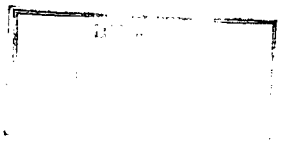

Development of *Yarrowia lipolytica* Expression Systems for Recombinant Proteins

by

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DECLARATION

I, Siyavuya Ishmael Bulani, hereby declare that the dissertation/thesis hereby handed for the qualification of Philosophiae Doctor in Biotechnology at the University of Free State, is my own independent work and that I have not previously submitted the same work for a qualification at/in another University/faculty. The University of Free State retains the copyrights of this dissertation.

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DEDICATION

This thesis is proudly dedicated to by late father, **Mongezi Richard Bulani**, who has lived not to see this day and my mother **Nophumzile Vivian Bulani**, who has been a pillar of strength throughout my academic journey.

"Nalo umqolo ndiwuqabelisisle"

LIST OF ABBREVIATIONS

AEP: alkaline extracellular protease

AIDS: acquired immune deficiency syndrome

Amp: ampicillin

CCL5: chemokine (C-C motif) ligand 5

CWP: cell wall protein

cDNA: complementary DNA

Cut: cutinase

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DsRed: *Discosoma* spp. red fluorescent protein

EDTA: ethylenediaminetetraacetic acid

EGFP: enhanced green fluorescent protein

Flo1p: flocculation protein 1

FDA: Food and Drug Administration

FPs: fluorescent proteins

FSCut: *Fusarium solani cutinase*

GFP: green fluorescent protein

GPI: glycosylphosphatidylinositol

GRAS: Generally Regarded As Safe

His: histidine

kDa: kilo dalton

Kana: kanamycin

LB: luria broth

Leu: leucine

Lip2ss: lip2 secretion signal

LTR: long terminal repeats

MALDI-TOF-MS: matrix-assisted laser desorption/ ionization time of-mass spectrometry

mRFP: monomeric red fluorescent protein

Nss: native secretion signal

OD_{600}: optical density at wavelength 600 nm

OD_{595}: optical density at wavelength 595 nm

OD_{410}: optical density at wavelength 450 nm

PIR: protein with internal repeats

PMF: peptide mass fingerprinting

pNP: *p*-nitrophenol

pNPB: *p*-nitrophenol butyrate

pNPP: *p*-nitrophenyl palmitate

RANTES: Regulated upon Activation, Normal T cell Expressed and presumably Secreted

rDNA: ribosomal DNA

Rpm: revolution per minute

SDS: sodium dodecyl sulphate

SDS–PAGE: sodium dodecyl sulphate polyacrylamide

Tags: triacylglycerols

TLL: *Thermomyces lanuginosus* lipase

Ura: uracil

YlCwp: *Yarrowia lipolytica* cell wall protein

YPD: yeast peptone dextrose

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

Chapter 1. Literature review: *Yarrowia lipolytica* expression systems

1.1 Introduction

Eukaryotic and prokaryotic organisms are essential production platforms for commercial production of proteins in the biotechnology sector (Nicaud *et al.*, 2002; Porro *et al.*, 2011). Bacterial expression systems, in particular *Escherichia coli*, have been used extensively for heterologous expression of recombinant polypeptides. The choice of prokaryotic cells for heterologous protein expression is a result of ease of genetic manipulation, availability of efficient genetic tools, high transformation efficiency and rapid growth rates (Bordes *et al.*, 2007). The *E. coli* expression system is well established with regards to its metabolic pathway and its ability to reach high levels of protein production (Panda, 2003). However, application of prokaryotic systems for recombinant production of proteins is limited by their inability to produce correctly folded eukaryotic proteins, absence of post-translational modifications, high throughput purification of recombinant proteins from inclusion bodies and high cell density fermentation (Panda, 2003; Kjaerulff and Jensen, 2005; Bordes *et al.*, 2007).

Although several therapeutic proteins can be produced using the prokaryotic expression systems, the majority of these proteins require post-translational modifications to retain the biological function of the protein. Glycosylation, in particular *N*-glycosylation, plays an important role in proper folding, pharmacokinetics and stability of a wide range of proteins (Helenius and Aebi, 2001). Most eukaryotic proteins are produced in their active biological form by mammalian cells. However, these cells are more difficult to handle compared to microorganisms as their production efficiency is low and media and costs of culture are high. In addition, retroviral contamination and the time required to prepare stable cell lines are regarded as bottlenecks for application of mammalian cell culture in heterologous protein expression (Choi *et al.*, 2003; Kjaerulff and Jensen, 2005).

Because of these drawbacks from bacterial and mammalian expression systems, yeast expression systems have gained considerable interest in the expression of recombinant proteins (Dominguez *et al.*, 1998). Unlike bacterial expression systems, yeast expression systems undergo post-translational processing and modification allowing production of correctly folded and active eukaryotic proteins (Buckholz and Gleeson, 1991). Yeast lack the presence of endotoxins, oncogenic and viral DNA (Porro *et al.*, 2005). Similarly, to bacterial systems, yeast systems retain the advantages of unicellular microorganisms such as rapid growth rates and ease of

genetic manipulation (Buckholz and Gleeson, 1991). As a result of these advantages, yeast expression systems provide an alternative to prokaryotic and mammalian cellular expression systems in providing cost efficient screening and production of biochemically modified products (Gellissen *et al.*, 2005). Yeast expression systems were initially developed for *Saccharomyces cerevisiae* and later for a number of other non-conventional yeasts (Hensing *et al.*, 1995). Although *S. cerevisiae* has GRAS (generally regarded as safe) status granted by the FDA (Food and Drug Administration), it is not an ideal host for high level expression of heterologous proteins as a result of technical problems in fermentation processes, low expression levels of heterologous proteins, poor plasmid stability and decrease in biomass yield due to ethanol formation. In addition, heterologous proteins produced using this yeast are often hyperglycosylated and their retention within the periplasmic space is complicated by the presence of partially degraded protein, which make downstream processes difficult (Nicaud *et al.*, 2002; Madzak *et al.*, 2004; Porro *et al.*, 2005).

These limitations presented by *S. cerevisiae* prompted the search for alternative hosts and development of expression systems using non-conventional yeasts (Buckholz and Gleeson, 1991; Dominguez *et al.*, 1998). Among the studied non-conventional yeasts, *Yarrowia lipolytica* was found to be one of the most attractive alternative hosts for heterologous protein production (Romanos *et al.*, 1992; Muller *et al.*, 1998). This strictly aerobic dimorphic yeast is used as a model for the study of protein production, dimorphism and degradation of hydrophobic substrates (Jaafar *et al.*, 2003; Fickers *et al.*, 2005; Amaral *et al.*, 2006). *Y. lipolytica* is widely used in industry for production of citric acid, peach flavour and single cell protein (Beckerich *et al.*, 1998). The genomic sequence data of *Y. lipolytica* is available, with the strains and genetic tools developed for protein production in this yeast (Madzak *et al.*, 2000; Nicaud *et al.*, 2002; Madzak *et al.*, 2004; Yue *et al.*, 2007). This chapter reviews current and potential *Y. lipolytica* expression systems focusing on the tools used for heterologous protein production in this yeast.

1.2 *Y. lipolytic:* a platform for protein production

Y. lipolytica is one amongst the highly attractive non-conventional yeasts platforms that are used as expression systems for heterologous protein production. The availability of a wide range of host strains, large number of genetic markers and molecular tools in *Y. lipolytica* (Madzak *et al.*, 2005) has made this yeast a platform

for efficient heterologous protein production (Nicaud *et al.*, 2002). More recently, this yeast has been engineering to be a platform for production of glycoproteins (De Pourcq *et al.*, 2012). *Y. lipolytica* is a prolific producer of a wide range of proteins such as proteases, phosphatases, lipases, RNase and esterase (Barth and Gaillardin, 1997). Under favourable inducing conditions, this yeast has been reported to secrete large amounts (1-2 g/l) of alkaline extracellular protease (AEP), an enzyme encoded by the *XPR2* gene (Tobe *et al.*, 1976; Ogrydziak and Scharf, 1982). More than 40 recombinant proteins ranging from six to 116 kDa from different sources have been successfully expressed in *Y. lipolytica* (Madzak *et al.*, 2005).

Y. lipolytica, unlike *S. cerevisiae* has been reported to have secretion machinery that resembles that of higher eukaryotes for post-translational modifications (Beckerich *et al.*, 1998; Boisrame *et al.*, 1998). Similarly, to higher eukaryotes, a co-translation mechanism is predominant in *Y. lipolytica.* This similarity gives *Y. lipolytica* an advantage for production of hererologous proteins, in contrast to *S. cerevisiae,* which has a posttranslational pathway (Boisrame *et al.*, 1998). Glycosylation pattern of the non-conventional yeast, *Y. lipolytica* has been reported to show similarity to that of mammalian as compared to *S. cerevisiae* (Madzak *et al.*, 2005). Unlike hyperglycosylation shown by *S. cerevisiae,* other yeasts such as *Y. lipolytica*, *Pichia pastoris* and *Hansenula polymorpha* have shown reduced hyperglycosylation (Grinna and Tschopp, 1989; Gellissen and Hollenberg, 1997; Song *et al.*, 2007). Furhermore *Y. lipolytica* mutant strains that can be used as a model for the N-linked glycosylation pathway in this yeast have been developed (Barnay-Verdier *et al.*, 2004).

1.2.1 Host strains

Y. lipolytica strains such as Po1d, Po1f, Po1g and Po1h (Table 1.1) used for protein production were derived from the wild type strain W29 by disruption of the *URA3* gene with the *S. cerevisiae SUC2* gene (Nicaud *et al.*, 1989; Barth and Gaillardin, 1997). Nicaud and co-workers (1989) further introduced deletions in the *LEU2* gene by a pop in/ pop out event and subsequently followed by deletion of the *XPR2* gene giving rise to Po1d strain. This strain is able to utilize sucrose as a sole carbon source and is one of the most used *Y. lipolytica* strains for heterologous protein production (Nicaud *et al.*, 1989; Le Dall *et al.*, 1994). The strain Po1f was obtained by deletion of the *AXP* gene, encoding for acid extracellular protease (AXP) from the Po1d strain (Madzak *et al.*, 2000). The Po1f strain was transformed with the pBR322-

URA3 vector to form a docking platform for direct integration of pBR322-based expression vectors to obtain the Po1g strain (Madzak *et al.*, 2000). The strain Po1h, derived from Po1f by gene conversion of *leu2-270* to *LEU2,* and its mother strain integrate expression cassettes from an auto-cloning vector non-homologously generating producing strains devoid of the bacterial moiety (Madzak *et al.*, 2004). Although *Y. lipolytica* has been compared to other expression hosts for heterologous protein production (Muller *et al.*, 1998), there have been no clear reports from literature about comparisons of the different *Y. lipolytica* strains (Madzak *et al.*, 2004).

Table 1.1 *Y. lipolytica* host trains

Strain	Genotype	Phenotype	Reference
W29	*MatA*	WT	Madzak *et al.* (2000)
Po1d	*MatA, leu2-270, ura3-302, xpr2-322*	Leu$^-$, Ura$^-$, ΔAEP, Suc$^+$	Le Dall *et al.* (1994)
Po1f	*MatA, leu2-270, ura3-302, xpr2-322, axp1-2*	Leu$^-$, Ura$^-$, ΔAEP, ΔAXP, Suc$^+$	Madzak *et al.* (2000)
Po1g	*MatA, leu2-270, ura3-302: : URA3, xpr2-322, axp1-2*	Leu$^-$, ΔAEP, ΔAXP, Suc$^+$pBR322	Madzak *et al.* (2000)
P01h	*MatA, ura3-302, xpr2-322, axp1-2*	Ura$^-$, ΔAEP, ΔAXP, Suc$^+$	Madzak *et al.* (2000)

1.2.2 *Y. lipolytica* expression vectors

Similarly to other yeasts, *Y. lipolytica* employs shuttle vectors for heterologous protein production (Madzak *et al.*, 2005). Auto-cloning shuttle vectors devoid of the bacterial DNA moiety have been constructed for protein production in *Y. lipolytica* strains (Nicaud *et al.*, 1998; Pignede *et al.*, 2000a). Both episomal replicative and integrative vectors are used for recombinant protein production in yeasts (Muller *et al.*, 1998). Replicative and integrative vectors can be transformed into *Y. lipolytica* strains using either lithium acetate (Xuan *et al.,* 1988) or electroporation (Fournier *et al.*, 1993). The lithium acetate method has been shown to be an ideal method for integrative vectors as it exhibits high transformation efficiencies. However, frequent recombination events between short repeated sequences of replicative vectors prevent its use for this type of vector. Hence, electroporation is more suitable for replicative vectors (Barth and Gaillardin, 1996).

Replicative vectors are unstable and are lost rapidly from the yeast cells in the absence of selective pressure (Dominguez *et al.*, 1998), which makes them a bottleneck for efficient industrial management (Madzak *et al.*, 2005). This type of vector is unattractive as an expression vector as their copy number is limited from

one to three copies per cell (Madzak *et al.*, 2000). Despite their inability to maintain high copy number, replicative vectors have been used successfully for protein production in *Y. lipolytica* (Muller *et al.*, 1998). In *Y. lipolytica* integration occurs mainly by homologous recombination of vectors directed to a target site, which results in high transformation frequencies (Xuan *et al.*, 1988). Single copy cross-over events of integrative vectors are highly stable and present in yeast cells in low copy numbers (Romanos *et al.*, 1992; Hamsa and Chattoo, 1994). The single copy vectors are directed to the pBR322 docking platform of the Leu$^-$ Po1g strain genome (Madzak *et al.* 2000) or using the *URA3* allele for single copy integration (Nicaud *et al.*, 2002). Integrative plasmids offer an advantage over replicative vectors, as they can integrate multiple copies and maximise gene expression. Multiple integrative vectors that integrate into the recipient strain genome by either homologously or non-homologously with multiple target sites using a defective marker have been constructed (Madzak *et al.*, 2004). Such vectors have been targeted to the ribosomal DNA (rDNA) cluster (Juretzek *et al.*, 2001), or the *Ylt1* long terminal repeats (LTRs) referred to as the zeta (Schmid-Berger *et al.*, 1994).

The defective marker *ura3d4* was employed for generating multiple integration in the fragment of the G unit of the rDNA with multiple integration existing mostly as tandem repeats and as dispersed copies on one or two sites (Le Dall *et al.*, 1994). The second class of vectors, zeta-based vectors, has been show to integrate homologously into *Ylt1* positive *Y. lipolytica* strains and non-homologously into Ylt1-free strains (Nicaud *et al.*, 1998; Juretzek *et al.*, 2001). Both Ylt1 positive and negative strains have been used for recombinant protein production when using multiple copy zeta-based vectors (Pignede *et al.*, 2000a; Nicaud *et al.*, 2002).

1.2.3 Selection markers

Of the many genes evaluated in *Y. lipolytica* as selection markers, only a handful have been successful fused (Madzak *et al.*, 2005). Although this yeast was found to be resistant to most commonly used antibiotics, it was reported to confer resistance to the bleomycin/phleomycin group and hygromycin B antibiotics when carrying expression vectors containing the relavant resistance gene (Gaillardin and Ribet, 1987; Cordero Otero and Gaillardin, 1996). In search for suitable selection markers, Nicaud *et al.* (1989) expressed the *S. cerevisiae SUC2* gene and subsequently evaluated it as a selection marker. Since Po1d and its derivatives are able to grow on

sucrose as a sole carbon source, this marker when used for selection allowed heterologous production of *S. cerevisiae* invertase in *Y. lypolytica* (Nicaud *et al.*, 1989). With the availability of *Y. lipolytica* strains harbouring non-reverting *leu2* and *ura3*, the most extensively used markers are *LEU2* and *URA3* auxotrophic markers (Barth and Gaillardin, 1996). Le Dall *et al.* (1994) designed defective alleles of the *URA3* marker gene by sequential deletion in the *URA3* promoter, which was essential for selection of transformants with multiple copies. The *ura3d4* allele, which retained only 6 bp upstream from the *URA3* start codon, was unable 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). The *ura3d4* defective selection marker has been used successfully for selection of multiple integration in both homologous and heterologous protein production (Pignede *et al.*, 2000a; Nicaud *et al.*, 2002).

1.2.4 Promoters

The *XPR2* promoter is one of the most studied promoters of *Y. lipolytica* for heterologous protein production in this yeast (Blanchin-Roland *et al.*, 1994; Madzak *et al.*, 1999; Madzak *et al.*, 2004). Although this strong promoter achieved success in driving expression of recombinant proteins, industrial application of *XPR2* promoter is limited by its complex regulation. The *XPR2* promoter is reported to be functional at pH above 5.5 on media lacking preferred carbon and nitrogen sources, with a high level of peptone pivotal for full induction (Ogrydziak *et al.*, 1977). Expression of AEP using the *XPR2* promoter rapidly destabilised and intoxicated *Y. lipolytica* transformants carrying high copy number of integrative plamids under induction conditions (Le Dall *et al.*, 1994).

The search for a better promoter that can be used industrially led to the development of a hybrid promoter, hp4d (Madzak *et al.*, 2000). Hp4d promoter retains the high transcriptional activity of a fully induced *XPR2* promoter. In addition, hp4d promoter is almost unaffected by environmental conditions and unlike the *XPR2* promoter does not require peptone for induction (Madzak *et al.*, 2000). The growth phase dependent, hp4d promoter, has been used successfully for driving heterologous proteins to the media and yeast cell surface (Nicaud *et al.*, 2002; Yue *et al.*, 2007). Other inducible promoters such as peroxisomal acyl-CoA oxidase 2 (*POX2*) and isocitrate lyase 1 (*ICL1*) (Pignede *et al.*, 2000a; Barth and Scheuber, 1993) and a

constitutive promoter, translational elongation factor EF-1 alpha (*TEF1*) (Muller *et al.*, 1998) have also been developed. A study by Juretzek *et al.* (2000) found promoters pICL1, p*POT1* and p*POX2* to be the strongest inducible promoters. Although these promoters are efficient for driving heterologous protein production, their industrial application can encounter some challenges (Juretzek *et al.*, 2000; Nthangeni *et al.*, 2004; Madzak *et al.*, 2004).

1.2.5 Secretion signals

Secretion of recombinant proteins into the culture supernatant is essential for limiting toxicity of over-expressed proteins on the host cells. In addition, secretion signals play an important role in expression of processed and post-translationally modified proteins (Kjaerulff and Jensen, 2005). Several homologous and heterologous secretion signals have been described for directing proteins to the extracellular medium in *Y. lipolytica* (Jolivalt *et al.,* 2005; Gasmi *et al.*, 2011). In *Y. lipolytica*, the *XPR2* secretion signal was the first signal reported for directing recombinant proteins and has been patented by the company Pfizer (Davidow *et al.*, 1987). The most used secretion signal is the *XPR2* pre-pro region (Barth and Gaillardin, 1996). Other secretion signals have been identified and used successfully for directing heterologous proteins to the culture supernatant, including *XPR2* pre-region (Swennen *et al.*, 2002), *Y. lipolytica* lipase 2 (*LIP2*) gene pre-pro region (Pignede *et al.*, 2000b), hybrid between *XPR2* and *LIP2* pre-pro regions (Nicaud *et al.*, 2002) and *LIP2* pre-region (Gasmi *et al.*, 2011).

1.3 *Y. lipolytica* cell surface displaying systems

Yeast is a suitable expression system that tolerates certain cell surface modifications and has become the ideal host for cell surface expression of heterologous proteins in biotechnology, pharmaceutical and medicine (Breinig and Schmitt, 2002). Cell surface display of heterologous proteins in yeast is widely reported in literature, although focus has been on certain yeast strains such *S. cerevisiae*.

1.4 Current systems used for cell surface display in *Y. lipolytica*

1.4.1 *Y. lipolytica* cell wall protein 1

Y. lipolytica cell wall protein 1 (YlCwp1), first identified by Jaafar and Zueco (2004), is a homologue of *S. cerevisiae* CWP1. Alignment of YlCwp1 amino acid sequence with that of *S. cerevisiae* shows 28.5 % overall identity and presence of several common features. YlCwp1 has a putative signal peptide with a possible peptidase site between positions 16 and 17 at the N-terminus, and a putative glycosylphosphatidylinositol (GPI)-attachment site at the C-terminus (Nuoffer *et al.*, 1993; Van der Vaart *et al.*, 1995; Jaafar and Zueco, 2004). The N-terminal signal sequence is essential for import into the endoplasmic reticulum (von Heijne, 1986). The GPI-attachment site is defined by an asparagine followed by glycine and alanine (NAG or NGA), with NGA in YlCwp1, followed by a hydrophobic carboxy-terminal region which gives it the overall hydropathy profile characteristic of a GPI-CWP (Jaafar and Zueco, 2004).

Other common features are the high content of serine and alanine and the presence of the motif DGQIQA close to the carboxy terminus, which is shared by other *S. cerevisiae* GPI-CWPs (Van der Vaart *et al.*, 1995; Jaafar and Zueco, 2004). The highly rich serine and/or threonine C-terminal half implies that the domains are highly *O*-glycosylated (Jaafar and Zueco, 2004). Wojciechowicz *et al.* (1993) suggested that this structure is essential for exposing the N-terminal binding domain on the surface of the cell wall. The N-terminal sequence of the ORF contains a putative GPI attachment signals (Jaafar and Zueco, 2004). Studies by Nuoffer *et al.* (1993) reported that asparagine is the most efficient anchor attachment site.

These features are similar to those of *S. cerevisiae* glucanase extractable cell wall mannoproteins, Cwp1, Cwp2 and Tip1 (Van der Vaart *et al.*, 1995). The protein of Cwp1p, Tp1p and Cwp2p all contain an asparangine followed by glycine and alanine near the carboxyl termini; the necessary polar and hydrophobic regions (Caras *et al.*, 1987; Caras *et al.*, 1989) are also present although no specific sequence is required (Caras and Weddell, 1989). Van der Vaart *et al.* (1995) reported a high degree of holomology between the hydrophobic regions.

YlCwp1Δ strains have shown a slight sensitivity to Congo red suggesting that YlCwp1 plays a role in the cell wall (Jaafar and Zueco, 2004). The 60 kDa YlCwp1 is

localised in *Y. lipolytica* in a similar fashion as the 55 kDa Cwp1 of *S. cerevisiae* (Jaafar and Zueco, 2004). Induced expression of Cwp1p is not limited to a specific growth condition indicating that their expression might be under the control of different transcriptional activating networks (Damveld *et al.*, 2005).

The fist report of a cell surface display system in *Y. lipolytica* was published by Yue *et al.* (2007). For cell surface display on the cells of *Y. lipolytica,* the authors used a C-terminal anchor domain of YlCwp1 from *Y. lipolytica* to display EGFP and haemolysin. They found that the heterologous proteins were successfully displayed on the yeast cells and that the immobilised haemolysin retained its activity towards erythrocytes. The YlCwp1 has more recently been used in *Y. lipolytica* to display alkaline proteases for bioactive peptide production (Ni *et al.*, 2009) and alginate lyase for production of oligosaccharides (Liu *et al.*, 2009).

1.4.2 *S. cerevisiae* Flocculation protein 1

Flocculation gene (*FLO1*), localised in chromosome 1 (Teunissen *et al.*, 1993a) and linked to the centromere (Miki *et al.*, 1982), encodes for flocculation protein1 (*FLO*1p) (Van der Vaart *et al.,* 1995; Bony *et al.*, 1998). Flo1p is a CWP directly involved in the yeast non-sexual flocculation process (Teunissen *et al.*, 1993a; Shankar and Umesh-Kumar, 1994). The *FLO1* gene of *S. cerevisiae* has been cloned, sequenced and the protein isolated and purified to homogeneity (Javadekar *et al.,* 2000). The sequence is comprised of a large ORF that contains 14 putative *N*-glycosylation sites and a Ser and Thr rich C-terminus (46%), which are potential *O*-glycosylation sites (Lehle and Bause, 1984; Bony *et al.*, 1997). The Flo1p contains a hydrophobic C-terminal region that contains the recognition/attachment signal to the GPI anchor (Nuoffer *et al.,* 1993). This makes Flo1p a GPI-anchored CWP that is secreted through the secretary pathway similarly to other GPI-anchored mannoproteins (Bony *et al.*, 1997; Sundstron, 2002). The hydrophobic N-terminus region contains the secretion signal, three putative *N*-glycosylation sites and highly *O*-glycosylated, 22% content of Ser and Thr (Teunissen *et al.*, 1993b; Bony *et al.*, 1997). The Flo1p has four regions with repeated sequences which constitute 70% of the amino acids of the protein (Bidard *et al.*, 1995). The N-terminus has repetitive sequences (Teunissen *et al.*, 1993b) that might play a role as spacers to expose a reacting domain of Flo1p on the yeast cell surface (Bony *et al.*, 1997).

1.4.3 Role in yeast flocculation

*FLO*1p is regarded as the primary mannoprotein that causes strong flocculation in the budding yeast *S. cerevisiae* compared to other yeast flocculating proteins such as *FLO5* and *FLO11* (Govender *et al.,* 2008). Studies by Bony *et al.* (1998) suggest that cells poorly flocculate at the beginning of growth and the flocculation level increase progressively during exponential phase through the first hours of the stationary phase. The flocculation capacity is reported to be directly proportional to the number of tandem repeats present in the *FLO*1 gene (Smukalla *et al.*, 2008; Brown and Buckling, 2008). Studies by Bony *et al.* (1997) suggest that the Flo1p N-terminal region is the protein's active domain that is essential for flocculation. The mannose recognition site at the N-terminus of Flo1p that is important for flocculation has been described by Kobayashi *et al.* (1998). Cells of *S. cerevisiae* produce mannoprotein that binds to cell wall α-mannan carbohydrates of the adjacent cells via hydrogen bonding between α-mannan and the protein causing flocculation (Shankar and Umesh-Kumar, 1994). Flocculation is a calcium depended process (Miki *et al.*, 1982) that is highly specific to mannose (Javadekar *et al.,* 2000), except for the non-sensitive *GTS1* flocculation protein (Bossier *et al.*, 1997).

Flocculent yeasts are essential for production of alcohol because of the simple and efficient separation of yeast cells from fermenting mash (Ishida-Fujii *et al.*, 1998). The *FLO*1 gene has been introduced for expression in both nonflocculent laboratory and industrial *S. cerevisiae* strains successfully causing flocculation. Industrially such strains can be easily recovered for reuse from fermentation mash without any physical energy (Ishida-Fujii *et al.*, 1998; Smukalla *et al.*, 2008), that involves centrifugation and filtration that is time consuming and costly (Govender *et al.*, 2008; Zhao and Bai, 2009). Flocculation behaviour of industrial yeast can be engineered to optimise specific production processes (Govender *et al.*, 2008). More recently, the *FLO*1 gene from *S. cerevisiae* has been introduced in the ethanol producing *Kluyveromyces marxianus* for flocculation purposes (Nonklang *et al.*, 2009).

Studies by Ishida-Fujii *et al.* (1998) on industrial non-flocculent *S. cerevisiae* showed an increase in ethanol production after introduction of the *FLO*1 gene, which might result from alteration of the cell-surface structure caused by expression of the flocculent protein. Smukalla *et al.* (2008) suggested that cells might form flocs to protect cells from harmful compounds, including drugs such as antimicrobials or

resist stress conditions including ethanol production. Recently Beauvais *et al.* (2009) showed that flocs secrete an extracellular matrix (ECM) that is composed of glucose and mannose polysaccharides that surrounds flocculating cells. The ECM prevents large molecules coming into contact with the floc, but its role in drug resistance and ethanol production is not clear.

1.5 Potential systems for cell surface display in *Y. lipolytica*

1.5.1 *S. cerevisiae* α-agglutinin 1 protein

The *S. cerevisiae* cell surface binding protein α-agglutinin is composed of a single polypeptide encoded by the *AGα1* gene (Lipke *et al.*, 1989). The α-agglutinin gene has been isolated, cloned and the cell wall mannoprotein purified to homogeneity (Burke *et al.*, 1980; Hauser and Tanner, 1989). Examination of the *AGα1* open reading frame (ORF) indicates several features. Both the N and C-termini are hydrophobic and are likely to be involved in the localization of the protein on the cell surface. The glycoprotein has 12 potential N-linked glycosylation sites, with the majority located on the C-terminal half of the ORF (Lipke *et al.*, 1989; Lipke and Kurjan, 1992; Wojciechowicz *et al.,* 1993). The α-agglutinin C-terminal half contains also a cluster of serine (Ser) and threonine (Thr), which are potential O-glycosylation sites, that form 29% of the residues at the termini. As a result of the Ser and Thr-rich C-terminal half, α-agglutinin contains substantial amounts of endo-β-N-acetylglucosaminidase H (endo H) resistant carbohydrate, which might be O-linked saccharides (Lipke *et al.*, 1989; Lipke and Kurjan, 1992). α-Agglutinin analogs are composed of 30-50% carbohydrates that are N-linked to the glycoprotein and are removed by endo H. The N-linked carbohydrate is not required for activity as α-agglutinin is heat stable and resistant to reducing agents (Burke *et al.*, 1980; Lipke and Kurjan, 1992).

The C-terminal domain is required for anchoring the glycoprotein on the yeast cell surface via the GPI anchor (Wojciechowicz *et al.,* 1993; Lu *et al.*, 1994) whereas the N-terminal half of α-agglutinin contains the binding site for **a**-agglutinin. The binding domain resembles the immunoglobulin fold structures present in many mammalian adhesion proteins (Wojciechowicz *et al.,* 1993). The α-agglutinin binding subunit contains a single potential N-glycosylation site and a low level of Ser and Thr (Lipke *et al.*, 1989). Kapteyn *et al.* (1996) provided evidence that α-agglutinin and other cell

wall mannoproteins are covalently bound to β-1,6-glucan with β-1,3-glucan forming the complex (Kapteyn *et al*, 1996). Mature α-agglutinin from the yeast cell wall is resistant to SDS extraction, but released by β-glucanase suggesting that it is covalently bound to the glucan moiety of the cell wall (Schreuder *et al.*, 1993).

1.5.2 Role in yeast sexual adhesion

Yeast cells mate in a complex inducible system that involves agglutination, mating projection formation, cell fusion and nuclear fusion (Erdman *et al.*, 1998). The *S. cerevisiae* haploid MAT cells of **a** and α mating types express **a**- and α-agglutinins respectively, that are involved in sexual adhesion of the two mating types (Fehrenbacher *et al.*, 1978) in an irreversible manner (Zhao *et al.*, 2001). **a**-Agglutinin, a disulphide-linked oligomer consist of a large, hyper glycosylated subunit that mediates cell surface attachment (*Aga*2p), and small, less highly glycosylated subunit that binds to α-agglutinin (*Aga*1p) (Lipke and Kurjan, 1992; Cappellaro *et al.*, 1994). The **a**-agglutinin disulphide linkages are essential for maintaining Aga2p in its highly active form for the interaction with α-agglutinin (Shen *et al.*, 2001). These sexual agglutinins have been largely investigated on budding yeasts such as *Hansenula wingei*, *Pichia amethionina, S. kluyveri* and *S. cerevisiae* (Burke *et al.*, 1980).

Purified α-agglutinin has been shown to bind specifically to **a** cell, inhibiting its agglutinability. In addition, similarly to the unpurified, it functions in a pH range of 5-7, stable at pH range of 3-9 (Terrance *et al.*, 1987). Sexual adhesion of **a** and α-cells in the culture medium is mediated by sex pheromones, **a** and α-factor produced by the cells respectively, which affects the opposite mating type cells (Betz *et al.*, 1977; Betz and Duntze, 1979). Other cell wall proteins, Factor Induced Gene-1 and 2 (Fig1p and Fig2p), have been implicated in cell-cell interaction of the mating types. Overexpression of Fig2p is reported to cause α-cell-specific agglutinability (Erdman *et al.*, 1998; Jue and Lipke, 2002).

1.5.3 Protein with internal repeats

The protein with internal repeats (PIR) belongs to a small group of cell wall proteins (CWPs) that play an essential role as cell wall glycoproteins (Mazáň *et al.*, 2008). In

wild-type yeast cells PIR-CWPs form approximately 16% of the total amount of covalently linked CWPs (Kapteyn *et al.*, 1999a). Amongst yeast, *S. cerevisiae* PIR-CWPs have been extensively studied compared to those of other yeasts. PIR-CWP gene homologous to those of *S. cerevisiae* have been isolated and characterised from several yeasts, such as *Kluyveromyces lactis*, *Zygosaccharomyces rouxii* (Toh-E *et al.,* 1993), *Candida albicans* (Kandasamy *et al.*, 2000; Martínez *et al.*, 2004) and *Y. lipolytica* (Jaafar *et al.*, 2003), but have not been reported in the fission yeast *Schizosaccharomyces pombe*, suggesting that the PIR-CWP genes are conserved among budding yeasts (Sumita *et al.*, 2005).

PIR-CWPs were the first manoproteins found to be linked through a mild alkali sensitive linkage to cell wall glycans (de Groot *et al.*, 2005). These CWPs are linked directly to the β-1,3-glucan of the cell wall through alkaline sensitive linkages without any β-1,6-glucan serving as an interconnecting moiety (Kapteyn *et al.*, 1999a). The actual labile linkage between the protein moiety and β-1,3-glucan remains unknown, although studies by Kapteyn *et al.* (1999b) suggest that PIR-CWPs are immobilized to the cell surface either through a linkage between β-1,3-glucan and serine or threonine, or by the addition of β-1,3-glucan to existing side chains. The linkages of the PIR-CWPs to the saccharides side chains maybe *O*-linked (Mrsa *et al.*, 1997). Furthermore, the *O*-linkage side chains might be involved in the cross-linkage of the PIR-CWPs to other cell wall components (Kapteyn *et al.*, 1999b). These covalently linked CWPs are solubilized under very mild alkaline conditions, which leave the proteins intact after the *O*-chains have been cleaved off in a process called beta-elimination (Mrsa *et al.*, 1997; Kapteyn *et al.*, 1999b). The absence of the β-1,6-glucan linkage makes the PIR-CWPs resistant to extraction by β-1,6-glucanase, but easily released from the cell wall when treated with β-1,3-glucanase (Kapteyn *et al.*, 1999b). Recently Ecker *et al.* (2006) reported that ScPir4 is bound to the yeast cell wall via glutamine residue 74 within the highly conserved repetitive sequence QIGDGQ$_{74}$VQ.

PIR-CWPs do not have the GPI recognition signal and therefore lack the C-terminal GPI anchor (Mrsa *et al.*, 1997; Wang *et al.,* 2008). Similarly to the GPI anchored CWPs, PIR-CWPs have been found not to show any enzyme activity. However, the expression of PIR-CWPs has been reported to be highly upregulated in response to cell wall stress conditions that activate the cell wall integrity pathway (de Groot *et al.*, 2007). Both ScPir2 and CaPir2 have been observed to be produced at higher levels under conditions of cell wall weakening (Kapteyn *et al.*, 2000).

Over expression of PIR-CWPs has been found to increase resistance to plant antifungal protein osmotin, whereas simultaneous deletion of all PIR-CWP genes results in protein osmitin sensitive strain (Yun *et al.*, 1997). Deletion of PIR-CWPs has been reported to cause weakened, fragile and osmotically unstable cell walls which leads reduced levels of alkali-soluble β-1,3-glucan and increased levels of alkali-soluble chitin (Mazáň *et al.*, 2008). In addition, disruptions of PIR-CWP genes affect cell wall composition in a way that increases, only slightly, the resistance of the cells to agents that interfere with the synthesis of the cell wall or degrade it (Jaafar *et al.*, 2003). Mutants with deleted ScPir-CWPs (1, 2, 3 and 4) have decreased mating ability that might be due to the altered cell wall structure and increased sensitivity to calcofluor white (Mrsa and Tanner, 1999).

Unlike other members of the stress proteins such as *S. cerevisiae* Pir2-protein (ScPir2p) that is induced by heat, ScPir1p is not heat induced (Yun *et al.*, 1997). Studies by Toh-E *et al.* (1993) showed that disruption of each of the *S. cerevisiae* PIR-CWP genes does not confer sensitivity to heat shock on the cell whereas double disruption of ScPir1p and ScPir2p genes showed clear heat-shock-sensitive phenotype, which suggests that the PIR-CWP genes are essential for tolerance to heat shock. Although PIR-CWPs confer resistance to osmotin and stress in naturally resistant yeast strains, their function in the cell wall remains unknown (Yun *et al.*, 1997; Sumita *et al.*, 2005). Although the actual function of PIR-CWPs is unknown, they might be crucial as a cell wall compensatory mechanism that is activated when yeast cells experience decline in the level of β-1,6-glucan (Kapteyn *et al.*, 1999b). In addition, *Candida albicans* Pir1p has been suggested to be essential in the organisation of the cell wall as it is significantly expressed during the initial steps of protoplasm regeneration (Martínez *et al.*, 2004).

1.5.4 *Yarrowia lipolytica* protein with internal repeats 1

Compared to other CWPs, *S. cerevisiae* CWPs have been extensively studied and are used as a model for isolation of PIR-CWPs in other yeasts (Sumita *et al.*, 2005). PIR-CWPs were first discovered by Toh-E *et al.* (1993) in *S. cerevisiae*. *Y. lipolytica* Pir1-protein (YlPir1p), a homologue of *S. cerevisiae* (Sc) Pir4p, was discovered by Jaafar *et al.* (2003) and constitutes the first PIR-CWP identified in *Y. lipolytica*. The YlPir1p gene encodes for the 286 amino acids long Pir1p. Analysis of the YlPir1p amino acid sequence indicates the presence of a putative signal peptide that is

processed between amino acid 20 and 21 and a putative Kex2 cleavage site after amino acid 65 (Jaafar *et al.*, 2003) that is cleaved by the Kex2 protease (Moukadiri *et al.*, 1999; Wang *et al.,* 2008). The Kex2 cleavage site is conserved in all members of the PIR-CWP family, except for CaPir-CWPs (Martínez *et al.*, 2004), with the ScPir1p containing a Kex2 cleavage site after the first 60-70 N-terminal amino acids (Mrsa *et al.*, 1997).

ScPir1p is a highly *O*-glycosylated cell wall protein that is resistant to SDS extraction under reducing conditions and contains a potential *N*-glycosylation site (Mrsa *et al.*, 1997). In contrast to this, studies by Jaafar *et al.* (2003) showed that the mature YlPir1p does not contain any putative *N*-glycosylation site; however, it does contain a total of 38 serine and threonine residues making it an *O*-glycosylated mannoprotein. Sequence alignment of the PIR-CWPs from both *Y. lipolytica* and *S. cerevisiae* indicates that YlPir1p is more closely related to ScPir4p than to ScPir1p. These two β-mercaptoethanol extractable mannoproteins (YlPir1p and ScPir4p) behave in a similar way since their localization and apparent molecular weight are similar. Sequence analysis of the alignments revealed the absence of a characteristic repetitive motif from YlPir1p that is similar to the motif SQIGDGQVQAT at the N-termini, which is common to all PIR-CWPs of *S. cerevisiae* (Jaafar *et al.*, 2003). This highly conserved motif, with small codon variations, is repeated 8 times in ScPir1p and ScPir3p and 11 times in ScPir2p. The conserved motif is present only once in the ScPir4p amino acid sequence (Mrsa and Tanner, 1999; Jaafar *et al.*, 2003). In *C. albicans* a maximum of nine conserved repetitive amino acid sequences are present in CaPir1p, a homologue of ScPir4p (Martínez *et al.*, 2004).

Although the YlPir1p does not contain the repetitive motif, its carboxy terminal region has shown to be highly similar to those of *S. cerevisiae* PIR-CWPs. Another common structural feature among YlPir1p, ScPIR-CWPs and CaPir1p is the presence of four cysteines, a characteristic of ScPIR-CWPs, in the same position at the carboxy terminus (Jaafar *et al.*, 2003; Martínez *et al.*, 2004). This conserved feature could reflect the importance of disulphide bridges either in the secondary structure of the protein, in the formation of multimers, or in the attachment of PIR-CWPs to other components of the yeast cell wall (Jaafar *et al.*, 2003).

The ScPir1p gene encodes a protein containing a repeating unit of 18 to 19 amino acid residues repeated seven times in tandem (Toh-E *et al.*, 1993). Sumita *et al.* (2005) reported that deletion of the internal repetitive sequences from the *N*-terminus

of the ScPir1p affected localization of the protein to the bud scars. Furthermore, the strength of the linkage between ScPir1p and the cell wall depends on the number of repetitive sequence units. Whereas the ScPir1p repetitive sequences on the *N*-terminus are required for anchoring the protein to the cell wall, the C-terminal region of the protein is necessary for directing the protein to the bud scars.

ScPir1p, similarly to ScPir2p, is localized inside the chitin ring of bud scars. Although ScPir2p is localized inside the chitin ring of bud scars, it has also been reported to be expressed in other regions of the cell wall, which makes ScPir1p the only protein in the PIR family to be specifically expressed inside the bud scars. Because ScPir1p is not localized at the bud neck during the budding process but is present at bud scars after the completion of cell separation, it is conceivable that ScPir1p is required for reinforcement of the weakened cell wall following cell budding (Sumita *et al.*, 2005). Moukadiri *et al.* (1999) reported that ScPir4p is localized on the surface of growing buds and not on the surface of older mother cells. These findings contradicted those of Sumita *et al.* (2005) which reported that ScPir4p including ScPir4p are expressed uniformly on the cell surface and not present at the chitin ring of bud scars.

1.6 Aims of the study

With the large number of molecular tools available in *Y. lipolytica* this study looks at developing an efficient heterologous protein production system for this yeast. The overall aim of the research contained in this thesis was the development of a system to improve downstream processing of recombinant proteins expressed extracellularly and displayed on the cell wall of *Y. lipolytica.*

Study objectives:

1. Firstly, to evaluate *Y. lipolyica* as a host for production of *Fusarium solani pisi* cutinase using its native secretion signal and the pre-pro Lip2 secretion signal. Secondly, to evaluate the effect of the Histidine tag on the secretion and activity of cutinase expressed in *Y. lipolytica* using the two signal peptides.
2. Firstly, to evaluate *Y. lipolyica* as a host for production of lipase from *Thermomyces lanuginosus* from either a single copy or multicopies of its gene, using its native secretion signal and the pre-pro Lip2 secretion signal. Secondly, to evaluate the effect of the Histidine tag on the secretion and activity of lipase expressed in *Y. lipolytica* using the two signal peptides.
3. Develop a novel rDNA-based cell surface display system for *Y. lipolyica*
4. Cell surface display of *F. solani* cutinase and *T. lanuginosus* lipase on *Y. lipolyica*
5. Develop a novel cell surface system for expression of RANTES using mCherry as a reporter protein.

1.7 References

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Chapter 2. Heterologous expression and functional analysis of *Fusarium solani pisi* cutinase in *Yarrowia lipolytica*

2.1 Abstract

In this work, the capability of *Yarrowia lipolytica* pre-pro Lip2 secretion signal (Lip2ss) and the cutinase (Cut) native secretion signal (Nss) for directing the secretion of *Fusarium solani pisi* Cut (FSCut) to the extracellular medium was investigated in *Y. lipolytica*. The effect of a Histidine (His) tag on protein production and activity of the expressed FSCut was also investigated. Expression levels of FSCut in *Y. lipolytica* transformants were highly variable. The purified non-tagged FSCut directed by the Nss reached 13.1 mg per 100 ml, which was 2.5 fold higher than that observed for the C-terminally His-tagged FSCut directed by the same secretion signal and the non-tagged FSCut directed by the pre-pro Lip2ss. The purified non-tagged FSCut expression level was 5.6 fold higher than that of the C-terminally His-tagged FSCut directed by the pre-pro Lip2ss. Although the purified FSCut expression level directed by the Nss was higher, its specific activity was about 1.5 fold lower than the purified non-tagged FSCut secreted using the pre-pro Lip2ss. Biochemical characterization of all the purified proteins expressed using different approaches showed the highest hydrolytic activity at 35°C and pH 8.0 for the His-tagged protein whereas the non-tagged protein had a pH optimum of 10. The expressed FSCut showed a substrate preference for short chain fatty acids, with the highest activity towards tributyrin and *p*-NPB.

2.2 Introduction

Cutinases (EC 3.1.1.74) are hydrolytic enzymes that have the ability to degrade cutin, a major polymer of higher plants that contains an insoluble lipid-polyester composed of hydroxy and epoxy fatty acids (Purdy and Kolattukudy, 1975). Although the precise composition of cutin differs among species, the primary fatty acids are usually *n*-C_{16} and *n*-C_{18} that contain one to three hydroxyl groups (Kolattukudy, 1985; Carvalho *et al.*, 1999). Cutinases catalyse the hydrolysis of the cutin ester bonds into chloroform-methanol soluble cutin monomers (Fett *et al.*, 1992). The enzyme was first purified and characterized from *Fusarium solani pisi* grown on cutin as the sole carbon source (Purdy and Kolattukudy, 1975). In addition to its ability to hydrolyze cutin, a wide range of insoluble short and long chain triacylglycerols (TAGs) and soluble esters (Purdy and Kolattukudy, 1973), cutinase is also capable of hydrolysing fatty acid esters and emulsified TAGs as efficiently as lipases without any interfacial activation (Sarda and Desnuelle, 1958; Verger and de Haas, 1976). This enzyme therefore forms a link between 'true' esterases and 'true' lipases due to functional capabilities of both families and it is an efficient catalyst both in solution and at the lipidic interface (Martinez *et al.*, 1994). In addition, cutinases can be used as a model for more complex lipases, because their kinetic behaviour resembles that of 'true' lipases (Egmond and de Vlieg, 2000).

Because of their importance in pathogenesis, the majority of reported cutinases are from phytopathogenic fungi. Cutinases have been reported in *F. solani pisi* (Soliday *et al.*, 1984), *Aspergillus oryzae* (Maeda *et al.*, 2005), *Magnaportha grisea* (Sweigard *et al.*, 1992), *Collectotrichum* sp. (Chen *et al.*, 2007) and other fungi. While fungal cutinases are the most studied, there have been reports of cutinases in bacteria such as *Pseudomonas putida* (Sebastian *et al.*, 1987; Sebastian and Kolattukudy, 1988), *Thermobifida fusca* (Fett *et al.*, 1992) and *Thermoactinomyces vulgaris* (Fett *et al.*, 2000). More recently, Chen *et al.* (2008) reported on the gene responsible for expression of cutinase in *T. fusca*. The *T. fusca* gene was cloned and over-expressed in *Escherichia coli* with subsequent purification and characterization of the enzyme. A synthetic cDNA encoding for the cutinase from *F. solani pisi* has been expressed in *Saccharomyces cerevisiae* (Calado *et al.*, 2002), *E. coli* (Bandmann *et al.*, 2000), *Pichia pastoris* (Kwon *et al.*, 2009) and *Aspergillus awamori* (van Gemeren *et al.*, 1998). Cutinase remains one of the most interesting enzymes that can be used in a wide range of biotechnological sectors ranging from food, detergent and

chemical industries (Egmond and de Vlieg, 2000). The enzyme hydrolyses biodegradable plastic materials such as poly-butylene succinate and poly-butylene-*co*-adipate (Maeda *et al.*, 2005).

Yarrowia lipolytica is a strictly aerobic non-conventional yeast widely used in industry for production of citric acid, peach flavour and single cell protein (Barth and Gaillardin, 1996). This dimorphic yeast has been shown to secrete a wide range of proteins (alkaline or acid proteases, RNase and lipases) into the medium in amounts sufficient for industrial applications (Beckerich *et al.*, 1998). A series of tools for heterologous protein expression in *Y. lipolytica* have been developed (Madzak *et al.*, 2004). To the best of our knowledge there are no reports describing expression of FSCut or from any other source in *Y. lipolytica.* In this chapter for the first time the expression of FSCut by *Y. lipolytica* using both the native Cut secretion signal (Nss) and the pre-pro Lip2 secretion signal (Lip2ss) for directing the secretion of FSCut to the growth medium is reported. The effect of a His-tag on production and activity of the enzyme was also investigated.

2.3 Material and methods

2.3.1 Strains, plasmids and reagents

Escherichia coli XL10 Gold (Tet^r *D (mrcA) 183D (mrcCB-hsdSMR-mrr) 173endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacI^{q}ZDM15 Tn10* (Tet^r) Amy Cam^r]; Stratagene, USA) was used as host for plasmid construction and propagation of FSCut encoding gene. Cells were grown in Luria-Bertani (LB) broth or on agar plates (Sambrook *et al.*, 1989) supplemented with 50 µg/ml ampicillin (Amp). *Y. lipolytica* Po1g strain (*MatA, leu2-270, ura3-302::URA3, xpr2-322, axp1-2*; Madzak *et al.*, 2004) was used as a host for extracellular production of FSCut. The plasmid pKOV323 was used for the production of both His-tagged and non-tagged FSCut using both the pre-pro Lip2ss and the Cut Nss. PCR products were cloned into pGEM®-T Easy Vector (Promega, USA). Recombinant plasmid DNA was isolated from *E. coli* XL10 Gold cells either using the method of Berghammer and Auer (1993) for initial screening purposes or the Plasmid Isolation Kit (BioFlux) following the manufacturer's instructions. The *Y. lipolytica* transformants were selected on YNB-N_{5000} and grown in YPD. Chromogenic agar substrate plates used for selection of *Y. lipolytica* transformants expressing cutinase were prepared as described by Singh *et al.* (2006).

2.3.2 Construction of expression vectors

All standard DNA manipulation procedures were performed according to Sambrook *et al.* (1989). The entire *cut* gene encoding the open reading frame (ORF) including the native secretion signal (Nss) of FSCut (Gen Bank Accession No. K02640.1; Soliday *et al.*, 1984) was codon optimized for expression in *Y. lipolytica* (GeneArt, Regensbung, Germany). The *cut* gene was synthesised with its Nss (705 bp) at the N-terminal end flanked by a *Hind*III restriction site at the 5'. A His-tag was added at the C-terminus upstream of the stop codon (TAA), followed by an *Avr*II restriction site. The *cut* gene was released with *Hind*III and *Avr*II and ligated into the single copy pKOV323 expression vector digested with the same enzymes to generate pKOV323-Nss-*cut*-His (Fig. 2.1A).

To construct an expression vector that retained the *cut* Nss and no His-tag, the primers pKOV-F (5'-GTTTGCCAGCCACAGA-3') flanking the 3'-end of the hp4d

promoter, and Fs_p-R (5'-TTTCCTAGGTTAGTGGTGATGGTGGTGG TG-3'; *Avr*II site is underlined), which introduce a TAA stop codon, were used to amplify the gene from pKOV323-Nss-*cut*-His. Thermocycling reactions were carried out using the MJ Mini Personal Thermal Cycler (BIO-RAD). PCR amplifications were performed using Taq polymerase (Fermentas). A PCR reaction mixture was prepared containing 1/10 volume reaction buffer with magnesium chloride, 10 mM dNTPs, 1.0 µM each of primer, 0.625 U Taq polymerase, 5 µg of DNA and topped up to a total volume of 50 µl with distilled water. The thermal cycling conditions included an initial denaturation at 98°C for 30 sec, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 20 sec and extension at 72°C for 1 min, with a final extension step of 75°C for 5 min and held at 4°C. Following thermal cycling and agarose gel electrophoresis, PCR products were gel purified using the Gel Extraction Kit (BioFlux) according to the manufacturer's instructions. Purified products were subcloned into the pGEM®-T Easy vector, released with *Hind*III and *Avr*II and ligated into pKOV323 expression vector to generate pKOV323-Nss-*cut* (Fig. 2.1B).

To construct a *cut* gene under the pre-pro Lip2ss, the Nss was removed from the *cut* gene using plasmids pKOV323-Nss-*cut*-His and pKOV323-Nss-*cut* as templates for amplification of the *cut* gene with (637 bp) and without a His-tag (620bp) using the same PCR conditions as described above. The primer pair Fs_p-F1 (5'-AAAGGATCCATGCTGGGCCGAACCACCCGA-3'; *BamH*I site is underlined) and pKOV-R (5'-CGATATTCATTTATTAAAGTA-3') flanking the Lip2 terminator of the expression vector, were used to generate His-tagged amplicons from pKOV323-Nss-*cut*-His, whereas pKOV323-Nss-*cut* was used to generate non-tagged amplicons. Amplicons were gel purified, subcloned into pGEM®-T Easy vector and the inserts released with *BamH*I and *Avr*II and ligated in-frame downstream of the pre-pro Lip2ss of the pKOV323 expression vector linearized with the same enzymes to generate pKOV323-Lip2ss-*cut*-His and pKOV323-Lip2ss-*cut* (Fig. 2.1C and D respectively). The recombinant plasmids were used to transform *E. coli* XL 10 Gold cells grown on LB-Amp (50 µg/ml) agar plates and screened in 5 ml LB medium containing 50 µg/ml Amp.

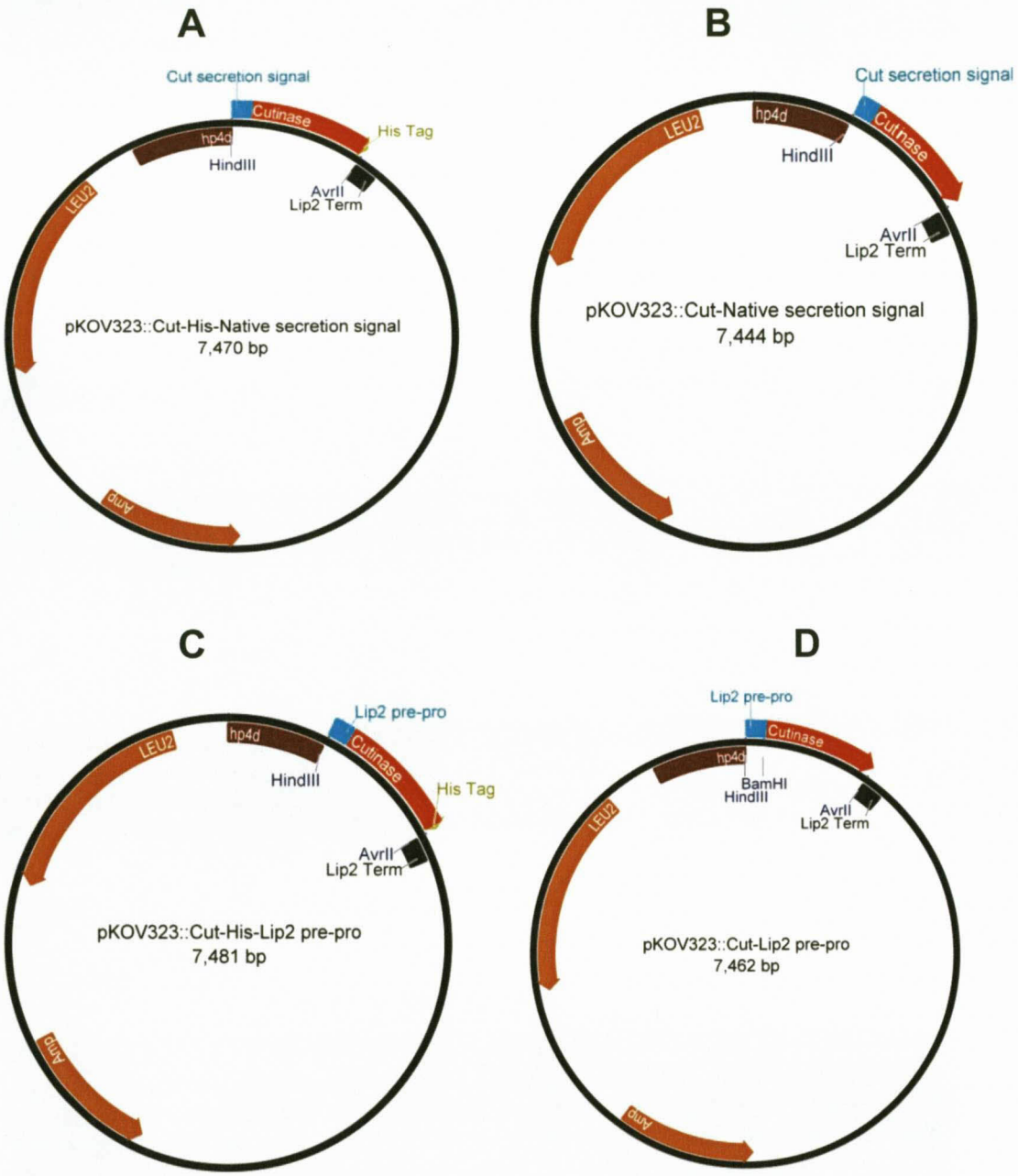

Figure 2.1: Schematic diagrams of *cut* gene in the expression plasmid pKOV323. The *cut* gene is under the Nss (A and B) or pre-pro Lip2ss (C and D). Diagrams A and C carry the His-tagged *cut* gene, whereas B and D carry the non-His tagged *cut* gene. The plasmid map was constructed using Geneious v5.5 (Drummond et al. 2011).

2.3.3 Yeast transformation

*Not*I linearized pKOV323-Lip2ss, pKOV323-Nss-*cut*-His, pKOV323-Nss-*cut*, pKOV323-Lip2ss-*cut*-His and pKOV323-Lip2ss-*cut* recombinant vectors were used to transform *Y. lipolytica* Po1g strain. Preparation of Po1g competent cells and transformation was performed as described by Xuan *et al.* (1988). For selection of Leucine$^+$ (Leu$^+$) clones, Po1g transformants were grown on selective YNB-N_{5000} media (0.17% YNB without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 1% glucose and 1.5% agar). Colonies were isolated from YNB-N_{5000} plates after 2 days of incubation at 28°C. Genomic DNA was extracted using the method of Chen *et al.* (1997) and used as a template for confirming the integration of the recombinant vectors into the yeast genome using pKOV410-F (5'-GTTTGCCAGCCACAGA-3') and pKOV410-R (5'-CGATATTCATTTATTAAAGTA-3') used in the amplification of the genes in the previous section. The PCR positive transformants were transferred to chromogenic plates [(1.0% trybutyrin sonically emulsified in 10 mM $CaCl_2$, 0.01% phenolphthalein, 2.0% agar and the pH adjusted to 7.3/7.4 using 0.1 N NaOH)] as described by Singh *et al.* (2006) and incubated for 24 h at 28°C. Preliminary studies on selection of transformants was done using chromogenic plates for screening several transformants and only the one forming the biggest halo on plates was selected for further investigation (data not shown). FSCut activity was estimated according to halo size around the colonies. The *Y. lipolytica* Po1g strains carrying pKOV323-Lip2ss, pKOV323-Nss-*cut*-His, pKOV323-Nss-*cut*, pKOV323-Lip2ss-*cut*-His and pKOV323-Lip2ss-*cut* expression cassettes were denoted Yl-L, Yl-FSCutNH, Yl-FSCutN, Yl-FSCutLH and Yl-FSCutL respectively.

2.3.4 Expression and purification of FSCut in *Y. lipolytica*

The Yl-L, Yl-FSCutNH, Yl-FSCutN, Yl-FSCutLH and Yl-FSCutL clones were inoculated into 25 ml of YPD medium and incubated overnight at 28°C with shaking at 200 rpm. When the culture reached an optical density at 600 nm (OD_{600}) of 3-6, the cells were re-suspended to an OD_{600} of 1.0 in 100 ml YPD and incubated at 28°C with shaking at 200 rpm. The OD_{600} was recorded and supernatant samples collected at 24 hour intervals over the cultivation period of 7 days. The recorded OD was used to assess whether expression of FSCut affected the growth patterns of *Y. lipolytica* Po1g strain. The cultures were centrifuged and supernatants analysed for total extracellular protein concentration.

After seven days, the remaining supernatant was collected for purification of the enzyme. The supernatants were concentrated and diafiltered with 20 mM Tris-HCl (pH 8.0) using an Amicon stirred ultrafiltration cell (Millipore, Billeria, MA, USA) with a Millipore ultrafiltration membrane of 30 kDa molecular weight limit (Separations, South Africa). The samples were further diafiltrated and concentrated using an ultrafiltration membrane of 10 kDa cut-off molecular weight limit.

2.3.5 Protein analysis

Protein concentrations of *Y. lipolytica* culture supernatant and the purified enzyme preparation were determined by the Bradford assay (1976) using the BIO-RAD protein dye reagent according to the manufacture's manual. Absorbance was measured at 595 nm employing Power Wave HT (BioTek) spectrophotometer. Bovine serum albumin (Roche, Mannheim, Germany) was used as a standard. To check the purity of the proteins, the purified protein samples were analysed on a 12% SDS-PAGE gel as described by Laemmli (1970).

2.3.6 FSCut activity

FSCut activity was measured using a pH titration method (Benzonana and Desnuelle, 1968) using tributyrin, glyceryl trioleate and glyceryl trioctanoate as substrates. A 1% solution of each of the substrates was emulsified through sonication (HD 2070 Bandelin, Sonopuls) in a solution containing 20 mM $CaCl_2$, 0.6 M NaCl, 1 mM sodium deoxycholate and 2.5% (w/v) gum Arabic. Enzyme solution (100 µg) was added, with liberated fatty acids from the TAG substrates titrated with 0.05 N NaOH to maintain a constant pH of 7.3-7.4 at 40°C. One unit (U) of FSCut activity was defined as the amount of enzyme releasing 1 µmol of fatty acid per minute under assay conditions. All assays were averaged from three independent experiments.

Spectrophotometric determination of the purified FSCut estereolytic activity was measured following hydrolysis of *para*-nitrophenyl butyrate (*p*-NPB) at 410 nm according to the methods of Pinsirodom and Parkin (2001) and Koschorreck *et al.* (2010), using a DU 800 spectrophotometer (Beckman Coulter). The assay mixture

contained 420 µM *p*-NPB prepared in assay buffer (0.l mM Tris-HCl pH 8.0; 0.5% (v/v) Triton X-100, and 0.1% (w/v) gum Arabic). To 1 ml of the substrate solution, 10 µl of the enzyme was added (5 ng protein). Hydrolysis of *p*-PNB followed by the release of *p*-nitrophenol showed an increase in absorbance at 410 nm and *p*-Nitrophenol was used as a standard. One unit (U) was defined as the amount of enzyme that releases 1 µmol of *p*-Nitrophenol per minute under assay conditions. All assays were averaged from three independent experiments done in duplicate with equal amounts of purified non-tagged and C-terminally His-tagged FSCut from the four transformants.

2.3.7 Enzymatic characterization of FSCut

The effect of pH and temperature on FSCut activity expressed in *Y. lipolytica* was determined by performing titrimetric assays measuring tributyrin hydrolysis by FSCut at different pH and temperatures. FSCut activity was measured at temperatures ranging from 20-60°C in 50 mM Tris-HCl buffer at pH 8.0. The pH dependence of FSCut was determined from 4 to 11 using Tris-HCl at 40°C. The substrate specificity of FSCut was determined by the titrimetric assay with TAGs of different chain lengths. The assay mixture contained 1% of TAG substrate and the assays were done at 40°C and pH 8.0. All assays were averaged from three independent experiments using 100 µg of supernatant and purified non-tagged and C-terminally His-tagged FSCut from the four transformants.

2.4 Results

2.4.1 Construction of single-copy vectors for expression of FSCut

Cut genes were cloned into pKOV323 in-frame with the Nss and the Lip2ss pre-pro region (Pignède *et al.*, 2000a, b) under the hp4d promoter (Madzak *et al.*, 2000). All the expression vectors harbouring the *cut* gene carried a growth-phase-dependent hybrid promoter hp4d and Lip2 terminator (Barth and Gaillardin, 1997). The FSCut producing strains were obtained by integrative transformation of *Y. lipolytica* Po1g strain with the four different *cut*-carrying vectors. The resulting transformants were screened by halo formation on chromogenic agar plates and PCR to confirm integration of the recombinant vectors into the yeast pBR322 docking platform using the primer pair pKOV410-F (5'-GTTTGCCAGCCACAGA-3') and pKOV410-R (5'-CGATATTCATTTATTAAAGTA-3'). The transformants forming the biggest halo were selected for further investigations. The *Y. lipolytica* transformants (YI-FSCutNH, YI-FSCutN, YI-FSCutLH and YI-FSCutL) positively confirmed by PCR to harbour the FSCut gene showed increased hydrolytic activity towards tributyrin on chromogenic activity plates compared to the control *Y. lipolytica* Po1g (YI-L). The transformants expressing FSCut produced larger halos on this medium after 24 hours of incubation than the control strain (Fig. 2.2). All the strains employing the homologous or heterologous secretion signals for secretion of non-tagged and C-terminally His-tagged FSCut exhibited higher-levels of lipolytic activity on the agar plates containing emulsified tributyrin compared to the control YI-L.

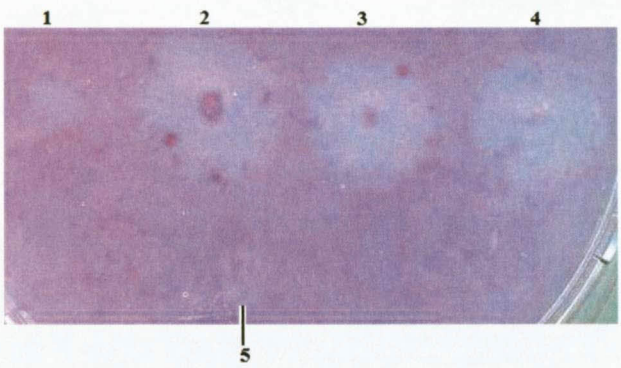

Figure 2.2: *Y. lipolytica* single copy transformants grown on chromogenic activity plates. The strains are grown on 2% agar plates containing emulsified 1% tributyrin and 0.01% phenolphthalein. *Y. lipolytica* transformants (1) YI-FSCutNH, (2) YI-FSCutN, (3) [YI-FSCutLH, (4) YI-FSCutL] and (5) Arrow indicating *Y. lipolytica* YI-L.

2.4.2 Heterologous expression of FSCut in Y. lipolytica

To ascertain whether expression of FSCut with and without His-tag directed by the Nss and the pre-pro Lip2ss for secretion affected the growth of *Y. lipolytica*, the strains expressing FSCut and YI-L were grown in liquid cultures under aerobic conditions on a rotary shaker (200 rpm, 28°C). Growth was determined by monitoring OD of the cultures at 600 nm at 24 hour intervals for 7 days. During the entire period of culture cultivation there were no significant differences in the growth profiles of *Y. lipolytica* cultures harbouring single copy genes of FSCut secreting non-tagged or tagged FSCut directed by either the Nss or the pre-pro Lip2ss (Fig. 2.3).

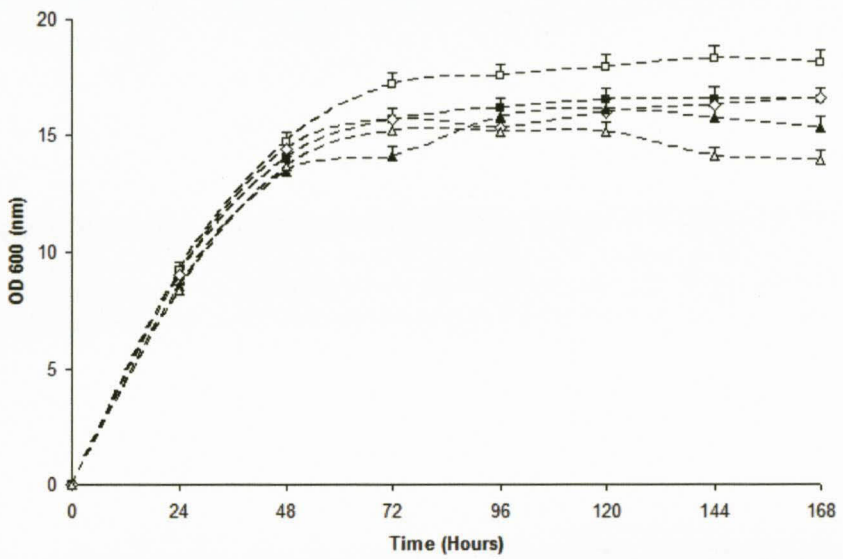

Figure 2.3: Comparison of the growth profiles of *Y. lipolytica* expressing FSCut. Strains were grown at 28°C in shake flasks (200 rpm) and growth was assessed by measuring absorbance at OD_{600} for a period of 168 hours at 24 hour intervals. *Y. lipolytica* cells: YI-FSCutNH (□); YI-FSCutN (■); YI-FSCutLH (Δ); YI-FSCutL (▲) and YI-L (◊). All measurements were done in duplicates.

Y. lipolytica Po1g expressing non-tagged FSCut directed by the Nss or the pre-pro Lip2ss for secretion produced similar amounts of total extracellular protein after 7 days of growth. The Nss and the pre-pro Lip2ss resulted in 33.2 and 30.4 mg of protein per 100 ml of culture supernatant, respectively (Fig. 2.4). Even though the growth pattern of *Y. lipolytica* strains expressing tagged and non-tagged FSCut was similar, quantitative analysis of the total protein showed differences. The total extracellular protein produced by both strains expressing non-tagged FSCut were 1.4

fold higher than the amount produced by the respective strains expressing C-terminally tagged FSCut directed by the Nss and the pre-pro Lip2ss. The total amounts of protein produced by these latter strains were 24.2 and 21.4 mg, per 100 ml of culture supernatant respectively (Fig. 2.4).

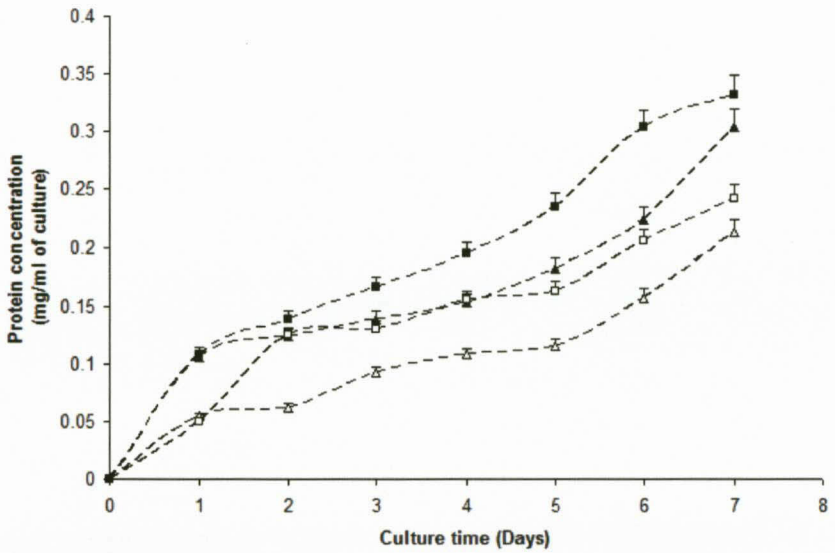

Figure 2.4: Comparison of total extracellular protein in different strains of *Y. lipolytica*. Total proteins were quantified at 24 hour intervals for a 168 hour period. *Y. lipolytica* transformants: YI-FSCutNH (□); YI-FSCutN (■); YI-FSCutLH (Δ); YI-FSCutL (▲) and YI-L (◊). All measurements were done in duplicates.

FSCut specific activity from crude samples (total culture supernatant) was similar in the *Y. lipolytica* cultures expressing non-tagged FSCut or C-terminally His-tagged FSCut directed by the pre-pro Lip2ss and non-tagged FSCut directed by the Nss for secretion (Table 2.1). SDS-PAGE analysis of the 24 hour interval samples revealed clear visible bands at ~20 kDa corresponding to FSCut (Fig. 2.5).

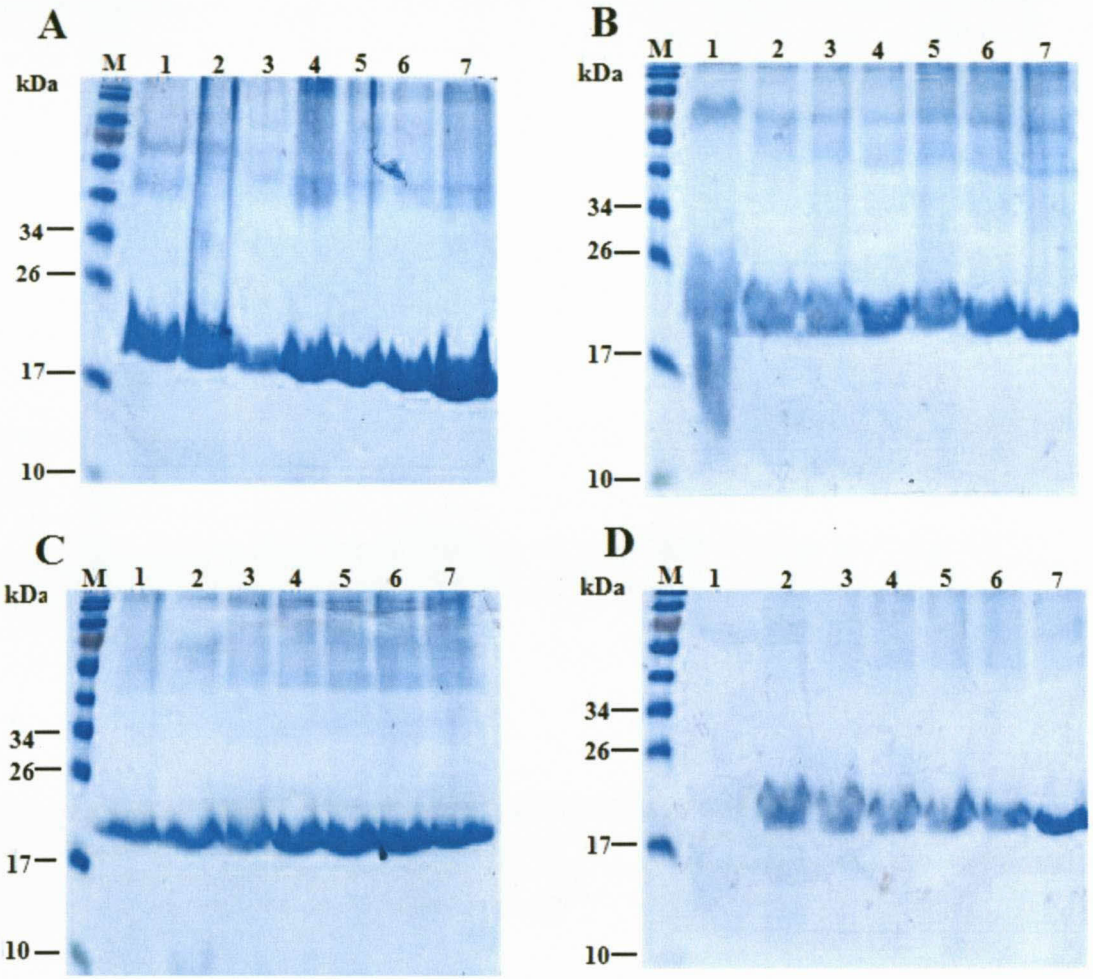

Figure 2.5: SDS-PAGE analysis of *Y. lipolytica* Po1g expressing tagged and non-tagged FSCut directed by homologous and heterologous secretion signals. The total protein culture supernatant samples were collected at 24 hour interval for 7 days and electrophoresed on 12% SDS-PAGE gels: (A) non-tagged FSCut directed by the Nss for secretion, (B) C-terminally tagged FSCut directed by the Nss for secretion, (C) non-tagged FSCut directed by the pre-pro Lip2ss for secretion, and (D) C-terminally tagged FSCut directed by the pre-pro Lip2ss for secretion. Lane M is a PageRuler stained protein ladder (Fermentas). The numbers depicted on top of the gel images, labelled 1-7 represent the cultivation period in days.

2.4.3 Purification and biochemical characterization

After 7 days of expression, FSCut was purified from 100 ml culture medium by ultrafiltration (Table 1). Expressed FSCut protein was purified using Amicon ultrafiltration membranes. The expression of FSCut under the Nss resulted in higher levels of expression than when the protein was expressed under the pre-proLip2ss. The non-tagged FSCut showed higher levels of expression than the His-tagged

version of the protein. The non-tagged purified FSCut directed by the Nss was 2.5 fold higher than the purified non-tagged FSCut directed by the pre-pro Lip2ss. In addition, this protein's expression levels were 2.3 fold higher than the purified C-terminally His-tagged FSCut directed by the Nss and 5.6 fold higher than the purified C-terminally His-tagged FSCut directed by the pre-pro Lip2ss. The amount of the purified non-tagged FSCut directed by the pre-pro Lip2ss was found to be similar to those of the purified C-terminally His-tagged FSCut directed by the Nss and 2.3 fold higher than the C-terminally His-tagged FSCut directed by the pre-pro Lip2ss. The purified C-terminally His-tagged FSCut directed by the Nss was 2.5 fold higher than the purified C-terminally His-tagged FSCut directed by the pre-pro Lip2ss.

Table 2.1 Purification of FSCut expressed in *Y. lipolytica*

Enzyme	Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification Fold
FSCut (Nss)	Culture supernatant	8898	33.2	268	100	1
	Purified	14135	13.1	1079	159	4.03
FSCut-His (Nss)	Culture supernatant	3703	24.2	153	100	1
	Purified	5390	5.84	923	146	6.03
FSCut (Lip2ss)	Culture supernatant	8907	30.4	293	100	1
	Purified	7817	5.44	1437	88	4.9
FSCut-His (Lip2ss)	Culture supernatant	6013	21.4	281	100	1
	Purified	2377	2.3	1016	40	3.6

100 µg of supernatant and purified FSCut was used to measure the activity of the enzyme at pH 8.0

When the purified FSCut was resolved on SDS-PAGE gel, only single protein bands were observed (Fig. 2.6).

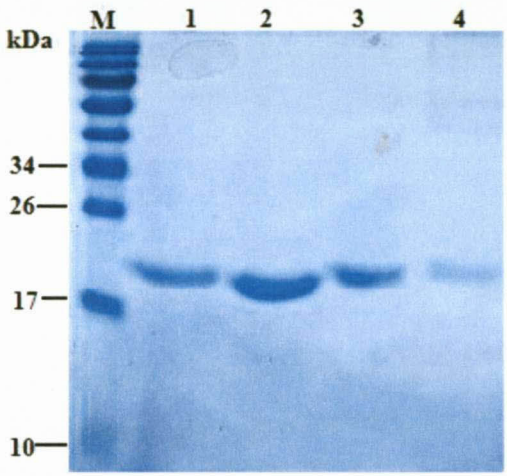

Figure 2.6: SDS-PAGE analysis of purified FSCut expressed in *Y. lipolytica* Po1g. The purified FSCut was analysed on 12% SDS-PAGE gels: Lane 1: purified C-terminally His-tag FSCut directed by Nss secretion, Lane 2: non-tagged purified FSCut directed by the Nss for secretion, Lane 3: non-tagged purified FSCut directed by the pre-pro Lip2ss for secretion and Lane 4: purified C-terminally His-tagged FSCut directed by the pre-pro Lip2ss for secretion. Lane M: PageRuler pre-stained protein ladder.

The activity of the purified FSCut was determined at pH 8.0 using tributyrin as a substrate. The non-tagged purified FSCut secreted using the pre-pro Lip2ss showed higher specific activity value (~1.5 fold) than the His-tagged purified FSCut when assayed using the titrimetric assay. The non-tagged purified FSCut secreted using the Nss showed similar specific activity value to those of the C-terminally His-tagged FSCut. The effects of pH (Fig. 2.7A) and temperature (Fig. 2.7B) on the activity of all purified FSCut was determined over a pH range of 4-11 and temperature range of 20-60°C. Both the purified tagged and non-tagged FSCut showed higher activity at temperatures ranging from 30-40°C compared to the temperatures below 30°C and above 40°C. Both the tagged and non-tagged FSCut showed optimal activity at 35°C. The effect of pH on the purified FSCut differed for both the tagged and non-tagged proteins. The purified His-tagged protein showed pH optimum at 8.0 whereas the purified non-tagged ones showed activity at pHs ranging from 8-10. Non-tagged FSCut showed optimal activity at pH 10. The His-tagged FSCut showed a drastic activity reduction at pH 10, indicating that His-tag affects FSCut at its pH optimum.

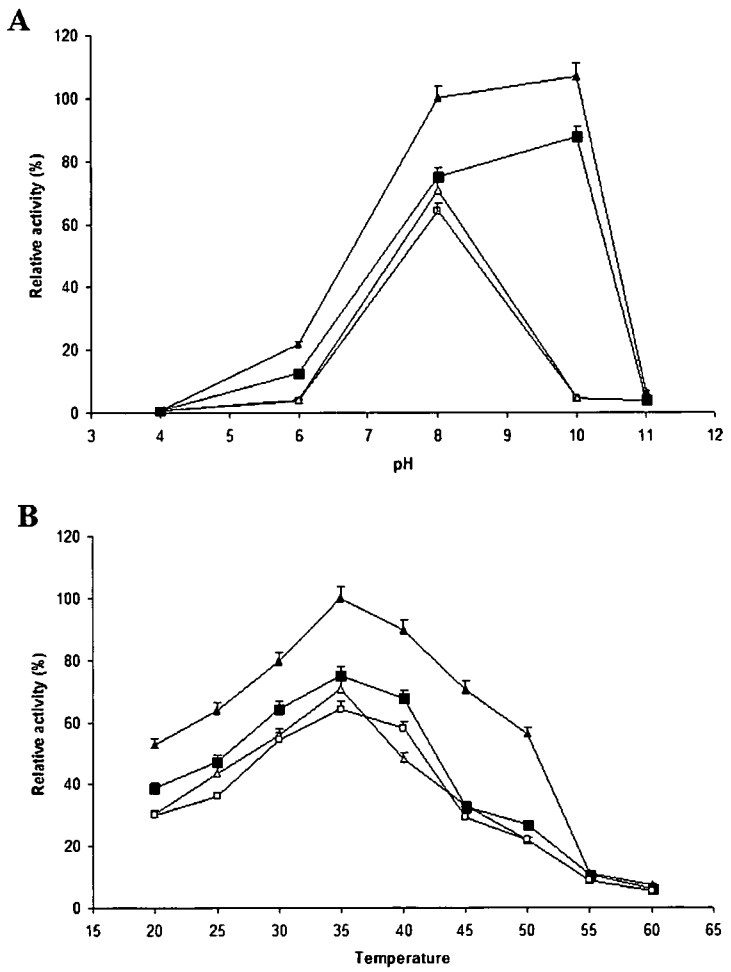

Figure 2.7: Effect of (A) pH and (B) temperature on purified non-tagged and His-tagged FSCut. FSCutN (■); FSCutNH (□); FSCutL (▲) and FSCutLH (Δ). Data are averages from three independent experiments.

Substrate specificity of FSCut was determined using TAGs of different chain lengths. The purified FSCut showed preference for tributyrin and *p*-PNB. Optimum hydrolysis of *p*-PNB was achieved using 5 ng of purified FSCut while only 100 µg of the protein was needed to optimally hydrolyse tributyrin. The specific activity of the purified non-tagged FSCut directed by the pre-pro Lip2ss for secretion was 1.6 fold higher than that of the non-tagged FSCut directed by the Nss for secretion when using *p*-PNB as a substrate (Fig. 2.8A). The non-tagged FSCut directed by the pre-pro Lip2ss further showed 2 fold higher specific activity than the C-terminally His-tagged directed by both secretion signals. The activity of FSCut decreased with an increase in chain length of the substrates, with the non-tagged FSCut showing the highest activity at its pH optimum when hydrolysing the long chain TAGs. His-tagged FSCut hydrolysed long chain TAGs optimally at pH8.0 (Fig. 2.8B) while the native protein hydrolysed the same substrate at pH 10 optimum (Fig. 2.8C).

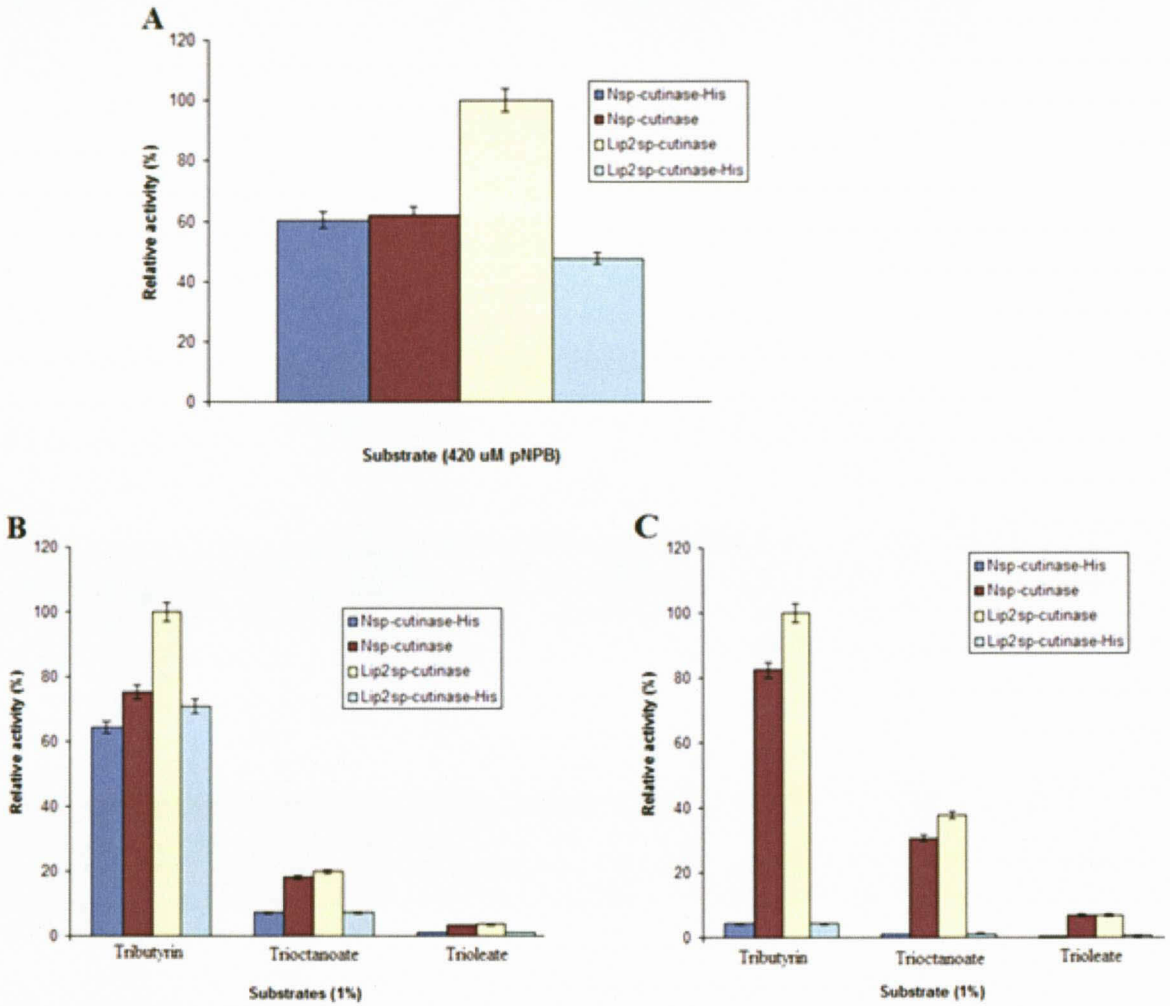

Figure 2.8: FSCut substrate specificity. (A) Hydrolysis of *p*-NPB at pH 8.0 and room temperature, (B) TAGs of different chain lengths hydrolysed at 40°C and pH 8.0, (C) TAGs of different chain lengths hydrolysed at 40°C and pH 10. Data are averages from three independent experiments.

2.5 Discussion

This chapter describes the expression of a *F. solani* cutinase in *Y. lipolytica* and its secretion into the culture medium. This dimorphic yeast has been shown to secrete a wide range of proteins into the medium in amounts sufficient for industrial application (Barth and Gaillardin, 1997; Beckerich *et al.*, 1998). *Y. lipolytica* has been used for large scale production of single cell proteins (Tsugawa *et al.*, 1969; Heslot, 1990) and expression of active heterologous proteins from different sources (Madzak *et al.*, 2004). This study employed both the *F. solani* native Cut secretion signal (Nss) and the pre-pro Lip2 secretion signal (Lip2ss) for directing the codon optimized recombinant protein to the secretory pathway. Optimization of coding sequences has been reported to be an essential tool for high level production of active heterologous proteins in different host systems (Uchijima *et al.*, 1988; Zhao and Chen, 2011; Wang *et al.*, 2011). Codon optimization has been shown by Gasmi *et al.* (2011) to increase production of hIFNα2b in *Y. lipolytica* 11 fold. The effect of His-tag on the expression levels and activity of expressed FSCut was also evaluated. The expression of FSCut did not affect the growth profile of *Y. lipolytica* (Fig. 2.3). Heterologous expression of non-tagged single copy FSCut in *Y. lipolytica* under the growth phase dependent hp4d promoter directed by the Nss resulted in about 330 mg of extracellular total protein per litre. This high level protein production can partly be attributed to a suitable secretion signal and the hp4d promoter, which facilitates protein production in any medium from the exponential to the stationary phase (Nicaud *et al.*, 2002; Gasmi *et al.*, 2011). These results are comparable to those obtained when the protein was expressed in *P. pastoris* using a mutlicopy plasmid (Kwon *et al.*, 2009; Koschorreck *et al.*, 2010). Similar expression levels were observed when FSCut was produced in *S. cerevisiae* using optimized fed-batch fermentation (Ferreira *et al.*, 2003).

Although single copy expression plasmids have been employed for production of FSCut, the high level total protein production demonstrates why *Y. lipolytica* remains an attractive host for protein production (Muller *et al.*, 1998). *Y. lipolytica* generally secretes heterologous proteins in amounts lower than it does homologous proteins. Production of most heterologous proteins in *Y. lipolytica* using shake flasks has been shown to reach 20 mg per litre (Madzak *et al.*, 2004). Lipase production in *Y. lipolytica* under the hp4d promoter harbouring a single copy has been reported to reach 1000 U/ml in shake flask and a strain harbouring multiple copies reached 2000

U/ml in shake flask (Pignède *et al.*, 2000b). The latter reached 11 500 U/ml in batch and 90 500 U/ml in fed batch (Nicaud *et al.*, 2002). FSCut production was found to be highly variable depending on the secretion signal used and whether the expressed FSCut was non-tagged or C-terminally His-tagged. Higher production of FSCut was achieved using the Nss for directing the protein to the extracellular medium (Table 2.1). The purified non-tagged FSCut directed by Nss was 2.5 fold higher than the purified FSCut directed by the pre-pro Lip2ss. Lower expression levels have beenreported by Gasmi *et al.* (2011) when the protein was directed by the pre-pro Lip2ss. It is essential to have several secretion signals since they differ widely in their potential to direct efficient secretion of heterologous proteins (Kjærulff and Jensen, 2005). In addition, optimization of the secretion signals has been reported to direct large scale production of onconase in *P. pastoris* (Zhao *et al.*, 2009).

Affinity tags greatly increase purification efficiency of the target proteins (Cunha *et al.*, 2003). The C-terminal His-tag affected the levels of FSCut expression in *Y. lipolytica*. In a previous study, secretion levels of FSCut under tryptophane-proline (WP) tags, namely $(WP)_2$ and $(WP)_4$, were reported to be lower than the levels obtained when the protein was expressed under its native secretion signal in *S. cerevisiae* (Cunha *et al.*, 2003). In this study, His-tag has been shown to affect levels of FSCut expression in *Y. lipolytica*. Fusion of His-tag to FSCut does not add any value in this instance, since diafiltration method using ultrafiltration membranes for purification can recover high purity FSCut in *Y. lipolytica*. Diafiltration was also shown to be sufficient in purifying FSCut expressed in *P. pastoris* (Kwon *et al.*, 2009). Therefore, His-tag should not be used in the case of FSCut because of its effect on the expression levels of the enzyme.

Both the secretion signals directed secretion of active recombinant tagged and non-tagged FSCut. However, the specific activity of His-tagged and non-tagged FSCut directed by the Nss were lower than that of the non-tagged FSCut directed by the pre-pro Lip2ss. Although native secretion signals are capable of directing expression of proteins in foreign hosts in sufficient amounts (Jolivalt *et al.*, 2005), they also have been reported to produce less active proteins. Studies by Madzak *et al.* (2005) reported that the native laccase secretion signal directed the secretion of a less efficient recombinant laccase compared to the *Y. lipolytica* laccase directed by either the *XPR2* pre or pre-pro. The specific activity of the purified non-tagged FSCut directed by the Nss was similar to that of the C-terminally His-tagged FSCut directed

by both the homologous and heterologous secretion signals. The similarity of the specific activity values of the purified non-tagged FSCut and the C-terminally His-tagged FSCut under the same assaying conditions have been reported by Kwon *et al.* (2009) using the N-terminal α-factor secretion signal under the control of the methanol-inducible *AOX1* promoter. Biochemical properties of the recombinant FSCut expressed in *Y. lipolytica* showed activity towards fatty acid esters and TAGs, with a high preference for short chain substrates. The preference of FSCut for short chain substrates is well documented (Purdy and Kolattukudy, 1973; Liu *et al.*, 2011). The C-terminally fused His-tag was found to lower the activity of the enzyme at its pH optimum. Hydrolysis of the substrates by His-tagged FSCut was observed to be higher at pH 8.0 compared to the pH 10 optimum of the non-tagged enzyme.

In conclusion, in this study, we have shown that *Y. lipolytica* can be used for production of FSCut using a single copy expression plasmid. It was also demonstrated that secretion signals play an important role in both secretion and activity of expressed FSCut. The high level expression and subsequent easy purification process make *Y. lipolytica* an efficient and cost effective host for large scale production of FSCut. Fermentation processes under both batch and fed-batch processes have been shown to increase product yields by several orders of magnitude in *Y. lipolytica*. It will be interesting to express FSCut in this yeast under such conditions in order to see if product yield would increase.

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Chapter 3. Cloning, expression from a single copy, and characterization of *Thermomyces lanuginosus* lipase in *Yarrowia lipolytica*

3.1 Abstract

Yarrowia lipolytica was evaluated as an expression host for production of *Thermomyces lanuginosus* lipase (TLL). The TLL encoding gene sequence was synthesised and codon optimised for expression in *Y. lipolytica*. The yeast pre-pro Lip2 and TLL native secretion signals (Nss) were used to direct the secretion of C-terminally histidine-tagged (His-tagged) and non-tagged TLL to the extracellular medium. Both secretion signals directed expression of functionally active TLL. The purified non-tagged TLL directed by native and yeast secretion signals for secretion showed similar specific activity and 1.6-fold higher activity that the His-tagged TLL directed by both signals. The His-tag affected TLL expression levels, its activity and thermostability. SDS-PAGE showed major and minor bands for the TLL directed by the Nss with molecular weights of 36 and 34 kDa, respectively. For TLL directed by the pre-pro Lip2ss a single band was observed on the SDS-PAGE with a molecular weight of 36 kDa. These differences in the protein band patterns were as a result of *N*-glycosylation on expressed TLL directed by the Nss. Characterization of all purified proteins expressed using different approaches showed the highest hydrolytic activity at 60°C and pH 9.0. TLL showed preference for medium chain fatty acids. The activities of all recombinant TLL were inhibited by triton-X100 and tween-80.

3.2 Introduction

Lipases (triacylgycerol acylhydrolases, EC 3.1.1.3) are ubiquitous carboxylesterases that catalyse the hydrolysis and synthesis of esters from triglycerides at the lipid-water interface (Schmid and Verger, 1998; Jaeger and Reetz, 1998). Lipases have a wide range of industrial applications such as wastewater treatment, hydrolysis and synthesis of various esters, formulation of detergents and production of biodiesel to name a few (Svendsen *et al.,* 1997; Schmid and Verger, 1998; Dharmssthiti and Kuhasuntisuk, 1999; Pandey *et al.,* 1999; Modi *et al.,* 2006). In the quest to develop a green chemical industry, stability and activity of lipases has limited their large scale application (Shu *et al.,* 2010). Among lipases from different sources, microbial lipases are of great interest due to their diverse catalytic activity, low cost associated with high production levels and relative ease of genetic manipulation. In addition, microbial lipases have broad substrate specificity, are not dependent on co-factors and are stable in organic solvents (Jaeger and Reetz, 1998). Lipases possess a common feature, the α/β-hydrolase fold, and as a result, they form part of the superfamily of serine hydrolases (Ollis *et al.,* 1992). The α/β-hydrolase fold usually consists of a small α-helix or loop, which is responsible for covering the active site pocket forming the closed conformation (Brady *et al.,* 1990). The open conformation is formed when the lipase is adsorbed to an interface allowing displacement of the α-helix, thus making the active site easily accessible to the substrate (Brzozowski *et al.,* 1991). The active site of lipases, similarly to that found in serine proteases, is composed of a catalytic triad of Ser, Asp and His (Brady *et al.,* 1990).

Thermomyces lanuginosus is a thermophilic fungus with the ability to grow at high temperatures of 50-60°C (Cooney and Emerson, 1964). The *T. lanuginosus* lipase (TLL) is amongst the most studied lipolytic enzymes (Adams and Deploey, 1978; Mogensen *et al.,* 2005; Gupta *et al.,* 2009). TLL contains three disulfide bonds and four tryptophan residues, of which Trp89 is located on the α-helix and has been found to be essential for efficient hydrolysis (Lawson *et al.,* 1994; Holmquist *et al.,* 1995). The 15 amino acids α-helix domain of TLL has been reported to show interfacial activation at the substrate-water interface during hydrolysis of its substrates due to the large conformation change of the lid (Lawson *et al.,* 1994; Derewenda *et al.,* 1994; Martinelle *et al.,* 1995; Salis *et al.,* 2003). TLL, similarly to *Talaromyces thermophilus* lipase, has shown the capability of hydrolysing a wide range of substrates with high affinity for medium chain (C_{10}-C_{12}) as compared to short

(C_4) and long chain (C_{18}) substrates (Mogensen *et al.,* 2005; Romdhane *et al.,* 2012). Immobilisation of TLL on different matrices has been extensively investigated for hydrolysis of different substrates (Aloulou *et al.,* 2007; Gupta *et al.,* 2009; Sorensen *et al.,* 2010). Although *T. lanuginosus* is ideal for production of thermostable lipases, its low level expression of thermophilic lipases makes it unfavourable for large scale industrial production (Zheng *et al.,* 2011). With the advent of genetic engineering technology, TLL has been expressed in *Aspergillus niger* (Prathumpai *et al.,* 2004), *Pichia pastoris* (Zheng *et al.,* 2011) and *Aspergillus oryzae* (Novozymes, personal communication). In Novozymes A/S Co., the recombinant lipase is produced as a fusion (hybrid) lipase between TLL and *Fusarium oxysporum* lipase (FOL) using the *A. oryzae* expression system. The hybrid lipase constitutes 1-284 amino acid residues from the N-terminal mature region of TLL and 285-339 amino acid residues from the N-terminal mature region of FOL. The fusion hybrid is mutated at position 113 from glycin to alanine, position 118 from aspartic acid to tryptophan and position 121 from glutamic acid to lysine (Novozymes, personal communication).

Yarrowia lipolytica has been identified is one of the most attractive yeasts for heterologous protein production (Muller *et al.,* 1998) and is used as a host for production of recombinant proteins from a wide range of sources (Madzak *et al.,* 2004). To the best of our knowledge there are no reports describing expression of TLL in *Y. lipolytica.* In this study we evaluate whether *Y. lipolytica* can be used as an expression system for TTL. Recombinant TLL was expressed in *Y. lipolytica* using both the native TLL secretion signal (Nss) and the pre-pro Lip2 secretion signal (Lip2ss) for directing the secretion of TLL to the growth medium. Furthermore, biochemical properties such as thermostability, effects of detergents, metal ion and solvent solution on the expressed TLL were evaluated.

3.3 Material and methods

3.3.1 Strains, plasmids and reagents

Escherichia coli XL10 Gold (Tet^r *D (mrcA) 183D (mrcCB-hsdSMR-mrr) 173endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacI^{q}ZDM15 Tn10* (Tet^r) Amy Cam^r]) was used as a host for plasmid construction and propagation of TLL encoding gene. Cells were grown in Luria-Bertani (LB) broth or on agar plates supplemented with 50 µg/ml ampicillin (Amp) (Sambrook *et al.,* 1989). *Y. lipolytica* Po1g strain (*MatA, leu2-270, ura3-302::URA3, xpr2-322, axp1-2*) was used as a host for single copy extracellular production of TLL (Madzak *et al.,* 2004). The single copy pBR322 docking platform plasmid pKOV323 was used for production of both His-tagged and non-tagged TLL using both the pre-pro Lip2ss and the TLL Nss. PCR products were cloned into pGEM®-T Easy Vector (Promega, USA). Recombinant plasmid DNA was isolated from *E. coli* XL10 Gold cells either using the method of Berghammer and Auer (1993) for initial screening purposes or the Plasmid Isolation Kit (BioFlux) following the manufacturer's instructions. *Y. lipolytica* Po1g transformants were selected on YNB-N_{5000} and grown in YPD. Chromogenic agar substrate plates used for selection of *Y. lipolytica* transformants expressing TLL were prepared as described by Singh *et al.* (2006).

3.3.2 Construction of *Y. lipolytica* expression vectors

All standard DNA manipulation procedures were performed according to Sambrook *et al.* (1989). The entire *tll* gene encoding the open reading frame (ORF) including the native secretion signal (Nss) of TLL (Gen Bank Accession No. AF054513.1) was codon optimized for expression in *Y. lipolytica* (GeneArt, Regensbung, Germany). The *tll* gene was synthesised with its Nss (894 bp) at the N-terminal end flanked by a *Hind*III restriction site at the 5'. A His-tag was added at the C-terminus upstream of the stop codon (TAA), followed by an *Avr*II restriction site. The *tll* gene was released with *Hind*III and *Avr*II and ligated into the single copy pKOV323 expression vector digested with the same enzymes to generate pKOV323-Nss-*tll*-His (Fig. 3.1A).

To construct an expression vector that retained the *tll* Nss and no His-tag, the primers pKOV-F (5'-GTTTGCCAGCCACAGA-3') flanking the 3'-end of the hp4d

promoter, and Fs_p-R (5'-TTTCCTAGGTTAGTGGTGATGGTGGTGG TG-3'; *Avr*II site is underlined), which introduced a TAA stop codon, were used to amplify the gene from pKOV323-Nss-*tll*-His. Thermocycling reactions were carried out using MJ Mini Personal Thermal Cycler (BIO-RAD). PCR amplifications were performed using Taq polymerase (Fermentas). A PCR reaction mixture was prepared containing 1/10 volume reaction buffer with magnesium chloride, 10 mM dNTPs, 1.0 µM each of primer, 0.625 U Taq polymerase, 5 µg of DNA and topped up to a total volume of 50 µl with distilled water. The thermal cycling conditions included an initial denaturation at 98°C for 30 sec, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 20 sec and extension at 72°C for 1 min, with a final extension step of 75°C for 5 min and held at 4°C. Following thermal cycling and agarose gel electrophoresis, PCR products were gel purified using a Gel Extraction Kit (BioFlux) according to the manufacturer's instructions. Purified products were subcloned into p-GEM-T® Easy vector and the insert (876 bp) released with *Hind*III and *Avr*II and ligated into pKOV323 expression vector to generate pKOV323-Nss-*tll* (Fig. 3.1B).

To construct a *tll* gene under the pre-pro Lip2ss, the Nss was removed from the *tll* gene using plasmids pKOV323-Nss-*tll*-His and pKOV323-Nss-*tll* as templates for amplification of the *tll* gene with and without a His-tag using the same PCR conditions as described above. The primer pair Fs_p-F1 (5'-AAAGGATCCATGCTGGGCCGAACCACCCGA-3'; *BamH*I site is underlined) and pKOV-R (5'-CGATATTCATTTATTAAAGTA-3') flanking the Lip2 terminator of the expression vector, were used to generate His-tagged amplicons (843 bp) from pKOV323-Nss-*tll*-His, whereas pKOV323-Nss-*tll* was used to generate non-tagged amplicons (825 bp). Amplicons were gel purified, subcloned into p GEM-T® Easy vector and the inserts released with *BamH*I and *Avr*II and ligated in-frame downstream of the pre-pro Lip2ss of the pKOV323 expression vector linearized with the same enzymes to generate pKOV323-Lip2ss-*tll*-His and pKOV323-Lip2ss-*tll* (Fig. 3.1C and D). The recombinant plasmids were used to transform *E. coli* XL 10 Gold cells grown on LB-Amp (50 µg/ml) agar plates and screened in 5 ml LB medium containing 50 µg/ml ampicillin.

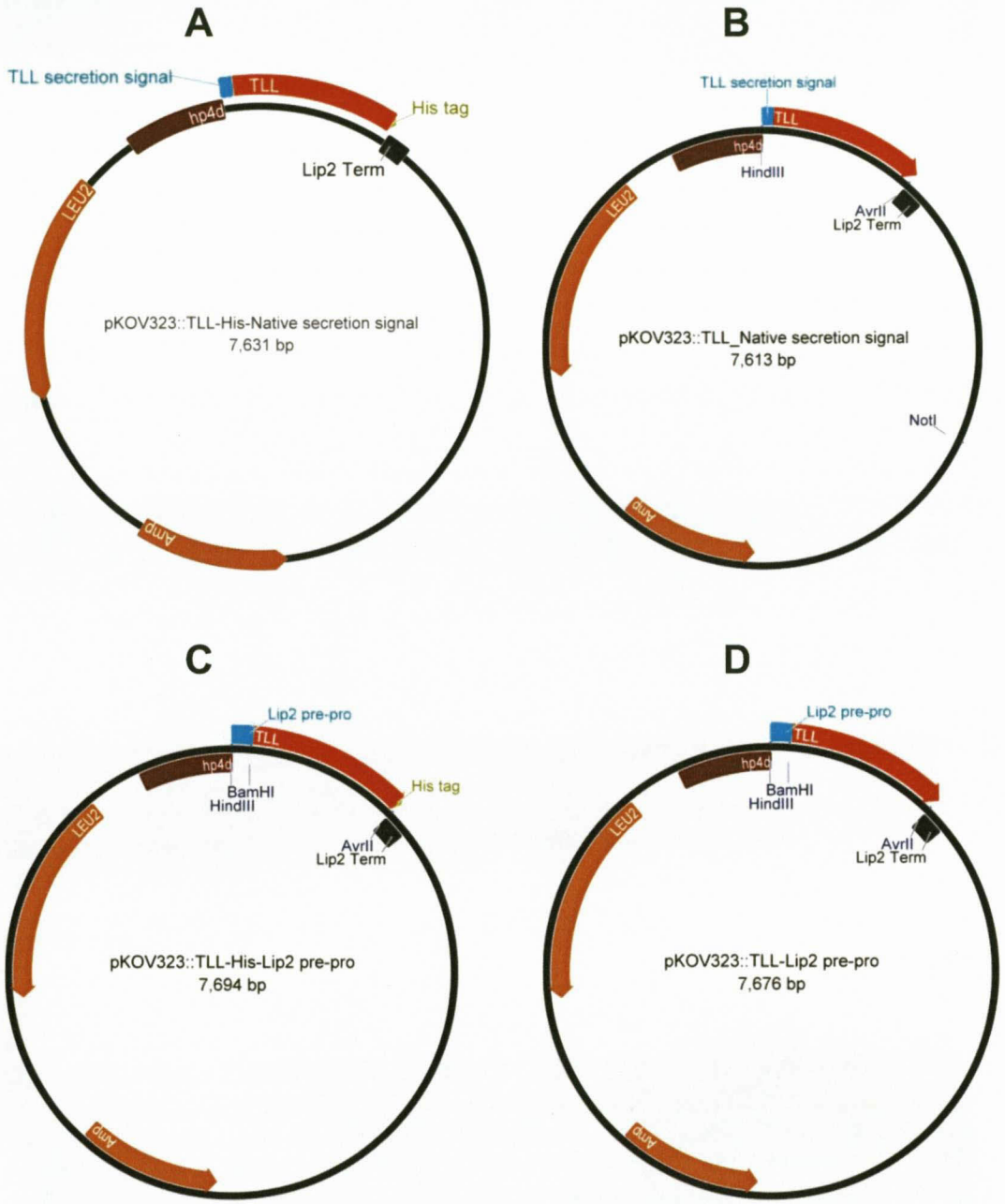

Figure 3.1: Schematic diagrams of *tll* gene in the expression plasmid pKOV323. The *tll* gene is under the Nss (A and B) or pre-pro Lip2ss (C and D). Diagrams A and C carry the His-tagged *tll* gene, whereas B and D carry the non-His tagged *tll* gene. The plasmid map was constructed using Geneious v5.5 (Drummond et al. 2011).

3.3.3 Yeast transformation

Prior to yeast transformation, recombinant vectors pKOV323-Lip2ss, pKOV323-Nss-*tll*-His, pKOV323-Nss-*tll*, pKOV323-Lip2ss-*tll*-His and pKOV323-Lip2ss-*tll* were linearized with *Not*I restriction enzyme in order to direct their integration at the pBR322 docking platform of *Y. lipolytica* Po1g strain. Preparation of Po1g competent cells and transformation was performed using the lithium acetate method as described by Xuan *et al.* (1988). For selection of Leucine$^+$ (Leu$^+$) clones, Po1g transformants were grown on selective YNB-N_{5000} media plates (0.17% YNB without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 1% glucose and 1.5% agar). Colonies were isolated from YNB-N_{5000} plates after 2 days of incubation at 28°C. Genomic DNA was extracted using the method of Chen *et al.* (1997) and used as a template for confirming the integration of the recombinant vectors into the yeast genome using primer pair pKOV410-F (5'-GTTTGCCAGCCACAGA-3') and pKOV410-R (5'-CGATATTCATTTATTAAAGTA-3'). The PCR positive transformants were inoculated on tributyrin chromogenic plates [(1.0% trybutyrin sonically emulsified in 10 mM $CaCl_2$, 0.01% phenolphthalein, 2.0% agar and the pH adjusted to 7.3/7.4 using 0.1 N NaOH)] as described by Singh *et al.* (2006) and incubated for 24 h at 28°C. Preliminary studies on selection of transformants was done using chromogenic plates for screening several transformants and only the one forming the biggest halo on plates was selected for further investigation. TLL activity was estimated according to halo size around the colonies. The *Y. lipolytica* Po1g transformants carrying single-copy expression cassettes of pKOV323-Nss-*tll*-His, pKOV323-Nss-*tll*, pKOV323-Lip2ss-*tll*-His, pKOV323-Lip2ss-*tll* and the control pKOV323-Lip2ss were denoted YI323N-TLLH, YI323N-TLL, YI323L-TLLH, YI323L-TLL and YI-323L respectively.

3.3.4 Expression and purification of TLL in *Y. lipolytica*

Yeast transformants (YI323N-TLLH, YI323N-TLL, YI323L-TLLH, YI323L-TLL and YI-323L) were inoculated into 25 ml of YPD medium and incubated overnight at 28°C with shaking at 200 rpm. When the culture reached an optical density at 600 nm (OD_{600}) of 3-6, the cells were re-suspended to an OD_{600} of 1.0 in 100 ml YPD and incubated at 28°C with shaking at 200 rpm. The cultures were grown in shake flasks under aerobic conditions. The OD_{600} was recorded and supernatant samples collected at 24 hour intervals over the cultivation period of 6 days. The recorded OD

was used to assess whether expression of TLL affected the growth patterns of *Y. lipolytica* Po1g strain.

After six days, the supernatant was collected by centrifugation and used for purification of TLL. Supernatants were diafiltered with 20 mM Tris-HCl (pH 8.0) using an Amicon stirred ultrafiltration cell (Millipore, Billeria, MA, USA) with a Millipore ultrafiltration membrane of 50 kDa molecular weight limit (Separations, South Africa). The samples were further diafiltrated and concentrated using an ultrafiltration membrane of 30 kDa cut-off molecular weight limit.

3.3.5 Protein analysis

Protein concentrations of *Y. lipolytica* culture supernatant and the purified enzyme preparation were determined by the Bradford assay (1976) using the BIO-RAD protein dye reagent according to the manufacture's manual. Absorbance was measured at 595 nm employing Power Wave HT (BioTek). Bovine serum albumin (Roche, Mannheim, Germany) was used as a standard. To check the purity of the proteins, the purified protein samples were analysed on a 12% SDS-PAGE gel as described by Laemmli (1970).

Double protein bands on SDS gel were excised and cut into small chips separately. The sample was treated as described by Webster and Oxley (2005) and digested overnight with porcine trypsin (Promega, Madison, USA). MALDI-TOF-MS was performed using a QSTAR® Elite mass spectrometer (Applied Biosystems Inc., Ontario, Canada). The generated PMF data was 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).

3.3.6 TLL activity

TLL activity was measured using a pH titration method (Benzonana and Desnuelle, 1968; Pinsirodom and Parkin, 2001) with tributyrin as substrates. Tributyrin (1%) was emulsified through sonication (HD 2070 Bandelin, Sonopuls) in a solution containing

20 mM $CaCl_2$, 0.6 M NaCl, 1 mM sodium deoxycholate and 2.5% (w/v) gum Arabic. TLL (100 µg) was added, with liberated fatty acids from the substrate titrated with 0.05 N NaOH to maintain a constant pH of 7.3-7.4 at 40°C. One unit (U) of TLL activity was defined as the amount of enzyme releasing 1 µmol of fatty acid per minute under assay conditions. All assays were averaged from three independent experiments.

3.3.7 Enzymatic characterization of TLL

The effect of pH and temperature on TLL activity expressed in *Y. lipolytica* was determined by performing titrimetric assays measuring tributyrin hydrolysis by TLL at different pH and temperatures. TLL activity was measured at temperatures ranging from 20-80°C in 50 mM Tris-HCl buffer at both pH 8.0 and 9.0. The pH dependence of TLL was determined from 4 to 11 using Tris-HCl, pH 9.0 at 60°C. The assay mixture contained 1% of tributyrin and the assays were done at 60°C and pH 9.0. All assays were averaged from three independent experiments using 100 µg of supernatant and purified non-tagged and C-terminally His-tagged TLL from the four transformants. For temperature stability, the purified TLL from the four strains were incubated at 60°C for up to 3 h in 50 mM Tris-HCl buffer, pH 9.0 and the residual activity measured at 1 h time intervals. Detergents and solvent solution were added to the substrate solution respectively and their effect on purified TLL was determined.

3.3.8 Substrate specificity

Substrate specificity of TLL was evaluated using 1% of tributyrin, glyceryl trioleate and glyceryl trioctanoate as substrates employing a pH titration method (Benzonana and Desnuelle, 1968; Pinsirodom and Parkin, 2001). Each of the substrates was emulsified through sonication (HD 2070 Bandelin, Sonopuls) in a solution containing 20 mM $CaCl_2$, 0.6 M NaCl, 1 mM sodium deoxycholate and 2.5% (w/v) gum Arabic. Enzyme solution (100 µg) was added, with liberated fatty acids from the TAG substrates titrated with 0.05 N NaOH to maintain a constant pH of 7.3-7.4 at 40°C. One unit (U) of TLL activity was defined as the amount of enzyme releasing 1 µmol of fatty acid per minute under assay conditions. All assays were averaged from three independent experiments with equal amounts of purified non-tagged and C-terminally His-tagged TLL.

3.4 Results

3.4.1 Construction of single-copy vectors for expression of TLL

The *tll* encoding gene was cloned into pKOV323 in-frame with the Nss and the Lip2ss pre-pro region (Pignède *et al.,* 2000a, b) under control of the hp4d promoter (Madzak *et al.,* 2000). All expression vectors harbouring the *tll* gene carried a growth-phase-dependent hybrid promoter hp4d and the Lip2 terminator (Barth and Gaillardin, 1996). TLL producing strains were obtained by integrative transformation of *Y. lipolytica* Po1g with four different *tll* expression cassettes. The resulting transformants were confirmed to be positive by screening several transformants on chromogenic agar plates and PCR using the using primer pair pKOV410-F (5'-GTTTGCCAGCCACAGA-3') and pKOV410-R (5'-CGATATTCATTTATTAAAGTA-3') (data not shown) suggesting integration of the recombinant vectors into the yeast pBR322 docking platform. *Y. lipolytica* transformants positively confirmed by PCR to harbour *tll* gene showed increased hydrolytic activity towards tributyrin on chromogenic activity plates compared to the controls cells YI-323L. Yeast transformants expressing TLL produced larger halos on agar activity plates after 24 hours of incubation than the control strain (Fig. 3.2). Strains employing homologous and heterologous secretion signals for secretion of non-tagged TLL exhibited higher-levels of lipolytic activity on the agar activity plates containing emulsified tributyrin compared to their respective strains expressing C-terminally His-tagged TLL.

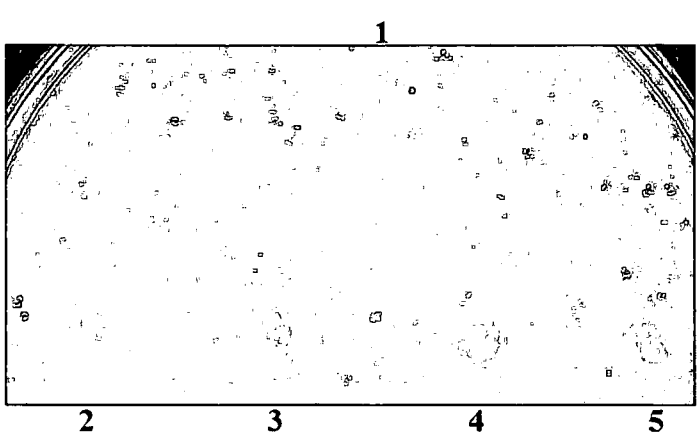

Figure 3.2: *Y. lipolytica* single copy transformants grown on chromogenic activity plates. The strains are grown on 2% agar plates containing emulsified 1% tributyrin and 0.01% phenolphthalein. *Y. lipolytica* cells: (1) YI-323L, (2) YI323N-TLL, (3) YI323N-TLL, (4) YI323L-TLLH and (5) YI323N-TLLH.

3.4.2 Heterologous expression of TLL in *Y. lipolytica*

The effect of TLL expression with and without a His-tag directed by the Nss and the pre-pro Lip2ss for secretion on the growth of *Y. lipolytica* Po1g strain was assessed by growing the strains expressing TLL and control cells in liquid cultures under aerobic conditions on a rotary shaker (200 rpm, 28°C). Growth profiles of the strains were measured at 24-hour intervals by monitoring OD of the cultures at 600 nm for 6 days. During the entire period of culture cultivation there were no differences in the growth profiles of *Y. lipolytica* cultures harbouring a single copy gene of TLL secreting His-tagged or non-tagged TLL directed by either the Nss or the pre-pro Lip2ss (Fig. 3.3).

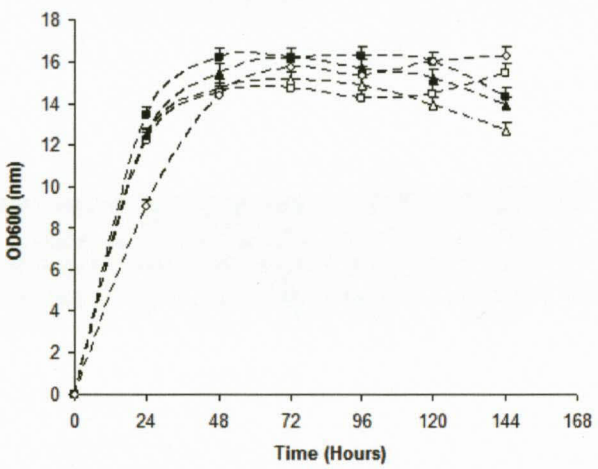

Figure 3.3: Comparison of the growth profiles of *Y. lipolytica* expressing TLL. Strains were grown at 28°C in shake flasks (200 rpm) and growth was assessed by measuring absorbance at OD_{600} for a period of 144 hours at 24-hour intervals. *Y. lipolytica* cells: YI323N-TLLH (Δ); YI323N-TLL (▲); YI323L-TLLH (□); YI323L-TLL (■) and YI-323L (◊). All measurements were done in duplicates.

Y. lipolytica Po1g expressing non-tagged TLL directed by the pre-pro Lip2ss or the Nss for secretion produced similar amounts of extracellular total protein. The Nss and the pre-pro Lip2ss resulted in 20.2 and 19 mg of protein per 100 ml of culture supernatant, respectively (Fig. 3.4). In contrast, the total protein produced by the strains expressing His-tagged TLL directed by the Nss and the pre-pro Lip2ss were 9.4 and 11.4 mg per 100 ml of culture supernatant, respectively (Fig. 3.4). The total protein produced by both strains expressing non-tagged TLL were about 2 fold higher than the total protein produced by the respective strains expressing C-terminally tagged TLL directed by the Nss and the pre-pro Lip2ss.

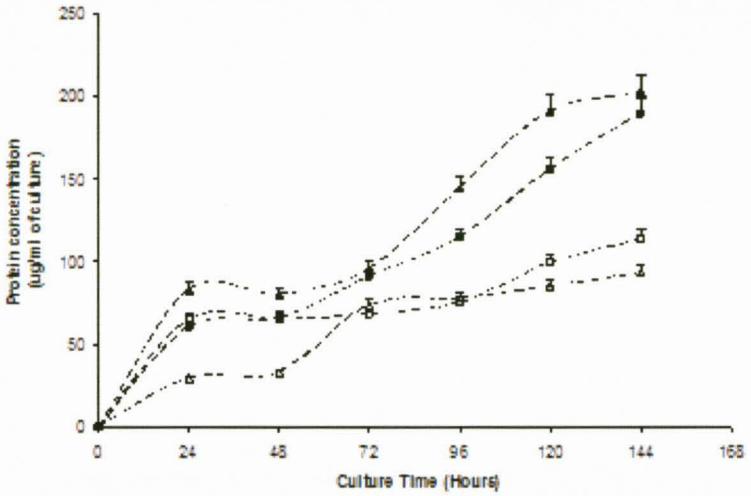

Figure 3.4: Comparison of total extracellular protein in different strains of *Y. lipolytica*. Total proteins were quantified at 24-hour intervals for a 144 hour period. *Y. lipolytica* cells: YI323N-TLLH (Δ); YI323N-TLL (▲); YI323L-TLLH (□) and YI323L-TLL (■). All measurements were done in duplicates.

TLL activity from total culture supernatant was similar in the *Y. lipolytica* cultures expressing non-tagged TLL directed by the pre-pro Lip2ss (216 U/ml) and the Nss (211 U/ml) for secretion, assayed using 1% tributyrin at the end of the culturing period (Fig. 3.5). C-terminally His-tagged TLL directed by the pre-pro Lip2ss (73 U/ml) and the Nss (63 U/ml) for secretion were about 1.7-fold lower than the non-tagged TLL directed by both secretion signals.

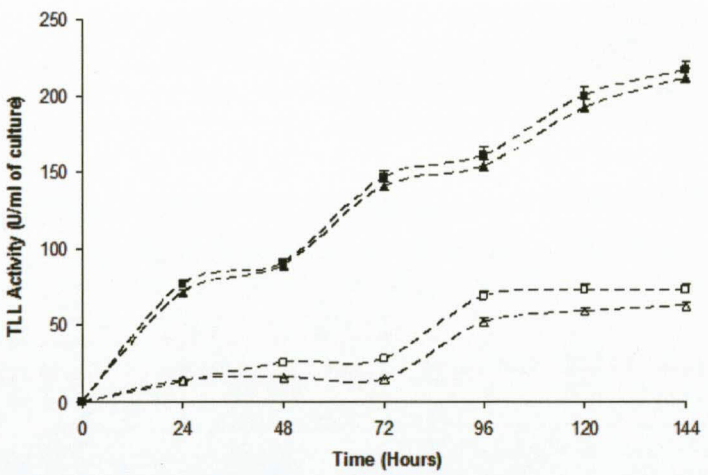

Figure 3.5: Comparison of TLL activity in the culture supernantant. Lipolytic activity of TLL was quantified at 24-hour intervals for a 144 hour period from the supernatant of *Y. lipolytica* cells: YI323N-TLLH (Δ); YI323N-TLL (▲); YI323L-TLL3H (□) and YI323L-TLL (■). Data are averages from three independent experiments.

SDS-PAGE analysis of the 24-hour interval samples revealed low levels of the His-tagged TLL directed by both signals (Fig. 3.6B, D). The non-tagged TLL directed by the Nss showed two bands (Fig. 3.6A), whereas a single band was observed for the non-tagged TLL directed by the pre-pro Lip2ss (Fig. 3.6C).

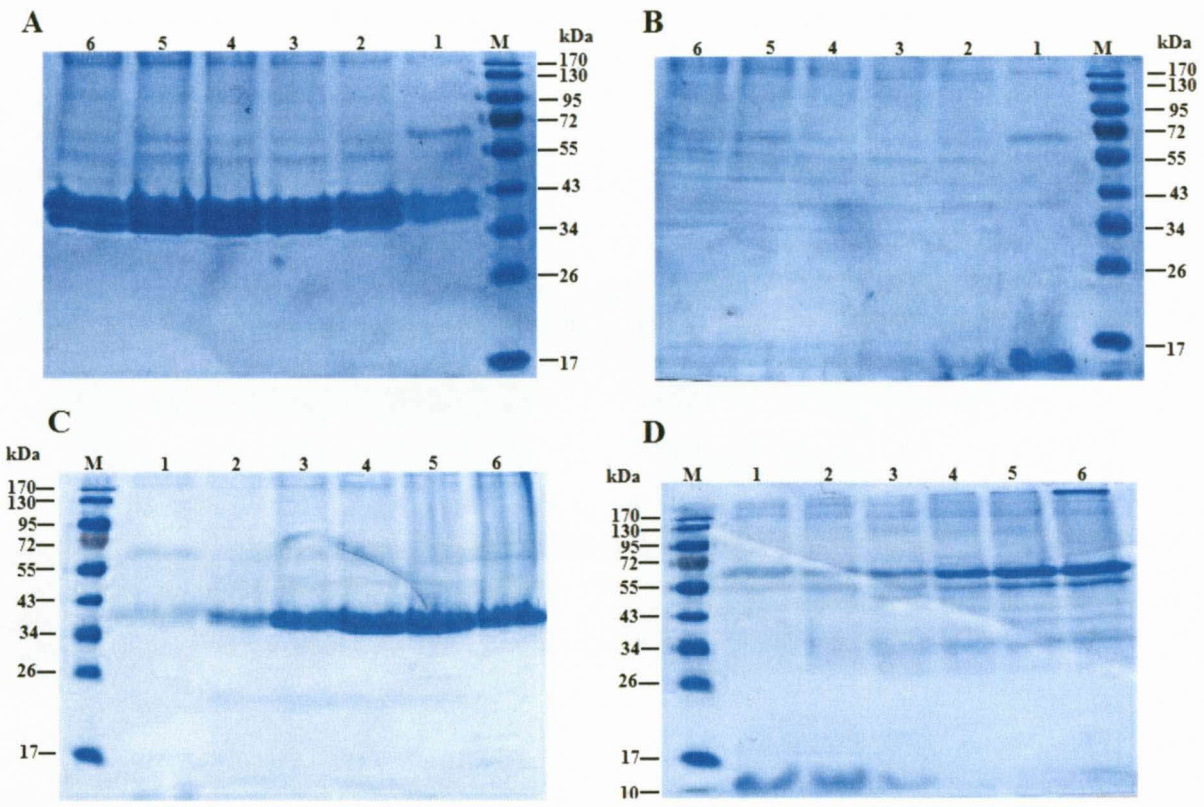

Figure 3.6: SDS-PAGE analysis of *Y. lipolytica* Po1g expressing tagged and non-tagged TLL directed by homologous and heterologous secretion signals. The total protein culture supernatant samples were collected at 24 hour interval for 6 days and electrophoresed on 12% SDS-PAGE gels: (A) non-tagged TLL directed by the Nss for secretion, (B) C-terminally tagged TLL directed by the Nss for secretion, (C) non-tagged TLL directed by the pre-pro Lip2ss for secretion, and (D) C-terminally tagged TLL directed by the pre-pro Lip2ss for secretion. Lane M is a PageRuler stained protein ladder (Fermentas). The numbers depicted on top of the gel images, labelled 1-7 represent the cultivation period in days.

3.4.3 Purification of TLL

After 6 days of expression, TLL was purified from 100 ml culture medium by ultrafiltration (Table 3.1). C-terminally His-tagged and non-tagged TLL were purified

using Amicon ultrafiltration membranes. The non-tagged TLL showed higher levels of expression than the His-tagged protein. The non-tagged purified TLL directed by the Nss was 1.3-fold higher than the purified non-tagged TLL directed by the pre-pro Lip2ss. In addition, the purified non-tagged TLL directed by the Nss was 7- and 5-fold higher than the purified C-terminally His-tagged TLL directed by the Nss and the pre-pro Lip2ss, respectively. The amount of the purified non-tagged TLL directed by the pre-pro Lip2ss was 5.6- and 4-fold higher than those of the purified C-terminally His-tagged TLL directed by the Nss the pre-pro Lip2ss, respectively.

Table 3.1 Purification of single copy TLL expressed in *Y. lipolytica*

Enzyme	Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification Fold
TLL (Nss)	Culture supernatant	23371.4	20.2	1157	100	1
	Purified	12885.6	8.4	1534	55.1	1.3
TLL-His (Nss)	Culture supernatant	3666	9.4	390	100	1
	Purified	1159.2	1.2	966	31.6	2.5
TLL (Lip2ss)	Culture supernatant	20007	19	1053	100	1
	Purified	10742.1	6.7	1603.3	53.7	1.5
TLL-His (Lip2ss)	Culture supernatant	3408.6	11.4	299	100	1
	Purified	1700.5	1.7	1000.3	49.9	3.4

100 µg of supernatant and purified TLL was used to measure the activity of the enzyme at pH 9.0

Similarly to the crude protein, two different profiles of TLL were observed when purified TLL was resolved on SDS-PAGE gel. Only a single protein band was observed from both tagged and non-tagged TLL directed by pre-pro Lip2ss (36 kDa), whereas two bands of approximately 36 and 34 kDa were observed from both tagged and non-tagged TLL by Nss (Fig. 3.7). To ascertain whether the double bands in lanes 3 and 4 on the SDS-PAGE gel were TLL, both bands in each lane were excised from the gel and subjected to MALDI-TOF-MS peptide mass fingerprinting. The generated data were analysed using Mascot BLAST and NCBI. BLAST searches of the identified peptide mass fingerprints from both bands gave 95% match to *Thermomyces lanuginosus* lipase (Gen Bank Accession number: CAD38229).

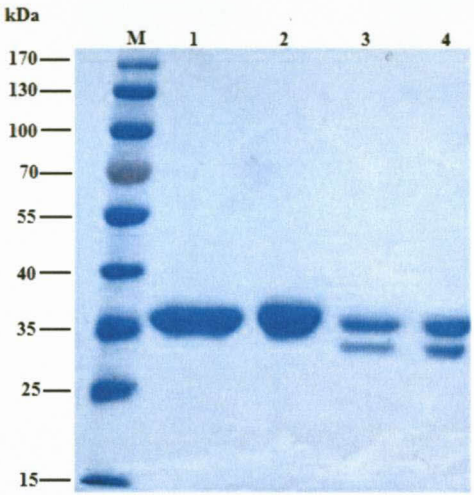

Figure 3.7: SDS-PAGE analysis of purified TLL expressed in *Y. lipolytica* Po1g strain. The purified TLL was analysed on 12% SDS-PAGE gel: Lane 1: non-tagged TLL directed by the pre-pro Lip2ss, Lane 2: His-tagged TLL directed by the pre-pro Lip2ss, Lane 3: non-tagged TLL directed by the Nss Lane 4: His-tagged TLL directed by Nss secretion. Lane M: PageRuler pre-stained protein ladder.

The protein sequences of TLL directed by both the homologous and heterologous secretion signals were analysed with *N*-glycosylation software (NetNGlyc 1.0, Technical University of Denmark) to predict potential *N*-glycosylation sites. The TLL protein sequence under the Nss showed a potential *N*-glycosylation site at the asparagine located at position 55 (Fig. 3.8). No potential *N*-glycosylation sites were shown when TLL was under the pre-pro Lip2ss. In addition, there were no *O*-glycosylation sites predicted for TLL protein sequences under Nss or the pre-pro Lip2ss.

MRSSLVLFFVSAWTALASPIRREVSQDLFNQFNLFAQYSAAAYCGKNNDAPAGT**NIT**CTGNACPEVEK
ADATFLYSFEDSGVGDVTGFLALDNTNKLIVLSFRGSRSIENWIGNLNFDLKEINDICSGCRGHDGFTSS
WRSVADTLRQKVEDAVREHPDYRVVFTGHSLGGALATVAGADLRGNGYDIDVFSYGAPRVGNRAFAE
FLTVQTGGTLYRITHTNDIVPRLPPREFGYSHSSPEYWIKSGTLVPVTRNDIVKIEGIDATGGNNQPNIPDI
PAHLWYFGLIGTCL

Figure 3.8: Predicted potential *N*-glycosylation site on TLL protein sequence. Asn-Xaa-Ser/Thr sequons in the sequence are highlighted in blue, whereas the Asparagine predicted to be *N*-glycosylated is highlighted in red. The bold letters show the TLL Nss and rest is the TLL mature sequence.

3.4.4 Biochemical characterization of TLL

The activity of purified TLL was determined at pH 9.0 using tributyrin as a substrate. The purified non-tagged TLL secreted using the Nss showed similar specific activity value to that of the purified TLL secreted using the pre-pro Lip2ss. Purified non-tagged TLL secreted using the pre-pro Lip2ss and Nss showed higher specific activity value (~1.6-fold) than the purified His-tagged TLL directed by both the Nss and the pre-pro Lip2ss. The effects of pH (Fig. 3.9A) and temperature (Fig. 3.9B) on the activity of all purified TLL was determined over a pH range of 4-11 and temperature range of 20-80°C. The effect of pH on the purified TLL differed for both the tagged and non-tagged proteins. Both purified His-tagged and non-tagged proteins showed activity at a pH range from 8-10. The His-tagged proteins showed optimum activity at pH 8.0 and retained its activity at pH 9.0, whereas the Non-tagged TLL showed optimal activity at pH 9.0. The His-tagged and non-tagged TLL retained good activity at pH 10.0, indicating that TLL functions at alkaline pH. At pH 11.0 a drastic decrease in the activity of TLL was observed, this points out that TLL functions efficiently at a pH of between 8 to 10. Both tagged and non-tagged TLL showed higher activity at temperatures ranging from 50-60°C. The non-tagged TLL retained about 60% of its original activity at 70°C with the activity of the His-tagged decreasing drastically at the same temperature retaining about 20% of its original activity. Both the tagged and non-tagged TLL showed optimal activity at 60°C (Fig. 3.9B).

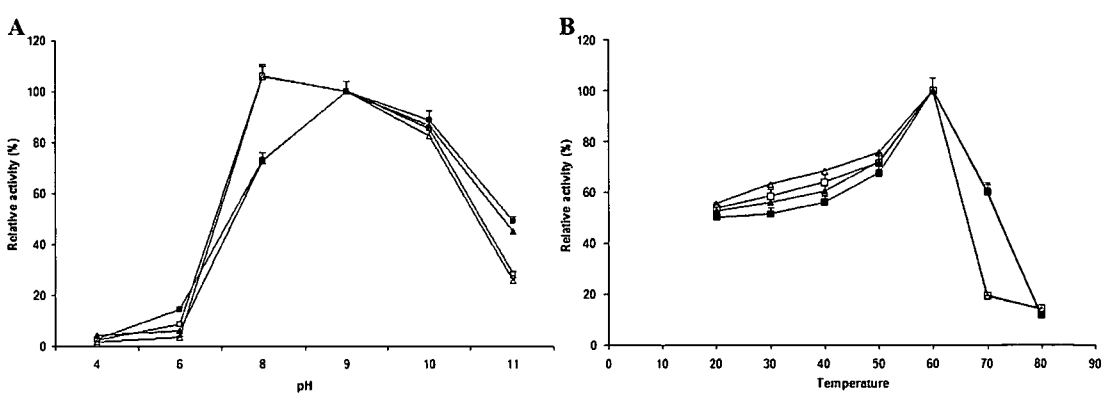

Figure 3.9: Effect of pH (A), temperature (B) on purified non-tagged and His-tagged TLL. YI323N-TLLH (Δ); YI323N-TLL (▲); YI323L-TLLH (□) and YI323L-TLL (■). Data are averages from three independent experiments.

Thermostability of both tagged and non-tagged purified TLL was evaluated after incubating the enzyme solution at 60°C for 1-3 h (Fig. 3.10). The non-tagged TLL directed by the Nss showed thermal stability of 73% after 1 h and 71% after both 2 and 3 h of its original activity. Non-tagged TLL directed by pre-pro Lip2ss showed 67, 65 and 63% thermal stability after 1, 2 and 3 h of its original activity respectively. The His-tagged TLL directed by both native and heterologous secretion signals showed less thermal stability. At 60°C, TLL directed by the Nss retained about 50% of its original activity after 1 h of incubation and after 3 h 38% were retained. For the thermal stability of His-tagged TLL directed by the pre-pro Lip2ss, the enzyme retained 47% of its original activity after 1 h of incubation at 60°C and 34% after 3 h.

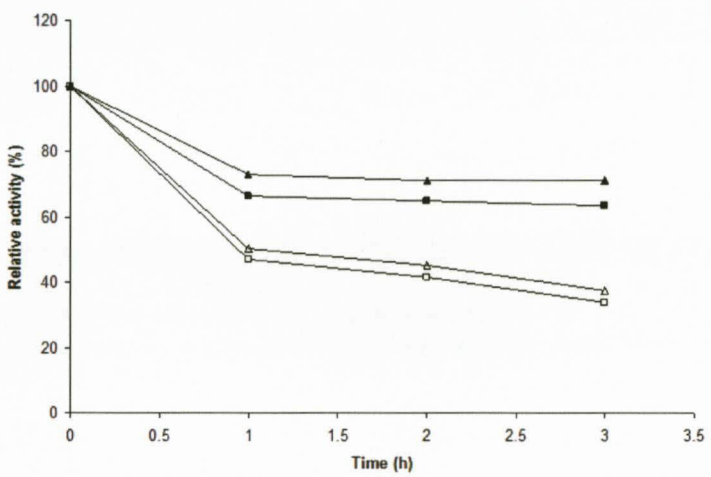

Figure 3.10: Thermostability of TLL expressed in *Y. lipolytica*. Purified TLL were incubated at 60°C for 1-3 h in 50 mM Tris-HCl buffer, pH 9.0. The activity at 60°C was defined as 100% for YI323N-TLLH (Δ); YI323N-TLL (▲); YI323L-TLLH (□) and YI323N-TLL (■).

The effects of detergents, metal ions and solvent solution on the activity of TLL are shown in Table 3.2. The activity of non-tagged TLL directed by the Nss was not affected by EDTA, whereas His-tagged TLL was slight affected by EDTA. The activity of both tagged and non-tagged TLL directed by the pre-pro Lip2ss was slightly less than both TLL directed by the Nss. The activity of non-tagged TLL directed by both secretion signals was affected by SDS showing a 40% relative activity, whereas the tagged TLL directed by both secretion signals was adversely affected by SDS. In the presents of DMSO the His-tagged TLL directed by both secretion signals exhibited better relative activity compared to the non-tagged TLL directed by both secretion signals. The non-ionic surfactants, Triton X-100 and Tween-80 exhibited a negative effect on the activity of all TLLs.

Table 3.2 Effect of detergents and solvent solution

Factor	Concentration	Lip2-TLL*	Lip2-TLL-His*	Nss-TLL*	Nss-TLL-His*
Control		100 ± 6.1	100 ± 7.0	100 ± 7.4	100 ± 2.4
EDTA	1 mM	91 ± 4.6	81 ± 4.0	107 ± 7.2	96 ± 6.4
SDS	1 mM	40 ± 5.7	17 ± 2.8	40 ± 8.6	17 ± 3.5
DMSO	10% (v/v)	83 ± 9.2	96 ± 10.6	92 ± 7.3	105 ± 8.1
Triton X-100	0.1% (v/v)	6 ± 1.1	1.4 ± 0.3	9 ± 2.4	2.2 ± 0.3
Tween-80	0.1% (v/v)	43 ± 3.4	5 ± 0.4	37 ± 4.8	4.5 ± 0.7

* Relative activity, SD = 3

3.4.5 Substrate specificity

Substrate specificity of TLL was determined using TAGs of different chain lengths (Table 3.3). Purified non-tagged TLL showed preference for tributyrin and triotanoate. The activity of non-tagged TLL decreased with an increase in the chain length of the substrates. The His-tagged TLL showed an increase in hydrolysis of the long chain substrate (trioleate) compared to the medium chain substrate (triotanoate).

Table 3.3 Substrate specificity

Substrate	Lip2-TLL *	Lip2-TLL-His*	Nss-TLL *	Nss-TLL-His*
1% Tributyrin	100 ± 6.1	100 ± 7.0	100 ± 7.4	100 ± 2.4
1% Trioleate	64 ± 2.1	83 ± 3.0	83 ± 7.1	95 ± 9.9
1% Triotanoate	94 ± 3.5	64 ± 7.1	97 ± 2.7	65 ± 1.4

* Relative activity, Data are given as means ± SD, *n*=3

3.5 Discussion

The pBR322 docking platform has been well studied for site directed integration in the genome of *Y. lipolytica* strains Po1e and Po1g (Madzak *et al.*, 2004, 2005). The pBR322 docking platform offers a precise targeting of single copy integration events generating transformants that are comparable with regards to copy numbers and integration site (Jolivalt *et al.*, 2005). More recently Duquesne and co-workers (2012) developed a new *Y. lipolytica* strain, JMY1212, by introducing a zeta based docking platform in yeast genome for reproducible protein expression levels. In this study, the single copy integrative plasmid pKOV323 directed to the pBR322 docking platform was used for heterologous expression of His-tagged and non-tagged TLL. Expression of both His-tagged and non-tagged TLL were directed by both homologous and heterologous secretion signals under the growth-phase dependent hybrid promoter, hp4d. Expression of TLL did not affect the growth profile of *Y. lipolytica* and these were similar to the control Po1g cells (Fig. 3.3). Po1g strain expressing heterologous proteins under the hp4d promoter has been reported to show similar growth profiles to those of the wild-type W29 strain (Madzak *et al.*, 2000).

Evaluation of total protein production showed significant protein level differences between the His-tagged and the non-tagged TLL produced by the yeast. Recombinant production of non-tagged TLL in *Y. lipolytica* directed by the Nss or the pre-pro Lip2ss resulted in about 200 and 190 mg of extracellular total protein per litre, respectively. The Po1g strains expressing the His-tagged recombinant TLL produced almost half of the total protein produced by the strains expressing non-tagged TLL, giving amounts of 80 and 110 mg/l for the Nss and pre-pro Lip2ss respectively. The decreased levels of total protein production in strains expressing the His-tagged TLL could be as a result of the additional tag since the strains expressing non-tagged TLL produced larger amounts of total proteins.

The expression of TLL in *Y. lipolytica* has a potential to be increased further. In particular, the use of a multicopy expression plasmid and optimization of expression conditions in a bioreactor can be an efficient way to improve TLL yields. Different heterologous proteins produced in *Y. lipolytica* have been reported to increase about 10-fold in production when multi-copy vectors are used and scaling-up using batch and fed-batch cultivation (Madzak *et al.*, 2004).

Both secretion signals directed secretion of active His-tagged and non-tagged TLL, with the non-tagged directed by both secretion signals however more active than their respective tagged TLL. Significant levels of non-tagged TLL activity were measured in the last day liquid cultures of cultivation demonstrating that *Y. lipolytica* is capable of producing active TLL. The activity of non-tagged TLL from total culture supernatant was similar for TLL directed by the pre-pro Lip2ss and the Nss, whereas the His-tagged TLL directed by the pre-pro Lip2ss and the Nss were about 1.7-fold lower. Studies by Jolivalt *et al.* (2005) reported higher activity in the culture medium from the laccase directed by the native secretion signal compared to the laccase directed by the yeast secretion signal.

The diafiltration method using ultrafiltration membranes for purification of both tagged and non-tagged TLL was found to be efficient in the recovery of TLL of high purity. Purified TLL amounts were found to be similar for the non-tagged TLL directed by both secretion signals and higher than the His-tagged TLL. Both His-tagged and non-tagged purified TLL directed by the Nss showed a major and a minar protein band on the SDS-PAGE gel. These two bands were confirmed by MALD-TOF-MS to be both TLL. The difference in molecular weight of TLL could be as a result of glycosylationas predicted by a potential *N*-glycosylation site on TLL protein sequence. The single *N*-glycosylation site predicted for the expression of TTL directed by Nss in *Y. lipolytica* is in agreement with the wild type TLL reported to be a mono-glycosylated lipase (Pinholt *et al.*, 2010). Bioinformatics analysis of a single band protein sequence of TLL expressed in *P. pastoris* predicted two potential glycosylation sites (Zheng *et al.*, 2011). Although the recombinant TLL directed by Nss in this study had a single *N*-glycosylation site, its molecular weight was higher than the wild type TLL and TLL expressed in *P. pastoris*, 31.4 and 33 kDa respectively (Zheng *et al.*, 2011). This could suggest that secretion signals used for heterologous protein expression could influence expression of single or double protein bands as they are essential in the prediction of potential glycosylation sites on the protein sequence.

Specific activity of purified TLL remained similar for the non-tagged TLL directed by both secretion signals and higher than the His-tagged TLL directed by both secretion signals. Similarly to the TLL expressed in *P. pastoris* (Zheng *et al.*, 2011), all the purified TLL expressed in *Y. lipolytica* showed an optimal activity at pH 9.0 and 60°C. TLL expressed in *Y. lipolytica* directed by both secretion signals was found to be more thermostable than the TLL expressed in *P. pastoris* (Zheng *et al.*, 2011). The

non-tagged TLL directed by the Nss showed the highest thermostability, retaining 71% of its original activity after 3 h of incubation at 60°C. The non-tagged TLL directed by pre-pro Lip2ss showed good thermostability, retaining 63% of its original activity after 3 h. Both His-tagged TLL showed less thermostability, retaining less than for 40% of their original activity after 3 h of incubation at 60°C. For most enzymatic reactions that occur between 40 and 60°C, thermostable thermophilic fungal enzymes are more attractive for industrial use (Maheshwari *et al.,* 2000). This means TLL expressed in *Y. lipolytica* can be of industrial importance. In addition, the posttranslational modification of TLL expressed in *Y. lipolytica* can be of value as glycosylation is suggested to improve thermostability of lipases (Hung *et al.,* 2011). The activity of tagged and non-tagged TLL directed by the Nss was less affected by EDTA and SDS as compared to both TLL directed by pre-pro Lip2ss. The activities of all recombinant TLL were adversely affected by triton-X100 and tween-80. Substrate specificity of the recombinant TLL expressed in *Y. lipolytica* showed higher preference for tributyrin chain substrates. Efficient hydrolysis of medium chain substrates by TLL is well documented (Mogensen *et al.,* 2005; Salis *et al.,* 2003; Romdhane *et al.,* 2012).

In conclusion, this study reported the cloning, expression, purification and characterization of TLL. Functionally active His-tagged and non-tagged TLL were successfully expressed in *Y. lipolytica* using yeast or TLL native secretion signals for directing secretion of the recombinant lipase. *Y. lipolytica* expressed TLL with different protein band profiles when TLL was directed with the two secretion signals. Recombinant TLL purified using a one-step diafiltration method showed maximum activity at pH 9.0 and a temperature of 60°C. Furthermore, the His-tag affected the expression levels, activity as well as the thermostability of the heterologously expressed enzyme.

3.6 References

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Chapter 4. High level expression and characterization of *Thermomyces lanuginosus* lipase in *Yarrowia lipolytica*

4.1 Abstract

Thermomyces lanuginosus lipase (TLL) encoding gene was codon-optimized, cloned and expressed in *Yarrowia lipolytica*. Expression cassettes were constructed using the rDNA multi-copy expression vector pKOV410, with the secretion of TLL evaluated as an inframe fusion to the pre-pro Lip2 secretion signal as well as the TLL native secretion signal (Nss). The His-tag was fused to the C-terminal of TLL and its effect on expression and activity was evaluated. High level protein production of the His-tagged and non-tagged TLL was obtained when the enzyme was directed by the pre-pro Lip2ss for secretion into the extracellular medium, with the non-tagged showing the highest levels of total protein production. About 74 mg of non-tagged TLL per 100 ml, which was approximately 2.5-fold higher than the His-tagged TLL, was purified using the diafiltration method from the strain harbouring the expression cassette with the pre-pro Lip2ss. This high level of purified non-tagged TLL was 5- and 44-fold higher than the purified non-tagged and His-tagged TLL from the strain harbouring the expression cassettes with the Nss, respectively. *Y. lipolytica* expressed active non-tagged and His-tagged TLL with temperature optima at 60°C and pH 9.0 optima. The purified non-tagged TLL directed by either secretion signal showed similar specific activity which was higher than the His-tagged specific activity. The His-tagg affected the activity and thermostability of the enzyme. The results reported in this study indicate that *Y. lipolytica* is prolific producer of TLL with potential large scale industrial production of the enzyme.

4.2 Introduction

Yeasts are ideal hosts for heterologous protein production due to their ability to reach high growth capacity, ease of unicellular organism manipulation and post-translational modification (Jolivalt *et al.,* 2005). These characteristics are essential when producing lipases of industrial interest. One of such important lipase in the detergent sector is the *Thermomyces lanuginosus* lipase (TLL). Efficient industrial application of lipases requires high level production, high activity and high stability of the enzyme (Shu *et al.,* 2010). Production of TLL by its native host has been reported (Adams and Deploey, 1978). Although *T. lanuginosus* is ideal for production of thermostable lipases, its low level expression of thermophilic lipases makes it unfavourable for large scale industrial production (Zheng *et al.,* 2011). Industrial production of lipases using the *Pichia pastoris* expression system is limited by concerns over safety and toxicity associated with the use of methanol as an inducer (Shu *et al.,* 2010).

Among the no-conventional yeasts, *Yarrowia lipolytica* remains one of the most attractive hosts for heterologous protein production (Muller *et al.,* 1998). A wide range of strains and vectors for heterologous protein expression and secretion have been developed in this dimorphic yeast (Nicaud *et al.,* 2002). *Y. lipolytica* has been found to be an efficient model organism to study early stages of the protein secretion pathway which are essential in the secretion of heterologous proteins (Beckerich *et al.,* 1998). This yeast has been reported to be a better host for expression of functionally active heterologous proteins (Madzak *et al.,* 2000). *Y. lipolytica* has been found to be an attractive alternative to *Saccharomyces cerevisiae* or *Aspergillus niger* for heterologous production of recombinant proteins (Muller *et al.,* 1998; Roth *et al.,* 2009). More recently, *Y. lipolytica* has been successfully used for simultaneous co-expression of heterologous proteins (Chuang *et al.,* 2010).

This chapter describes high level expression of TLL using an rDNA multi-copy expression vector. Heterologous expression of TLL was under the control of the hp4d promoter and directed by either the pre-pro Lip2ss or the TLL Nss secretion into the liquid medium. The developed expression system evaluated the effect of his-tag on production and activity of TLL.

4.3 Material and methods

4.3.1 Strains, plasmids and reagents

Escherichia coli XL10 Gold (Tet^r *D (mrcA) 183D (mrcCB-hsdSMR-mrr) 173endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacI^{q}ZDM15 Tn10* (Tet^r) Amy Cam^r]) was used as host for plasmid construction and propagation of TLL encoding gene. Cells were grown in Luria-Bertani (LB) broth or on agar plates supplemented with 50 µg/ml ampicillin (Amp) or 100 µg/ml kanamycin (Sambrook *et al.,* 1989). *Y. lipolytica* Po1f strain (*MatA, leu2-270, ura3-302, xpr2-322, axp1-2*) was used as host for multiple copy extracellular production of TLL (Madzak *et al.,* 2000). Multi-copy plasmid, pKOV410 was used for production of both His-tagged and non-tagged TLL using both the pre-pro Lip2ss and the TLL Nss. PCR products were cloned into pGEM®-T Easy Vector (Promega, USA). Recombinant plasmid DNA was isolated from *E. coli* XL10 Gold cells either using the method of Berghammer and Auer (1993) for initial screening purposes or the Plasmid Isolation Kit (BioFlux) following the manufacturer's instructions. *Y. lipolytica* Po1f transformants were selected on YNB-casamino and grown in YPD. Chromogenic agar substrate plates used for selection of *Y. lipolytica* transformants expressing TLL were prepared as described by Singh *et al.* (2006).

4.3.2 Construction of multi-copy expression vectors

All standard DNA manipulation procedures were performed according to Sambrook *et al.* (1989). The *tll* open reading frame (ORF) including the native secretion signal (Nss) of TLL (Gen Bank Accession No. AF054513.1) was codon optimized for expression in *Y. lipolytica* (GeneArt, Regensbung, Germany). The *tll* gene was synthesised with its Nss (894 bp) at the N-terminal end flanked by a *Hind*III restriction site at the 5'. A His-tag was added at the C-terminus upstream of the stop codon (TAA), followed by an *Avr*II restriction site. The *tll* gene was released with *Hind*III and *Avr*II and ligated into the multiple copy pKOV410 expression vector digested with the same enzymes to generate pKOV410-Nss-*tll*-His (Fig. 4.1A).

To construct an expression cassette that retained the *tll* Nss but without a His-tag, the primers pKOV-F (5'-GTTTGCCAGCCACAGA-3') flanking the 3'-end of the hp4d

promoter, and Fs_p-R (5'-TTTCCTAGGTTAGTGGTGATGGTGGTGG TG-3'; *Avr*II site is underlined), which introduced TAA stop codon, were used to amplify the gene from pKOV410-Nss-*tll*-His. Thermocycling reactions were carried out as described previously (chapter 3). Following thermal cycling and agarose gel electrophoresis, PCR products were gel purified using a Gel Extraction Kit (BioFlux) according to the manufacturer's instructions. Purified products were subcloned into p-GEM-T® Easy vector and the insert (876 bp) released with *Hind*III and *Avr*II and ligated into pKOV410 expression vector to generate pKOV410-Nss-*tll* (Fig. 4.1B).

To construct a *tll* gene under the pre-pro Lip2ss, the Nss was removed from the *tll* gene using plasmids pKOV410-Nss-*tll*-His and pKOV410-Nss-*tll* as templates for amplification of the *tll* gene with and without a His-tag using the same PCR conditions as described previously (chapter 3). The primer pair Fs_p-F1 (5'-AAAGGATCCATGCTGGGCCGAACCACCCGA-3'; *BamH*I site is underlined) and pKOV-R (5'-CGATATTCATTTATTAAAGTA-3') flanking the Lip2 terminator of the expression vector, were used to generate His-tagged amplicons (843 bp) from pKOV410-Nss-*tll*-His, whereas pKOV410-Nss-*tll* was used to generate non-tagged amplicons (825 bp). Amplicons were gel purified, subcloned into pG GEM-T® Easy vector and the inserts released with *BamH*I and *Avr*II and ligated in-frame downstream of the pre-pro Lip2ss of the pKOV410 expression vector linearized with the same enzymes to generate pKOV410-Lip2ss-*tll*-His and pKOV410-Lip2ss-*tll* (Fig. 4.1C and D). The recombinant plasmids were used to transform *E. coli* XL 10 Gold cells grown on LB-Kana (30 µg/ml) agar plates and screened in 5 ml LB medium containing 30 µg/ml kanamycin.

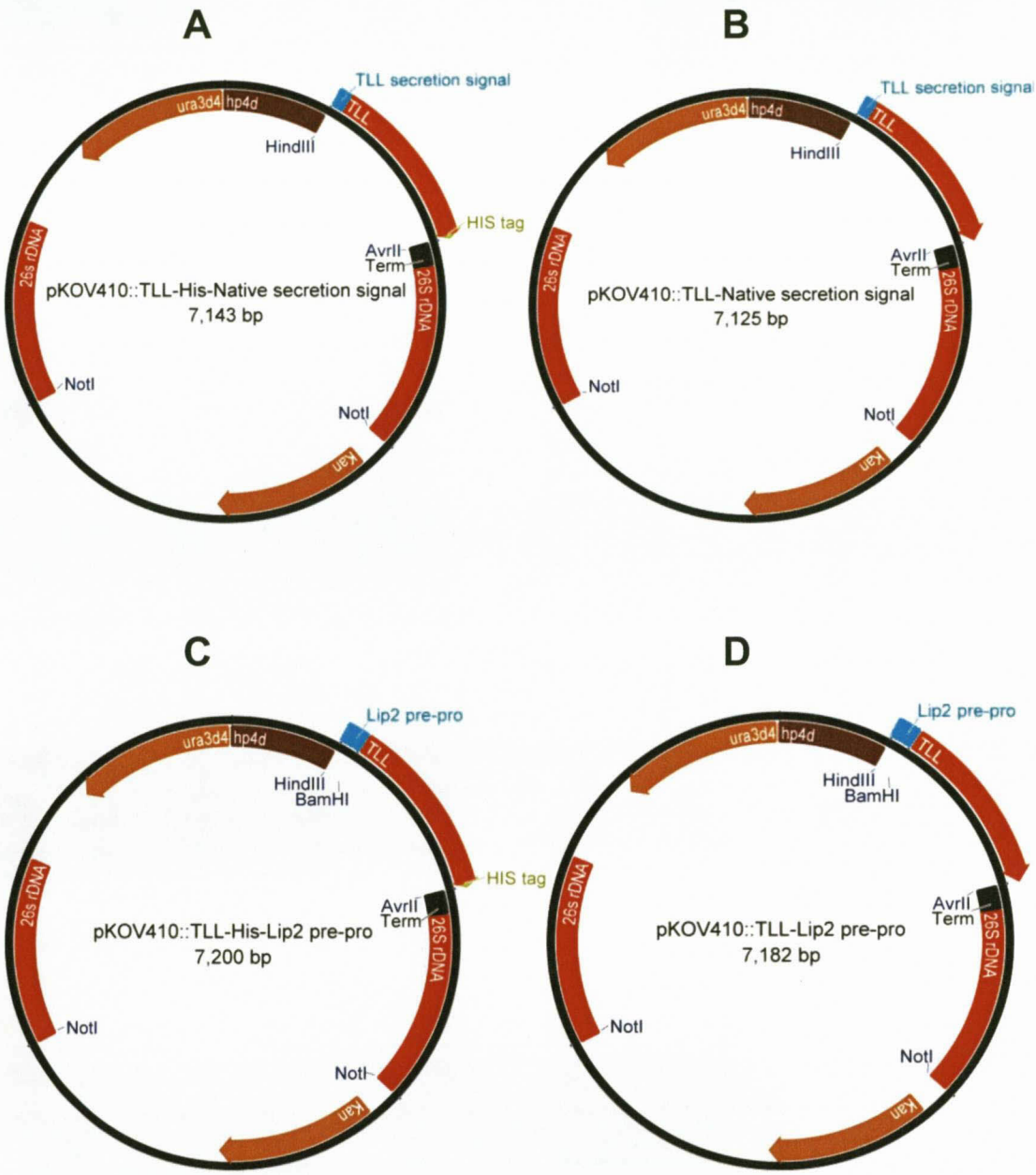

Figure 4.1: Schematic diagrams of *tll* gene in the expression plasmid pKOV410. The *tll* gene is under the Nss (A and B) or pre-pro Lip2ss (C and D). Diagrams A and C carry the His-tagged *tll* gene, whereas B and D carry the non-His tagged *tll* gene. The plasmid map was constructed using Geneious v5.5 (Drummond et al. 2011).

4.3.3 Transformation of *Y. lipolytica*

The plasmids pKOV410-Nss-*tll*-His, pKOV410-Nss-*tll*, pKOV410-Lip2ss-*tll*-His and pKOV410-Lip2ss-*tll* were digested with *Not*I and the expression cassettes separated from the bacterial moiety by agarose gel electrophoresis. The expression cassettes

were used to transform *Y. lipolytica* Po1f strain. Preparation of Po1f competent cells and transformation was performed using the lithium acetate method as described by Xuan *et al.* (1988). For selection of Uracil$^+$ (Ura$^+$) clones, Po1f transformants were grown on selective YNB-casamino acid media plates (0.17% YNB without amino acids and ammonium sulfate, 1% glucose, 0.1% casamino acids, 0.1% sodium glutamate and 1.5% agar). Colonies were isolated from YNB-casamino plates after 1-3 weeks of incubation at 28°C. Genomic DNA was extracted using the method of Chen *et al.* (1997) and used as a template to confirming the integration of the recombinant vectors into the yeast genome using pKOV410-F (5'-GTTTGCCAGCCACAGA-3') and pKOV410-R (5'-CGATATT CATTTATTAAAGTA-3') primers. The PCR positive transformants were inoculated on tributyrin chromogenic plates [(1.0% trybutyrin sonically emulsified in 10 mM $CaCl_2$, 0.01% phenolphthalein, 2.0% agar and the pH adjusted to 7.3/7.4 using 0.1 N NaOH)] as described by Singh et al. (2006) and incubated for 48 h at 28°C. TLL activity was estimated according to halo size around the colonies. The *Y. lipolytica* Po1f transformants carrying multiple copy expression cassettes of pKOV410-Nss-*tll*-His, pKOV410-Nss-*tll*, pKOV410-Lip2ss-*tll*-His, pKOV410-Lip2ss-*tll* and the control pKOV410-Lip2ss were denoted YI410N-TLLH, YI410N-TLL, YI410L-TLLH, YI410L-TLL and YI-p410L respectively.

4.3.4 Expression and purification of TLL

Yeast transformants, YI410N-TLLH, YI410N-TLL, YI410L-TLLH, YI410L-TLL and YI-p410L, were inoculated into 25 ml of YPD medium and incubated overnight at 28°C with shaking at 200 rpm. When the culture reached an optical density at 600 nm (OD_{600}) of 3-6, the cells were re-suspended to an OD_{600} of 1.0 in 100 ml YPD and incubated at 28°C with shaking at 200 rpm. The cultures were grown in shake flasks under aerobic conditions. The OD_{600} was recorded and supernatant samples collected at 24-hour intervals over the cultivation period of 6 days. The recorded OD was used to assess whether expression of TLL affected the growth patterns of *Y. lipolytica* Po1f strain. The cultures were centrifuged and supernatants analysed for total extracellular protein concentration.

After six days, the remaining supernatant was collected for purification of the enzyme. The supernatants were concentrated and diafiltered with 20 mM Tris-HCl (pH 8.0) using an Amicon stirred ultrafiltration cell (Millipore, Billeria, MA, USA) with a Millipore ultrafiltration membrane of 50 kDa molecular weight limit (Separations,

South Africa). The samples were further diafiltrated and concentrated using an ultrafiltration membrane of 30 kDa cut-off molecular weight limit.

4.3.5 Protein analysis

Protein concentrations of *Y. lipolytica* culture supernatant and the purified enzyme preparation were determined by the Bradford assay (1976) using the BIO-RAD protein dye reagent according to the manufacture's manual. Absorbance was measured at 595 nm employing Power Wave HT (BioTek). Bovine serum albumin (Roche, Mannheim, Germany) was used as a standard. To check the purity of the proteins, the purified protein samples were analysed on a 12% SDS-PAGE gel as described by Laemmli (1970).

4.3.6 TLL activity

TLL activity was measured using a pH titration method (Benzonana and Desnuelle, 1968; Pinsirodom and Parkin, 2001) with tributyrin as substrates. Tributyrin (1%) was emulsified through sonication (HD 2070 Bandelin, Sonopuls) in a solution containing 20 mM $CaCl_2$, 0.6 M NaCl, 1 mM sodium deoxycholate and 2.5% (w/v) gum Arabic. TLL (100 µg) was added, with liberated fatty acids from the substrate titrated with 0.05 N NaOH to maintain a constant pH of 7.3-7.4 at 40°C. One unit (U) of TLL activity was defined as the amount of enzyme releasing 1 µmol of fatty acid per minute under assay conditions. All assays were averaged from three independent experiments.

4.3.7 Characterization of TLL

The effect of pH and temperature on TLL activity was determined by performing titrimetric assays measuring tributyrin hydrolysis by TLL at different pH and temperatures. TLL activity was measured at temperatures ranging from 20-80°C in 50 mM Tris-HCl buffer at both pH 8.0 and 9.0. The pH dependence of TLL was determined from pH 4 to 11 using Tris-HCl, pH 9.0 at 60°C. The assay mixture contained 1% of tributyrin and the assays were done at 60°C and pH 9.0. All assays were averaged from three independent experiments using 100 µg of supernatant and purified non-tagged and C-terminally His-tagged TLL from the four transformants. For

temperature stability, the purified TLL from the four strains were incubated at 60°C for up to 3 h in 50 mM Tris-HCl buffer, pH 9.0 and the residual activity measured at 1 h time intervals. Detergents and solvent solution were added to the substrate solution respectively and their effect on purified TLL was determined.

4.3.8 Substrate specificity

Substrate specificity of TLL was evaluated using 1% of tributyrin, glyceryl trioleate and glyceryl trioctanoate as substrates employing a pH titration method (Benzonana and Desnuelle, 1968; Pinsirodom and Parkin, 2001). Each of the substrates was emulsified through sonication (HD 2070 Bandelin, Sonopuls) in a solution containing 20 mM $CaCl_2$, 0.6 M NaCl, 1 mM sodium deoxycholate and 2.5% (w/v) gum Arabic. Enzyme solution (100 µg) was added, with liberated fatty acids from the TAG substrates titrated with 0.05 N NaOH to maintain a constant pH of 7.3-7.4 at 40°C. One unit (U) of TLL activity was defined as the amount of enzyme releasing 1 µmol of fatty acid per minute under assay conditions. All assays were averaged from three independent experiments with equal amounts of purified non-tagged and C-terminally His-tagged TLL from the four transformants.

4.4 Results

4.4.1 Construction of multi-copy vectors for expression of TLL

The *tll* encoding gene was cloned into pKOV410 in-frame with the Nss and the Lip2ss pre-pro region (Pignède *et al.,* 2000a, b) under the hp4d promoter (Madzak *et al.,* 2000). All expression vectors harbouring the *tll* gene carried a growth-phase-dependent hybrid promoter hp4d and Lip2 terminator (Barth and Gaillardin, 1996). TLL producing strains were obtained by integrative transformation of *Y. lipolytica* Po1f with the four different *tll* expression cassettes. The resulting transformants were confirmed to be positive by PCR (data not shown) confirming integration of the recombinant vectors into the yeast genome. *Y. lipolytica* transformants positively confirmed by PCR to harbour *tll* gene showed increased hydrolytic activity towards tributyrin on chromogenic activity plates compared to the controls cells. Yeast transformants expressing TLL produced larger halos on agar activity plates after 48 hours of incubation than the control strain (Fig. 4.2). Strains employing homologous and heterologous secretion signals for secretion of non-tagged TLL exhibited higher-levels of lipolytic activity on the agar activity plates containing emulsified tributyrin compared to their respective strains expressing C-terminally His-tagged TLL.

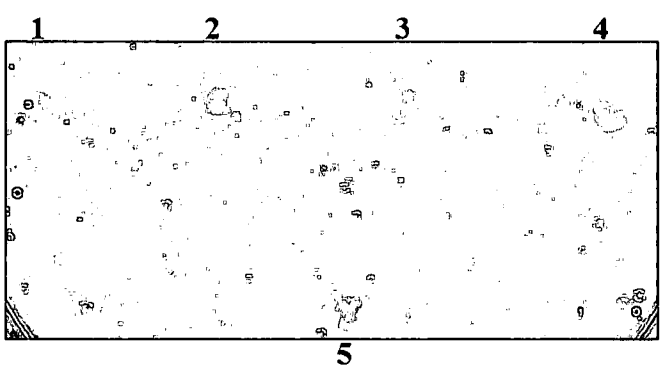

Figure 4.2: *Y. lipolytica* multicopy copy transformants grown on chromogenic activity plates. The strains were grown on 2% agar plates containing emulsified 1% tributyrin and 0.01% phenolphthalein. *Y. lipolytica* cells: (1) YI410N-TLLH, (2) YI410N-TLL, (3) YI410L-TLLH, (4) YI410L-TLL and (5) YI-p410L.

4.4.2 Heterologous expression of TLL in *Y. lipolytica*

To ascertain whether multiple copy expression of TLL with and without His-tag directed by the Nss and the pre-pro Lip2ss for secretion affected the growth of *Y. lipolytica*, the strains expressing TLL and control cells (YI-p410L) were grown in liquid cultures under aerobic conditions on a rotary shaker (200 rpm, 28°C). Growth was determined by monitoring OD of the cultures at 600 nm at 24-hour intervals for 6 days. During the entire period of culture cultivation, there were no differences in the growth profiles of *Y. lipolytica* cultures expressing non-tagged TLL directed by either the Nss or the pre-pro Lip2ss, His-tagged TLL directed by the pre-pro Lip2ss and the control (Fig. 4.3). The YI410N-TLLH cells expressing His-tagged TLL directed by the Nss showed growth impairment, with about 2–fold decrease in the growth of the cells compared to other cells expressing TLL and control cells.

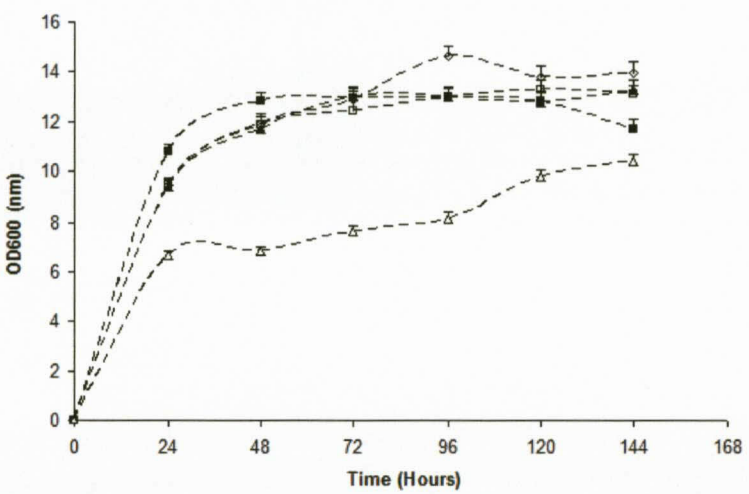

Figure 4.3: Comparison of the growth profiles of *Y. lipolytica* expressing TLL. Strains were grown at 28°C in shake flasks (200 rpm) and growth was assessed by measuring absorbance at OD_{600} for a period of 168 hours at 24-hour intervals. *Y. lipolytica* cells: YI410N-TLLH (△); YI410N-TLL (▲); YI410L-TLLH (□) and YI410L-TLL (■) and YI-p410L (◊).All measurements were done in duplicates.

Expression of recombinant TLL was highly variable between the strains using the developed strategy. *Y. lipolytica* Po1f strains expressing non-tagged and His-tagged TLL directed by the pre-pro Lip2ss for secretion produced the highest amounts of total protein compared to the yeast strains expressing non-tagged and His-tagged TLL directed by the Nss. Yeast strains expressing non-tagged and His-tagged TLL directed by the pre-pro Lip2ss resulted in 105 and 67 mg of total protein per 100 ml of culture supernatant, respectively (Fig. 4.4). In contrast, the total amounts of protein

produced by the strains expressing non-tagged and His-tagged TLL directed by the Nss were 21 and 11 mg, respectively (Fig. 4.4).

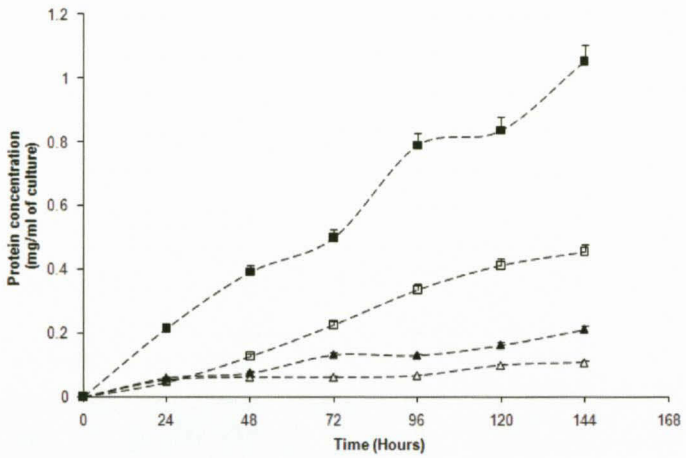

Figure 4.4: Comparison of total extracellular protein in different strains of *Y. lipolytica*. Total proteins were quantified at 24-hour intervals for a 144 hour period. *Y. lipolytica* cells: YI410N-TLLH (Δ); YI410-TLL (▲); YI410L-TLLH (□) and YI410L-TLL (■).All measurements were done in duplicates.

TLL activity from total culture supernatant was higher in the *Y. lipolytica* cultures expressing non-tagged TLL directed by the pre-pro Lip2ss (409 U/ml) and the Nss (270 U/ml) for secretion, assayed using 1% tributyrin at the end of the culturing period (Fig. 4.5). C-terminally His-tagged TLL directed by the pre-pro Lip2ss (237 U/ml) and the Nss (104 U/ml) for secretion were about 1.7-fold lower than the non-tagged TLL directed by both secretion signals.

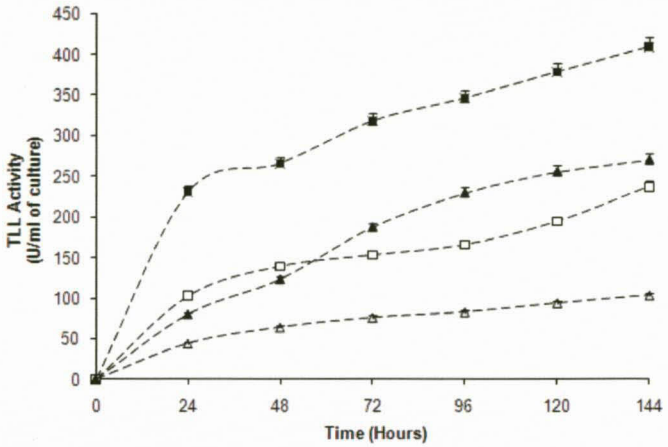

Figure 4.5: Comparison of TLL activity in the culture supernatant. Lipolytic activity of TLL was quantified at 24-hour intervals for a 144 hour period from the supernatant of *Y. lipolytica* cells: YI410N-TLLH (Δ); YI410N-TLL (▲); YI410L-TLLH (□) and YI410L-TLL (■). Data are averages from three independent experiments.

SDS-PAGE analysis of the 24-hour interval samples revealed lower levels of the His-tagged TLL directed by the Nss (Fig. 4.6B). The non-tagged TLL directed by the Nss showed two bands (Fig. 4.6A), whereas a single band was observed for the tagged and non-tagged TLL directed by the pre-pro Lip2ss (Fig. 4.6C, D).

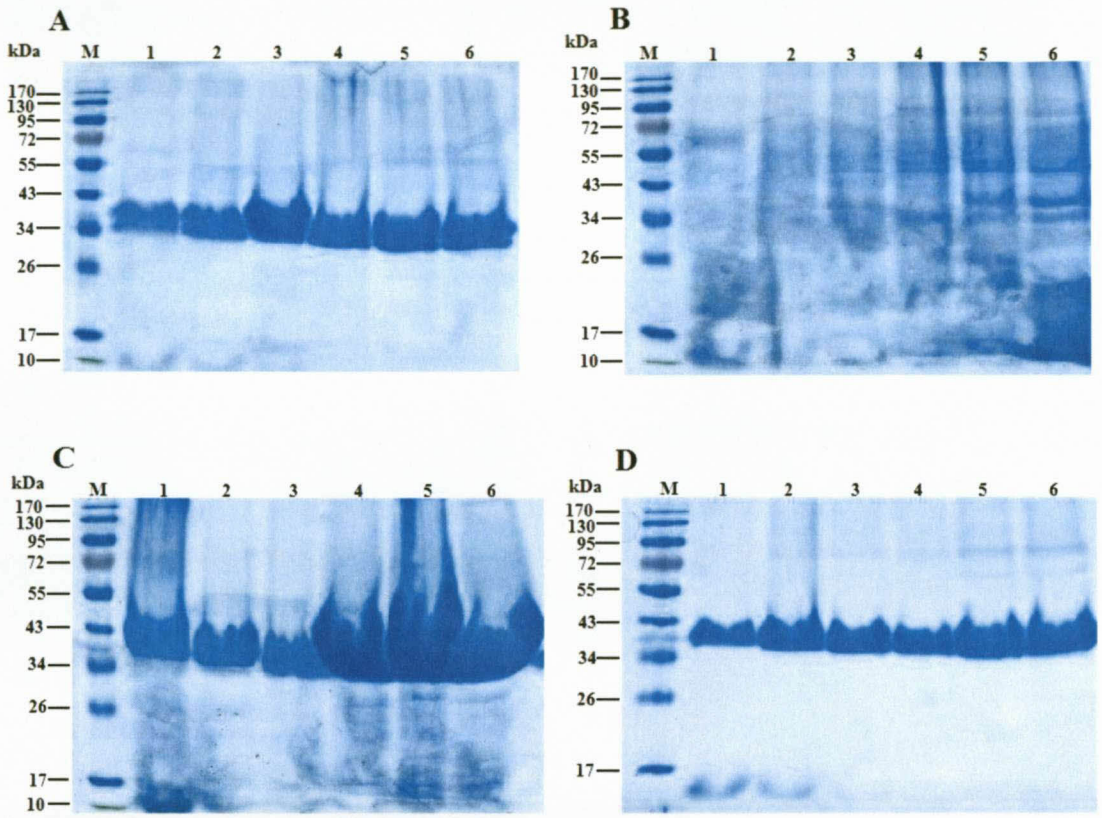

Figure 4.6: SDS-PAGE analysis of *Y. lipolytica* Po1f expressing tagged and non-tagged TLL directed by homologous and heterologous secretion signals. The total protein culture supernatant samples were collected at 24-hour intervals for 6 days and electrophoresed on 12% SDS-PAGE gels: (A) non-tagged TLL directed by the Nss for secretion, (B) C-terminally tagged TLL directed by the Nss for secretion, (C) non-tagged TLL directed by the pre-pro Lip2ss for secretion, and (D) C-terminally tagged TLL directed by the pre-pro Lip2ss for secretion. Lane M is a PageRuler stained protein ladder (Fermentas). The numbers depicted on top of the gel images, labelled 1-7 represent the cultivation period in days.

4.4.3 Purification of TLL

After 6 days of expression, TLL was purified from 100 ml culture medium by ultrafiltration (Table 4.1). C-terminally His-tagged and non-tagged TLL were purified using Amicon ultrafiltration membranes. The non-tagged and His-tagged TLL directed by the yeast secretion signal showed higher levels of expression than both the TLL proteins directed by the Nss, with the non-tagged protein directed by the pre-pro Lip2ss produce in higher levels. The non-tagged purified TLL directed by the pre-pro Lip2ss was 5-fold higher than the purified non-tagged TLL directed by the Nss for

secretion. In addition, the purified non-tagged TLL directed by the pre-pro Lip2 was 44- and 2.5-fold higher than the purified C-terminally His-tagged TLL directed by the Nss and the pre-pro Lip2ss, respectively. The amount of the purified His-tagged TLL directed by the pre-pro Lip2ss was 17- and 2-fold higher than those of the purified C-terminally His-tagged and non-tagged TLL directed by the Nss, respectively. Although the purified non-tagged TLL was lower than those directed by the pre-pro Lip2ss for secretion, it was found to be 9-folds higher than the His-tagged TLL directed by the same secretion signal.

Table 4.1 Purification of TLL expressed in *Y. lipolytica*

Enzyme	Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification Fold
TLL (Nss)	Culture supernatant	25235.6	21.1	1196	100	1
	Purified	23611.1	14.7	1606.2	93.6	1.3
TLL-His (Nss)	Culture supernatant	5424.9	10.7	507	100	1
	Purified	1720.9	1.7	1012.3	31.7	2
TLL (Lip2ss)	Culture supernatant	157573	105.4	1495	100	1
	Purified	136346	74.2	1837.3	86.5	1.2
TLL-His (Lip2ss)	Culture supernatant	52392.9	66.7	785.5	100	1
	Purified	34119.9	29.6	1152.7	65.1	1.5

100 µg of supernatant and purified FSCut was used to measure the activity of the enzyme at pH 9.0

Similarly to the crude protein, two different profiles of TLL were observed when purified TLL was resolved on SDS-PAGE gel. Only a single protein band was observed from both tagged and non-tagged TLL directed by pre-pro Lip2ss (36 kDa), whereas two bands of approximately 36 and 34 kDa were observed from both tagged and non-tagged TLL by Nss (Fig. 4.7). In the previous chapter, both bands were confirmed to be TLL.

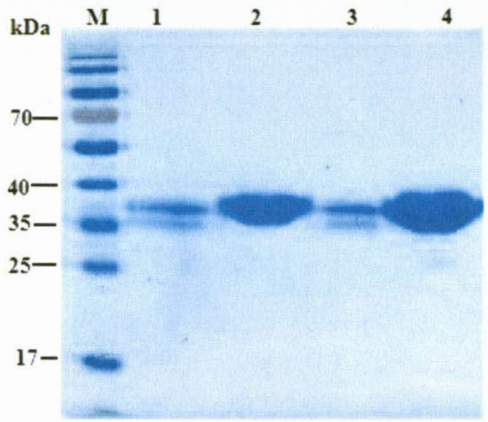

Figure 4.7: SDS-PAGE analysis of purified TLL expressed in *Y. lipolytica* Po1f strains. The purified TLL was analysed on 12% SDS-PAGE gel: Lane 1: His-tagged TLL directed by the pre-pro Nss, Lane 2: His-tagged TLL directed by the pre-pro Lip2ss, Lane 3: non-tagged TLL directed by the Nss and Lane 4: non-tagged TLL directed by the pre-pro Lip2ss. Lane M: PageRuler pre-stained protein ladder.

4.4.4 Biochemical characterization of TLL

The activity of purified TLL was determined at pH 9.0 using tributyrin as a substrate. The purified non-tagged TLL directed by the Nss or the pre-pro Lip2ss showed similar specific activity. Purified non-tagged TLL secreted using the pre-pro Lip2ss showed higher specific activity (~1.6- and 1.8-fold) than the purified His-tagged TLL directed by both the pre-pro Lip2ss and the Nss respectively. The effects of pH (Fig. 4.8A) and temperature (Fig. 4.8B) on the activity of all purified TLL was determined over a pH range of 4-11 and temperature range of 20-80°C. The effect of pH on the purified TLL was found to be similar for both the tagged and non-tagged proteins. Both purified His-tagged and non-tagged proteins showed activity at pHs ranging from 8-10. The His-tagged proteins showed optimum activity at pH 8.0 and retained its activity at pH 9.0, whereas the Non-tagged TLL showed optimal activity at pH 9.0. The His-tagged and non-tagged TLL retained good activity at pH 10.0, indicating that TLL functions at alkaline pH. At pH 11.0 a drastic decrease in the activity of TLL was observed, this points out that TLL functions efficiently at pH between 8 to 10. Both tagged and non-tagged TLL showed higher activity at temperatures ranging from 50-60°C. The non-tagged TLL retained about 60% of its original activity at 70°C with the activity of the His-tagged decreasing drastically at the same temperature retaining

about 20% of its original activity. Both the tagged and non-tagged TLL showed optimal activity at 60°C (Fig. 4.8B).

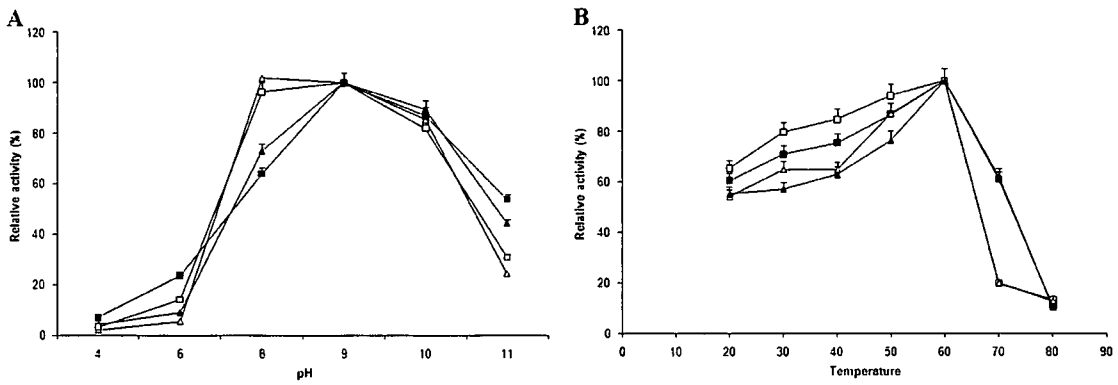

Figure 4.8: Effect of pH (A), temperature (B) on purified non-tagged and His-tagged TLL. YI410N-TLLH (Δ); YI410N-TLL (▲); YI410L-TLLH (□) and YI410L-TLLH (■). Data are averages from three independent experiments.

Thermostability of both tagged and non-tagged purified TLL was evaluated after incubating the enzyme solution at 60°C for 1-3 h (Fig. 4.9). The non-tagged TLL directed by the Nss showed thermal stability of 72% after 1 h and 60% after 3 h of its original activity. Non-tagged TLL directed by pre-pro Lip2ss showed stable thermal stability of 64% after 1 and 3 h of its original activity. The His-tagged TLL directed by both homologous and heterologous secretion signals showed less thermal stability. At 60°C, the His-tagged TLL directed by the Nss retained about 50% of its original activity after 1 h of incubation and after 3 h it reached about 32%. For the thermal stability of His-tagged TLL directed by the pre-pro Lip2ss, the enzyme retained 44% of its original activity after 1 h of incubation at 60°C and 34% after 3 h.

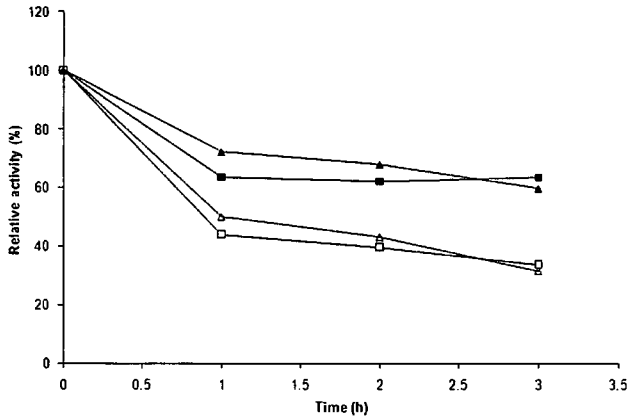

Figure 4.9: Thermostability of TLL expressed in *Y. lipolytica*. Purified TLL were incubated at 60°C for 1-3 h in 50 mM Tris-HCl buffer, pH 9.0. The activity at 60°C was defined as 100% for YI410N-TLLH (Δ); YI410-TLL (▲); YI410-TLLH (□) and YI410L-TLL (■).

The effects of detergents, metal ions and solvent solution on the activity of TLL are shown in Table 4.2. The activity of non-tagged TLL directed by the Nss was slightly enhanced and was not affected by EDTA, whereas His-tagged TLL was slight affected by EDTA. The activity of both tagged and non-tagged TLL directed by the pre-pro Lip2ss were slightly less than those of both TLL directed by the Nss. The activity of non-tagged and His-tagged TLL directed by both secretion signals was negatively affected by SDS. In the presents of DMSO the His-tagged TLL directed by both secretion signals exhibited better relative activity compared to the non-tagged TLL directed by both secretion signals. The non-ionic surfactant, Triton X-100 adversely affected the activity of all TLLs. In addition, the activity of both His-tagged TLL was also adversely affected by Tween-80. Unlike the His-tagged TLLs, the non-tagged were moderately affected by Tween-80 with the TLL directed by the pre-pro Lip2ss showing better activity compared to that directed by the Nss.

Table 4.2 Effect of detergents and solvent solution

Factor	Concentration	Lip2-TLL*	Lip2-TLL-His*	Nss-TLL*	Nss-TLL-His*
Control		100 ± 8.5	100 ± 7.9	100 ± 2.7	100 ± 274
EDTA	1 mM	94 ± 1.6	85 ± 1.6	105 ± 6.9	94 ± 4.2
SDS	1 Mm	54 ± 4.0	23 ± 6.4	3.9 ± 9.1	16 ± 4.2
DMSO	10% (v/v)	79 ± 9.7	91 ± 1.2	89 ± 4.9	101 ± 5.7
Triton X-100	0.1% (v/v)	11 ± 8.0	2.7 ± 2.6	7 ± 4.6	2 ± 0.9
Tween-80	0.1% (v/v)	77 ± 4.0	9 ± 6.4	58 ± 9.3	7 ± 1.1

* Relative activity, Data are given as means ± SD, *n*=3

4.4.5 Substrate specificity

Substrate specificity of TLL was determined using TAGs of different chain lengths (Table 4.3). Purified non-tagged TLL showed preference for tributyrin and triotanoate. The activity of non-tagged TLL decreased with an increase in the chain length of the substrates. The His-tagged TLL showed an increase in hydrolysis of the long chain substrate (trioleate) compared to the medium chain substrate (triotanoate).

Table 4.3 Substrate specificity

Substrate	Lip2-TLL *	Lip2-TLL-His*	Nss-TLL *	Nss-TLL-His*
1% Tributyrin	100 ± 8.5	100 ± 7.9	100 ± 2.7	100 ± 2.7
1% Trioleate	63 ± 1.2	83 ± 4.9	70 ± 7.1	91 ± 9.2
1% Triotanoate	93 ± 1.0	63 ± 9.6	93 ± 4.9	63 ± 2.8

* Relative activity, Data are given as means ± SD, *n*=3

4.5 Discussion

The rDNA cluster has been reported as a target integration site for selection of transformants carrying multi-copy integrations when using a defective marker (Erhardt and Hollenberg, 1983). Application of *LEU2d* marker showed an increase of up to 200 copies of the expression cassette in *Saccharomyces cerevisiae* transformants when directed to the rDNA (Lopes *et al.,* 1989). Le Dall et al. (1994) reported a similar multi-copy integration system for increased copy numbers in *Y. lipolytica*. The authors constructed an rDNA targeted vector containing *ura3* gene as a selection marker that produced yeast transformants carrying up to 60 copies of the expression cassette. In this study, the rDNA targeted plasmid pKOV410 was used for heterologous expression of TLL under the control of the growth phase dependent hybrid promoter hp4d. The vector carried the defective *ura3d4* gene as a marker, which is required to alleviate the uracil auxotrophy of the yeast for multi-copy integration (Juretzek *et al.,* 2001). Either a TLL Nss or *Y. lipolytica* pre-pro Lip2ss were used to target His-tagged and non-tagged recombinant protein to the secretion pathway.

Growth profile of Po1f strains carrying the expression cassettes were not affected by expression of TLL, except for the strain expressing His-tagged TLL directed by the Nss which showed growth impairment. In our previous study using single copy integration the His-tagged TLL directed by the Nss did not affect the growth profile of the strain (Chapter 3) suggesting that an increase in copy number of TLL coupled with the His-tag using the Nss has a negative effect on the specific growth rate (Calado *et al.,* 2002). Expression of non-tagged TLL directed by the pre-pro Lip2ss in *Y. lipolytica* using the multi-copy expression vector resulted in about 742 mg of purified protein per litre. To our knowledge this is the first study to report such high level of purified recombinant TLL. The purified His-tagged TLL directed by the pre-pro Lip2ss, although lower than the non-tagged TLL directed by the same secretion signal, reached 300 mg per litre. Under optimal conditions *Y. lipolytica* has been reported to produce 1-2 g per litre of an alkaline extracellular protease (Barth and Gaillardin, 1996). Both tagged and non-tagged TLL were produced at high levels when the proteins were directed by the pre-pro Lip2ss compared to the same proteins directed by the Nss.

Expression of endo-1,4-β-mannanase in *Y. lipolytica* transformants containing similar copy numbers gave different expression levels in shake flasks suggesting that the integration site plays an important role (Roth *et al.*, 2009). Some of the other factors reported to influence protein production include codon optimization of genes and the nature of the secretion signal used (Gasmi *et al.*, 2011). It would be interesting to determine the copy number of the expression cassette between the strains and evaluate whether the high-level expression of TLL is a result of the copy numbers or the integration site. Optimization of some genes has shown reduction in protein production (Griswold *et al.*, 2003; Yuankai *et al.*, 2012).

The work on identification (peptide mass fingerprinting) and glycosylation of the recombinant TLL is well discussed in Chapter 3. Functionally active non-tagged and His-tagged TLL was expressed in *Y. lipolytica.* The non-tagged TLL directed by both secretion signals was more active than the His-tagged. In this study, *Y. lipolytica* demonstrated to be a platform for high level production of active TLL. Production of TLL in this yeast can further be increased using a bioreactor.

4.6 References

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Chapter 5. Development of a novel rDNA based plasmid for enhanced cell surface display on *Yarrowia lipolytica*

5.1 Abstract

In this study, a novel rDNA based plasmid was developed for display of heterologous proteins on the cell surface of *Yarrowia lipolytica* using the C-terminal end of the glycosylphosphatidylinositol (GPI) anchored *Y. lipolytica* cell wall protein 1 (YlCWP1). mCherry was used as a model protein to assess the efficiency of the constructed plasmid. *Y. lipolytica* transformants harbouring the expression cassettes showed a purple colour phenotype on selective YNB-casamino plates as compared to control cells indicating that mCherry was displayed on the cells. Expression of mCherry on cells of *Y. lipolytica* was confirmed by both fluorescent microscopy and flow cytometry. Furthermore, SDS-PAGE analysis and matrix-assisted laser desorption/ionization (MALDI)-time-of (TOF)-mass spectrometry (MS) peptide mass fingerprinting (PMF) confirmed that the protein cleaved from the yeast cells using enterokinase was mCherry. Efficient cleavage of mCherry reported in this work offers an alternative purification method for displayed heterologous proteins on *Y. lipolytica* cells using the plasmid constructed in this study. The developed displaying system offers great potential for industrial production and purification of heterologous proteins at low cost.

5.2 Introduction

Since the first development of a cell surface display system on bacteriophage by Smith (1985), various yeast cell surface displaying systems have been developed for expression of heterologous proteins (Sergeeva *et al.,* 2006). Yeast cell surface display has been used as a method of choice for expression of heterologous proteins. This is because yeast cell surface display is convenient, shows ease of handling of displayed heterologous proteins and has been found to be comparatively stable against environmental changes (Inaba *et al.,* 2010). In addition, the advantage of yeast cell surface display over bacterial display is that yeast has a post-translational modification system that resembles the mammalian system for efficient processing and folding of proteins (Kondo and Ueda, 2004). Cell surface displaying systems in yeast, in particular *Saccharomyces cerevisiae*, have been studied extensively (Kondo and Ueda, 2004; Furukawa *et al.,* 2006). Although *S. cerevisiae* emerged as the most favourable microorganism for displaying heterologous proteins (Kondo and Ueda, 2004), hyperglycosylation of expressed proteins has remained a major drawback (Gemmill and Trimble, 1999). Hyperglycosylation of heterologous proteins has a potential to affect protein activity (Wang *et al.,* 2007). Other yeasts, such as *Pichia pastoris* have been reported to express heterologous proteins with reduced glycosylation (Choi *et al.,* 2003). More recently, cell surface display of active heterologous proteins in both *Y. lipolytica* and *P. pastoris* has gained momentum.

Amongst the non-conventional yeasts, *Y. lipolytica* remains one of the most attractive hosts for heterologous protein production (Muller *et al.,* 1998). A series of molecular tools for heterologous protein expression in *Y. lipolytica* have been developed (Nicaud *et al.,* 2002; Madzak *et al.,* 2004). Yue *et al.* (2007) constructed a surface displaying vector for immobilization of proteins on *Y. lipolytica*. The vector carries zeta elements (LTRs from Ylt1 retrotransposon), which allows it to integrate either by homology in *Y. lipolytica* strains carrying Ylt1, or by non-homologous integration into Ylt1-free strains (Nicaud *et al.,* 1998; Pignede *et al.,* 2000a; Juretzek *et al.,* 2001). The zeta based plasmid employs the C-terminal end of the *YlCWP1* for cell surface display of proteins. A wide range of heterologous proteins have been successfully displayed on *Y. lipolytica* cell surface using the zeta-based displaying plasmid (Ni *et al.,* 2009; Liu *et al.,* 2009, 2010; Yu *et al.,* 2010). More recently, studies by Yuzbasheva *et al.* (2011) reported five genes encoding YlCWP. All identified proteins

were used successfully to display active Lip2 lipase on *Y. lipolytica* employing a zeta-based plasmid.

As an alternative approach, in this study a new vector for cell surface display on *Y. lipolytica* cells was developed. The construction of the displaying vector is based on the rDNA autocloning pKOV410. The vector integrates homologously into the yeast ribosomal cluster. The new plasmid employs the growth-phase dependent promoter, hp4d, for heterologous expression of proteins (Madzak *et al.,* 2004), the pre-pro Lip2 secretion signal (Pignede *et al.,* 2000a, b) for directing secretion and the Lip2 terminator (Barth and Gaillardin, 1996). In this study, we successfully constructed a novel rDNA based plasmid for enhanced cell surface display of heterologous proteins on *Y. lipolytica* using YlCWP1 as a membrane anchor. The ability of the new rDNA based plasmid to display heterologous proteins on *Y. lipolytica* cell surface was tested using the fluorescent protein mCherry as a reporter. The yeast transformants displaying mCherry showed colour change on YNB-casamino selective plates and culturing medium. The displayed mCherry was confirmed by fluorescence microscopy and flow cytometry. In addition, mCherry was easily cleaved from the yeast cells and detected using SDS-PAGE and mass spectrometry.

5.3 Material and methods

5.3.1 Strains and media

Escherichia coli XL10 Gold cells (Stratagene) were used for cloning and plasmid propagation. *E. coli* transformants were grown in 5.0 ml Luria-Bertani (LB) broth or agar plates at 37°C overnight (Sambrook *et al.,* 1989). When necessary, 30 µg/ml of kanamycin or 50 µg/ml of ampicillin was added. *Y. lipolytica* Po1f strain (*MatA, leu2-270, ura3-302, xpr2-322, axp1-2*; Madzak *et al.,* 2000) was used as host for cell surface display. In addition, *Y. lipolytica* Po1f strain genomic DNA was used for amplification of the C-terminal end of the gene encoding glycosylphosphatidylinositol anchored cell wall protein (*GPI-CWP1*). Yeast transformants were selected on YNB-casamino acid plates (0.17% YNB without amino acids and ammonium sulfate, 1% glucose, 0.1% casamino acids, 0.1% sodium glutamate and 1.5% agar). For expression of immobilised proteins on *Y. lipolytica* cell wall, the yeast was grown in 100 ml YPD (1% yeast extract, 2% bacto-peptone and 2% glucose). For solid media, 1.5% agar was added.

5.3.2 Plasmids

The plasmid pKOV410 (Fig. 5.1), an rDNA based multi-copy expresson vector was constructed at the Department of Microbial, Biochemical and Food Biotechnology, University of Free State, South Africa. Plasmid pRSET-B harbouring the gene encoding mCherry was kindly supplied by Dr. Lucy Moleleki of the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa. All sub-clonings of PCR products were done using pGEM®-T Easy vector (Promega, Madison, USA).

5.3.3 PCR amplification

Thermocycling reactions were carried out using MJ Mini Personal Thermal Cycler (BIO-RAD). PCR amplifications were performed using *Taq* polymerase (Fermentas). A PCR reaction mixture was prepared containing 1/10 volume reaction buffer with magnesium chloride, 10 mM dNTPs, 1.0 µM each of primer, 0.625 U *Taq* polymerase, 5 µg of DNA and topped up to a total volume of 50 µl with distilled water.

The thermal cycling conditions included an initial denaturation at 98°C for 30 sec, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 20 sec and extension at 72°C for 1 min, with a final extension step of 75°C for 5 min and held at 4°C.

5.3.4 DNA extraction, purification, restriction digestion and transformation

Yeast total genomic DNA from *Y. lipolytica* was extracted as described by Sambrook *et al.* (1989). Plasmids from *E. coli* transformants were isolated using Plasmid Isolation Kit (BioFlux) according to the manufacturer's instructions. PCR products were gel purified using Gel Extraction Kit (BioFlux) according to the manufacturer's instructions. Bacterial transformants were plated out onto LB agar plates containing 30 µg/ml of kanamycin or 50 µg/ml of ampicillin. *Y. lipolytica* transformation was performed as described by Xuan *et al.* (1988).

5.3.5 Construction of recombinant vector for surface display of mCherry

Primers for amplification of *YlCWP1* C-terminal end were designed based on the sequence of the gene (GeneBank Accession number: AY084077). The *YlCWP1* gene was amplified from the genomic DNA of *Y. lipolytica* Po1f strain using the forward primer Cwp1_F (5'-GGTACCATT**AAGCTT**ATGGGCAACGGTTACGCCGT-3'; underlined and bold bases indicate a *Kpn*I and *Hind*III sites, respectively) and the reverse primer Cwp1_R (5'-GGGCCTAGGCAATTAAGCTGTAATGAGGAG-3'; underlined bases indicate an *Avr*II site). PCR amplification was done as described above. PCR products (369 bp) were separated by agarose gel electrophoresis and recovered using a Gel Extraction Kit (BioFlux). Purified PCR products were subcloned into pGEM®-T Easy vector (Promega, USA) following the manufacturer's instructions and transformed into *E. coli* XL10 Gold. Recombinant vectors harbouring PCR products were extracted from *E. coli* transformants and purified using a Plasmid Isolation Kit (BioFlux).

To construct a recombinant cell surface displaying vector containing *mcherry*, the primers for amplification of the gene encoding mCherry were designed according to the sequence of the gene (GeneBank Accession number: HM771696) and published work by Lagendijk *et al.* (2010) with primers mC-F (5'-AAAGGTACCGGAATGGTGA

GCAAGGGCGAG-3'; underlined bases indicate a *Kpn*I site) and mC-R: (5'-TTTAAGCTTTAC**CTTGTCGTCGTCGTC***ATCGAT*TTTCTTGTACAGCTCGTCCAT-3'; underlined bases indicate a *Hind*III site, bold bases encode enterokinase cleavage sequence and italic bases indicate a *Cla*I site). PCR amplification was performed as described above. The plasmid pRSET-B was used as a template for amplification of *mcherry*. PCR products (756 bp) were digested with *Kpn*I and *Hind*III and ligated into pGem-*YlCWP1* digested with the same enzymes. The ligation reaction mixture was transformed into *E. coli* XL10 Gold. Recombinant vectors harbouring fusion genes were extracted from *E. coli* transformants and purified as described above. The resulting plasmid harbouring *mcherry* was named pGem-*cherry-YlCWP1*. The plasmid was subsequently digested with *Kpn*I and *Avr*II and digests ligated into pKOV410 digested with the same enzymes and transformed into *E. coli* XL10 Gold. Generated plasmid harbouring the fusion gene *mcherry-YlCWP1* was named pKOV410-*mcherry-YlCWP1* (Fig. 5.1).

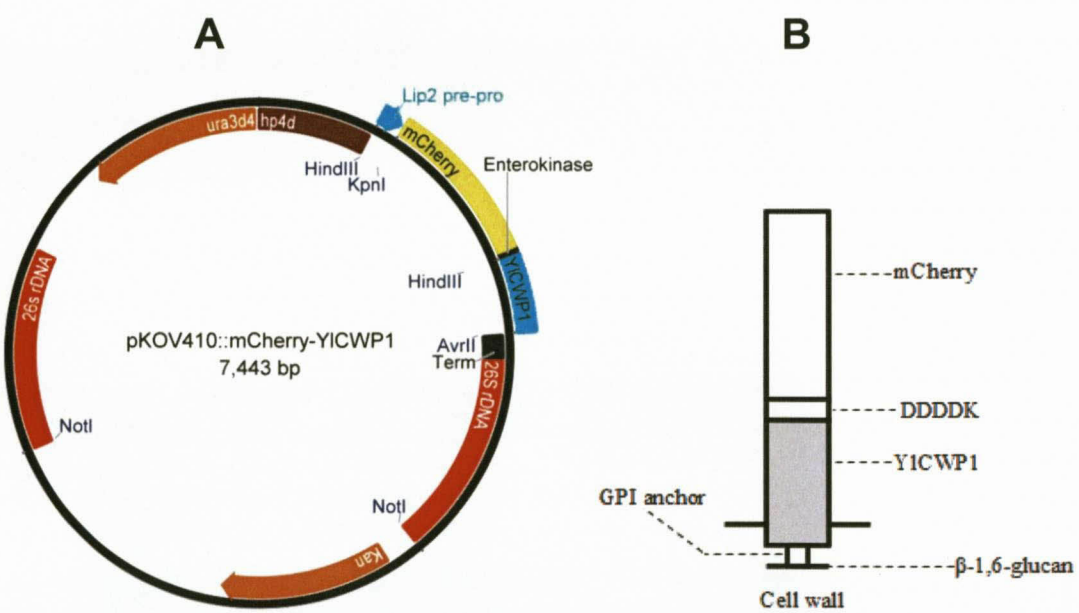

Figure 5.1: Schematic plasmid map of pKOV410-*mCherry-YlCWP1* expression vector and of the cell wall fusion proteins. (A) *mCherry-YlCWP1* fusion gene in plasmid pKOV410-*mCherry-YlCWP1* under the transcriptional control of hp4d promoter, pre-pro Lip2 for secretion of fusion proteins and terminator (Term). The plasmid contains *ura3d4* and *KanR* markers for selection. (B) mCherry-YlCWP1 covalently binds to β-1,6-glucan in the cell wall of *Y. lipolytica* through glycosylphosphatidylinositol (GPI) anchor. The plasmid map was constructed using Geneious v5.5 (Drummond *et al.*, 2011).

5.3.6 Yeast transformation

Recombinant plasmids pKOV410-*mcherry-YlCWP1* and pKOV410-*YlCWP1* were digested with *Not*I and the expression cassettes separated from the bacterial moiety by agarose gel electrophoresis. Bands of interest were recovered using a Gel Extraction Kit (BioFlux) and transformed into *Y. lipolytica* Po1f by lithium acetate method (Xuan *et al.,* 1988). Transformants were selected on YNB-casamino plates and isolated after 1 to 3 weeks of incubation at 28°C. Genomic DNA was extracted using the method of Chen *et al.* (1997) and used as a template to confirm integration of the expression cassettes into the yeast genome as described previously. The primer pair mC-F and Cwp1_R was used to amplify *mcherry-YlCWP1* fusion gene (data not shown) to check the integration of the fusion gene in yeast genome. The yeast transformants carrying pKOV410-*mcherry-YlCWP1* expression cassettes were denoted as Yl-mch1 and those carrying pKOV410-*YlCWP1* were denoted as Yl-p410.

5.3.7 Culture conditions

Yeast transformants, Yl-mch1 and Yl-p410 were inoculated into 25 ml of YPD medium and incubated overnight at 28°C with shaking at 200 rpm. When the culture reached an optical density at 600 nm (OD_{600}) of 2-3, cells were re-suspended to an OD_{600} of 1.0 in 100 ml YPD and incubated at 28°C with shaking at 200 rpm from 96 hours. Cultures were grown in shake flasks under aerobic conditions.

5.3.8 Analysis using fluorescence microscopy and flow cytometry

For detection of mCherry displayed on the cell wall of *Y. lipolytica* using YlCWP1, yeast cells in the culture medium were collected and washed three times by centrifugation at 16000 x *g* for 2 min at 4°C using phosphate-buffered saline (PBS pH 7.4). Yeast cells were visualised under fluorescence microscope (Olympus) at 492 nm and photographed. Following fluorescence detection, cells were analysed using flow cytometer (FACS Calibur, Becton Dickinson). Approximately 30 000 yeast cells were analysed for each sample and the data analysed using FlowJo.

5.3.9 Cleavage and identification of the displayed mCherry

Cells (1 ml) of Yl-mch1 and Yl-p410 cultivated for 96 h were harvested and washed three times by centrifugation at 16000 x *g* for 2 min with enterokinase buffer (20 mM Tris-HCl pH 8.0, 2 mM $CaCl_2$, 50 mM $NaCl_2$). Washed cells were resuspended into 1 ml of enterokinase buffer and 4 ng/ml of enterokinase (New England Biolabs, USA) was added to the cell suspension. The mixture was incubated at 16°C for 24 hours and 200 µl of the supernatant precipitated with acetone. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 12% polyacrylamide gel under denaturing conditions (Laemmli 1970).

The protein band at approximately 25 kDa on SDS gel was excised and cut into small chips. The sample was treated as described by Webster and Oxley (2005) and digested overnight with porcine trypsin (Promega, Madison, USA). MALDI-TOF-MS was performed using a QSTAR® Elite mass spectrometer (Applied Biosystems Inc., Ontario, Canada). The generated PMF data was 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).

5.4 Results

5.4.1 Immobilization of mCherry protein on *Y. lipolytica* cell surface

To evaluate the rDNA-based vector using YlCWP1 GPI-anchored protein for cell surface display on *Y. lipolytica* cell wall, a multi-copy plasmid for the display of mCherry as a model protein was constructed (pKOV410-*mcherry-YlCWP1*, Fig. 5.1A). For displaying mCherry on the cell surface of *Y. lipolytica*, its encoding sequence containing an enterokinase cleavage site at its C-terminal was fused to the *N*-terminal of the *YlCWP1* encoding sequence. The *mcherry* and *YlCWP1* fusion gene was linked by an enterokinase cleavage site (DDDDK) which is essential for cleavage of mCherry protein from the yeast cell surface after expression. The fusion gene was inserted into the multi-cloning site of the multi-copy plasmid, pKOV410, downstream of the pre-pro Lip2 secretion signal under the control of hp4d promoter. The translated fusion sequence is as shown in figure 5.2.

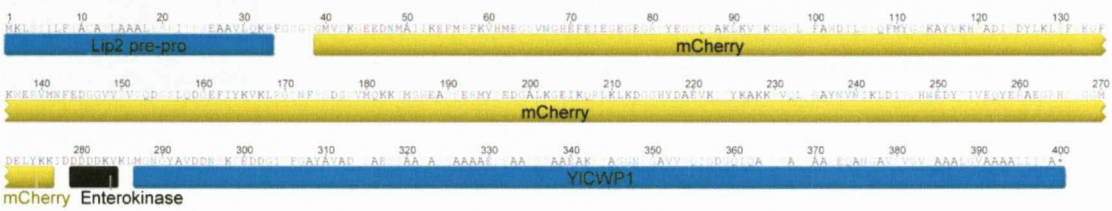

Figure 5.2: Amino acid sequence of the mCherry-Ylcwp1 fusion and the lip2 secretion signal. The diagram shows position of the proteins and the enterokinase cleavage site. The alignment was constructed using Geneious v5.5 (Drummond *et al.*, 2011).

The expression cassettes were transformed into *Y. lipolytica* Po1f using the method of Xuan *et al.* (1988). *Y. lipolytica* transformants carrying *mCherry* were confirmed by PCR (data not shown). Yl-mch1 transformants growing on YNB-casamino selective plates showed a purple colour (Fig. 5.3A) compared to Yl-p410 control transformants (Fig. 5.3B). The change in colour of Yl-mch1 transformants indicated that mCherry was displayed on the *Y. lipolytica* cells. Yl-mch1 transformed cells exhibited reddish colour even when grown in YPD liquid medium (Fig. 5.3C) in comparison to the control cells (Fig. 5.3D).

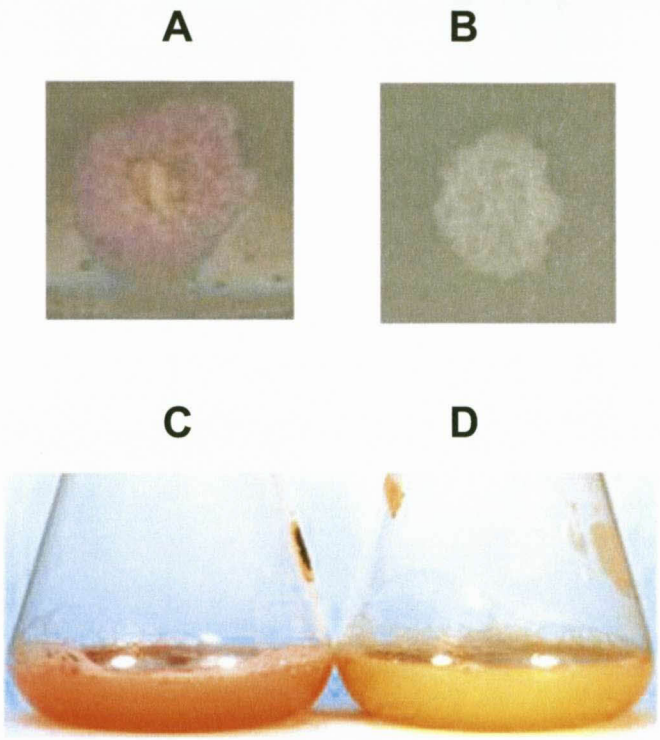

Figure 5.3: Effect of displayed mCherry on the *Y. lipolytica* transformants and culture media. (A) Photograph of cells carrying mCherry-YlCWP1, the cells showed a pinkish-reddish colour. (B) Photograph of the control cells carrying YlCWP1, cells did not show any colour change. (C) Photograph of shake flask culture media containing *Y. lipolytica* displaying mCherry proteins, the culture medium showed a change in colour during the cultivation period which was a result of the displayed mCherry. (D) Photograph of the shake flask culture media containing *Y. lipolytica* control cells, culture media did not show any change in colour during the cultivation period.

Successful display of mCherry on the cell surface of cultivated *Y. lipolytica* transformants was also confirmed by fluorescent microscopy. Yl-p410 cells were used as control. Fluorescence results in Fig. 5.4 showed a strong fluorescence indicating that Yl-mch1 cells successfully displayed mCherry (Fig. 5.4B) whereas no fluorescence was observed on the control cells (Fig. 5.4D). Quantitative expression of mCherry on Yl-mch1 cells was analysed using flow cytometer (Fig. 5.4E). About 75% of Yl-mch1 cells expressed mCherry on their surface. These results strongly suggest that mCherry was successfully expressed on the cell surface of *Y. lipolytica*.

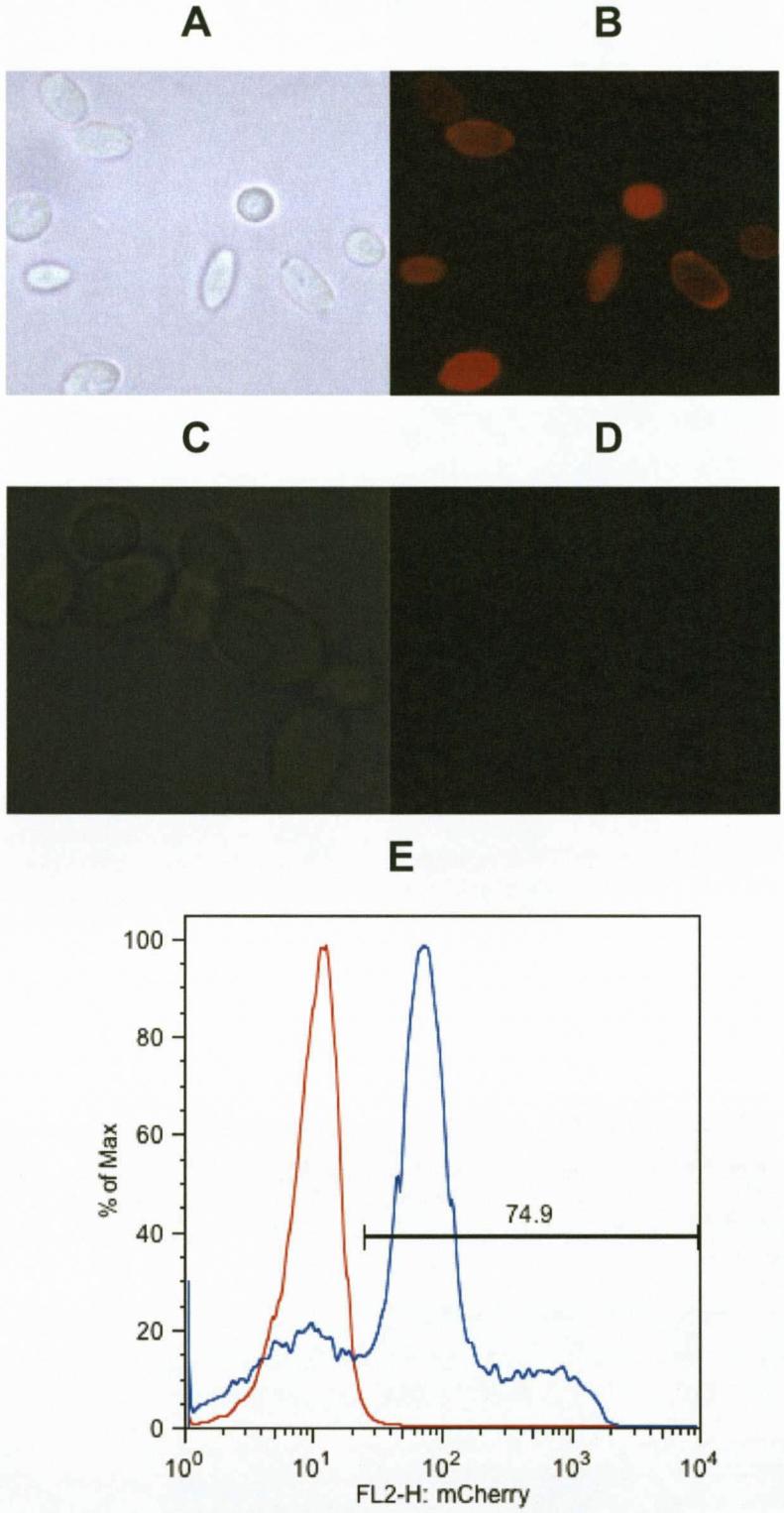

Figure 5.4: Microscopic and flow cytometric photographs of *Y. lipolytica* cells transformed with pKOV410-*mCherry-YlCWP1* (A & B) and pKOV410-*YlCWP1* (C & D) expression cassettes. *Y. lipolytica* cells in A and C were photographed under visible light and the cells in B and D were photographed under UV light (492 nm). Flow cytometry histograms shown in (E) depict the mean fluorescent signal of mCherry displayed on *Y. lipolytica* cells (in blue) and control cells (in red).

5.4.2 Cleavage of mCherry displayed on *Y. lipolytica* cells

In order to obtain free mCherry, Yl-mch1 cells were treated with enterokinase. The Yl-mch1 cells after treatment with enterokinase retained the purple colour phenotype. An approximately 25 kDa protein was detected on SDS-PAGE (Fig. 5.5). To ascertain that the observed protein band on the SDS-PAGE was mCherry, the band was subjected to MALTI-TOF-MS peptide mass fingerprinting. The generated data was analysed using Mascot BLAST and NCBI. BLAST searches of the 23 identified peptide mass fingerprints gave 95% match to a synthetic monomeric red fluorescent protein (Gen Bank Accession number: AAV52164). These results indicate that mCherry was successfully displayed on *Y. lipolytica* cells. Additionally, the displayed mCherry could be removed from the cell surface by treatment with enterokinase.

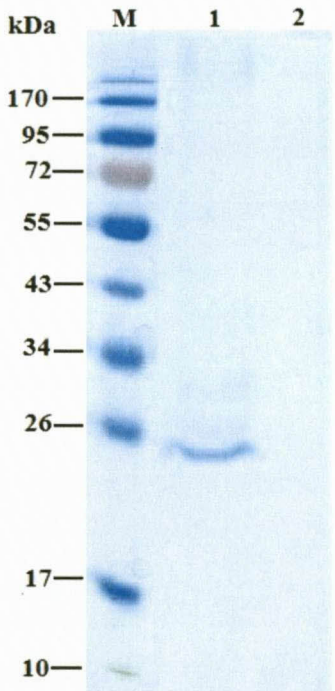

Figure 5.5: SDS-PAGE analysis of mCherry cleaved from *Y. lipolytica* cell surface. Lane M: Prestained protein molecular weight marker (NEB); Lane 1: m-Cherry cleaved from *Y. lipolytica* cells with enterokinase; Lane 3: *Y. lipolytica* control cells treated with enterokinase.

5.5 Discussion

Cell surface display has shown great potential for various applications, such as whole-cell biocatalysis and combinatorial library construction (Furukawa *et al.,* 2006). Its application in *Y. lipolytica*, which has been reported to secrete a wide range of proteins (Beckerich *et al.,* 1998), could be essential for immobilization of active heterologous proteins. In this study, we constructed a novel rDNA based plasmid for surface display of heterologous proteins on *Y. lipolytica*. The plasmid uses the *ura3d4* allele for homologous multiple integration into the rDNA cluster (Juretzek *et al.,* 2001). Similar to the zeta-based plasmid constructed by Yue *et al.* (2007) for cell surface display of heterologous proteins on *Y. lipolytica*, the constructed rDNA based displaying plasmid in this study uses the strong recombinant growth phase dependent hp4d promoter (Madzak *et al.,* 2004). The hp4d promoter has traits optimal for heterologous protein expression as it operates almost unaffected by environmental conditions such as pH, carbon and nitrogen sources and presence of peptone (Madzak *et al.,* 1995, 2000). In addition, the expression cassette used to transform *Y. lipolytica* is devoid of a bacterial moiety including antibiotic resistance genes as a result retaining its GRAS (generally regarded as safe) status (Nicaud *et al.,* 1998; Pignede *et al.,* 2000a). Because of these characteristics, the plasmid pKOV410 was used to construct a novel plasmid for enhanced cell surface display on *Y. lipolytica* using the GPI-anchored *YlCWP1*.

The efficiency of the displaying plasmid for enhanced display of heterologous proteins was demonstrated using mCherry as a model protein. When *Y. lipolytica* was transformed with the expression cassettes, purple transformants were observed on the YNB-casamino selective plates compared to the *Y. lipolytica* negative control transformants (Fig. 5.3). This colour change on the transformants served as a quick visual indication that mCherry was displayed on the yeast cell surface. The purple colour was observed on cells grown on both solid agar plates and liquid YPD medium, respectively (Fig. 5.3A, C). Similar results were first reported by Keppler-Ross *et al.,* (2008) in *S. cerevisiae* overexpressing codon optimised enhanced monomeric red fluorescent protein (EmRFP). In addition, Gerami-Nejad *et al.* (2009) constructed a synthetic codon optimised monomeric red fluorescent protein from *Discosoma sp.* (DsRed) that produced transformants detectable at colony level based on colour. Recent studies by Wu *et al.* (2011) demonstrated a change in colour of *E. coli* cells displaying enhanced green fluorescent protein (EGFP) after

induction with IPTG. However, the fluorescence was insufficient to change the colour of the growth medium to green. Studies by Kuroda *et al.* (2009) did not report transformants colour changes when the DsRed-monomer was displayed on *S. cerevisiae* cell surface. To our knowledge, the purple colour change observation in the Yl-mch1 engineered in this study constitutes the first report of such an effect of displayed mCherry on both solid and liquid medium.

Yl-mch1 cells exhibited red fluorescence under fluorescent microscopy, but no red fluorescence was observed on the control cells (Fig. 5.4). The fluorescent protein, mCherry, emits in the red wavelengths of the visible spectrum (Castro-Longoria *et al.,* 2010). Previous studies using the GPI-anchored YlCWP1 have reported successful display of heterologous proteins on *Y. lipolytica* cell surface (Yue *et al.,* 2007; Ni *et al.,* 2009; Liu *et al.,* 2009, 2010; Yu *et al.,* 2010). The expression level of displayed mCherry on *Y. lipolytica* cell wall was evaluated using flow cytometry (Fig. 5.4E). The results indicate that approximately 75% of Yl-mch1 cells displayed mCherry. These results can not be compared to those of Yue *et al.*, (2007) that reported 100% cells displaying eGFP as no quantitative analysis using flow cytometry was done but relied on microscopy for quantitative analysis. Following treatment of the cells with enterokinase, free mCherry was detected on SDS-PAGE (Fig. 5.5) and confirmed by MALDI-TOF-MS peptide mass fingerprinting. Most studies have been unable to report detection of free heterologous protein on SDS-PAGE after cleavage of the displayed proteins on cells. Previous studies have relied on western blot analysis for detection of cleaved proteins (Wang *et al.,* 2007, 2008; Jiang *et al.,* 2007). Because of enhanced expression of mCherry on the cell surface of *Y. lipolytica*, high levels of mCherry were observed on the yeast cells after cleavage with enterokinase as indicated by the purple colour phenotype. This incomplete cleavage of mCherry from the cell surface is probably due to inefficient proteolysis and could require optimization for complete cleavage. Accessibility of displayed heterologous proteins on yeast cells for cleavage has been investigated by Kuroda *et al.* (2009) using DsRed-monomer as a model on *S. cerevisiae* cells. More recently, Wu *et al.* (2011) reported detection of free recombinant EGFP on SDS-PAGE. Unlike the studies by Wu *et al.* (2011) which purified EGFP using the histidine tag, the mCherry in this study did not require any purification as it was cleaved as a single dominant protein.

In this study, we have developed a method for high-level expression of mCherry on *Y. lipolytica* cell surface using an rDNA based cell surface displaying plasmid. Because of colour development and visual detection of the transformed colonies, the rDNA-based plasmid together with mCherry could be used as for visual screening in identifying new cell wall proteins in *Y. lipolytica*. This method will be similar to that used for selection of white and blue bacterial colonies associated with the disruption of *lacZ*-encoded β-galactosidase and those reported by Keppler-Ross *et al.* (2008) and Gerami-Nejad *et al.* (2009). The constructed plasmid offers an alternative approach for the purification of heterologous proteins displayed on *Y. lipolytica* cell wall. Displayed proteins can be cleaved with enterokinase to obtain pure proteins without the need to purify using chromatographic methods. The developed system is highly efficient for downstream processing of displayed heterologous proteins. Cell surface display has great potential for various applications such as whole-cell biocatalysis and combinatorial library construction (Furukawa *et al.*, 2006). Application of cell surface display on *Y. lipolytica* could be essential for immobilization and purification of displayed heterologous proteins at lower costs. The developed displaying system offers great potential for industrial expression and purification of heterologous proteins.

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Chapter 6. Cell surface display of active *Fusarium solani pisi* cutinase and *Thermomyces lanuginosus* lipase on *Yarrowia lipolytica*

6.1 Abstract

Fusarium solani cutinase (FSCut) and *Thermomyces lanuginosus* lipase (TLL) encoding gene sequences were cloned into the rDNA based displaying plasmid and displayed on *Yarrowia lipolytica* Po1f cell surface using YlCWP1 as an anchor. Halo assay using 1% tributyrin agar plates revealed that both lipolytic enzymes were expressed on the yeast cell surface after 24 h incubation at 28°C. Displayed FSCut and TLL showed high level activity towards *p*-nitrophenyl palmitate as compared to the control cells, 137.7 ± 2.5, 94.3 ± 1.9 and 1.2 ± 0.01 U/g (dry cell), respectively. Both displayed enzymes showed optimal temperature and pH at 35°C and pH 9.0. The activity of displayed FSCut was not adversely affected by both EDTA and Ca^{2+} suggesting FSCut is not calcium dependent, whereas SDS, Triton X-100 and Tween-80 adversely inhibited the activity of displayed FSCut. The activity of displayed TLL was increased three-fold by Ca^{2+} but inhibited by EDTA, suggesting that the displayed TLL is calcium dependent. Although, the activity of displayed TLL was slightly enhanced by DMSO, its activity was adversely inhibited by SDS, Triton X-100 and Tween-80. Both displayed enzymes exhibited a high degree of thermostability.

6.2 Introduction

In recent years, the yeast cell surface display system has been of interest for display and expression of functionally active heterologous proteins. A yeast surface display system is an important tool for increasing affinity, specificity and stability of displayed heterologous proteins (Boder and Wittrup, 1997). A wide range of yeast cell surface displaying proteins have been used as anchors for display of different heterologous proteins (Kondo and Ueda, 2004; Jiang *et al.*, 2007; Yue *et al.*, 2007; Khasa *et al.*, 2011). Yeast surface display of enzymes as whole-cell catalysts has been found to be more attractive than traditional immobilisation of enzymes as they require simple genetic manipulation and no purification (Shibasaki *et al.*, 2009; Liu *et al.*, 2010a, b). In addition, cell surface display system compared to traditional immobilisation system has shown some advantages such as enzyme stability and lower cost associated with downstream processing (Washida *et al.*, 2001; Matsumoto *et al.*, 2002). Therefore, enzymes displayed on the yeast cell surface have a potential to be effective whole-cell biocatalysts with great potential in bioconversion processes (Su *et al.*, 2010a; Inaba *et al.*, 2010; Chen *et al.*, 2011).

Lipases (EC 3.1.1.3) and cutinases (EC 3.1.1.74) represent the most important class of lipolytic industrial biocatalysts (Jaeger and Reetz, 1998; Egmond and de Vlieg, 2000). In biotechnology, cell surface display of lipases has presented many applications as whole-cell catalysts with potential industrial application (Su *et al.*, 2010b; Chen *et al.*, 2011; Yuzbasheva *et al.*, 2011). *Yarrowia lipolytica, a* non-conventional dimorphic yeast, is known for secretion of lipases such as Lip2, Lip7 and Lip8 (Pignede *et al.*, 2000; Fickers *et al.*, 2005; Fickers *et al.*, 2011). These lipases have been successfully displayed on *Saccharomyces cerevisiae's* (Liu *et al.*, 2010a, b) and *Pichia pastoris's* (Jiang *et al.*, 2007; Su *et al.*, 2010b; Zhang *et al.*, 2010; Zhao *et al.*, 2011) cell surface. Although lipases have been of interest as whole-cell biocatalysts, only the study of Yuzbasheva *et al.* (2011) has reported display of Lip2 on *Y. lipolytica*. As a prolific producer of lipases, *Y. lipolytica* holds a potential as a whole-cell biocatalyst that can be used in detergent and food industries. In this chapter, an rDNA based displaying plasmid was assessed for the display of functionally active *Fusarium solani pisi* cutinase (FSCut) and *Thermomyces lanuginosus* lipase (TLL) on *Y. lipolytica* cells using YlCWP1 as an anchor. The biochemical properties such as thermostability, effects of detergents,

metal ion and solvent solution on displayed His-tagged FSCut and TLL were evaluated.

6.3 Material and methods

6.3.1 Strains plasmids and media

Escherichia coli XL10 Gold (Stratagene) was used as a host for recombinant DNA manipulation and was grown in 5.0 ml Luria-Bertani medium or agar plates at 37°C overnight containing 30 µg/ml of kanamycin or 50 µg/ml of ampicillin. For solid media, 1.5% agar was added. *Y. lipolytica* Po1f strain (*MatA, leu2-270, ura3-302, xpr2-322, axp1-2*) was used as host for cell surface display (Madzak *et al.*, 2000). For selection of Uracil$^+$ (Ura$^+$) clones, yeast host cells were selected on YNB-casamino acid plates (0.17% YNB without amino acids and ammonium sulfate, 1% glucose, 0.1% casamino acids, 0.1% sodium glutamate and 1.5% agar). Yeast transformants were aerobically cultivated in 100 ml YPD (1% yeast extract, 2% bacto-peptone and 2% glucose). The plasmids, pGem-*YlCWP1* was constructed in our laboratory and plasmid pKOV410 was used previously for cell surface display (Bulani *et al.*, 2012). The plasmids pCUT and pTLL harbouring cutinase (*cut*) gene and lipase (*tll*) gene, respectively, were purchased from GeneArt (Germany). All sub-clonings of PCR products were done using pGEM®-T Easy vector (Promega, Madison, USA). Chromogenic agar substrate plates used for selection of *Y. lipolytica* transformants expressing FSCut and TLL were prepared as described by Singh *et al.* (2006).

6.3.2 Construction of plasmids

All standard DNA manipulation procedures were performed according to Sambrook *et al.* (1989). The open reading frames (ORF) of the C-terminally histidine (His) tagged FSCut and TLL encoding sequences were codon optimised and synthesised at GeneArt (Regensbung, Germany) for expression in *Y. lipolytica*. FSCut ORF encoding sequence (*cut*) was amplified from the plasmid pCUT using the forward primer FSCut_F 5'-AAAGGATCCATGCTGGGCCGAACCACCCGA-3' (underlined bases encode *BamH*I site) and the reverse primer FSCut_R 5'-TTTAAGCTTCGTGGTGATGGTGGTGGTG-3' (underlined bases encode *Hind*III site). For amplification of TLL ORF encoding sequence (*tll*), the plasmid pTLL was used as a template using the forward primer TLL_F 5'-AAAAGGTACCATGTCTCC CATCCGACGA-3' (underlined bases encode *Kpn*I site) and reverse primer TLL_R 5'- TTTAAGCTTCGTGGTGATGGTGGTGGTG-3' (underlined bases encode *Hind*III

site). Polymerase chain reaction (PCR) was carried out using MJ Mini Personal Thermal Cycler (BIO-RAD). A PCR reaction mixture was prepared containing 1/10 volume reaction buffer with magnesium chloride, 10 mM dNTPs, 1.0 µM each of primer, 0.625 U *Taq* polymerase, 5 µg of DNA and topped up to a total volume of 50 µl with distilled water. Thermal cycling conditions included an initial denaturation at 98°C for 30 sec, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 20 sec and extension at 72°C for 1 min, with a final extension step of 75°C for 5 min and held at 4°C. Amplified fragments of *cut* and *tll* were digested with *BamH*I and *Hind*III, *Kpn*I and *Hind*III, respectively, and jump-cloned into pGem-*YlCWP1* to generate pGem-*cut*-*YlCWP1* and pGem-*tll*-*YlCWP1*. The plasmids pGem-*cut*-*YlCWP1* and pGem-*YlCWP1* were digested with *BamH*I and *Avr*II, whereas the plasmid pGem-*tll*-*YlCWP1* was digested with *Kpn*I and *Avr*II, and ligated into pKOV410 digested with the same enzymes to generate pKOV410-*cut*-*YlCWP1*, pKOV410-*YlCWP1* and pKOV410-*tll*-*YlCWP1* respectively (Fig. 6.1).

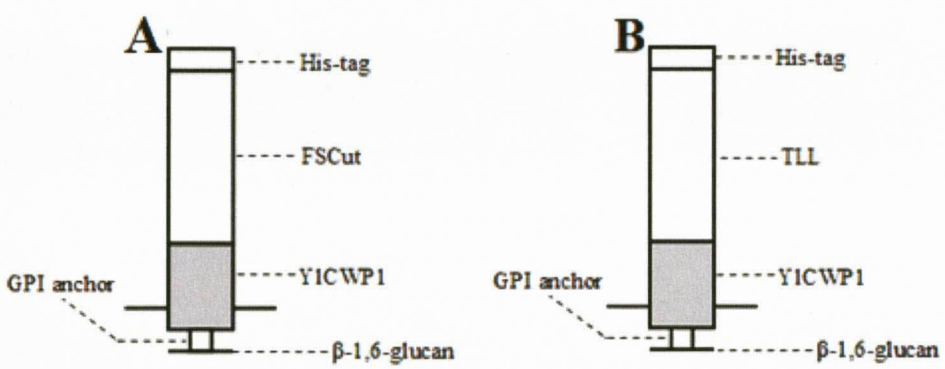

Figure 6.1: Construction of the cell surface displaying plasmids for C-terminally His-tagged FSCut and TLL. Schematic diagrams of (A) FSCut-YlCWP1p and (B) TLL-YlCWP1p fusion proteins covalently bound to β-1,6-glucan on the cell wall of *Y. lipolytica* through glycosylphosphatidylinositol (GPI), respectively.

6.3.3 Yeast transformation

Plasmids pKOV410-*cut*-*YlCWP1*, pKOV410-*tll*-*YlCWP1* and pKOV410-*YlCWP1* were digested with *Not*I and the expression cassettes separated from the bacterial moiety by agarose gel electrophoresis. Expression cassettes were transformed into *Y. lipolytica* Po1f by lithium acetate method (Xuan *et al.*, 1988) and spread on YNB-casamino selective medium plates. Transformants were isolated after 1 to 3 weeks of incubation at 28°C. Genomic DNA was extracted using the method of Chen *et al.*

(1997) and used as a template to confirm integration of the expression cassettes into the yeast genome as described previously. The primer pair FSCut-F (5'-AAAGGATCCATGCTGGGCCGAACCACCCGA-3') and Cwp1_R (5'-GGGCCTAGG CAATTAAGCTGTAATGAGGAG-3') was used to amplify *cut-YlCWP1* fusion gene. The primer pair TLL_F (5'-AAAAGGTACCATGTCTCCCATCCGACGA-3') and Cwp1_R was used to amplify *tll-YlCWP1* fusion gene. Yeast transformants carrying pKOV410-*cut-YlCWP1* and pKOV410-*tll-YlCWP1* expression cassettes were denoted as Yl-FSCut and Yl-TLL respectively, whereas those carrying pKOV410-*YlCWP1* (negative control) were denoted as Yl-p410.

6.3.4 Culture conditions

Yeast transformants Yl-FSCut, Yl-TLL and Yl-p410 were inoculated into 25 ml of YPD medium and incubated overnight at 28°C with shaking at 200 rpm. When the culture reached an optical density at 600 nm (OD_{600}) of 2-3, the cells were re-suspended to an OD_{600} of 1.0 in 100 ml YPD and incubated at 28°C with shaking at 200 rpm for 96 hours. Cultures were grown in shake flasks under aerobic conditions. Yeast cells were collected by centrifugation at 6,000 x *g* at 4°C for 10 min and washed three times with distilled water. The cell suspension was adjusted to an OD_{600} of 3.0 in distilled water. Following centrifugation of 1 ml of the cell suspension, the dry cell weight was measured by drying the pellet in an oven at 70°C until a constant weight was reached.

6.3.5 Halo assay

Yl-FSCut, Yl-TLL and Yl-p410 transformants were inoculated on tributyrin chromogenic plates [(1.0% trybutyrin sonically emulsified in 10 mM $CaCl_2$, 0.01% phenolphthalein, 2.0% agar and the pH adjusted to 7.3/7.4 using 0.1 N NaOH)] as described by Singh *et al.* (2006) and incubated for 24 h at 28°C. The activity of the displayed lipolytic enzymes was estimated by the halos formed around the yeast colonies.

6.3.6 Detection of displayed FSCut and TLL activity

Activity of the C-terminally His-tagged FSCut and TLL was determined spectrophotometrically using *p*-nitrophenyl palmitate (*p*-NPP) (Vorderwulbecke *et al.*, 1992). The substrate *p*-NPP stock solution was prepared by dissolving 15 mg of *p*-NPP in 5 ml of isopropanol. After emulsifying the solution by sonication (HD 2070 Bandelin, Sonopuls), the assaying substrate was prepared by adding 1 ml of the stock solution into 9 ml of 50 mM Tris-HCl, pH 9.0 buffer drop wise to get an emulsion that remained stable for 2 h. The reaction was carried out in 1 ml of the substrate solution for 5 min at 40°C after adding 100 µl cell suspensions (OD_{600} of 3.0), 100 µg dry weight cells. For determination of lipolytic activity in the supernatant, 100 µl of each culture supernatant was used. The liberated *p*-nitrophenol from the hydrolysis of *p*-NPP was measured at 410 nm using DU 800 spectrophotometer (Beckman Coulter). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol *p*-nitrophenol per min. All assays were done in duplicates.

6.3.7 Characterization of displayed FSCut and TLL

The optimum temperature of FSCut and TLL activity was measured at different temperatures from 20-60°C at pH 9.0 in 50 mM Tris-HCl buffer. The pH optimum of the lipolytic enzymes was determined from pH 5.0-10.0 at 35°C using 50 mM Tris-HCl buffer. For temperature stability, yeast cells were incubated at 25, 30, 35, 40 and 45°C for up to 3 h in 50 mM Tris-HCl buffer, pH 9.0 and the residual activity measured at 1 h time intervals. Metal ions, detergents and solvent solution were added to the substrate solution respectively and their effects on displayed FSCut and TLL determined.

6.4 Results and discussion

6.4.1 Construction of *Y. lipolytica* cell surface displaying plasmids

The plasmids pKOV410-*cut-YlCWP1* and pKOV410-*tll-YlCWP1* were successfully used for surface display of FSCut and TLL on *Y. lipolytica* cells, respectively (Fig. 6.1). The mature encoding sequences of FSCut and TLL containing a His-tag at the 3' end were fused to the 5' end of the gene encoding the C-terminal end of YlCWP1 GPI-anchored protein. The fusion genes were inserted downstream of the pre-pro Lip2 secretion signal under the control of hp4d promoter in the rDNA based multi-copy plasmid, pKOV410. The constructed expression cassettes were integrated into the genome of *Y. lipolytica* Po1f strains. Functional expression of FSCut and TLL was assessed by spotting yeast cells harbouring the respective expression cassettes onto chromogenic agar plates containing 1% tributyrin (Singh *et al.*, 2006). Clear halos were observed around yeast colonies harbouring FSCut (Yl-FSCut) and TLL (Yl-TLL) (Fig. 6.2) after 24 h incubation at 28°C. No halo was formed around the control cells (Yl-p410). These results indicated that active FSCut and TLL were displayed on the cell surface of *Y. lipolytica*. Studies by Yuzbasheva *et al.* (2011) reported display of lip2 on *Y. lipolytica* cells using a zeta-based plasmid, in this study an rDNA plasmid previously used for display of mCherry (Bulani *et al.*, 2012) was employed for displaying functionally active His-tagged FSCut and TLL proteins.

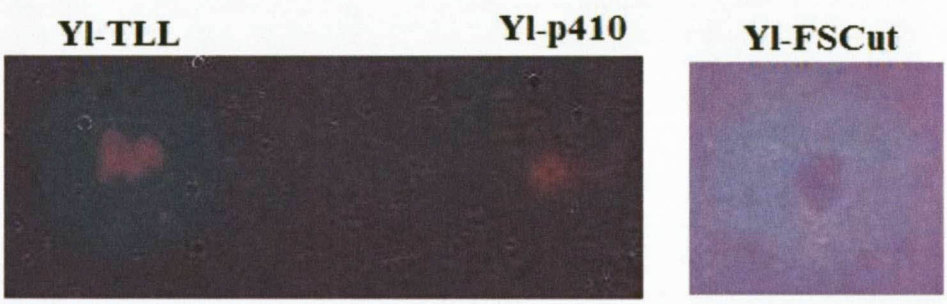

Figure 6.2: Halo formation of Yl-FSCut- and Yl-TLL on 1% tributyrin chromogenic agar plates. Clear halos were observed around Yl-FSCut and Yl-TLL, no halos were observed around Yl-p410.

6.4.2 Activity assay of displayed FSCut and TLL

Hydrolytic activity of displayed FSCut and TLL were determined using *p*-NPP as a substrate (Liu *et al.*, 2010a). The measured hydrolytic activity of the whole cells Yl-FSCut, Yl-TLL and Yl-p410 were 137.7 ± 2.5, 94.3 ± 1.9 and 1.2 ± 0.01 U/g (dry cell) after 96 h of cultivation at 28°C, respectively. This high difference in lipolytic activity of yeast cells displaying Yl-FSCut and Yl-TLL compared to the control cells (Yl-p410) further confirmed that FSCut and Yl-TLL were displayed on the yeast cells. The level of activity of displayed FSCut towards *p*-NPP was comparable to that of *Alternaria brassicicola* Cut engineered by site-directed mutagenesis for improved activity of the enzyme and expressed in *P. pastoris* (Koschorreck *et al.*, 2010). FSCut displayed on the surface of *S. cerevisiae* cells using α-agglutinin and on *E. coli* cells using EstA, has been reported to exhibit good activity towards *p*-nirophenyl butyrate and *p*-nirophenylcaprylate, respectively (Schreuder *et al.*, 1996; Becker *et al.*, 2005). Hydrolytic activity towards *p*-NPP has also been reported for TLL immobilised in polyvinyl alcohol (PVA) alginate beads (Cruz-Ortiz *et al.*, 2011) and free TLL towards *p*-nitrophenol butyrate (Mogensen *et al.*, 2005).

Lipolytic activity was also found in the supernatant of the yeast cultures. The measured hydrolytic activity of the supernatant from Yl-FSCut, Yl-TLL and Yl-p410 were 0.092 ± 0.004, 0.053 ± 0.001 and 0.015 ± 0.001 U/ml, respectively. Considerable amounts of lipolytic activity have been shown in the growth medium of *Y. lipolytica* cells displaying the lipase Lip2 (Yuzbasheva *et al.*, 2011), whereas negligible lipase activity in the supernatant of *S. cerevisiae* and *P. pastoris* displaying lipases have been reported (Liu *et al.*, 2010a, b; Su *et al.*, 2010b). Studies by Yuzbasheva *et al.* (2011) using Ylcwp1, Ylcwp3 and Ylcwp6 for displaying Lip2 suggest that these cell wall proteins have low cell wall retention efficiency. In addition *Y. lipolytica* is a reported to produce native lipases (Pignede *et al.*, 2000; Fickers *et al.*, 2005) hence lipolytic activity could be detected on Yl-p410.

6.4.3 Characterization of displayed FSCut and TLL

Yl-FSCut and Yl-TLL cells were characterised for application as whole-cell biocatalysts. The activity of displayed FSCut and TLL was determined from 25 to 60°C. The highest activity for both displayed lipolytic enzymes was observed at 35°C. The optimum temperature of the displayed FSCut was consistent with that of FSCut

expressed to the extracellular medium in *Y. lipolytica* (chapter 2). The optimum temperature of displayed TLL was not consistent to those expressed in *Y. lipolytica* to the extracellular medium (Chapter 3 and 4). At their optimum temperature, the displayed FSCut showed higher activity compared to the displayed TLL. The activity of displayed FSCut dropped sharply above 35°C retaining about 12% of its activity at 60°C (Fig. 6.3A). Although, displayed YI-TLL showed lower activities between 25 to 40°C than those of displayed FSCut, it maintained activities above 80% between 40 and 55°C, retaining about 50% of its activity at 60°C (Fig. 6.3A).

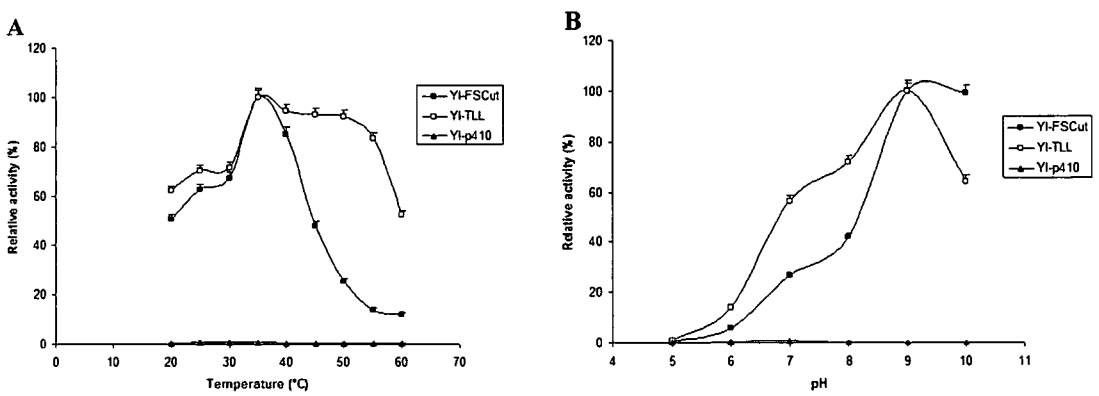

Figure 6.3: Effect of (A) temperature and (B) pH on the activity of surface displayed FSCut and TLL. The activity at 35°C was defined as 100% for the displayed, (■) FSCut [137.7 U/g (dry cell)], (□) TLL [94.3 U/g (dry cell)] and (▲) YI-p410 [1.2 U/g (dry cell)]. All measurements were done in duplicates.

The effect of pH on the displayed FSCut and TLL was determined over a pH range of 5-10. Displayed FSCut and TLL showed optimal activity at pH 9.0 (Fig. 6.3B). FSCut showed its highest activity at more alkaline conditions, exhibiting no loss of activity at pH 10.0 as compared to TLL, which retained about 60% of its activity. In Chapter 2, pH optimum of the expressed FSCut in *Y. lipolytica* was affected by the presence of the His tag. His-tagged FSCut showed an optimum activity at pH 8.0 and non-tagged FSCut showed its optimum activity at pH 9.0. The optimal pH of the displayed FSCut was consistent with that of non-tagged FSCut (Chapter 2). The His-tag on the displayed FSCut did not affect the enzyme negatively compared to when the enzyme was expressed in the extracellular medium using *Y. lipolytica* as a host. Unlike FSCut whose pH optimum is affected by the presence of a His-tag, displayed TLL expressed in *Y. lipolytica* retained its pH optimum at pH 9.0. This result is consistent with the result observed when TLL is expressed as a tagged enzyme.

These results suggest that the His-tag on the displayed FSCut and TLL does not affect the folding conformation of the enzymes and subsequently its activity. The effect of the His-tag on the activity of heterologous proteins displayed on *Y. lipolytica* has been shown to vary for different proteins. The activity of His-tagged alkaline protease and alginate lyase displayed on *Y. lipolytica* were reported to be higher than those of the same enzymes without a His-tag displayed on the same yeast (Ni *et al.*, 2009; Liu *et al.*, 2009). Contrary to the increased activity of the His-tagged alkaline protease and alginate lyase, the activity of acid protease without a His-tag displayed on *Y. lipolytica* was found to be much higher than that of displayed acid protease containing a His-tag (Yu *et al.*, 2010).

Thermostability of both displayed lipolytic enzymes was evaluated after incubating the cells between 25-45°C for 1-3 h (Fig. 6.4). The whole cell YI-FSCut showed high stability of displayed FSCut between 20-35°C (Fig. 6.4A). FSCut on *Y. lipolytica* cells remained consistent and retained above 98% of its original activity at both temperatures 25 and 30°C after 3 h. At 35°C, the FSCut retained about 93% of its original activity after 1 h of incubation and after 3 h it decreased to approximately 90%. Displayed FSCut showed low thermostability at 40 and 45°C, retaining 40 and 10%, respectively, of its original activity after incubation for 1h with complete inactivation after 3h at 45°C. For the thermostability of displayed TLL, TLL retained 73, 64 and 86% of its original activity for 3 h at 25, 30 and 35°C, respectively. Only 60 and 46% of the activity was left when the cells displaying TLL were incubated for 3 h at 40 and 45°C, respectively (Fig. 6.4B).

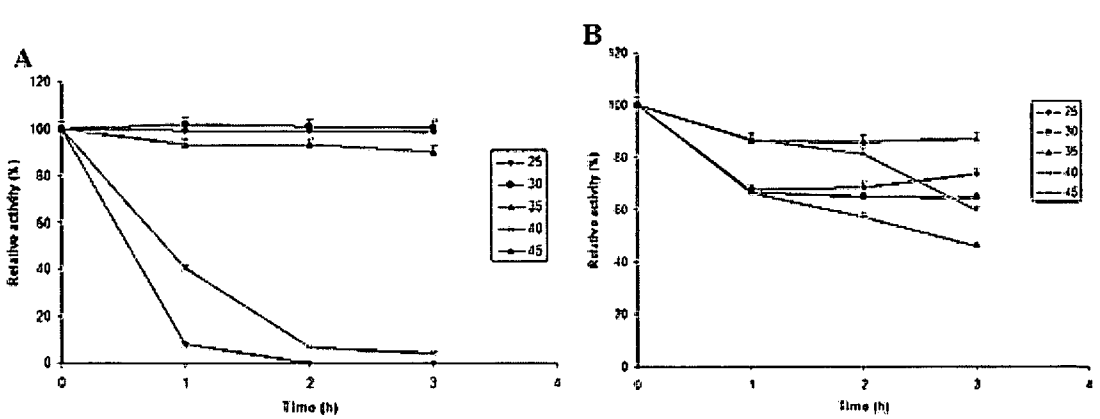

Figure 6.4: Thermostability of displayed FSCut and TLL. The displayed (A) FSCut and (B) TLL were incubated at 35-45°C for 1-3 h in 50 mM Tris-HCl buffer, pH 9.0. The activity at 35°C was defined as 100% for the displayed FSCut [137.7 U/g (dry cell)] and TLL [94.3 U/g (dry cell)]. All measurements were done in duplicates.

The effects of detergents, metal ions and solvent solution on the activity of displayed FSCut are shown in Table 6.1. The activity of displayed FSCut was slightly enhanced by Ca^{2+} suggesting that displayed FSCut is not Ca^{2+} dependent as it showed high activity in the absence of Ca^{2+} (Egmond and van Bemmel, 1997). The activity of the displayed FSCut was slightly affected by DMSO and EDTA. SDS and the non-ionic surfactants, Triton X-100 and Tween-80, exhibited a negative effect on the activity of displayed FSCut.

Table 6.1 Effect of detergents and solvent solution on displayed FSCut

Factor	Concentration	Relative activity (%)[a]
Control		100 ± 1.8
$CaCl_2$	1 mM	107.5 ± 0.71
EDTA	1 mM	77.1 ± 2.33
SDS	1 mM	31.6 ± 2.1
Triton X-100	0.1% (v/v)	8.5 ± 1.0
Tween-80	0.1% (v/v)	0.3 ± 0.07
DMSO	10% (v/v)	82.8 ± 3.96

Original activity was set as 100% [139.5 ± 2.5 U/g (dry cell)]. All measurements were done in duplicates.
[a]Average ± SD.

The effects of detergents, metal ions, and solvent solution on the activity of displayed TLL are shown in Table 6.2. The activity of displayed TLL was increased three-fold by the presence of Ca^{2+} ions. This suggests that the displayed TLL is Ca^{2+} dependent. Similar results were reported by Zheng *et al.* (2011) on TLL expressed in *Pichia pastoris*. The activity of a thermophilic L2 lipase from *Bacillus* sp. expressed in *P. pastoris* increased five-fold in the presence of Ca^{2+} (Sabri *et al.*, 2009). The activity of displayed TLL was slightly enhanced by DMSO, but EDTA, SDS and the non-ionic surfactants, Triton X-100 and Tween-80 inhibited the activity of displayed TLL. The inhibition by detergents exerted on lipolytic enzymes is a result of the detergents blocking binding of the enzyme at the lipid substrate interface (Aloulou *et al.*, 2007).

Table 6.2 Effect of detergents and solvent solution on displayed TLL

Factor	Concentration	Relative activity (%)[a]
Control		100 ± 2.1
$CaCl_2$	1 mM	310.1 ± 9.0
EDTA	1 mM	31.1 ± 0.2
SDS	1 mM	21.5 ± 0.9
Triton X-100	0.1% (v/v)	13.9 ± 2.3
Tween-80	0.1% (v/v)	1.8 ± 0.6
DMSO	10% (v/v)	106.8 ± 5.0

Original activity was set as 100% [94.3 ± 1.9 U/g (dry cell)]. All measurements were done in duplicates. [a]Average ± SD.

6.5 Conclusions

In this study, functionally active C-terminally His-tagged FSCut and TLL were successfully displayed on the cell surface of *Y. lipolytica* using YlCWP1 as an anchor protein. High level functionally active FSCut and TLL displayed on *Y. lipolytica* cells was observed compared to the His-tagged cell free enzymes. These results indicate that *Y. lipolytica* may be used for industrial expression C-terminally His-tagged FSCut and TLL. This opens way for the use of *Y. lipolytica* as whole-cell catalysts. Recent studies have shown that addition of a linker greatly increases the activity of displayed lipases (Liu *et al.*, 2010a, b). The addition of a linker for increasing the activity of displayed FSCut and TLL on *Y. lipolytica* should be explored.

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Chapter 7. Construction of a system for expression and detection of RANTES/ CCL5 displayed on *Yarrowia lipolytica* using mCherry as a reporter protein

7.1 Abstract

Fluorescent proteins (FPs) are used extensively in cell biology for monitoring localization and expression of proteins in a wide range of hosts. In this study, we constructed an alternative expression system to the system currently employed for expression of heterologous proteins fused to fluorescent proteins to the extracellular medium. A versatile plasmid was developed for heterologous expression of peptides on the cell surface of *Yarrowia lipolytica* using the C-terminal half of the GPI-anchored YICWP1. Using the constructed system, RANTES fused to the N- or C-terminal of mCherry on the cell surface of *Y. lipolytica* was successfully expressed. Yeast transformants displaying the fusion protein were screened visually by colour change. Positive transformants showed a purple colour change compared to the negative control cells. The displayed RANTES was determined by fluorescent and flow cytomeric detection of its fusion partner, mCherry. In addition, the expressed RANTES on the yeast cells was cleaved using enterokinase and cell free RANTES confirmed by SDS-PAGE, MALDI-TOF-MS peptide fingerprinting, western analysis and ELISA.

7.2 Introduction

The wild type red fluorescent protein from *Discosoma* species (DsRed) is a tetramer responsible for the red colouration around the oral disk of a coral of the *Discosoma* sp. (Tsien 1998; Baird *et al.*, 2000; Vrzheshch *et al.*, 2000). DsRed has been identified as an attractive chromophore with potential application in biotechnology and cell biology as a fusion partner that would complement or substitute green fluorescent protein (GFP) (Matz *et al.*, 1999). GFP and its variants are used extensively as indicators for localization of protein expression and as donor/receptor pairs for fluorescence resonance energy transfer (Mizuno *et al.*, 2001). Since its discovery, DsRed has in most studies been paired with GFP for localization of fusion proteins (Courdavault *et al.*, 2011). Although, DsRed exhibits a range of advantages such as high extinction co-efficiency, resistance to photobleaching and pH extremes, its major drawbacks have been strong oligomerization and slow maturation (Baird *et al.*, 2000). The second generation of red fluorescent proteins involved engineered DsRed by direct evolution to a monomeric red fluorescent protein (mRFP1) that shows rapid maturation and displays minimal emission when excited at wavelengths optimal for GFP (Campbell *et al.*, 2002). The next generation of red fluorescent monomers, monomeric Cherry (mCherry) was developed by Shaner *et al.* (2004). mCherry has a more complete maturation, more tolerance to N-terminal fusions and has over 10-fold increased photostability compared to mRFP1 (Shaner *et al.*, 2004).

Application of fluorescent proteins (FPs) as reporter proteins in the evaluation of different cell wall proteins for cell surface display on microorganisms has become widespread (Wang *et al.*, 2007; Yue *et al.*, 2007). Biological studies have largely used enhanced GFP (EGFP) as a versatile reporter (Kusser and Randall, 2003) and mCherry to a lesser extent (Kuroda *et al.*, 2009). Their use has been extended to development of immunoassays and biosensors for antibodies (Pavoor *et al.*, 2009; Guo *et al.*, 2010). Fluorescent proteins are used as fusion partners for extracellular expression of heterologous proteins (Skosyrev *et al.*, 2003a; Naumann *et al.*, 2011). Such fusions have not been reported for expression of heterologous proteins on the cell surface of bacteria or yeast.

In our previous study, we reported high-level expression of mCherry displayed on *Y. lipolytica* cells (Bulani *et al.*, 2012). This study investigated the potential of expressing heterologous proteins fused to mCherry and displayed on *Yarrowia lipolytica* cell

surface. RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted) was used as a model protein for cell surface based expression system. RANTES, a cytokine that belongs to the family of C-C chemokines, plays an essential mediatory role in inflammatory processes (Song *et al.*, 2000). RANTES is ubiquitously produced by different cell types under different conditions (Schall *et al.*, 1988; von Luettichau *et al.*, 1996; Hornung *et al.*, 2001). RANTES is a potent chemoattractant of monocytes, T lymphocytes, eosinophils, basophils and NK cells (Schall *et al.*, 1990; Kameyoshi *et al.*, 1992; Rot *et al.*, 1992; Dahinden *et al.*, 1994; Taub *et al.*, 1995). It has been shown to inhibit human immunodeficiency virus-type 1 (HIV-1) viral replication (Feng *et al.*, 1996). Because of its potential as a therapeutic peptide for cancer and AIDS, RANTES is an important pharmaceutical target (Nelson and Krensky, 1998; Huang *et al.*, 2007).

In this work, we report an alternative expression system to the current method used for expression of heterologous proteins fused to EGFPs or other FPs to the extracellular medium (Naumann *et al.*, 2011). The proposed expression system involves displaying fusion proteins on the cell surface of *Y. lipolytica* using the rDNA based pKOV410-*mcherry-YlCWP1* displaying plasmid. We successfully expressed RANTES fused to mCherry on the cell surface of *Y. lipolytica* cells. Yeast transformants displaying the fusion protein were screened by purple colour change. The displayed RANTES was determined by fluorescent and flow cytomeric detection of its fusion partner, mCherry. Expression of RANTES on *Y. lipolytica* cell surface was further confirmed by proteolytic cleavage and identification of the cell free RANTES using various methods.

7.3 Material and methods

7.3.1 Strains and media

Y. lipolytica Po1f strain (*MatA, leu2-270, ura3-302, xpr2-322, axp1-2*) was used as a host for cell surface display (Madzak *et al.*, 2000). *Y. lipolytica* Po1f strain harbouring pKOV410-*mcherry-YlCWP1* expression cassettes (Yl-mch1) for displaying mCherry was used a positive control for expression of mCherry. *Y. lipolytica* Po1f strain harbouring pKOV410-*YlCWP1* expression cassettes (Yl-p410) was served as a negative control. Yeast host cells were selected on YNB-casamino acid plates (0.17% YNB without amino acids and ammonium sulfate, 1% glucose, 0.1% casamino acids, 0.1% sodium glutamate and 1.5% agar). Yeast transformants were aerobically cultivated in 100 ml YPD (1% yeast extract, 2% bacto-peptone and 2% glucose). *Escherichia coli* XL10 Gold (Stratagene) was used as a host for recombinant DNA manipulation and was grown in 5.0 ml Luria-Bertani medium or agar plates at 37°C overnight containing 30 µg/ml of kanamycin or 50 µg/ml of ampicillin (Sambrook *et al.*, 1989). For solid media, 1.5% agar was added.

7.3.2 Plasmids

The plasmids, pGem-*mcherry-YlCWP1* and cell surface displaying plasmid pKOV410-*mcherry-YlCWP1* were constructed in this laboratory (Bulani *et al.*, 2012). RANTES was codon optimised for expression in *Y. lipolytica* and synthesised by GeneArt (Germany). All sub-clonings of PCR products were done using pGEM®-T Easy vector (Promega, Madison, USA).

7.3.3 Construction of plasmids

Polymerase chain reaction (PCR) was carried out using MJ Mini Personal Thermal Cycler (BIO-RAD). The plasmid containing codon optimised RANTES, pCCL5, was used as template for amplification of RANTES. Two strategies were developed for fusion of RANTES to mCherry. The first strategy involved fusing RANTES to the N-terminal end of mCherry, while the second strategy involved fusing RANTES to the C-terminal end of mCherry. For ligation of RANTES to the N-terminal of mCherry, the

forward primer R1_1F (5'-AAAGGATCCAGCAGGGCCCGCCGCTGA-3'; underlined bases indicate a *BamH*I site) and reverse primer R1_1R (5'-TTTGGTACC**CTTGTCA TCGTCATC**CTTAGACATCTCCAGGGAGT-3'; underlined bases indicate a *Kpn*I site and bold bases encode enterokinase cleavage site [DDDDK]) were used. PCR products (245 bp) were digested with *BamH*I and *Kpn*I and ligated to the 5'-end of *mcherry* in plasmid pKOV410-*mcherry-YlCWP1* digested with the same enzymes followed by transformation into *E. coli* XL10 Gold cells. The generated plasmid harbouring the fusion gene was named pKOV410-*RANTES1-mcherry-YlCWP1* (Fig. 7.1A).

For ligation of *RANTES* to the C-terminal of *mcherry* the forward primer R1_F (5'-GGTATCGATAAG**GATGACGATGACAAG**-3'; underlined bases indicate a *Cla*I site and bold bases encode enterokinase cleavage site) and reverse primer R1_R (5'-TTTAAGCTT**CTTGTCATCGTCATC**CTTAGACATCTCCAGGGAGT-3'; underlined bases indicate a *Hind*III site and bold bases encode enterokinase cleavage site) were used to amplify the peptide. PCR products (261 bp) were digested with *Cla*I and *Hind*III and ligated at the 3'-end of *mcherry* in plasmid pGem-*mcherry-YlCWP1* digested with the same enzymes and transformed into *E. coli* XL10 Gold to generate pGem-*mcherry-RANTES-YlCWP1*. The cloning vector harbouring the three genes (*mcherry-RANTES-YlCWP1*) was subsequently digested with *Kpn*I and *Avr*II and the fusion construct mcherry-*RANTES-YlCWP1* ligated into pKOV410 digested with the same enzymes. The ligation reaction was transformed into *E. coli* XL10 Gold and the generated plasmid harbouring the fusion gene was named pKOV410-*mcherry-RANTES-YlCWP1* (Fig. 7.1C). All PCR products were gel purified using Gel Extraction Kit (BioFlux) and recombinant plasmids isolated from *E. coli* transformants using Plasmid Isolation Kit (BioFlux) according to the manufacturer's instructions.

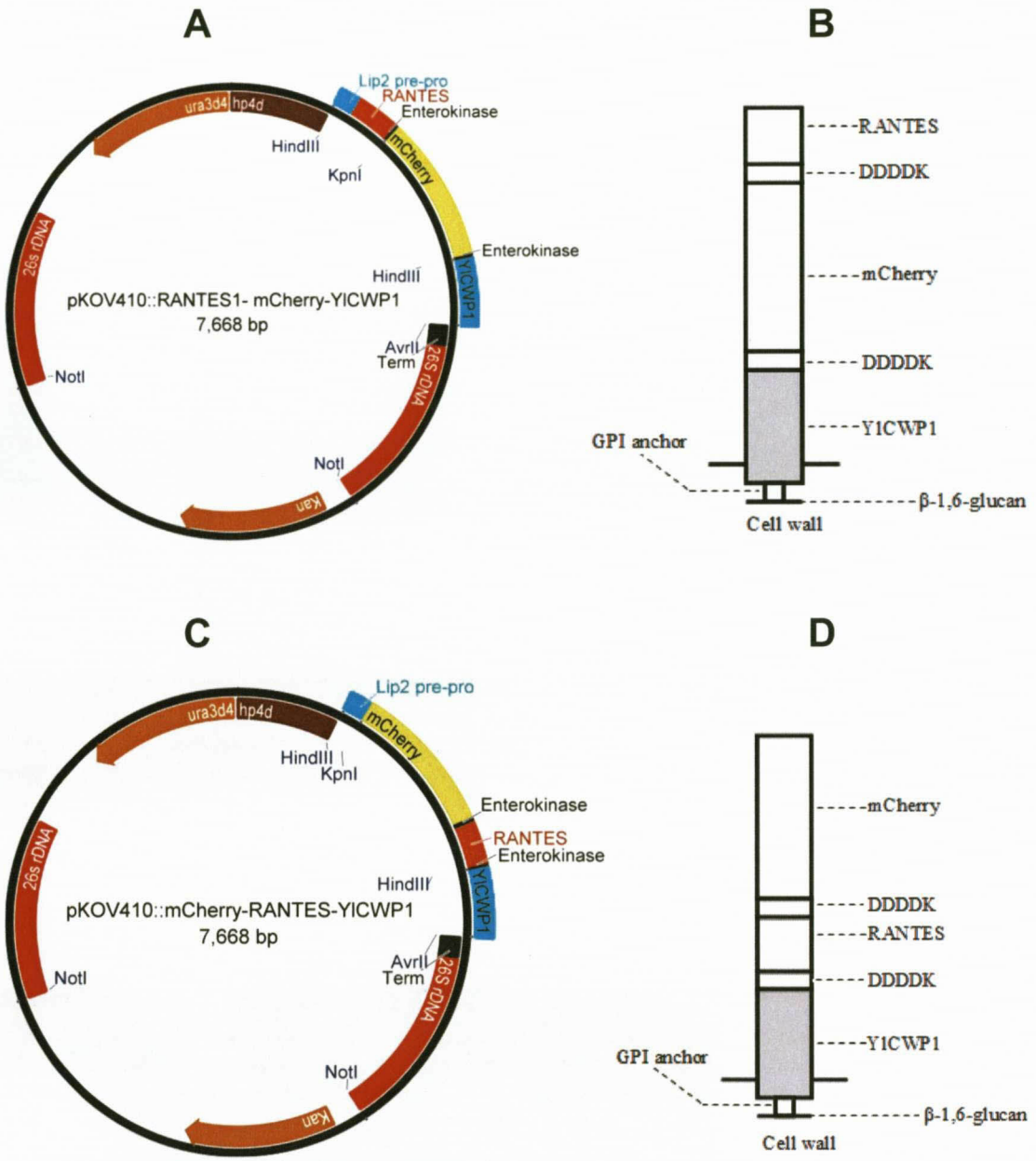

Figure 7.1: Construction of the plasmids pKOV410-*RANTES-mcherry-YlCWP1* and pKOV410-*mcherry-RANTES-YlCWP1* for expression of RANTES on *Y. lipolytica* cells. (A) *RANTES-mcherry-YlCWP1* and (C) *mcherry-RANTES-YlCWP1* fusion genes in plasmid pKOV410. (B) and (D): schematic diagrams of the fusion proteins covalently bound to β-1,6-glucan on the cell wall of *Y. lipolytica* through GPI. The plasmid map was constructed using Geneious v5.5 (Drummond et al. 2011).

7.3.4 Yeast transformation

Recombinant plasmids pKOV410-*YlCWP1*, pKOV410-*RANTES-mcherry-YlCWP1* and pKOV410-*mcherry-RANTES-YlCWP1* were digested with *Not*I and the expression cassettes separated by agarose gel electrophoresis. The expression cassettes were transformed into *Y. lipolytica* Po1f by lithium acetate method (Xuan *et al.*, 1988). The transformants were spread on YNB-casamino selective medium plates and transformants isolated after 1 to 3 weeks of incubation at 28°C. Yeast transformants carrying pKOV410-*RANTES-mcherry-YlCWP1* expression cassettes were denoted as Yl-mch2 whereas those carrying pKOV410-*mcherry-RANTES-YlCWP1* were denoted as Yl-mch3 and the control cells carrying pKOV410-*YlCWP1* were denoted Yl-p410.

7.3.5 Culture conditions

Yeast transformants Yl-mch2, Yl-mch3 and Yl-p410 were inoculated into 25 ml of YPD medium and incubated overnight at 28°C with shaking at 200 rpm. When the culture reached an optical density at 600 nm (OD_{600}) of 2-3, cells were re-suspended to an OD_{600} of 1.0 in 100 ml YPD and incubated at 28°C with shaking at 200 rpm for 96 h under aerobic conditions.

7.3.6 Analysis of fusion protein using fluorescence microscopy and flow cytometry

RANTES-mCherry fusion protein displayed on the cell surface of *Y. lipolytica* using YlCWP1 as an anchor were detected from yeast cells after culturing for 96 h. The yeast cells were harvested and washed three times by centrifugation at 16000 x *g* for two min at 4°C using phosphate-buffered saline (PBS pH 7.4). Cells were visualised under fluorescence microscope (Olympus) at 492 nm and photographed. Following fluorescence detection, cells were analysed using flow cytometer (FACS Calibur, Becton Dickinson). Approximately 30 000 yeast cells were analysed for each sample and the data analysed using FlowJo. Yl-p410 cells were used as negative control.

7.3.7 Cleavage and identification of displayed RANTES

Y. lipolytica cells cultivated for 96 h were harvested (1.0 ml) and washed three times by centrifugation at 16000 x *g* for 2 min with enterokinase buffer (20 mM Tris-HCl pH 8.0, 2 mM $CaCl_2$, 50 mM $NaCl_2$). Washed cells were resuspended into 1.0 ml of enterokinase buffer supplemented with 4 ng/ml of enterokinase (New England Biolabs, USA). The mixture was incubated at 16°C for 24 h after which 200 µl of supernatant was precipitated with acetone. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 12% polyacrylamide gel under denaturing conditions (Laemmli, 1970).

Protein bands on the SDS gel were excised and cut into small chips. The samples were treated as described by Webster and Oxley (2005) and digested overnight with porcine trypsin (Promega, Madison, USA). MALDI-TOF-MS was performed using a QSTAR® Elite mass spectrometer (Applied Biosystems Inc., Ontario, Canada). The generated PMF data was 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).

7.3.8 Western blot analysis

Following proteolytic cleavage of the fusion proteins from *Y. lipolytica* cell wall, the supernatant was applied on a 12% SDS-PAGE gel according to the method of Laemmli (1970). Proteins separated on the gel were electroblotted onto a nitrocellulose membrane at 100 V for 1 h at room temperature. The membrane was blocked with 5% (w/v) non-fat milk in PBS for 1 h at room temperature. The blocking buffer was discarded and the membrane incubated with rabbit IgG anti-DDDDK polyclonal antibody (Abcam, UK) diluted 1:2500 and kept at room temperature for 45 min with shaking. The membrane was washed three times for 5 min each time with PBS and incubated with HRP-conjugated secondary antibody (1:5000) at room temperature for 45 min with shaking. The membrane was washed three times for 5 min each time with PBS and stained with TMB liquid substrate for peroxidise.

7.3.9 Enzyme-linked immunosorbent assay (ELISA)

To measure the level of cleaved RANTES, ELISA plate (Dynex Technologies, Chantilly, VA) was coated for 16 h at 4°C with 5 µg/ml of monoclonal anti-human CCL5/RANTES MAB678 (R & D Systems®, Minneapolis, Minnesota). After washing the plate three times with wash buffer (1 x PBS + 0.1% (v/v) Tween 20), the plate was incubated with blocking buffer (phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 and 5% (w/v) dry milk to eliminate non-specific binding for 2 h on a rotary shaker and followed by three washes using wash buffer. A standard curve was prepared in triplicate by making serial dilutions (ranging from 10 to 0.078 ng/ml) of pure RANTES protein (278RN-rhCCL5/RANTES), coated onto the ELISA microtiter plate and incubated for 2 h and washed three times with wash buffer. All test samples (supernatants from cells cleaved with enterokinase) were added in triplicate to the ELISA plate and incubated for 2 h and washed three times with wash buffer. Biotinylated anti-human CCL5/RANTES BAF278 secondary antibody (250 ng/ml) in blocking buffer was added and incubated for 2 h on a shaker. After washing the plate three times, Streptavidin-HRP (horseradish peroxidase) was added and incubated for 20 min on a shaker. The plate was then washed three times and 3,3,5,5-tetramethylbenzidine (TMB) liquid substrate for peroxidase was added and incubated for 15 min at room temperature. The reaction was terminated with 1 M H_2SO_4. Absorbance was measured at 450 nm with ELISA reader (uQuant Microplate Spectophotometer, Model MQX200, Bio–Tek Instruments, Inc., Winooski, VT). A standard curve was used to calculate the concentration of RANTES present in the samples.

7.4 Results

7.4.1 Construction of plasmids for cell surface display of RANTES

In the previous study (Chapter 5) we reported enhanced expression of mCherry on *Y. lipolytica* cells using an rDNA based displaying plasmid. In this study, the plasmid pKOV410-*mcherry-YlCWP1* was evaluated for expression of RANTES on the cell surface of *Y. lipolytica*. Two strategies were devised to ligate *RANTES* to the 5'- or 3'-end of *mcherry*. Firstly, the RANTES encoding gene was amplified from the plasmid pCCL5. The reverse primer bases were designed to encode enterokinase recognition site (DDDDK) and ligated at the 5'- end of *mcherry* in the plasmid pKOV410-*mcherry-YlCWP1* as shown in Fig. 7.1A. Secondly, the gene encoding RANTES was amplified from the plasmid pCCL5 with both primers incorporating DDDDK at the 5'- and 3'- end of the amplicon and subsequently ligated at the 3'- end of *mcherry* in the plasmid pGem-*mcherry-YlCWP1* and the fusion genes ligated into pKOV410 as shown in Fig. 7.1C. The amino acid sequence of the fusion proteins is shown in figure 7.2, indicating the position of each protein/ peptide and the enterokinase cleavage sequence. The expression cassettes obtained from pKOV410-*YlCWP1*, pKOV410-*RANTES-mcherry-YlCWP1* and pKOV410-*mcherry-RANTES-YlCWP1* expression vectors were transformed into *Y. lipolytica* using the lithium acetate method (Xuan *et al.*, 1988).

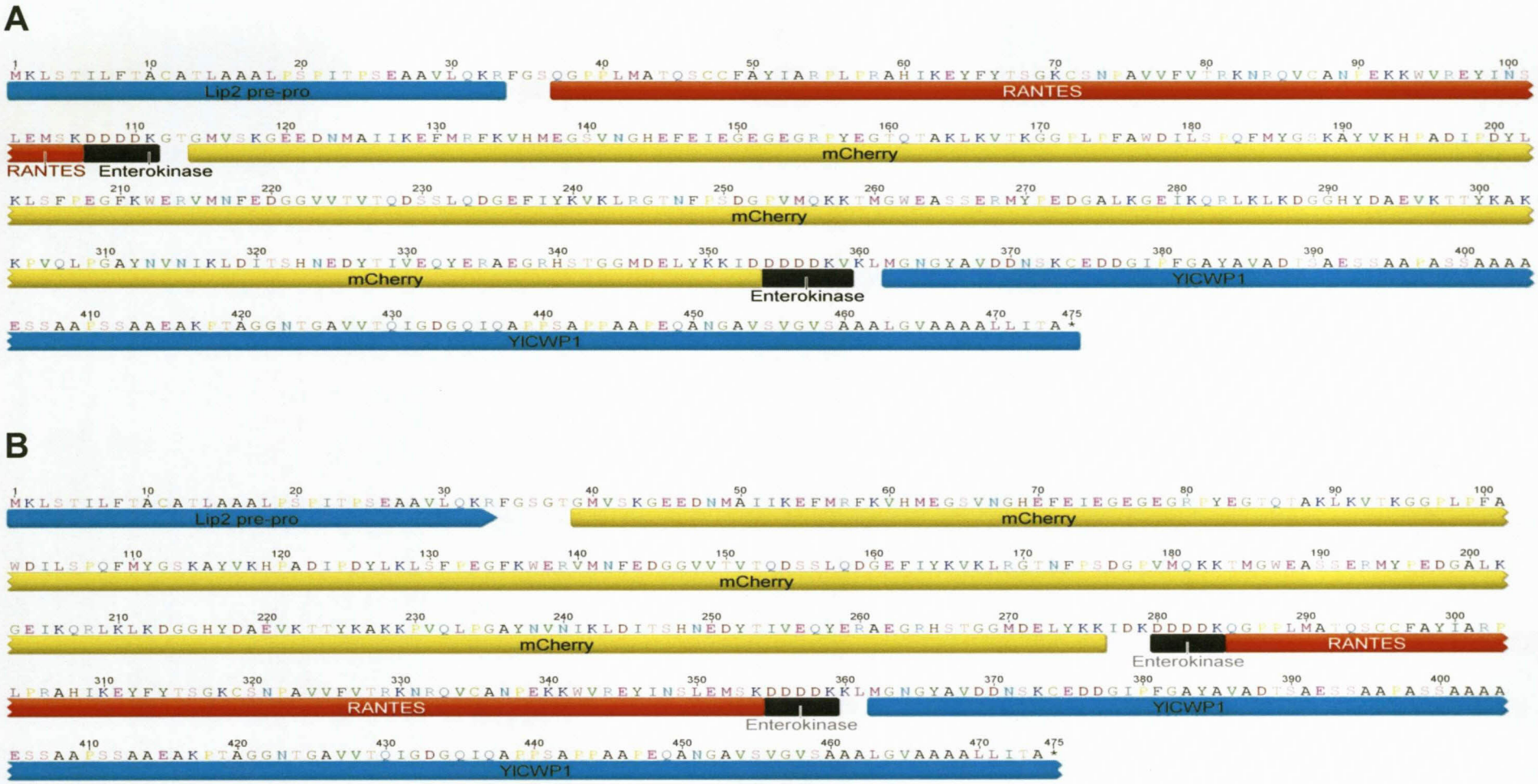

Figure 7.2: Amino acid sequence of the fusion proteins downstream the lip2 secretion signal. (A) RANTES-mCherry-YlCwp1, (B) mCherry-RANTES-Ylcwp1 fusion. The diagrams show position of the proteins and the enterokinase cleavage site.

Similarly to the yeast transformants displaying mCherry (Yl-mch1) which showed purple transformants on the selective YNB-casamino plates (Chapter 5), the *Y. lipolytica* cells harbouring RANTES fused to the N-terminal end (Yl-mch2) and C-terminal end (Yl-mch3) of mCherry protein showed purple yeast transformants indicating that the fusion proteins were displayed on *Y. lipolytica* cells (Fig. 7.3A).

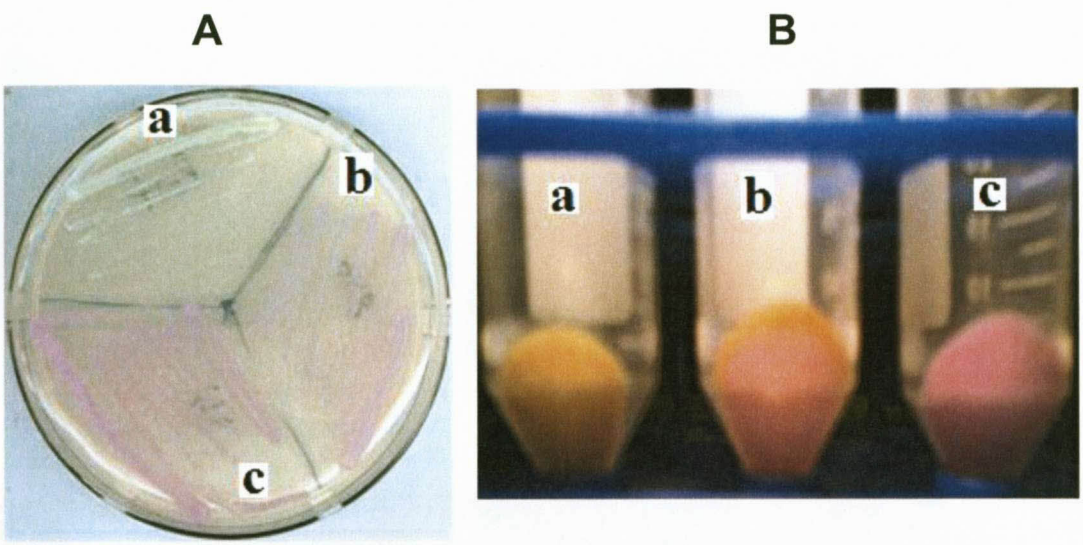

Figure 7.3: Effect of displayed mCherry and RANTES fusion proteins on the *Y. lipolytica* transformants. Photographs of *Y. lipolytica* plate (A) and wet cell pellets (B). (a) Yl-p410 control cells showing no colour change, (b) Yl-mch2 cells showing both purple and non-colour change for the wet pellets (c) Yl-mch3 cells showing a purple colour change.

7.4.2 Fluorescence microscopy and flow cytometry

To confirm the presence of the RANTES-mCherry fusion protein on *Y. lipolytica* cell surface further, the yeast transformants carrying the expression cassettes were grown in YPD media for 96 h. The Yl-mch2 cells as shown in figure 7.3 B showed two different population (cells displaying the fusion protein and cell the cell devoid of the fusion protein), whereas the Yl-mch3 cells showed a single population of cells. Successful display of RANTES-mCherry on the cell surface of the cultivated *Y. lipolytica* transformants was confirmed by fluorescence microscopy. Both Yl-mch2 and Yl-mch3 cells exhibited a strong red fluorescence (Fig. 7.4B and D, respectively). However, the Yl-p410 cells showed no fluorescence (Fig. 7.4E).

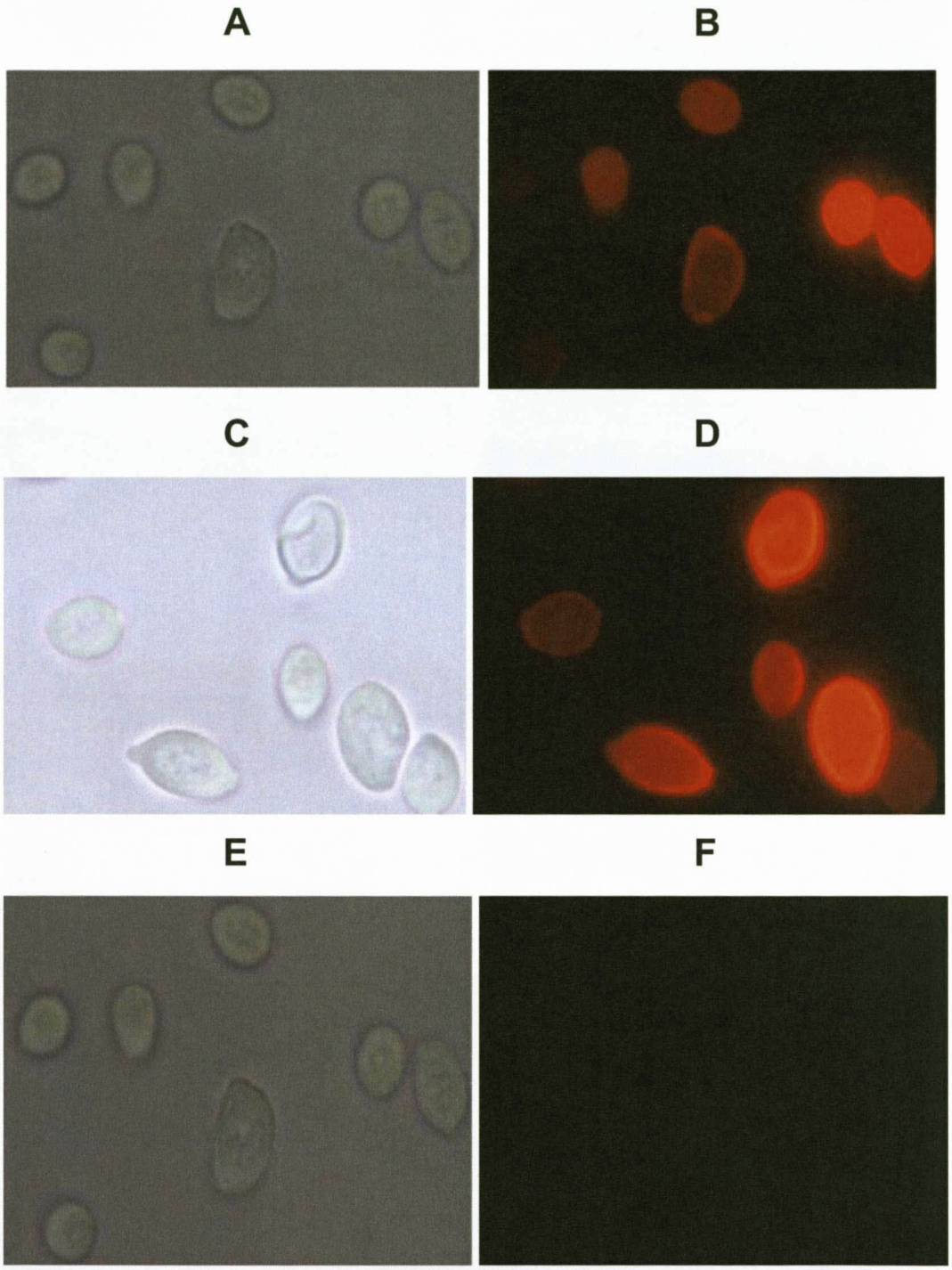

Figure 7.4: Microscopic photographs of *Y. lipolytica* cells. (A and B) Yl-mch2, (C and D) Yl-mch3 and (E and F) Yl-p410 cells. *Y. lipolytica* cells in A, C and E were photographed under visible light whereas cells in B, D and F were photographed under UV light (492 nm).

Quantitative expression of mCherry on *Y. lipolytica* cells was analysed using flow cytometry (Fig. 7.5). Similarly, to the wet pellet cells (Fig 7.3B) Flow cytometric analysis showed two populations of Yl-mch2 cells. About 79% of Yl-mch2 cells exhibited fluorescence with 53% of that showing high fluorescence as compared to 24% which showed lower fluorescence (Fig. 7.5A). About 21% of Yl-mch2 cells showed no fluorescence. About 98% of Yl-mch3 cells expressed mCherry on their surface (Fig. 7.5B). These results strongly suggest that RANTES-mCherry was successfully expressed on the cell surface of *Y. lipolytica*.

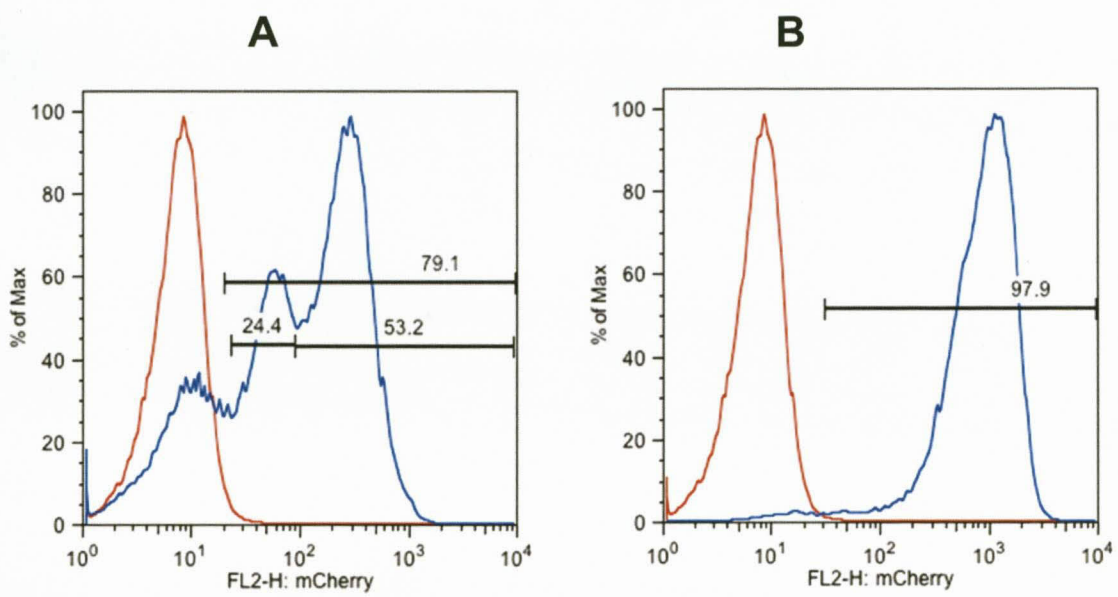

Figure 7.5: Flow cytometric photographs of *Y. lipolytica* cells. (A) Yl-mch2 and (B) Yl-mch3. Flow cytometric histograms depict the mean fluorescent signal of Yl-mch2 and Yl-mch3 cells (in blue) and Yl-p410 cells (in red).

7.4.3 Expression of RANTES

In order to confirm that RANTES was expressed and displayed on the *Y. lipolytica* cell wall, the cultivated cells of Yl-mch2 and 3 were treated with enterokinase. Supernatants of the proteolytic reaction were subjected to SDS-PAGE analysis for detection of RANTES and mCherry (Fig. 7.6A). However, even after treating the cell suspensions with enterokinase, the purple colouration on the cells remained. Protein bands cleaved from both Yl-mch2 and 3 cells showing similar molecular weight to that of Yl-mch1 mCherry (this band has been confirmed in our previous work as mCherry in Chapter 5) were observed on the gel (lanes 2 and 3). To ascertain

whether the observed protein bands on the SDS-PAGE gel were RANTES and mCherry, the bands were cleaved from the gel. Following treatment of the cleaved bands, the samples were subjected to MALTI-TOF-MS peptide mass fingerprinting. BLAST searches of the 36 identified peptide mass fingerprints from the bands denoted as mCherry gave a 95% match to a synthetic monomeric red fluorescent protein (GenBank Accession number: AAV52164). The 10-kDa band denoted as RANTES identified three peptide mass fingerprints. BLAST searches of the mass fingerprints gave 95% match to a synthetic *Homo sapiens* chemokine (C-C motif) ligand 5 construct (Gen Bank Accession number: AAP36897). Free RANTES from Yl-mch3 cells was further confirmed by western blot analysis (Fig. 7.6B) as this peptide still retains the enterokinase cleavage site. RANTES from Yl-mch2 was used as a negative control and western blot could not detect the peptide using DDDDK antibodies as this fragment did not contain the cleaved site. Quantitative analysis of free RANTES from Yl-mch2 using ELISA gave 4.39 $\pm$ 0.187 ng/ml, whereas 7.45 $\pm$ 0.255 ng/ml RANTES was detected from the supernatant of Yl-mch3. These results strongly indicate that RANTES was successfully displayed on *Y. lipolytica* cells. Additionally, the displayed RANTES could be removed from the cell surface and its fusion partner by treatment with enterokinase.

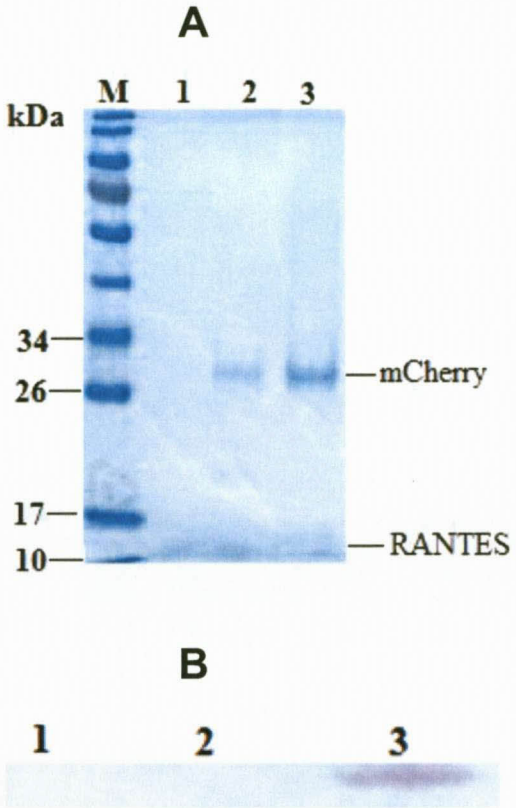

Figure 7.6: SDS-PAGE analysis of mCherry and RANTES from *Y. lipolytica* cell surface. (A) Lane M: Prestained protein molecular weight marker (NEB); Lane 1: supernatant from Yl-p410 cells treated with enterokinase; Lane 2: supernatant from Yl-mch2 cells treated with enterokinase; Lane 3: supernatant from Yl-mch3 cells treated with enterokinase. (B) Western blot analysis of RANTES cleaved from the *Y. lipolytica* cells. Lane 1: supernatant from Yl-p410 cells; Lane 2: supernatant from Yl-mch2 cells; Lane 3: supernatant from Yl-mch3 cells showing RANTES.

7.5 Discussion

Fluorescent proteins (FPs) are used extensively in cell biology for monitoring localization and expression of proteins in a wide range of hosts (Castro-Longoria *et al.*, 2010; Courdavault *et al.*, 2011). Their application as fusion partners in localization studies of proteins has been essential in examining kinetics, interaction and mobility of expressed proteins (Slaughter *et al.*, 2007; Castro-Longoria *et al.*, 2010). Different FPs have been codon-optimized for use as reporter proteins for a variety of live-cell imaging methods (Leroch *et al.*, 2011). One such FP, EGFP, has been used as a fusion partner and reporter protein for expression of antimicrobial peptides (Skosyrev *et al.*, 2003a, b). In this study, the efficiency of an rDNA based plasmid carrying mCherry and the GPI-anchored YlCWP1 was assessed for cell surface display of peptides on *Y. lipolytica* cells using RANTES as a model peptide and mCherry as a reporter protein for selection of yeast transformants. The generated data showed that fusion of RANTES to mCherry and its display on *Y. lipolytica* cell surface results in high level expression of RANTES. We compared the effect of fusing RANTES to the N- and C-terminals of mCherry and demonstrated that expression of RANTES was achieved using both the C- and N-terminals of mCherry. mCherry has been shown to have an increased tolerance for both N- and C-terminal fusion of proteins (Shaner *et al.*, 2004). Although RANTES was fused to both terminals of mCherry and successfully displayed on yeast cells, the N-terminus proved to be a better fusion site with regards to the number of cells exhibiting fluorescence as shown by the flow cytometric data. It is unclear if fusion of RANTES to the C-terminus of mCherry on cell surface affected its expression. Other FPs such EGFP, which allows for the construction of both N- and C-terminal fusions (Tsien, 1998; Tabuchi *et al.*, 2010) should be assessed to test whether heterologous protein fused to its N-terminal and displayed of *Y. lipolytica* using the strategy developed in this study will give similar results.

Studies in our lab have not been successful in expression of recombinant RANTES. Heterologous expression of RANTES has been reported in *E. coli* and eukaryotic cells (HEK293T). Its expression in *E. coli* required a tRNA supplementation strategy because of the presence of rare codons, which made expression unsuccessful in this host (Arathy *et al.*, 2011). Expression of recombinant chemokines in *E. coli* has been limited by formation of inclusion bodies, which results in low expression levels of both insoluble and soluble proteins (Cho *et al.*, 2008). EGFP fusion proteins have been

shown to form inclusion bodies when overexpressed in *E. coli* at 37°C (Skosyrev *et al.*, 2003 b). As a result, Cho *et al.* (2008) reported expression of recombinant RANTES in *E. coli* using mannose-binding protein and glutathione-S-transferase as fusion partners. Since expression of RANTES in *E. coli* is associated with formation of inclusion bodies (Skosyrev *et al.*, 2003b) and separation from other co-expressed proteins (Cho *et al.*, 2008), the expression system developed in this study offers an alternative production of RANTES as yeast do not form inclusion bodies and there is no problem of co-expression.

Yl-mch2 and 3 cells retained the purple phenotypic colour formation previously observed from Yl-mch1 (Chapter 5) on both solid agar plates and liquid YPD medium (Fig. 7.3). The phenotypic purple colour formation was used for selection of yeast transformants displaying RANTES-mCherry fusion protein. In contrast to Yl-p410 cells which showed no fluorescence when observed under fluorescent microscope, Yl-mch2 and 3 cells exhibited strong red fluorescence. In Chapter 5, we reported that 75% of Yl-mch1 cells displayed mCherry, whereas in this study 79% and 98% of Yl-mch2 and 3 cells expressed mCherry, respectively. Yl-mch2 and 3 cells showed increased expression levels of mCherry when compared to Yl-mch1 cells (Chapter 5) analysed using flow cytometry. In this study, RANTES seems to have functioned as a linker and thus increased the expression of mCherry on *Y. lipolytica* cells and as a result increased its own expression (Ylmch3). Different linker proteins have been reported to greatly increase expression and activity of displayed proteins (Washida *et al.*, 2001; Breinig and Schmitt, 2002; Liu *et al.*, 2010).

Data accumulated in this work provide evidence that there is a strong correlation between the colour of the transformants, fluorescence, and expression of the fusion proteins. Keppler-Ross *et al.* (2008) have also showed such a correlation. Following treatment of Yl-mch2 and 3 cells with enterokinase, free RANTES, and mCherry were detected on SDS-PAGE and confirmed by MALDI-TOF-MS PMF. In addition, western blot analysis and ELISA confirmed expression of RANTES. Because of high-level expression of RANTES-mCherry fusion on the cell surface of *Y. lipolytica*, RANTES-mCherry fusion was observed on the yeast cells after cleavage with enterokinase as indicated by the purple colour phenotype. The incomplete cleavage of RANTES-mCherry fusion from yeast cell surface will require optimization for complete cleavage.

In literature, purification of recombinant RANTES has largely depended on histidine tag or other chromatographic purification methods (Cho *et al.*, 2008; Vangelista *et al.*, 2010). The system used for expression of RANTES in this study does not require any chromatographic purification as RANTES can be cleaved from the yeast cells. Thus, the current method reduces the number of steps that are required for downstream processing of expressed peptides. It remains to be seen if this method can be scaled up and thus making it attractive for industrial applications.

In conclusion, these results suggest that RANTES can be produced using the developed cell surface display expression system. It remains to be seen if the developed methods can be extended to other peptides of similar size to the one used in this study. The purple colour change of the yeast expressing RANTES provides convenient and rapid way of screening transformants harbouring expressed heterologous proteins. The developed expression system offers a simplified process for downstream processing of fusion proteins. Optimization of the proteolytic cleavage of RANTES can be monitored visually and purification processes do not require chromatographic methods, thus, reducing time and costs. The reduction of costs associated with PCR and immunofluorescent reagents, ease of purification and reduction in time required for downstream processing make this method attractive for screening and industrial production of peptides.

7.6 References

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Chapter 8. Concluding Remarks

Production of recombinant proteins is a research field that has attracted interest in both research and commercial sectors. High-level expression of recombinant proteins is essential for industrial production of proteins with applications in the pharmaceutical or biotechnology sectors. Large scale production of recombinant proteins is often limited by many factors including, choice of expression host, availability of genetic markers and molecular tools, codon usage and characteristics of the target protein. These limitations serve as drawbacks in obtaining efficient recombinant proteins at low costs.

Different expression system ranging from yeasts, insect cells, mammalian cells, bacterial cells, transgenic animals and plants have been established for heterologous protein production. Yeast expression platforms emerged as the most powerful expression systems due to their ease of genetic manipulation, presence of efficient protein processing and post-translational modification pathways. *Yarrowia lipolytica,* dimorphic yeast with a GRAS status, emerged as the most attractive host for heterologous protein production. Although a wide range of expression systems for production of heterologous proteins are available, the most challenging limitations are the methods and costs associated with downstream processing of produced proteins that can be used industrially. For any expression system to be valuable for industrial application, it has to demonstrate efficient heterologous protein production with minimum costs on protein purification and other downstream processes.

The objective of this study was firstly to evaluate *Y. lipolytica* as a host for efficient heterologous protein production using the already available genetic tools of this yeast. The industrially important enzyme cutinase from *Fusarium solani pisi* (FSCut) was used to evaluate factors affecting its high-level expression and its activity in this host. The histidine (His) tag, one of the most widely used tags as a target for protein

purification, was placed under scrutiny for its effect on protein expression and activity. In addition, secretion signals, both native and heterologous to the FSCut, were evaluated for their role in protein expression and activity of the recombinant protein. The employed expression system allowed for the direct comparison of single copy integrants for evaluating each recombinant protein without differences in copy numbers and transcriptional activities. Expression levels and activity of tagged and non-tagged recombinant FSCut directed by the two secretion signals differed greatly. This study raised some questions concerning factors such as tags and secretion signal that play a role in protein expression and purification. Application of the multi-copy expression cassettes available for expression of FSCut in *Y. lipolytica* and fermentation processes still needs to be investigated.

The studies on FSCut led to the evaluation of *Y. lipolytica* as host for production of lipase from *Thermomyces lenuginosus* (TLL), another important enzyme in the detergent industry. Two expression systems looking at two different yeast strains and target sites were evaluated for expression of TLL. Single copy and multi-copy expression systems were assessed for production of the His-tag and non-tagged TLL in *Y. lipolytica* using different secretion signals for directing the recombinant protein to the extracellular medium. Similar to the expression of FSCut, expression levels and activity of the recombinant lipase were also highly variable. High-level expression of TLL were observed making this yeast a candidate for industrial production of this enzyme. In both the expression of FSCut and TLL, lower activity of the enzymes was observed when the enzymes contained a His-tag. A single method for purification was used which allowed easy downstream processing of the recombinant proteins.

Secondly, a cell surface expression system for heterologous protein production was also developed. Using an rDNA based plasmid; a novel cell surface displaying system was constructed for *Y. lipolytica*. This system employs the YlCwp1 for displaying heterologous proteins. The developed system proved to be efficient for enhanced protein production when using mCherry as reporter protein. A phenotypic characteristic of the displayed mCherry serve as a tool for selecting positive transformants. The developed displaying system offers great potential for industrial production and purification of heterologous proteins at low costs. This was evident when mCherry was used as a model protein as no chromatographic methods were required for protein purification.

Development of a cell surface displaying system for both His-tagged FSCut and TLL on the *Y. lipolytica* cells provided a complete study of heterologous protein production of the enzymes in this yeast. The enzymes exhibited better activity when displayed on the yeast cells compared to when targeted to the extracellular environment. Such a holistic approach in protein expression shows the importance of exhausting all expression tools available.

The third and final objective of this thesis was to exploit the developed cell surface display system for construction of fusion proteins and application ofmCherry as a reporter protein for expression of peptides, such as RANTES. Studies for expression of this therapeutic peptide in *Y. lipolytica* as an unfused protein conducted in our lab were not successful previously. The peptide was only successfully expressed when it was fused to the over-expressed Lip2 protein. Challenges were encountered when the peptide was removed from its fusion partner and purification required chromatographic methods. In this study, a novel cell surface displaying system was developed for expression of RANTES using mCherry as a reporter protein. This system takes advantage of the highly expressed mCherry and exploits it for expression of fusion proteins on the yeast cells. Using the displaying system, RANTES fused to the N- and C-terminal of mCherry was successfully expressed. This system has a potential in expression of peptides targeted to the yeast cell surface for efficient downstream processing.

This thesis led to the development of many transformants containing expression cassettes that are influenced by different factors for protein production. The strategy used to express the hydrolytic proteins provides understanding of the factors influencing production of FSCut and TLL. For expression of both hydrolytic enzymes evidence of a simple purification process were discussed and problems of using His-tags raised. Development of a novel cell surface displaying system and the use of this system as an expression platform for production of RANTES, makes this system a potential expression system for therapeutic peptides.

Chapter 9. Summary

The first aim of this study was to evaluate *Yarrowia lipolytica* as a host for heterologous protein production of functionally active histidine (His) tagged and non-tagged *Fusarium solani pisi* cutinase (FSCut) directed by the pre-pro Lip2 secretion signal (Lip2ss) and the native cutinase secretion signal (Nss). Single copy plasmid, pKOV323, directed to the pBR322 docking platform was used for expression of FSCut. The expression levels of FSCut in the Po1g strain were highly variable, with the purified non-tagged FSCut higher and more active that the His-tagged FSCut. The His-tag affected the expression levels and activity of the recombinant FScut. In addition, the His-tag influenced the enzyme activity when assayed at its optimal pH.

The second aim of the study was to evaluate *Y. lipolytica* as a host for expression of *Thermomyces lenuginosus* lipase (TLL). Similarly to FSCut, the effect of the TLL Nss and the Lip2ss were evaluated for directing the secretion of His-tagged and non-tagged TLL. The plasmid pKOV323 and the multi-copy plasmid, pKOV410, were used for expression of TLL in the strains Po1g and Po1f strains, respectively. Both secretion signals directed expression of functionally active TLL. The His-tag affected TLL expression levels, its activity, and thermostability. The expressed TLL showed differences in band patterns on the SDS gel. This study demonstrated that secretion signals play an important role in both secretion and activity of recombinant proteins. In addition, *Y. lipolytica* has a potential to be used for large-scale industrial production of FScut and TLL.

Characterization of all purified proteins expressed using different approaches showed the highest hydrolytic activity at 60°C and pH 9.0. High-level protein production of the His-tagged and non-tagged TLL was obtained when the enzyme was directed by the pre-pro Lip2ss for secretion into the extracellular medium, with the non-tagged showing the highest levels of total protein production. The results reported in this

study indicate that *Y. lipolytica* is prolific producer of TLL with potential large-scale industrial production of the enzyme.

For the third aim of this study, a novel rDNA based plasmid was developed for display of heterologous proteins on the cell surface of *Y. lipolytica* using the C-terminal end of the glycosylphosphatidylinositol (GPI) anchored *Y. lipolytica* cell wall protein 1 (YlCWP1). This system employed mCherry as a model protein. High-level display of mCherry on the yeast cell was shown by changes in the phenotypic colour of the cells and culture medium. The displayed mCherry was cleaved from the yeast cells using enterokinase. The developed cell surface displaying plasmid was used to display functionally active His-tagged FSCut and TLL on *Y. lipolytica* cell surface. The FSCut and TLL displayed on *Y. lipolytica* cells were more active that the His-tagged cell free enzymes expressed in *Y. lipolytica*. The display of active hydrolytic enzymes on the cells of *Y. lipolytica* demonstrated that this yeast have a potential industrial application as whole-cell catalyst.

The last aim of this study takes advantage of the plasmid used for over-expressing mCherry displayed on *Y. lipolytica* cell surface. Using the novel constructed system, RANTES fused to mCherry on the cell surface of *Y. lipolytica* cell surface was successfully expressed. The developed expression system offers a simplified process for downstream processing of fusion proteins.