at 600nm than *Y. lipolytica* Po1d (Figure 3.15).

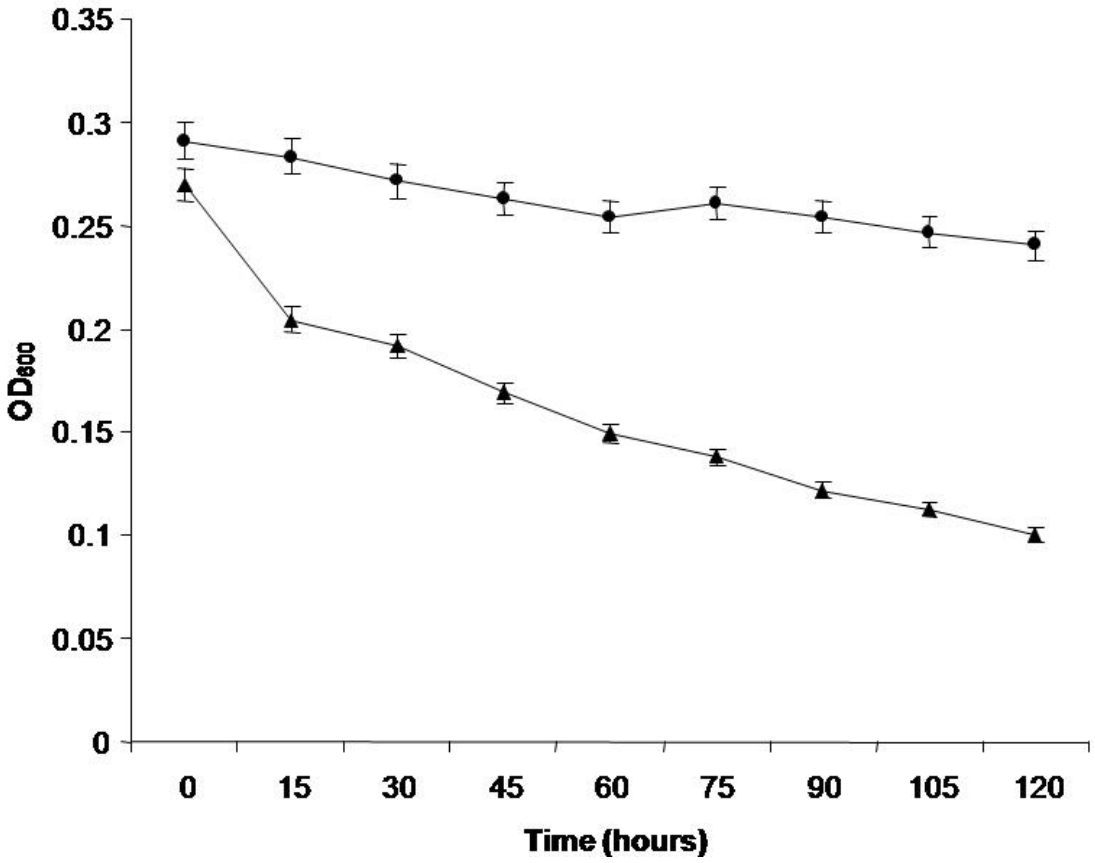

Figure 3.15. Zymolyase sensitivity of *Y. lipolytica* Yl12 cells (▲) when compared to *Y. lipolytica* Po1d (●) cells. Cells exponentially growing on YPD medium at 28ºC were treated zymolyase. At intervals after the addition of the enzyme, the absorbance was measured after dilution in water. Shown are the means ± standard deviations of three independent experiments with three samples per experiment.

3.3.7. The effect of ylGPI7 deletion on cell separation

It has been observed that GPI biosynthesis, particularly the addition of ethanolamine phosphate (EtNP) to the glycan portion of the GPI anchor by the *ylGPI7* gene product, is involved in the control of cell separation *via* modification of daughter-specific cell wall assembly proteins (Fujita *et al*., 2004). To establish if disruption of *ylGPI7* in *Y. lipolytica* affects the budding pattern of the cells, *Y. lipolytica* Po1d and Yl12 were cultured in YPD media and incubated at 28 and 37ºC with shaking. The cultures were subjected to live cell imaging using the Zeiss microscope. It was apparent that two daughter cells could not easily separate from the mother cell with the *Y. lipolytica* Yl12 *ylGPI7* null mutant strain at 37°C as compared to the *Y. lipolytica* Po1d control cells (Figure 3.16).

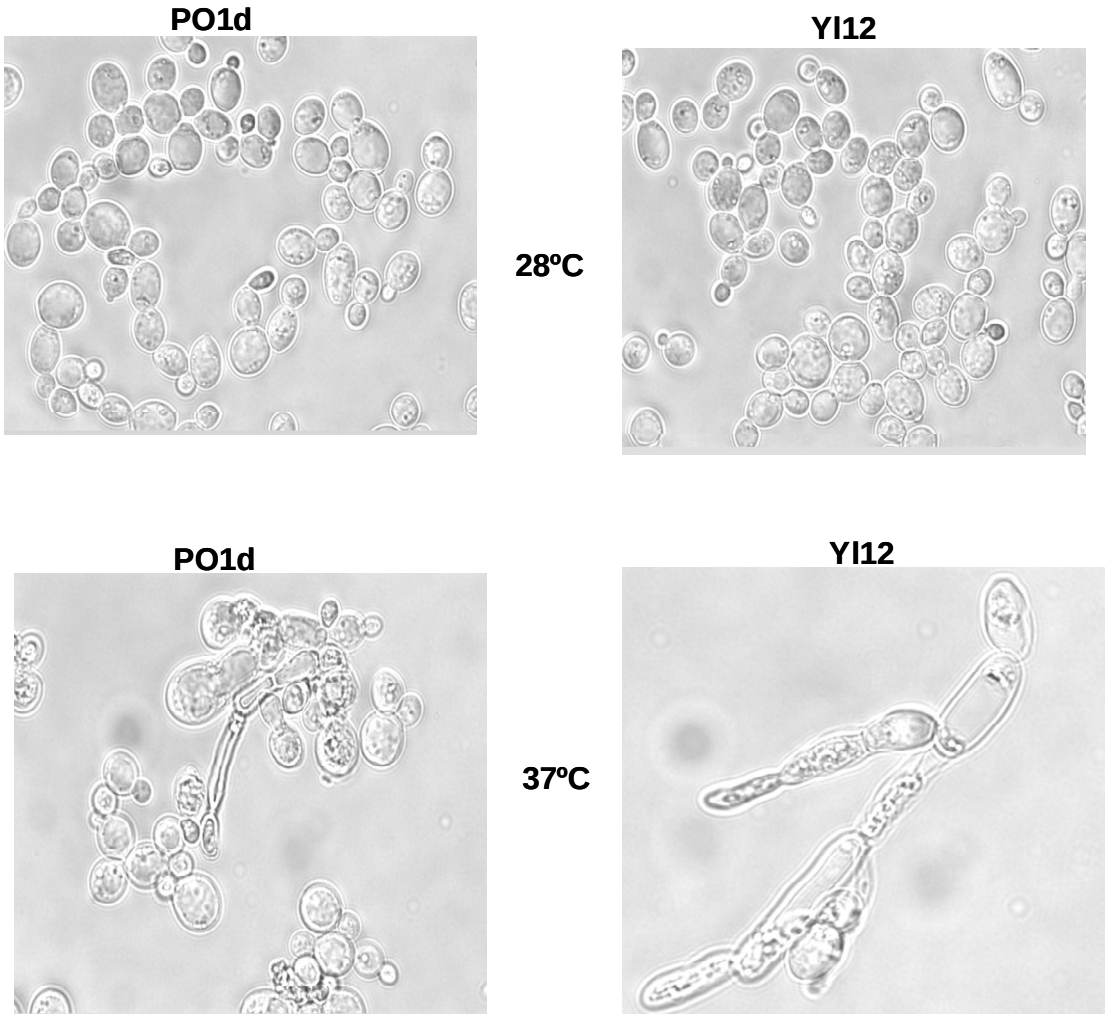

Figure 3.16. A Carl Zeiss microimage profile of actively growing cells from the cultures of *Y. lipolytica* Po1d and Yl12 strains. The strains were grown in YPD at 28 and 37°C.

3.3.8. The effect of GPI7 deletion on lipase production in shake cultures

The growth profile and lipase production by *Y. lipolytica* Po1d and Yl12 strains was studied in YPDO liquid medium which contains olive oil. Both the *Y. lipolytica* Po1d and Yl12 strains grew similarly as judged by OD measurements (Figure 3.17A). However, extracellular lipase activity of *Y. lipolytica* Po1d was less than 0.1 $U.mL^{-1}$ as compared to *Y. lipolytica* Yl12 which accumulated the maximum of 0.7 $U.mL^{-1}$ lipase activity after 70 hours of cultivation (Figure 3.17A). Three genes encode for extracellular lipase activity in *Y. lipolytica* LIP2 (Pignede *et al*., 2000a, b), LIP7 and LIP8 (Fickers *et al*., 2005b), but low levels of lipase are observed from the wild type strain Po1d strain (Pignede *et al*., 2000a). The SDS-PAGE analysis of the supernatants revealed accumulation over time of the protein band of about 38.5 kDa, the expected size of the extracellular lipase protein from *Y. lipolytica* (Figure 3.17B).

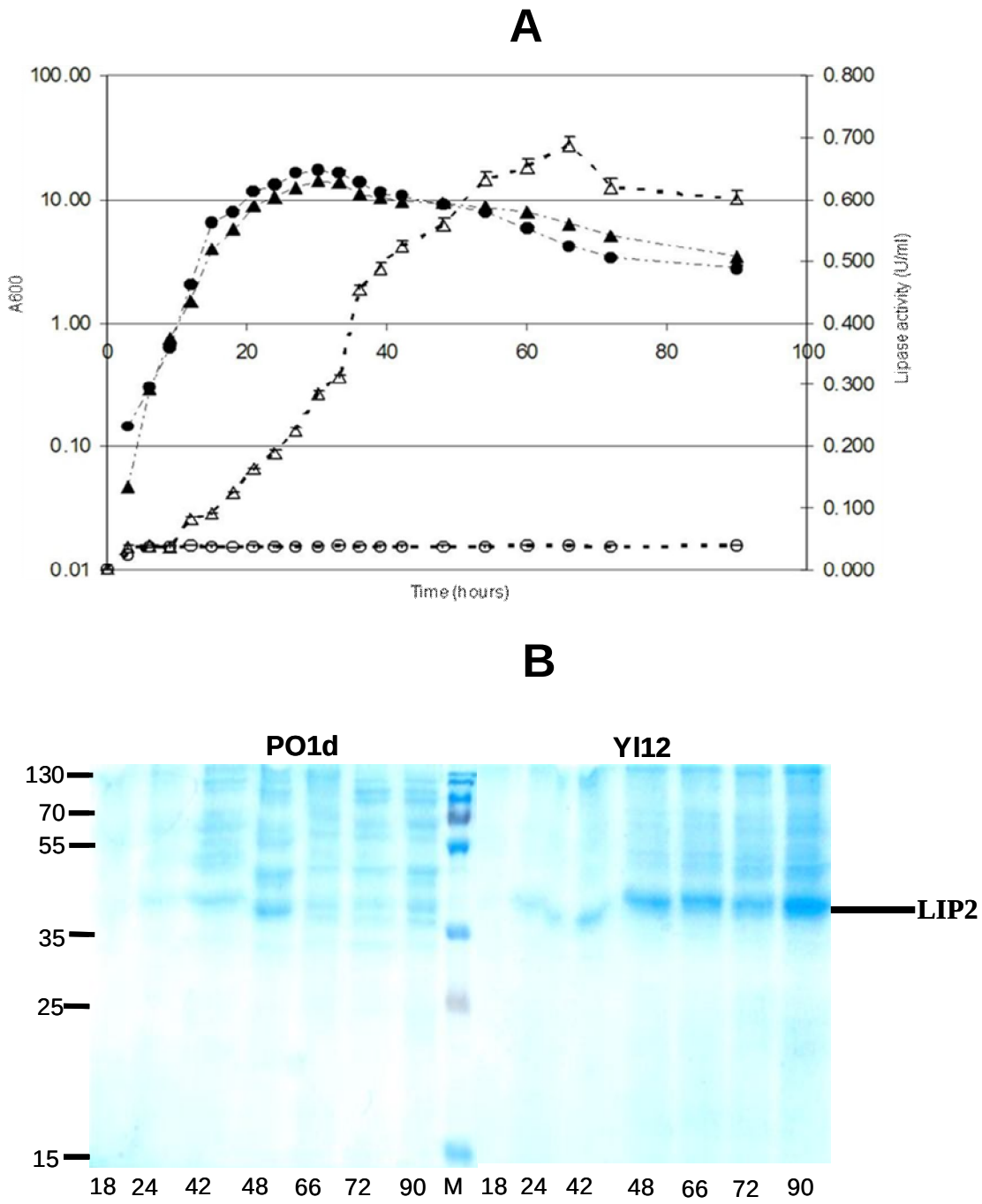

Figure 3.17. Production of LIP2 by *Y. lipolytica* YI12 in YPDO. (**A**) Growth and lipase activity in rich YPDO medium. Cell growth of the *Y. lipolytica* Po1d is shown by solid circles while that of *Y. lipolytica* YI12 is shown by solid triangles. (**B**) Extracellular lipase accumulation in YPDO medium. The lipase activity profile of *Y. lipolytica* Po1d is represented by open circles while that of *Y. lipolytica* YI12 is represented by open triangles. Samples (10 µl of crude supernatant) were resolved by SDS-PAGE (12.5%). Sizes of prestained PageRuler^{TM} protein ladder (Fermentas) molecular weight standards in kDa (lane M) are indicated on the left. The arrow marks the 38.5 kDa band representing LIP2.

3.3.9. The effect of GPI7 deletion on the extracellular release intracellular proteins

3.3.9.1. Extracellular EH activity

The *Y. lipolytica* YI25HmA strain (Maharajh *et al*., 2008) has been genetically engineered to express intracellularly the epoxide hydrolase (EH) from *Rhodotorula araucariae*, which is normally a microsomal enzyme. The derivative of *Y. lipolytica* YI25HmA deleted for *GPI7* and denoted *Y. lipolytica* YI25HmAΔGPI7 was constructed in this study. The extracellular release of EH by YI25HmA and YI25HmAΔGPI7 in shake flasks was investigated by assaying EH activity with 1,2-epoxyhexane as the substrate. GC analysis revealed a peak which corresponded to 1,2-hexanediol only when the substrate was treated with the extracellular fraction derived from the culture of the *Y. lipolytica* YI25HmAΔGPI7 (Figure 3.18). The results indicated the presence in the extracellular medium of EH activity in the culture of the *Y. lipolytica* YI25HmAΔGPI7 strain.

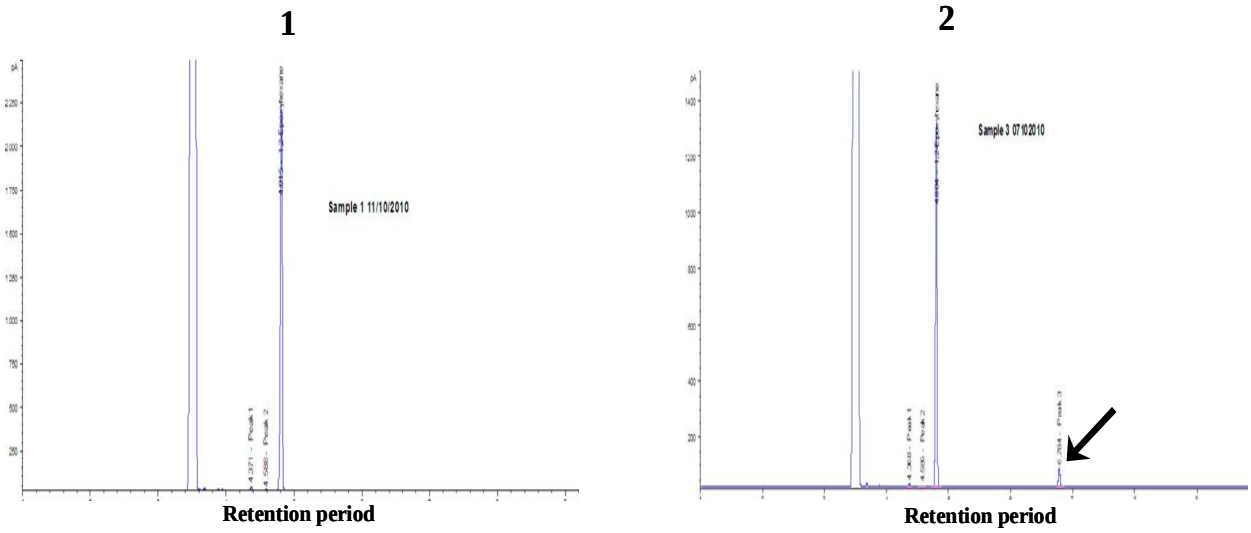

Figure 3.18. Analysis of EH production by *Y. lipolytica* YI25HmA and YI25HmAΔGPI7. Crude extracellular fractions were mixed with 1,2-epoxyhexane and analysed by GC. The chromatograms are illustrating the GC peaks generated by *Y. lipolytica* (**1**) YI25HmA and (**2**) YI25HmAΔGPI7. The arrow is indicating the 1.2-hexanediol peak.

3.3.9.2. Extracellular protein quantification

Y. lipolytica YI25HmA and YI25HmAΔGPI7 were cultured in YPD and their growth profiles and extracellular protein production patterns were compared. The *Y. lipolytica* YI25HmA and YI25HmAΔGPI7 showed similar growth pattern as reflected by OD_{600} measurements (Figure 3.19). Analysis of the total extracellular protein from the strains using extracellular supernatants derived from cultures of *Y. lipolytica* YI25HmA and YI25HmAΔGPI7 revealed that the YI25HmAΔGPI7 produced about 0.6

µg.mL^{-1} extracellular protein as compared to 0.3 µg.mL^{-1} obtained with Yl25HmA (Figure 3.19).

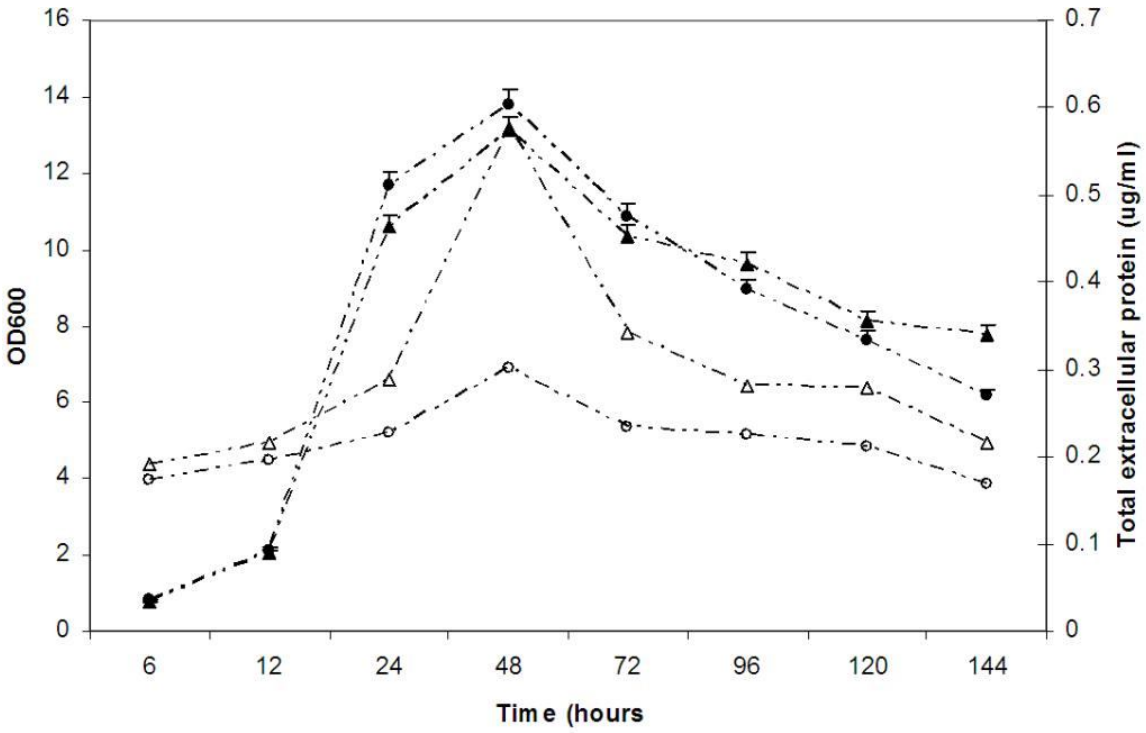

Figure 3.19. Extracellular protein accumulation in the cultures of *Y. lipolytica* Yl25HmA and Yl25HmAΔGPI7. The solid circles (●) are representing the growth profiles of *Y. lipolytica* Yl25HmA while that of Yl25HmAΔGPI7 is represented by solid triangles (▲). Extracellular protein accumulation from *Y. lipolytica* Yl25HmA is shown by open circles (○) while that from Yl25HmAΔGPI7 is shown by open triangles (Δ). The data represent the mean ± standard deviations of three independent experiments.

3.4. Discussion

The *zeta*-based cassette for random mutagenesis of the *Y. lipolytica* genome has been used previously for the identification of mutants affected in the utilization of hydrophobic substrates (Mauersberger *et al*., 2001; Thevenieau *et al*., 2007). This study employed the *zeta*-based random mutagenesis technique to identify *Y. lipolytica* mutant strains with enhanced extracellular protein production using LIP^{++} as a reporter phenotype. Three clones, *Y. lipolytica* Yl5, Yl6 and Yl10, transformed with the *zeta*-based MTC resulted in enhanced production of extracellular lipase when assessed on agar medium containing tributyrin. The genetic analysis of the mutants revealed that the lipase hyperproduction phenotype is caused by insertions at different loci in the genome as indicated by the different hybridization patterns obtained with Southern blotting, suggesting the presence of multiple loci that regulate extracellular lipase production.

The SSA-PCR for genome walking was used to identify the *zeta*-based MTC insertion points. The technique was successful only with the *Y. lipolytica* YI10 mutant clone. Sequence and BLAST search analysis revealed that the insertion of the MTC occurred between ORFs encoding GPI7 and PK. It was expected that the *PK* gene is affected as the integration of the MTC occurred at a locus that is near the promoter region of the *ylPK* gene. Quantitative RT-PCR was employed to identify the ORF whose changed expression resulted in extracellular lipase hyperproduction phenotype. The mRNA transcript level of *GPI7* was found to be significantly reduced in comparison to mRNA transcript level of the *ylPK* gene indicating that the disruption of the *GPI7* was likely to result in enhanced extracellular lipase production. The *GPI7* ORF was deleted in the *Y. lipolytica* Po1d strain to create the *Y. lipolytica* YI12 strain and the analysis of extracellular lipase production on tributyrin agar plate and in liquid shake cultures confirmed that the disruption of *GPI7* does indeed enhance extracellular lipase production. This was interesting since GPI7 is known to play a role during the biosynthesis of the GPI-anchor by adding phospho-ethanolamine to the second mannose residue (Benachour *et al*., 1999; Orlean and Menon, 2007).

The deletion of the ORF encoding *ylPK* was attempted several times without success. PK proteins mediate signal transduction processes in eukaryotes which play pivotal roles in the regulation of essential biological processes such as cell cycle progression, cellular morphogenesis, cell development, and cellular response to environmental changes (Chen *et al*., 2003). This could explain the failure to delete the *ylPK* gene within the *Y. lipolytica* genome as some of these genes are essential for cell viability and therefore impossible to delete (Bimbó *et al*., 2005). The *ylPK* gene identified in this study could therefore be one of the essential genes within the *Y. lipolytica* genome whose deletion is lethal to the yeast cell.

The mutants of *Y. lipolytica* with enhanced capabilities to produce extracellular lipase activity have been described in literature (Mauersberger *et al*., 2001; Fickers *et al*, 2003; Thevenieau *et al*., 2007). However, the genes responsible for the increased capacities of the *Y. lipolytica* strains to over-produce the extracellular lipase activity on solid or liquid media have not yet been reported in literature. *Yarrowia lipolytica* mutants disrupted in the *GPI7* gene have been described in literature, but the authors did not investigate lipase production by these mutants (Richard *et al*., 2001). GPI7 is known for its role in the synthesis of cell wall and cell membrane proteins. Its deletion results in defects on the biosynthetic pathways thereby affecting the integrity of the

cell wall (Benachour *et al*., 1999). *GPI7* mutations in *S. cerevisiae* and *Candida albicans* were also found to affect cell wall anchorage (Richard *et al*., 2002). Consistent with the disruption of cell wall integrity through deletion of *GPI7*, the *Y. lipolytica* Yl12 was more sensitive to cell wall hydrolysing enzymes (van der Vaart *et al*., 1995). In addition it also displayed defects in cell separation, showed by a daughter cell-specific growth defect at the non-permissive temperature (Fujita *et al*., 2004) and also exhibited hypersensitivity to Calcofluor white and Congo red which are known to interfere with yeast cell wall composition (Zhang *et al*., 2008; Richard *et al*., 2001). While the *Y. lipolytica* Yl12 and Po1d strains grew similarly in shake cultures, the *Y. lipolytica* Yl12 strain produced 7 times more extracellular lipase activity in liquid shake culture than the control *Y. lipolytica* Po1d strain. The lipase activity production studies were correlated with SDS-PAGE data on supernatants collected from the extracellular medium of *Y. lipolytica* Yl12 and Po1d cultures grown in medium containing olive oil. A protein band of about 38.5 kDa which corresponded to the size of the LIP2 protein accumulated over time as judged by SDS-PAGE analysis.

To investigate the mode of *GPI7* action in the enhancement of protein production, a *Y. lipolytica* YlHmA25 strain expressing intracellular EH enzyme under the hp4d promoter constructed by Maharajh *et al*., (2008) was used. The gene encoding GPI7 was deleted from the *Y. lipolytica* YlHmA25 strain to create *Y. lipolytica* YlHmA25ΔGPI7. The extracellular fractions derived from growth cultures of *Y. lipolytica* YlHmA25 and *Y. lipolytica* YlHmA25ΔGPI7 were used to assay for EH activity. GC analysis revealed that the extracellular fraction from *Y. lipolytica* YlHmA25ΔGPI7 resulted in the conversion of the 1,2-epoxyhexane substrate to 1,2 hexanediol as compared to YlHmA25 with which no activity was obtained in the extracellular supernatant. This data demonstrated the presence of EH activity in the extracellular supernatant of the *Y. lipolytica* YlHmA25ΔGPI7 culture. In addition, the *Y. lipolytica* YlHmA25ΔGPI7 accumulated more total extracellular protein than *Y. lipolytica* YlHmA25, an indication that the strain accumulates more proteins extracellulary. Taken together, the results suggested that the disruption of *GPI7* affects the integrity of the yeast cell wall which in turn results in leakage or "enhanced" secretion of proteins to the extracellular. The indication that GPI7 acts at protein secretion level is further suggested by the finding that both the LIP2 and EH reporter proteins used in the study resulted in the enhanced release to the extracellular despite the genes being under different promoters, the native *pLIP2* and the quasi constitutive hp4d, respectively. The mutants of *Zygosaccharomyces bailii*,

S. cerevisiae and *Pichia pastoris* defective in the *Gas1* gene encoding a beta-1,3-glucanosyltransglycosylase protein which plays an important role in cell wall construction and in the determination of its permeability was also reported to result in super secretion of proteins (Passolunghi *et al*., 2010). These observations suggest that protein secretion in yeast could be enhanced by disruption of selected cell wall proteins. There are several proteins that contribute to the structure of the cell wall. The mutant strains from this study whose affected genes could not be identified might also be affected in biosynthesis of cell wall structural proteins.

The surprising discovery was the finding that the insertion of the MTC within the DNA sequence between the *ylPK* and *ylGPI7* genes in *Y. lipolytica* Po1d strain affected the expression of the adjacent *ylGPI7* gene, despite the DNA fragment at the insertion point not encoding for any deducible ORF nor corresponding to any known transcription factor. However, Druker and co-workers (2004) reported on a retrotransposon having both upstream and downstream effects on transcription at the site of insertion in eukaryotic cells caused by alternative polyadenylations of the long terminal repeats (LTR) of the retrotransposon. In addition, studies in plants have shown that retrotransposons can influence the expression of adjacent host genes by altering the methylation status of the retrotransposon LTR (Kashkush and Khasdan, 2007). The *zeta*-based MTC described in this study carries the LTR derived from the *Y. lipolytica* retrotransposon *Ylt*1 and is capable of integrating randomly within the *Y. lipolytica* genome (Schmid-Berger *et al*., 1994, Mauersberger *et al*., 2001). The effect on the GPI7 gene could be as a result of fortuitous integration of the MTC near the GPI7 ORF and affecting negatively the gene expression through a yet to be establish *cis*-acting mechanism. The *zeta*-based MTC is increasingly being used in the isolation of *Y. lipolytica* mutants and there should be caution in the implication of genes affected in the derived mutants. Integrative expression systems based on *zeta* elements have also been described in literature and the different insertion sites of the *zeta* element could result in different expression levels of the target protein.

In conclusion, a GPI7 null mutant of *Y. lipolytica* was shown to possess enhanced extracellular lipase production probably through affecting the cell wall integrity thereby allowing leakage or enhanced secretion of the lipase protein to the cell extracellular. This GPI7 mutant strain denoted *Y. lipolytica* Yl12 holds potential for application as a host in the production of heterelogous proteins due to its enhanced capacity to secrete proteins to the extracellular and its growth profiles that remain unaltered at least in liquid growth cultures.

3.5. References

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Disruption of the gene encoding OCH1 in the *GPI7* null mutant *Yarrowia lipolytica* strain

4.1. Introduction

In eukaryotic cells, transmembrane and secreted proteins undergo several posttranslational modifications during their maturation to attain their full biological function (Hamilton and Gerngross, 2007). *N*-Glycosylation is one of these modifications; and it is essential for proper folding, pharmacokinetic stability, and efficacy for a large number of proteins (Helenius and Aebi, 2001). *N*-glycosylation is initiated in the endoplasmic reticulum (ER) lumen, were a core oligosaccharide composed of $Glc_3Man_9GlcNAc_2$ is transferred to the asparagine of the consensus sequence Asn-X-Ser/Thr of the nascent polypeptide chain. Before exiting the ER, the three glucose residues and one mannose residue are removed from the core oligosaccharide to create $Man_8GlcNAc_2$.

Yeast and mammals share these initial steps of *N*-glycosylation in the ER. However in the Golgi apparatus, the pathways diverge notably between mammals and yeast (Hamilton *et al*., 2003). In *S. cerevisiae*, the *N*-linked oligosaccharides assembled on glycoproteins include hypermannose structures with outer chains that may contain up to 200 mannose units (Dean and Pelham, 1990). Elongation of the outer chain is initiated by the OCH1 protein, which adds the first α-1,6-linked mannose to the core N-linked oligosaccharides upon arrival in the Golgi apparatus (Hamilton *et al*., 2003). Other yeast species, such as *Pichia pastoris*, *Hansenula polymorpha* and *Schizosaccharomyces pombe*, also use the OCH1 protein to extend the mannose outer chain of N-glycans (Kim *et al*., 2006). Consequently, the elimination of the OCH1 encoding gene has been performed to eliminate the yeast-specific outer-chain mannosylation (Hamilton *et al*., 2003; Kim *et al*., 2006). Glycoengineering of the *P. pastoris* yeast to produce proteins with human-like glycans was accomplished by inactivation of the *OCH1* gene followed by overexpression of an α-1,2-mannosidase retained in the endoplasmic reticulum as well as *N*-acetylglucosaminyltransferase I and β-1,4-galactosyltransferase retained in the Golgi apparatus (Vervecken *et al*., 2004). The engineered strain synthesized a nonsialylated hybrid-type *N*-linked oligosaccharide structures on its glycoproteins yielding up to 90% homogeneous protein-linked oligosaccharides (Vervecken *et al*., 2004).

The objective of this study was to establish if the *Y. lipolytica GPI7* null mutant strain constructed in Chapter 2 is amenable to glycoengineering. This was established through deletion of the *YlOCH1*, a key gene in the yeast glycosylation pathway and assessment of growth performance in liquid shake cultures. *YlOCH1* encodes an enzyme that initiates the outer-chain elongation by adding the first α-1,6-linked

mannose core oligosaccharide in yeast (Figure 4.1) (Song *et al*., 2007). When this first α-1,6-mannose is added, additional α-1,6-mannosyltransferases extend the α-1,6-chain, which then becomes the substrate for α-1,2-mannosyltransferase, as well as phospho-mannosyltransferases that adds yet more mannose sugars to the growing *N*-glycan structure (Dean, 1999). Disruption of the gene encoding OCH1 is the initial step towards constructing a yeast strain capable of producing human-like glycan structures (Hamilton *et al*., 2003). In this study, the nature of glycans produced by the *OCH1* deleted *Y. lipolytica GPI7* null mutant was studied in comparison to the glycan intermediates produced in mammalian cells.

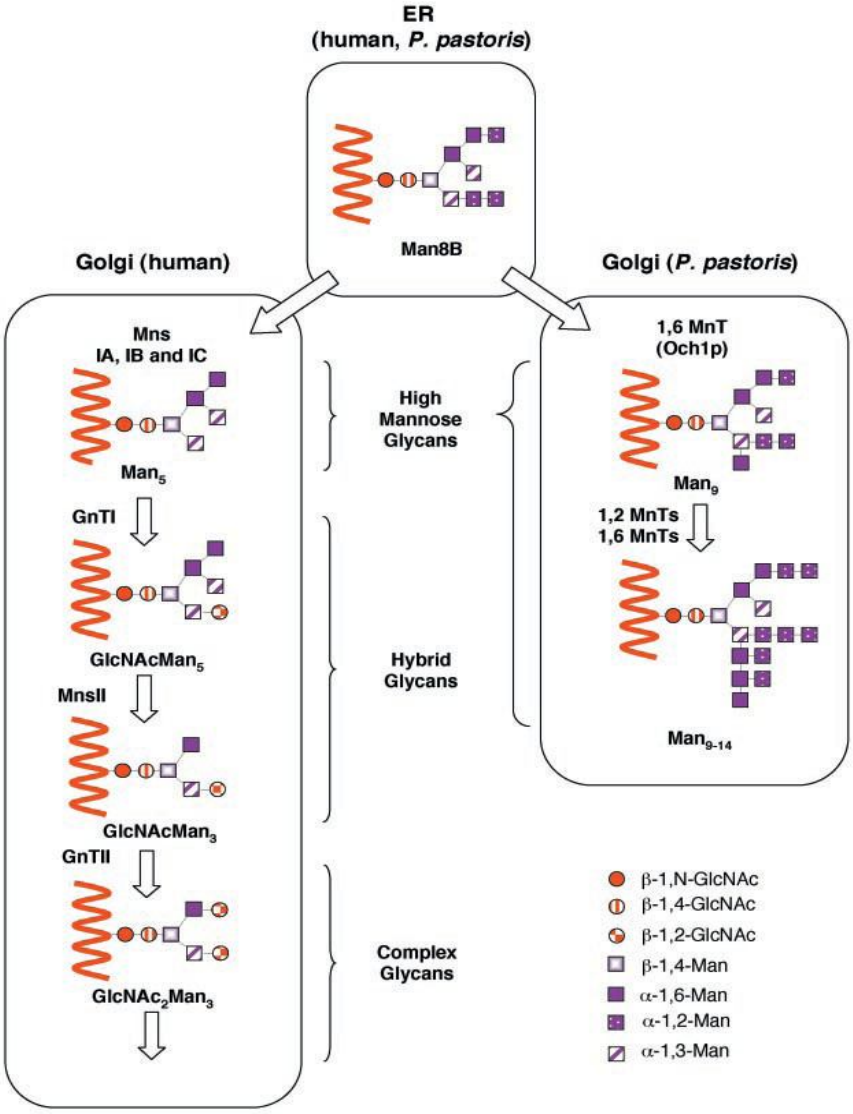

Figure 4.1. A diagram illustrating N-linked glycosylation pathway in humans and in yeasts, using *P. pastoris* as an example. Mns, α-1,2-mannosidase; MnsII, mannosidase II; GnT1, α-1,2-*N*-acetylglucosaminyltransferase I: GnTII,α-1,2-*N*-acetylglucosaminyltransferase II; MnT, mannosyltransferase (Taken from Hamilton *et al*., 2003).

4.2. Materials and Methods

4.2.1. Strains, media and growth conditions

Escherichia coli Xl10-gold (Stratagene, La Jolla, United States) used for plasmid preparation was grown in Luria-Bertani (LB) medium (Sambrook *et al*., 1989). The *Y. lipolytica* strains used in the study are listed in Table 3.1. The yeast media YPD ($g.L^{-1}$, glucose 10, yeast extract 10 and peptone 10) was used to culture the *Y. lipolytica* strains. For solid media, 1.5% (w/v) agar was added. Yeasts were transformed as described previously by the lithium acetate method (Le Dall *et al*., 1994), and clones were selected on YPD-hygromycin (YPD-Hyg) [600 $\mu g.mL^{-1}$ Hygromycin B (Roche Diagnostics, Mannheim, Germany)] at 28°C.

Table 4.1 Plasmid, strains and oligonucleotide primers used in the study

Plasmids, strains, primers	Genotype, primer (5´- 3´)	Reference, source, purpose
Plasmid		
pOCHI	pGemT-Easy containing the *YlOCH1* deletion cassette	This study
Yarrowia lipolytica strains		
Yl12	*MatA*, *leu2-270*, *ura3-302*, *xpr2-322,* ∆*GPI7*	This study
Yl13	*MatA*, *leu2-270*, *ura3-302*, *xpr2-322,* ∆*GPI7*, *ΔOCHI*	This study
Primers		
OCHIpF	TGAGCGTTATCTTACTCACTTTC	Deletion
OCHIpR	CATTACCCTGTTATCCCTAGGTGTGACGAAGTATCGAG	Deletion
OCHItF	CTAGGGATAACAGGGTAATGAGAGATTCGAGGGCAAAG	Deletion
OCHItR	CCTAGGCTATTAGTGGTGGTGGTGATGG	Deletion
OCHISc	GGCAGGCGTTGTATTCTCCCGAGACG	Screening
HygBR	CGTACACAAATCGCCCGCAGAAGCG	Screening

The underlined segment is representing the *I-Sce*-I recognition sequence

4.2.2. Construction of the deletion vector

4.2.2.1. Construction of the promoter-terminator cassette

The *OCH1* gene was identified by BLAST searches using ClustalW (Altschul *et al*., 1997). *Yarrowia lipolytica* Po1d was used as a source of genomic DNA as essentially described by Labuschagne and Albertyn (2007). The genomic DNA was used as a template to amplify the ~1 kbp promoter and terminator regions upstream and downstream of the *YlOCH1* using Taq DNA polymerase (New England Biolabs, Beverly, MA). The oligonucleotides used to amplify the *YlOCH1* promoter and terminator regions are listed in Table 3.1. Amplification of the *OCH1* promoter region was done using primers OCHIpF and OCHIpR, while the terminator region was amplified with primers OCH1tF and OCH1tR. The amplifications were done using the

following thermal cycling reactions initial denaturation of 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 80 s. This was followed by a final extension of 72°C for 10 min.

The generated amplicons were gel purified and pooled together based on sticky-end PCR (SEP) that facilitates the introduction of an I-*Sce*I site between the promoter and terminator regions (Fickers *et al*., 2003), to constitute the PT cassette. In a final reaction volume of 50 µl, 100 ng of the promoter and terminator amplicons were fused, and 4 mm of the OCHIpF and OCHItR (Table 3.1) were used for the amplification of the PT. The SEP thermal cycling reaction was as follows; 94°C for 2 min, 41°C for 5 min, 72°C for 10 min, followed by 35 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 2.5 min. This was followed by a final elongation at 72°C for 10 min. The PT amplicon was gel purified and subcloned into pGemT-Easy followed by transformation of *E. coli* for plasmid propagation. The plasmids were sequenced (Inqaba Biotech, Pretoria, South Africa). Correctness of the plasmids was confirmed by restriction analysis. JMP115 (Fickers *et al*., 2003) was digested with I-*Sce*I and the ~1.6 kbp fragment was gel purified and ligated into I-*Sce*I digested and dephosphorylated pGemT-Easy-PT. This resulted in a plasmid that was confirmed by restriction analysis and denoted pOCHI (Figure 4.3).

4.2.2.2. Transformation of the deletion cassette

The *YlOCH1* deletion vector (pOCHI) was used as a template for the amplification of the deletion cassettes. Oligonucleotides OCH1pF and OCHItR were used in a standard PCR to amplify the total deletion cassette. The thermal cycling condition included initial denaturation of 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 4 min, followed by final elongation of 10 min at 72°C. Transformation of *Y. lipolytica* Yl12 was done using 1 µg of the PCR product.

4.2.2.3. PCR deletion screening

Transformants demonstrating acceptable hygromycin B resistance after transformation were subjected to a second round of selection on hygromycin B containing plates where after a single colony of each was used to inoculate 5 ml YPD and cultivated at 28ºC while shaking for 2 days. Genomic DNA was isolated as described by Labuschagne and Albertyn (2007) and used as templates in a PCR screen using primer pairs OCH1F and HygBR. OCH1F is located upstream to the forward promoter of the *OCH1* gene, respectively while the HygBR is located within the hygromycin gene. The PCR amplification conditions were as follows: initial

denaturation of 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 61°C for 30 s, 72°C for 2 min, followed by final elongation of 5 min at 72°C.

4.2.2.4. Selection marker recycling

The *Y. lipolytica* Yl12 strain deleted for *OCH1* gene denoted was transformed with the pQQR2 plasmid (Fickers *et al.*, 2003) and selected on YNB_{5000} medium ($g.L^{-1}$; 1.7 Yeast nitrogen base (without amino acids or ammonium sulfate), 5 ammonium sulphate, 10 glucose) and incubated at 28°C. Transformants were replica-plated on YPD-Hyg and YNB_{5000} supplemented with casamino acids. A hygromycin sensitive colony was selected and denoted *Y. lipolytica* Yl13.

4.2.3. Growth and phenotype analysis of Y. lipolytica Yl12 and Yl13

To establish if the deletion of *OCH1* affects the growth of the mutant, the strains were culture in YPD for 18 h. The sensitivity of *Y. lipolytica* Yl13 to Calcofluor white and Congo red as compared to Yl12 was tested by growing the strains on YPD supplemented with these substrates. Single colonies were selected from YPD plates and suspended in YPD broth. Serial dilutions of the suspension, $10^{-1,}$ $10^{-2,}$ 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were made. Aliquots (5 µl) from each dilution were streaked on YPD agar plates containing Calcofluor white (20 $\mu g.mL^{-1}$) and Congo red (15 $\mu g.mL^{-1}$). The plates were incubated for 48 h at 28ºC for temperature sensitivity assay on YPD.

4.2.4. Extracellular protein production

Single colonies from *Y. lipolytica* Yl12 and Yl13 growing on a YPD agar plate incubated at 28°C for 48 h were transferred to a 500 ml Erlenmeyer shake flask (pH 6.8) containing 100 ml YPDO medium. The YPDO medium comprised ($g.L^{-1}$; 20 peptone, 20 glucose, 10 yeast extract, 10 olive oil). The culture flask was subsequently incubated on a rotary shaker at 28°C at 200 rpm. During the cultivation, growth was monitored by taking 2 ml samples at regular time intervals. The strains were cultured for 66 hours.

4.2.5. Extraction of extracellular proteins from the culture supernatants

Yarrowia lipolytica Yl12 and Yl13 cultures were centrifuged for 30 min at 12000 rpm in a bench top centrifuge at 4ºC. Supernatants were transferred to another tube and the proteins were extracted by acetone precipitation. Briefly, 1/3 vol. of 100% acetone was added to the supernatants and incubated for 1 h at 4 ºC. Precipitates were collected by centrifugation at 12000 rpm for 20 minutes. They were washed twice, first with 10 mM HCl/acetone and then acetone. Precipitated proteins were

allowed to dry at 55°C and were resuspended in 100 µl of 10 mM sodium phosphate buffer (pH 7.0).

4.2.6. Glycan analysis

4.2.6.1. Deglycosylation reactions

A microlitre of N-glycosidase (Roche Diagnostics, Mannheim, Germany) was added to 100 µl of protein solution (approximately 50 µg in 10 mM sodium phosphate, pH 7.0). IGEPAL CA-630 (25 µl) was added and the mixtures were incubated for a minimum of 16 h at 37ºC in a heating block. The N-glycosidase digestion mixtures were loaded onto Strata C18 cartridges (Phenomenex, Torrance, USA) and the flowthrough was collected. Cartridges were washed twice with 200 µl of water in order to collect additional glycans. 2-AB labelling reagent was prepared by dissolving 43 mg of 2-AB in 1 ml of 30% acetic acid/DMSO to which 68 mg sodium cyanoborohydride was added. Dried glycans were resuspended in 10 µl of 2-AB labelling reagent and incubated at 70ºC for 2 hours. Labelled glycans were purified using AccubondII CYANO cartridges (Agilent, Santa Clara, USA) that were equilibrated in 95% acetonitrile. Following loading of the samples onto equilibrated cartridges, and a wash with 95% acetonitrile, glycans were eluted in 600 µl 50% acetonitrile, and dried in a SpeedVac.

4.2.6.2. MALDI/TOF analysis

Glycans were re-suspended in 10 µl MS-grade H_2O, vortexed and sonicated in a water-bath sonicator for 5 min. Samples were mixed, 1:1 (v/v), with 10 $mg.mL^{-1}$ DHB (2, 5-Dihydroxybenzoic acid) matrix re-suspended in 10% acetonitrile. The glycan-matrix mixture was spotted in duplicate on a ground steel plate. A gentle stream of cold air from a hair dryer was used to assist sample drying. Data was acquired in reflector mode using an accelerating voltage of 20 kV. Samples were ionised using a nitrogen laser (λ = 337 nm). Instrument calibration was performed using a polysaccharide standard that covers the mass range of interest 1000 – 3500 Da. Data, calibration, smoothing and base-line subtraction was performed using FlexAnalysis software. Peak allocation/identification was performed using Glycoworkbench (Ceroni *et al*., 2008).

4.3. Results and discussion

4.3.1. Construction of the promoter-terminator cassettes

The deletion cassette for the *Y. lipolytica OCH1* gene was constructed using the DNA sequence flanking the open reading frame of the *OCH1* gene. The regions corresponding to ~1kb upstream to the initiation codon (promoter region) and ~1kb downstream of the termination codon (terminator region) for the *YlOCH1* gene were successfully amplified by PCR (Figure 4.2A). The promoter and terminator regions had the I-*Sce*I restriction site at the 3′ and 5′ ends, respectively. The promoter-terminator fusion was created by PCR amplification using sense and antisense primers of promoter and terminator regions with the 1 kbp PCR fragments as templates. This resulted in the amplification of a 2 kbp DNA fragment representing the promoter-terminator fusion (Figure 4.2B). The promoter-terminator PCR product was cloned into pGemT-Easy to create pGemT-ylPro-Ter plasmid. The DNA fragment which included the hygromycin B resistance gene (*hph*) was transferred by I-*Sce*I digestion from JMP115 to the pGemT-ylPro-Ter resulting in the plasmid denoted pOCH1 (Figure 4.3). The pOCH1 plasmid was digested with *Not*I which confirmed the presence of the 3700 bp *YlOCH1* and the ~3000 bp corresponding to the pGemT-Easy backbone (Figure 4.3).

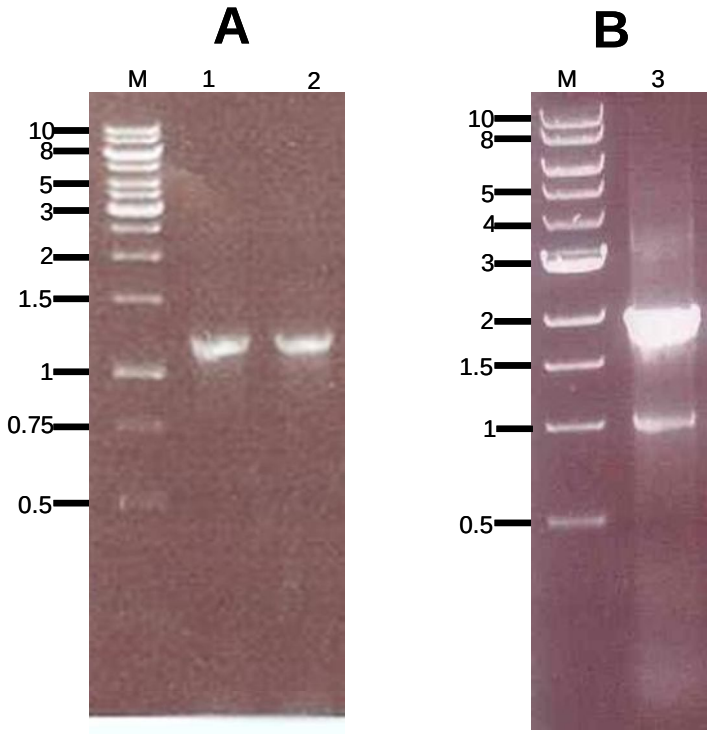

Figure 4.2. Agarose gel (1% w/v) showing the PCR products of the separate (**A**) ~1 kbp and (**B**) fused ~2 kbp) promoter and terminator regions of *Y. lipolytica OCHI* (lanes 1, 2 and 3). M = 2-Log DNA ladder (NEB) in kbp.

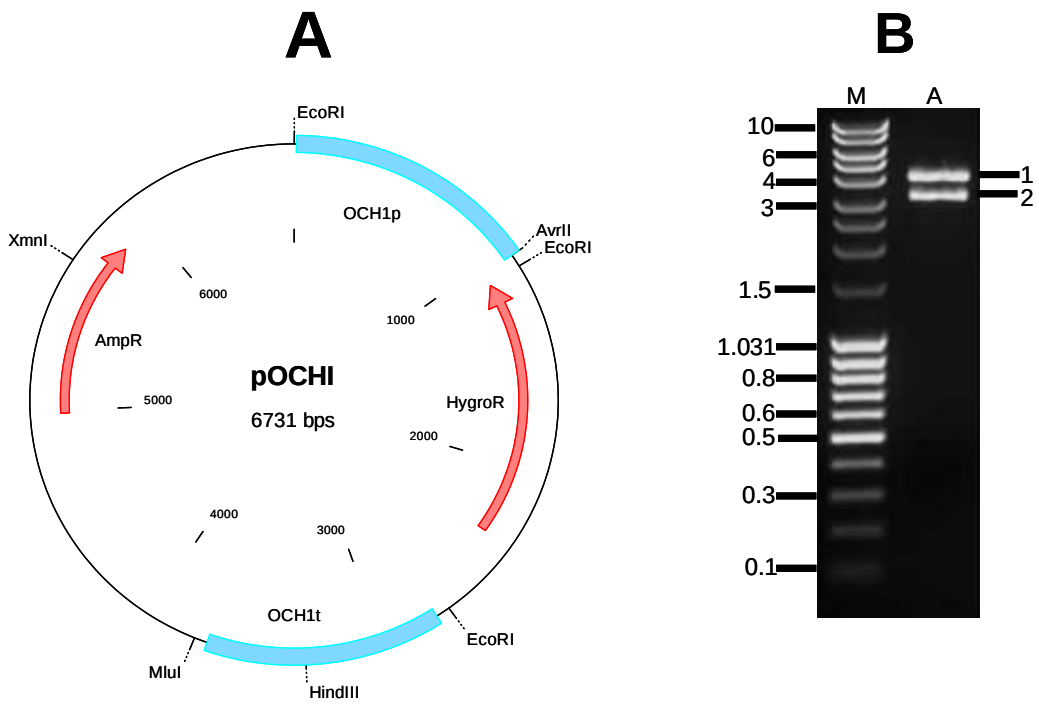

Figure 4.3. Construction of the *OCH1* deletion vector. (**A**) Schematic diagram of the pOCH1 vector for the disruption of the *Y. lipolytica OCHI* genomic region. (**B**) A 1% (w/v) agarose gel showing the pOCH1 construct digested with *Not*I. Digestion with this enzyme resulted in the release of *YlOCH1* deletion cassette of lane 1 (3.7 kbp from the pGemT-Easy backbone of 3.0 kbp. M = Fermentas MassRuler™ DNA Ladder Mix in kbp.

4.3.2. Deletion of YlOCH1

The deletion cassette corresponding to the promoter and terminator region of the *YlOCH1* separated by the gene encoding hygromycin resistance was amplified by PCR and used to transform the *Yarrowia lipolytica* Yl12 *GPI7* null mutant strain. The transformed colonies were selected on hygromycin B containing YPD plates. The correct transformant was identified by PCR where a sense primer based on the sequence upstream to the 1 kbp promoter region of the *YlOCH1* was used together with the antisense primer based on the hygromycin gene sequence contained within the deletion cassette. The expected DNA band of ~1.8 kbp was obtained on agarose gel electrophoresis with the clone denoted *Y. lipolytica* Yl13, and as expected, there was no PCR amplification with the DNA obtained from the *Y. lipolytica* Yl12 strain (Figure 4.4). Further confirmation of the deletion of the *YlOCH1* gene in *Y. lipolytica* Yl13 was confirmed by Southern blot analysis using the DIG-labelled *YlOCH1* DNA fragment as a probe. DNA hybridisation could only be obtained with the DNA derived from *Y. lipolytica* Yl12 indicating the absence of the gene within the Y*. lipolytica* Yl13 genome (Figure 4.5).

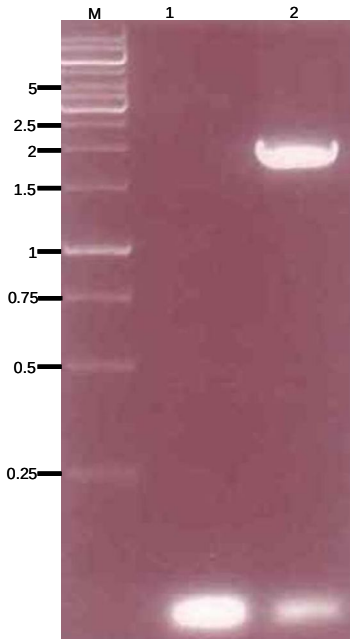

Figure 4.4. A 1% (w/v) agarose gel showing the amplicons generated by the *YOCH1* target specific primers. A fragment of ~1.8 kbp (lane 2) was obtained with *Y. lipolytica* Yl13 as compared to no product with *Y. lipolytica* Yl12 (lane 1 - not transformed with the deletion cassette) indicating disruption of the *OCH1* gene by the *YlOCH1* deletion cassette. M = the Generuler^{TM} (NEB) DNA ladder in kbp.

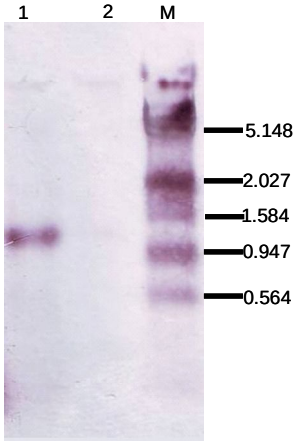

Figure 4.5. Southern blot of the *Y. lipolytica* Yl12 (lane 1) and Yl13 (lane 2) strains with the *YlOCH1* ORF as the probe. The genomic DNA of the strains was digested with *Eco*RI. M = the *Hind*III and *Eco*RI cut lambda DNA in kbp.

4.3.3. Marker rescue

A *Y. lipolytica* Yl12 strain transformed with the *OCH1* deletion cassette was transformed with the pRRQ2 plasmid carrying the Cre-Lox recombination system which facilitates excision of the DNA sequence flanked by lox sites. The hygromycin resistance gene within the deletion cassette is flanked by lox sites which enables excision of this gene. One derivative of *Y. lipolytica* Yl12 was found to have lost the hygromycin resistance when plated on hygromycin containing plates. The colony which had become hygromycin sensitive was denoted *Y. lipolytica* Yl13.

4.3.4. Growth and glycan profiles

The growth profiles of the *Y. lipolytica* Yl12 and Yl13 strains were studied in liquid shake cultures. Interestingly, the double deletion mutant (*OCH1* and *GPI7*) grew as well as the *GPI7* deleted strain. *Yarrowia lipolytica* Yl12 and Yl13 demonstrated similar growth profiles and displayed comparable maximum optical density when cultured in YPDO medium (Figure 4.6).

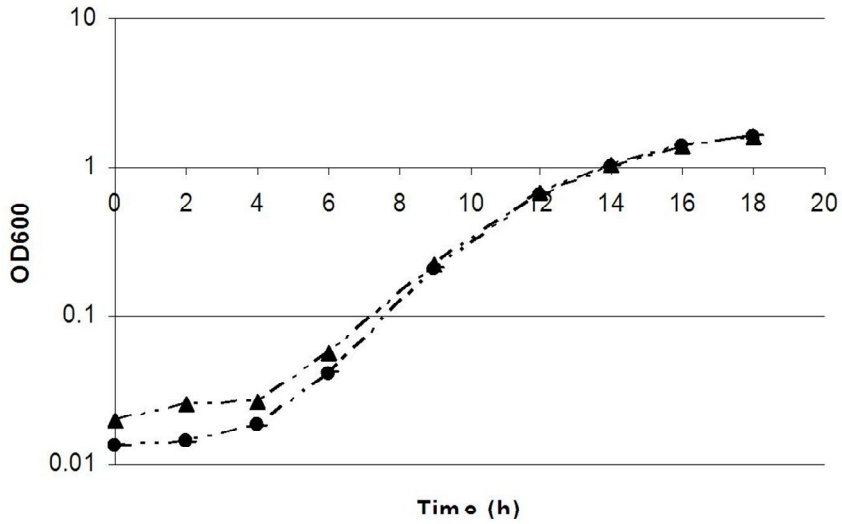

Figure 4.6 The growth pattern of *Y. lipolytica* Yl12 and Yl13. The growth profile of *Y. lipolytica* Yl12 is represented by solid circles while that of *Y. lipolytica* Yl13 is shown by solid triangles.

Song and co-workers (2007) revealed that deletion of *YlOCH1* results in a yeast mutant sensitive to Congo red and Calcofluor white, suggesting that the *YlOCH1* mutant has alterations in its cell wall composition, probably in mannoprotein. It was observed that a *YlOCH1:GPI7* double-deletion mutant displayed a growth phenotype similar to that of the *YlGPI7* mutant (Figure 4.7). This suggests that deleting *OCH1* in

a strain where there is already an alteration in the cell wall composition did not affect its growth pattern.

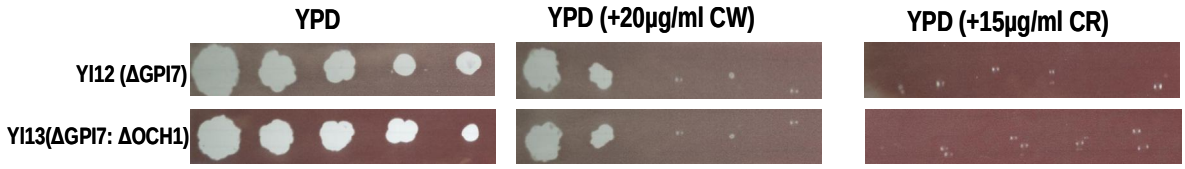

Figure 4.7. Phenotypic analysis of the Yl12 and Yl13 mutant strains. The Yl12 and Yl13 mutant cells were grown in YPD, and 5 µl of serial (1/10) dilutions of each strain was spotted on YPD plates containing 20 $\mu g.mL^{-1}$ Calcofluor white (CW), or 15 $\mu g.mL^{-1}$ Congo red (CR). The plates were incubated for 2 days at 28°C.

The glycan profiles of the extracellular proteins derived from the *Y. lipolytica* Yl12 and Yl13 cultures grown in YPDO medium were analyzed by MALDI-TOF mass spectrometry following cleavage and release of glycans with the *N*-glycosidase enzyme. The glycan profile of glycans extracted from the *Y. lipolytica* Yl12 showed the most abundant glycan being $Man_9GlcNAc_2$ (Figure 4.8A). Glycans corresponding to $Man_{10}GlcNAc_2$ and $Man_{12}GlcNAc_2$ were also detected. There is no evidence of the kind of hypermannosylation reported for *S. cerevisiae* where the core glycan structure could consist up to 200 mannose residues (Dean and Pelham, 1990; Vervecken *et al*., 2004). The disruption of the *YlOCH1* gene resulted in $Man_8GlcNAc_2$ being the most prevalent glycan extracted from the culture of *Y. lipolytica* Yl13 strain (Figure 4.8B). These results are significant for the glycoengineering of *Y. lipolytica* given that in mammalian cells the $Man_8GlcNAc_2$ N-glycosylated is the core glycan structure that exits from the endoplasmic reticulum to the Golgi apparatus for trimming into $Man_5GlcNAc_2$ (Kornfeld *et al*., 1985). The results indicated that it is not absolutely necessary to delete the *YlOCH1* gene to enable glycoengineering of the *Y. lipolytica* strain as no hypermannosylation was observed. The most abundant glycans are $Man_8GlcNAc_2$ and $Man_9GlcNac_2$ which are acceptable substrates of the *Trichoderma reesei* α-1,2-mannosidase I widely used in the engineering of yeast glycosylation pathway to produce $Man_5GlcNAc_2$, a prerequisite intermediate for the synthesis of complex glycans in human cells (Van Petegem *et al*., 2001; Vervecken *et al.*, 2004).

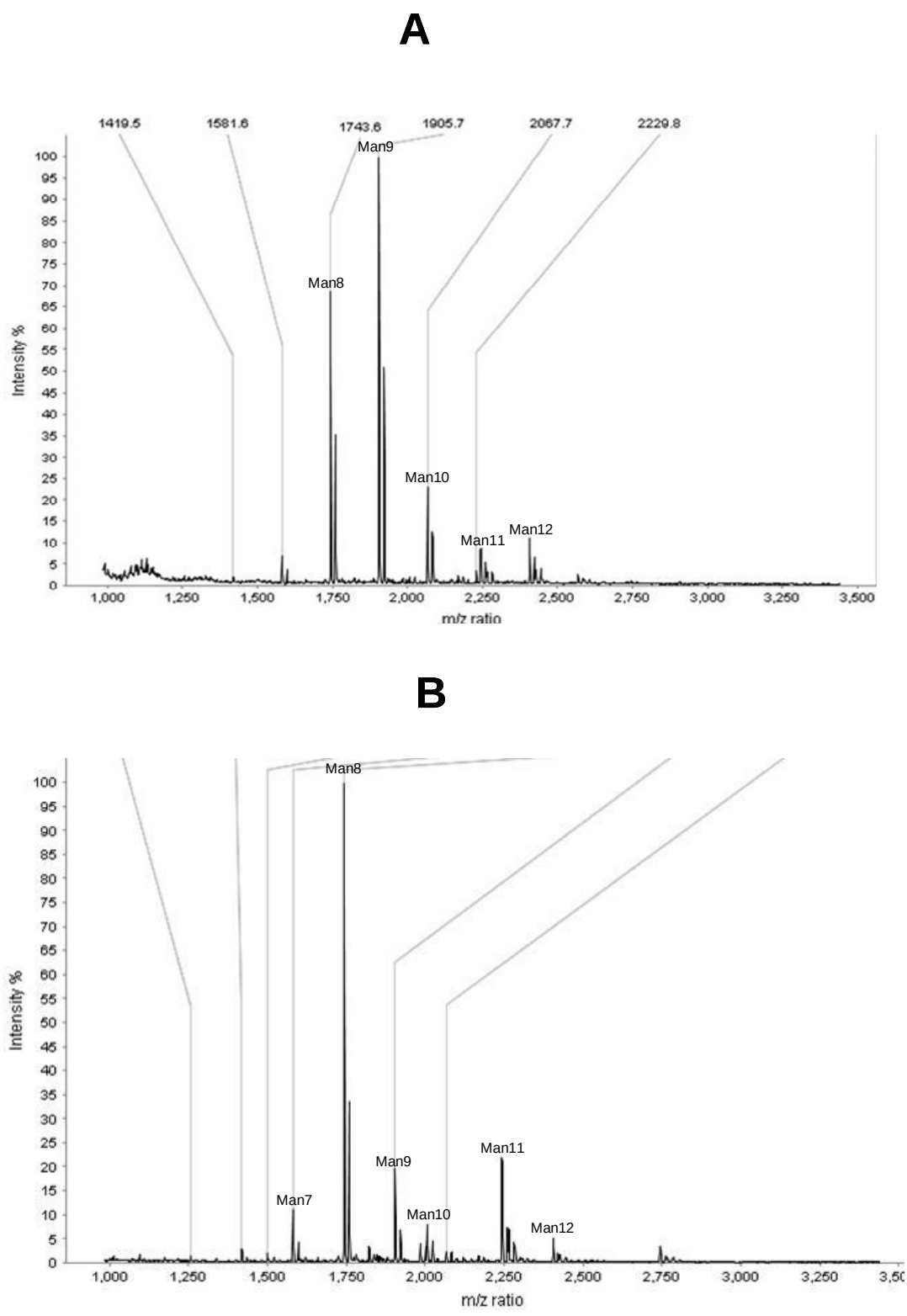

Figure 4.8. MALDI-TOF MS analysis of *N*-linked oligosaccharides assembled on extracellular proteins extracted from *Y. lipolytica* YI12 and YI13 cultures. Mass spectra were analyzed in the positive reflector mode for detection of neutral sugars released from the proteins secreted from *Y. lipolytica* (**A**) YI12 and (**B**) YI13. MALDI/TOF profiles of each of the glycan pools are shown and the assignment of peaks is indicated.

In conclusion, the gene encoding OCH1 was successfully deleted from the *Y. lipolytica* Yl12, a *GPI7* null mutant strain with enhanced ability to produce extracellular lipase. The yeast strain was denoted *Y. lipolytica* Yl13 and it grew as well as the parental *Y. lipolytica* Yl12 under standard growth conditions in shake cultures. The deletion of the *YlOCH1* gene resulted in the production of extracellular proteins mainly carrying $Man_8GlcNAc_2$ glycans. The *Y. lipolytica* strain used did not show hypermannosylation of the expressed proteins as the *Y. lipolytica* Yl12 strain produced proteins that contained mainly $Man_9GlcNAc_2$ glycans, an intermediate readily accepted by mannosidase I for trimming into $Man_5GlcNAc_2$ glycans. The results provided *Y. lipolytica* as a good candidate for glycoengineering to enable production of therapeutic proteins with human-like glycans.

4.5. References

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Concluding remarks

With regard to protein production, yeasts offer considerable advantages over alternative prokaryotic and mammalian cell systems (Dominguez *et al*., 1998). They combine the ease of genetic manipulation and fermentation of a microorganism with the capability to secrete and modify foreign proteins according to a general eukaryotic scheme. In addition, they provide low-cost screening and production systems for efficiently processed and modified proteins. In most cases, yeasts meet safety prerequisites as they do not harbour pyrogens, pathogens or viral inclusions. This provides yeasts as an option for the production of biopharmaceuticals suitable for application in humans and animals. However, secretory expression of heterologous proteins in yeast has attracted a lot attention due to several bottlenecks that limit yield. Several studies on yeast secretion systems have thus focused on the engineering of the host strains, vector systems, and glycosylation as well as the fermentation process (Gellisen *et al*., 2005).

Yarrowia lipolytica is a yeast that has been considered a potential candidate for production of heterologous proteins and knowledge gained from concentrated efforts to understand its physiology, genetics and molecular biology is available in literature (Nicaud *et al*., 2002, Madzak *et al*., 2004). This yeast is considered to be non-pathogenic and has GRAS (Generally Regarded As Safe) status and has been used in the production of lipase and citric acid at industrial scale. *Yarrowia lipolytica* is also known to be a prolific secretor of extracellular enzymes, notably proteases, lipases, esterases and RNase. The pathways leading to the assimilation of hydrophobic substrates such as *n*-alkanes, fatty acids, fats and oils by *Y. lipolytica* have been the subject of extensive studies with the aim of enhancing extracellular protein production (Nicaud *et al*., 2002).

Some of the factors that affect heterologous protein production in *Y. lipolytica* include the host strain and its cultivation, properties of the target protein, the vector system, promoter choice, codon usage, translation signals, processing and folding, as well as secretion. Molecular engineering of the secretory target proteins to optimize their properties for effective secretion is difficult and crucial in most cases. Consequently, current studies on the improvement of cell specific production of heterologous proteins have shifted to focus on host selection and engineering (Idiris *et al*., 2010). Protein-based host selection and promoter/signal peptide optimization are the simplest strategies, and often result in increased yields. However, many proteins are

still produced only at comparatively low levels even though the transcription or translation level of the target protein is optimized sufficiently for overexpression in the most suitable host system.

With its GRAS status, *Y. lipolytica* is an attractive host for the production of therapeutic peptides. The objective of this Ph.D study was to develop a *Y. lipolytica* recombinant protein secretory system. The targets are therapeutic peptides with application in humans. Initial attempts to express peptides in *Y. lipolytica* were carried using a RANTES (Regulated upon Activation Normal T cell Expressed and Secreted). RANTES belongs to the family of chemokines and plays a primary role in the inflammatory immune response via its ability to chemoattract leucocytes and modulates their function. The expression of RANTES under hp4d was not successful in *Y. lipolytica*. This was probably due to the observation that direct expression of peptides could result in their degradation by limited proteolysis (Owens and Heutte, 1997). Moreover, it is possible that expression of the peptide could be lethal to the host cell (Schmoldt *et al*., 2005).

Thus, co-expression as a fusion to a protein that is easily expressed and secreted by *Y. lipolytica* was hypothesised as probably the best method to express peptides in *Y. lipolytica*. To this end, a gene encoding an endogenous protein that is expressed to high levels in *Y. lipolytica* was selected. *Yarrowia lipolytica* produces large quantities of extracellular LIP2. LIP2, an extracellular lipolytic enzyme from *Y. lipolytica*, is a well-characterized enzyme which has been extensively used in structure-function studies (Pignède *et al*., 2000). The LIP2-RANTES fusion protein was directed to the extracellular of the host cell and its purification was relatively easy as the protein accumulated in the medium. A unique enterokinase cleavage site introduced at the junction of LIP2 and RANTES facilitated the release of RANTES from the fusion protein by enterokinase cleavage. The RANTES I peptide was functional, presumably suggesting that folding of the fusion protein and intramolecular disulfide bond formation of the peptide occurred as the protein passed through the secretory pathway.

Although 3D-modelling predicted a LIP2-RANTES structure that exposes the enterokinase and the Histidine tag at the junction, and there also was purification and cleavage of the fusion protein, the expression of the fusion protein can be further enhanced by reducing the size of the LIP2 reading frame. This would hypothetically result in a protein structure that would facilitate improved cleavage of the peptide

from LIP2 thereby minimising contamination of the purified peptide by LIP2 fragments as a result of unspecific cleavage by enterokinase.

To further enhance the ability of *Y. lipolytica* as a host for extracellular therapeutic peptide production, a second objective of the study was to engineer the strain for enhanced production of extracellular proteins. For this to be achieved, a *zeta* based mutagenesis system which gives random integration in a *Y. lipolytica* strain devoid of the Ylt1 retrotransposon was used to construct mutants with enhanced abilities to produce extracellular lipolytic activity. Previously, random integration of the *zeta* mutagenesis cassette was used to generate mutants affected in hydrophobic substrate utilization by *Y. lipolytica* (Mauersberger *et al*., 2001; Fickers *et al*., 2005, Thevenieau *et al*., 2007). In this study, three mutants were selected from the 2000 screened transformants on the basis of enhanced extracellular lipase production as compared to the wild type strain. The integration locus of the mutagenesis cassette was identified in one of the mutants and *GPI7* was identified as the gene responsible for the lipolytic activity hyperproduction phenotype. In addition, deletion of the gene encoding GPI7 displayed a similar lipase hyperproduction phenotype as compared to the *zeta* insertion mutant.

Δ*GPI7* cells are hypersensitive to Calcofluor White and Congo red, substrates that interact with various polysaccharides and affect the assembly of chitin microfibrils in yeasts. This suggests that *GPI7* deletion affected GPI protein transport, remodeling, as well as cell wall anchorage and integrity (Benachour *et al*., 1999; Richard *et al*., 2002). In yeasts *GPI7* deletion confers pleiotropic phenotypes such as changes in budding patterns and cell wall structure and composition. This consequently results in the mislocalization of cell wall-targeted proteins that accumulated in the growth medium (Plaine *et al*., 2008). Lipase accumulated to high levels in the supernatant of *GPI7* deleted strain, suggesting a “leaky” cell wall. A “porous” cell wall was also observed when *GPI7* deletion resulted in the release of epoxide hydrolase, a protein designed for intracellular expression to the extracellular. It is expected, but still need to be demonstrated, that GPI7 deleted strains expressing recombinant LIP2-peptide fusions will also give enhanced extracellular expression of these fusion proteins. It would also be interesting to establish the mechanism of action by which *GPI7* deletion result in “leaky” cell walls. This would in turn address the effect of GPI anchoring on the integrity of the cell wall.

Protein glycosylation is a major post-translational modification process in the yeast secretory pathway and confers an advantage in the secretory production of heterologous proteins that require glycosylation for proper folding and biological activity (Hamilton *et al*., 2003). However, for the production of therapeutic glycoproteins intended for use in human and animals, yeasts have been less useful because of their inability to modify proteins with human or animal glycosylation structures. This suggests that the properties of posttranslational glycosylation in each yeast host system and its optimization must be considered. To overcome this challenge in yeast glycosylation (mainly N-type), "humanizing" of the yeast glycosylation systems has become the current main strategy for glycoengineering of yeast species.

Glycoengineering in yeast was started by deleting some yeast-specific glycosyltransferases and introducing many other genes responsible for human-like sugar-nucleotide synthesis (Hamilton and Gerngross, 2007). It was found that formation of the yeast-specific, large outer chain is initiated by α-1,6-mannosylatransferase that is encoded by the *OCH1* gene, deletion of which blocks outer chain elongation. The deletion of the *OCH1* gene resulted in blockage of outer chain elongation of glycans on the proteins produced by the *Y. lipolytica* strain (Song *et al*., 2007). $Man_8GlcNAc_2$ was found to be the most abundant glycan structure as compared to the wild type strain were $Man_9GlcNAc_2$ is more prevalent. This suggests that, since outer chain elongation of the protein is blocked, the strain can thus be engineered further by introducing other genes responsible for the formation of human-like glycan structures. Therefore, to generate a *Y. lipolytica* strain that will be useful for production of therapeutic peptides with human-like glycan structures, the *Y. lipolytica GPI7* null mutant was also deleted of *OCH1*. LC-MS analysis showed that the double deletion mutant produced, as expected, $Man_8GlcNAc_2$ as the most abundant glycan structure, while the *GPI7* deleted strain produced $Man_9GlcNAc_2$ as the major glycan. The double deletion of *GPI7* and *OCH1* did not affect the growth pattern of *Y. lipolytica*, suggesting that the strain could still accumulate high biomass resulting in high level production of proteins.

By constructing a LIP2-based secretory vector system and generating a *GPI7* null mutant capable of enhanced production of extracellular proteins as well as deleting the OCH1 encoding gene, the study generated a *Y. lipolytica* secretory protein production system which might in future be useful for enhanced production of recombinant therapeutic peptides.

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Summary

Yarrowia lipolytica is a non-conventional yeast which is considered to be suitable for production of industrially important proteins at commercial scale. The yeast is non pathogenic and is generally regarded as safe and as such can be used to produce biotechnological products for human consumption and applications. The genetic tools for the manipulation of the yeast have been developed over the years. This includes chemical and genetic based mutagenesis techniques to develop and isolate *Y. lipolytica* strains with enhanced properties in the production of endogenous and heterologous proteins. Protein expression systems comprised of a variety of inducible and constitutive promoters are available; secretion signals for extracellular production of proteins and a choice of replicative or integrative expression systems have also been developed. Auxotrophic and antibiotic based selectable markers are available some of which result in single or multiple copies of the integrative expression cassettes containing the target gene. The *Y. lipolytica* yeast can utilise a wide variety of carbon sources and its fermentation processes in bioreactors are well established.

The property of *Y. lipolytica as a* prolific producer of the endogenous extracellular Lip2p was exploited for the development of an expression system for production of therapeutic peptides. To this end, the 38 amino acid long RANTES I peptide with therapeutic applications in the treatment of HIV/AIDS was selected as a model peptide for co-expression with Lip2p. The sequence encoding the peptide was cloned downstream of the complete *LIP2* gene. The therapeutic peptide was separated from the *LIP2* gene by the sequences encoding for 6X His and the DDDDK sequence recognized by the enterokinase proteolytic enzyme. The expression cassette was under the hp4d quasi constitutive promoter with the rDNA as a target for multi copy integration using the defective *Ura3d4* integration cassette. The amount of RANTES I produced by the expression system was estimated to be 0.274 $\mu g.L^{-1}$ as determined by the CCL5/RANTES immunoassay method. The RANTES I peptide produced in this study was reactive to polyclonal antibodies raised against human RANTES and functionality assays showed that the peptide inhibited the binding of the pseudoviral particles to the TZM-bl cells through the CD4/CCR5 receptors.

Another section of the study sought to identify genetic elements responsible for enhanced secretion of the extracellular Lip2p in *Y. lipoyltica*. Lipase hyperproducing mutants were generated using the *zeta* based mutagenesis system which integrates randomly within the *Y. lipolytica* genome. One mutant denoted *Y. lipolytica* Y12 which showed lipase hyperproducing phenotype was found to be disrupted in the function of the *GPI7* gene, a protein encoding a polypeptide of 830 amino acids. The GPI7 protein is 33% identical to the *S. cerevisiae* GPI7 known to play a role in the maintenance of the yeast cell wall integrity. Consistent with the disruption of cell wall integrity through deletion of GPI7, the *Y. lipolytica* strain was more sensitive to cell wall hydrolysing enzymes, had defects in cell separation and a daughter cell-specific growth defect at the non-permissive temperature and exhibited hypersensitivity to Calcofluor white and Congo red which are known to interfere with yeast cell wall composition. While the *Y. lipolytica* Yl12 and the ”wild-type” Po1d strains grew similarly in shake flask cultures, the *Y. lipolytica* Yl12 strain produced 7 times more extracellular lipase activity in liquid shake flask cultures than the control *Y. lipolytica* Po1d strain. To investigate the mode of *GPI7* action in the enhancement of protein production, a *Y. lipolytica* YlHmA25 strain expressing intracellular epoxide hydrolase under control of the hp4d promoter was deleted for the *GPI7* gene to create a *Y. lipolytica* YlHmA25ΔGPI7 strain. The YlHmA25ΔGPI7 strain contained epoxide hydrolase activity in the extracellular in contrast to the *Y. lipolytica* YlHmA25. In addition, the *Y. lipolytica* YlHmA25ΔGPI7 accumulated more total extracellular protein than *Y. lipolytica* YlHmA25 an indication that the strain accumulates more proteins extracellulary in comparison with the *Y. lipolytica* YlHmA25 strain. Taken together, the results suggested that the disruption of *GPI7* affects the integrity of the yeast cell wall which in turn results in leakage or “enhanced” secretion of proteins to the extracellular.

The challenge in developing a host system with broad applications in heterologous production of proteins including therapeutics lies in the elimination of the endogenous hyper-mannosylated yeast glycans. The *OCH1* gene encoding a protein with α-1,6-mannosyltransferase activity was deleted from the *GPI7* deleted mutant of *Y. lipolytica*. The *OCH1* and *GPI7* double deletion mutant of *Y. lipolytica* showed similar growth patterns to the *GPI7* null mutant. Glycan analyses revealed that the most abundant glycans are the $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ which are acceptable substrates of the *Trichoderma reesei* α-1,2-mannosidase I the enzyme widely used in the engineering of yeast glycosylation pathway to produce $Man_5GlcNAc_2$, a prerequisite intermediate for the synthesis of complex glycans in human cells. The

OCH1 and *GPI7* double deletion mutant of *Y. lipolytica* is a good candidate for further glycoengineering to enable production of extracellular therapeutic proteins with human-like glycans.

Opsomming

Yarrowia lipolytica is ‘n onkonvensionele gis wat beskou word as geskik vir die produksie van proteïene van industriële belang op kommersiële skaal. Die gis is nie-patogenies en word algeneem beskou as veilig (generally regarded as safe) en kan gevolglik gebruik word vir die produksie van biotegnologiese produkte vir menslike verbruik. Die genetiese gereedskap vir die manupilasie van gis is oor die jare ontwikkel. Dit sluit in chemies- en geneties-gebasseerde mutagenese tegnieke om *Y. lipolytica* rasse te ontwikkel en isoleer met verbeterde eienskappe vir produksie van endogene en heteroloë proteïene. Proteïenuitdrukkingsisteme, bestaande uit ‘n verskeidenheid induseerbare en konstitutiewe promotors, is beskikbaar; sekresieseine vir ekstrasellulêre produksie van proteïene asook ‘n keuse van replikatiewe of integrerende uitdrukkingsisteme is ook al ontwikkel. Ouksotrofiese en antibiotika gebasseerde selekteerbare merkers is beskikbaar, waarvan sommige lei tot integrasie van enkel of veelvuldige kopieë van die integrerende uitdrukkingskasette wat die teikengeen bevat. Die *Y. lipolytica* gis kan ‘n wye verskeidenheid koolstofbronne benut en het ‘n goed vasgestelde fermentasieproses in bioreaktors.

Die eienskap van *Y. lipolytica* as ‘n effektiewe produseerder van endogeniese ekstrasellulêre Lip2p is gebruik vir die ontwikkeling van ‘n uitdrukkingsisteem vir die produksie van terapeutiese proteïene. Vir hierdie doel is die 38 aminosuur lange RANTES I peptied, met terapeutiese toepassings in die behandeling van HIV/VIGS, gekies as ‘n model peptied vir ko-uitdrukking met die Lip2p. Die volgorde wat kodeer vir die peptied is stroom-af van die volledige *LIP2* geen gekloneer. Die terapeutiese peptied is geskei van die *LIP2* geen deur die volgordes wat kodeer vir 6X His en die DDDK volgorde wat deur enterokinase ensiem herken word. Die uitdrukkingskaset is onder beheer van die hp4d quasi-konstitutiewe promotor met die rDNA as ‘n teiken vir veelvuldige integrasie deur gebruik te maak van die defektiewe Ura3d4 integrasiekaset. Die hoeveelheid RANTES I geproduseer deur hierdie uitdrukkingsisteem is beraam as 0.274 $\mu g.L^{-1}$ soos bepaal deur die CCL5/RANTES immuno-essai metode. Die RANTES I peptied geproduseer in hierdie studie het reageer met poliklonale teenliggame opgewek teen menslike RANTES en aktiwiteitstoetse het gewys die peptied verhoed binding van die pseudovirale partikels aan die TZM-bl selle deur die CD4/CCR5 reseptore.

‘n Ander gedeelte van die studie het beoog om genetiese elemente te identifiseer wat verantwoordelik is vir verbeterde sekresie van die ekstrasellulêre LIP2 in *Y. lipolytica.* Lipase hiperproduserende mutante is gegenereer deur die zeta-gebasseerde mutagenese sisteem te gebruik wat ewekansig in die *Y. lipolytica* genoom integreer. Een mutant, genaamd *Y. lipolytica* Y12, wat die lipase hiperproduserende fenotipe toon is gevind om ontwrig te wees in die funksie van die *GPI7* geen wat kodeer vir ‘n polipeptied van 830 aminosure. Die ontwrigte GPI7 proteïen het ‘n 33% similaritiet met die *Saccharomyces cerevisiae* GPI7 wat bekend is om ‘n rol te speel in die instandhouding van die integriteit van die gis selwand. Ooreenstemmend met die ontwrigting van die selwand integriteit deur verwydering van *GPI7* was die *Y. lipolytica* ras meer sensitief vir selwand hidroliserende ensieme, het defekte gehad in selskeiding met ‘n dogtersel-spesifieke groeidefek by die nie-permissiewe temperatuur en hipersensitiwiteit getoon teenoor Calcofluor wit en Kongo rooi, wat bekend is om in te meng met die samestelling van die gis selwand. Alhoewel *Y. lipolytica* Y12 en wilde tipe Po1d eenders gegroei het in skudfleskulture, het Y12 sewe keer meer extrasellulêre aktiwiteit getoon in hierdie kulture as die kontrole, Po1d. Om die werking van GPI7 in die verhoging van proteïen produksie te ondersoek is ‘n *Y. lipolytica* YlHmA25 ras, wat intrasellulêre epoksied hidrolase onder beheer van die hp4d promotor produseer, se *GPI7* geen verwyder om ‘n *Y. lipolytica* YlHmA25ΔGPI7 ras te produseer. Die YlHmA25ΔGPI7 ras het ekstrasellulê epoksied hidrolase aktiwiteit getoon in teenstelling met *Y. lipolytica* YlHmA25. Verder het *Y. lipolytica* YlHmA25ΔGPI7 meer totale ekstrasellulêre proteïen opgehoop as *Y. lipolytica* YlHmA25, ‘n aanduiding dat hierdie ras meer proteïene ekstrasellulêr ophoop as *Y. lipolytica* YlHmA25. Saamgevat dui die resultate aan dat die ontwrigting van *GPI7* die integriteit van die gis selwand beïnvloed wat gevolglik ‘n lekkasie of “verhoogde” sekresie van proteïene na die ekstrasellulêre omgewing veroorsaak.

Die uitdaging in die ontwikkeling van ‘n gasheersisteem met wye toepassings vir heteroloë produksie van proteïene, insluitende terapeutiese proteïene, lê in die eliminering van endogene hiper-mannosileerde gis glikane. Die *OCH1* geen, wat kodeer vir ‘n proteïen met α-1,6-mannosieltransferase aktiwiteit, was verwyder van die *GPI7*-verwyderde mutant van *Y. lipolytica.* Die *OCH1* en *GPI7* dubbel-delesie mutant het eenderse groeipatrone getoon as die *GPI7* nul mutant. Glikaan analise wys $Man_8GlcNAc_2$ and $Man_9GlcNac_2$ is die volopste glikane, wat aanvaarbare substrate is vir *Trichoderma reesei* α-1,2-mannosidase I, die ensiem wat algeneem gebruik word in die manipulasie van die gis glikosileringsweg om $Man_5GlcNAc_2$ te

produseer, ‘n vereiste tussenganger vir die sintese van komplekse glikane in menslike selle. Die *OCH1* en *GPI7* dubbel-delesie mutant van *Y. lipolytica* is ‘n goeie kandidaat vir verdere gliko-manipulasie vir die produksie van ekstrasellulêre terapeutiese proteïene met menslike glikane.