

**Elucidating the role of growth rate on the production
of a fusion protein under regulation of the hp4d
promoter by *Yarrowia lipolytica***

by

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Declaration

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Research Output

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Abstract

Exenatide (Byetta[®]) is a type 2 diabetic drug which decreases the blood glucose level. Treatment using this drug is expensive due to its costly production by chemical synthesis therefore it is not an affordable drug of choice. A potential cost effective alternate for the production of exenatide is the use of recombinant production technology. *Yarrowia lipolytica*, a dimorphic yeast, was genetically engineered to produce the exenatide peptide (Exe, 39 amino acids) as a fusion to Lip2 (lipase) protein under the regulation of the hp4d promoter by the CSIR. The regulation of the promoter has, until recently, not been elucidated and is currently reported to be growth phase dependent. In order to optimise the conditions for the production of the fused Lip2:Exe peptide precursor, the regulation of the promoter needed to be understood.

In this study, the regulation of the hp4d promoter was established and a fed-batch fermentation strategy for the production of the fused Lip2:Exe precursor was developed. A *Y. lipolytica* strain (YIEx-gly) producing the Lip2:Exe peptide was cell-banked (to ensure stability of the production organism and repeatability of inoculation of fermenters) and the cell-bank was validated for production of the fused peptide. A transcript profile of the recombinant strain harbouring an expression vector encoding the Lip2:Exe under control of the hp4d promoter was determined using an optimised mRNA sandwich hybridisation methodology. Batch fermentation (1.2 l) was used to monitor production profiles during growth of *Y. lipolytica* followed by continuous fermentation (1 l) to determine the effect of growth rate on the transcription levels of the product under regulation by the hp4d promoter.

The synthetic hp4d promoter was found to be growth rate dependent which was confirmed by quantifying the amount of total protein produced during fed-batch fermentations (10 l) at different growth rates. A 60 % increase in production yields was achieved by using the optimised growth rate of 0.02 h⁻¹. This validated that the hp4d is growth rate dependent and not growth phase dependent as reported in literature. A strategy for the recombinant production of pharmaceutical peptides and proteins, under regulation of the hp4d promoter, using *Y. lipolytica* as a host, was therefore established. This research has paved the way for recombinant production of proteins at a lower cost therefore impacting on the health and economy of South Africa, by providing the public with potentially cheaper, affordable pharmaceutical drugs due to an alternative production strategy.

Opsomming

Exenatide (Byetta[®]) is a middle wat vir die behandeling van tipe 2 diabetes gebruik word deur die bloed glucose konsentrasie te verlaag. Hierdie behandeling is egter baie duur aangesien dit chemiesvervaardig word en dus nie bekostigbaar vir alle pasiënte is nie. 'n Bekostigbare alternatiewe vervaardigingsmetode is die gebruik van rekombinante produksie. *Yarrowia lipolytica* is 'n dimorfiese gis wat geneties gemanipuleer is om exenatide (Exe, amino sure) as 'n fusieproduk te produseer met die Lip2 (lipase) proteïen onder regulering van die hp4d promoterte produseer. Die regulering van die promoter is onbekend endit word aanvaar dat uitdrukking afhanklik is van die groeifase. Om produksie van die Lip2:Exe peptiedvoorloper te optimaliseer is dit belangrik om die regulering van die promoter te verstaan.

In die studie is die regulering van die hp4d promoter bepaal en 'n voerstrategie vir die produksie van die Lip2:Exe peptiedvoorloper in voer-lotfermentasie ontwikkel. 'n *Y. lipolytica* kloon (YIEx-gly) wat die Lip2:Exe peptiedvoorloper produseer is in 'n selbank gepreserveer om die stabiliteit van die produksie organisme en herhaalbaarheid van die inokulasie van die fermenteerders te verseker) en die selbank is getoets vir produksie van die fusiepeptied. 'n Transkripsie profiel van 'n positiewe kloon wat die hp4d uitdrukkings vector, (pKOV410:Lip2:Exe) bevat, is bepaal deur van 'n geoptimaliseerde mRNA toebroodjie hibridisasie metode gebruik te maak. Lotfermentasies (1.2 l) was gebruik om die produksieprofiel gedurende groei van *Y. lipolytica* te bepaal. Kontinue fermentasie (1 l) is gebruik om die effek van groeitempo op die transkripsievlakke van die produk onder regulering van die hp4d promoter te bepaal.

Dit is vasgestel dat die regulering van die sintetiese hp4d promoter onderhewig is aan die groei tempo van die organisme. Dit is bevestig deur die bepaling van die hoeveelheid totale proteïen wat geproduseer is in 10 l voer-lotfermentasies wat teen verskillende groeitempos uitgevoer is. 'n Sestig persent toename in produksie is teweeggebring deur die organisme teen 'n groeitempo van 0.02 h^{-1} te laat groei. Dit het bevestig dat die regulering van die hp4d promoter onderhewig is aan die groeitempo en nie die groeifase soos in literatuur genoem is nie. 'n Strategie vir die rekombinante produksie van farmaseutiese peptiede en proteïen onder beheer van die hp4d promoter deur *Y. lipolytica* is dus ontwikkel. Hierdie navorsing lê die grondslag vir goedkoper rekombinante produksie van proteïen. Hierdie tegnologie kan 'n

positiewe invloed hê op gesondheid en die ekonomie van Suid Afrika deur die daarstelling van potensieel goedkoper, beksotigbare farmaseutiese medisyne.

Abbreviations

ARS:	Autonomously Replicative Sequence
BBTP:	2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole phosphate
BBT:	2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole
BIDC:	Biomanufacturing Industry Development Centre
Bp:	base pair
BSA:	Bovine Serum Albumin
CHO:	Chinese hamster ovary cells
CSIR:	Council for Scientific and Industrial Research
DCW:	Dry Cell Weight
DIG:	Digoxigenin
DNA:	Deoxyribonucleic acid
DO:	Dissolved Oxygen
ECS:	Entrokinase Cleavage Site
Exe:	Exenatide
FDA:	Food and Drug Administration
GRAS:	Generally Regarded As Safe
His:	Histidine

HIV:	Human Immunodeficiency Virus
hp4d:	hybrid promoter derived (four tandem repeats) from the <i>XPR2</i> promoter
IPA:	Isopropyl Alcohol
leu:	leucine
<i>LEU2</i> :	β -isopropylmalate dehydrogenase
Lip2:	Endogenous lipase
mRNA:	messenger RNA
OD ₆₆₀ :	Optical density at wavelength 660 nm
PCA:	Plate Counting Agar
pNP:	<i>p</i> -nitrophenol
pNPP:	<i>p</i> -nitrophenyl palmitate
RNA:	Ribonucleic acid
slpms:	Standard liters per minute
UAS1:	Upstream Activating Sequence
Ura:	Uracil
<i>XPR2</i> :	Alkaline extracellular protease
YPD:	Yeast Peptone and Dextrose

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Chapter 1: Introduction and Hypothesis of Study

1.1 Introduction

The complex processes required for chemical synthesis of peptides have been studied for several decades providing crucial understanding necessary for production of proteins in the pharmaceutical industry. This has played an important role in the discovery of drugs necessary for the treatment of diseases. One of the shortcomings in the multi-national pharmaceutical industry is the costly chemical synthesis of drugs. On average, drugs like Enfuvirtide (Fuzeon[®]), a Human Immunodeficiency Virus (HIV) fusion inhibitor, requires 106 steps to manufacture (Bray, 2003). This in turn has a large impact on the consumer, especially for a third-world country such as South Africa. There is, however, a cost effective alternative for pharmaceutical peptide manufacturing that exists known as recombinant production.

Heterologous gene expression has, for decades, been used for the production of pharmaceutical peptides and enzymes such as insulin, mammalian chymosin and Penicillin G acylase (Cowan, 1996, Yang *et al.*, 2001, Ferrer-Miralles *et al.*, 2009; Martínez *et al.*, 2012). There are several expression systems that are commonly used such as bacteria, yeast, plants, mammalian cells and transgenic animals. When choosing a host system, one needs to consider the following: cell growth characteristics of the organism, the expression levels, secretion location (intracellular, extracellular or both), posttranslational modifications and the characteristics of the recombinant protein of interest (Goeddel, 1990; Hodgson, 1993; Makrides, 1996).

Yeast expression systems, in particular *Yarrowia lipolytica*, have widely generated interest in the field of recombinant protein production. Several *Y. lipolytica* processes have been classified as GRAS (Generally Regarded As Safe) by the American Food and Drug Administration (Madzak *et al.*, 2000). A large number of molecular tools are available for recombinant protein expression by *Y. lipolytica* and it has been described as one of the most promising yeasts available as a host for heterologous protein production (Müller *et al.*, 1998).

A number of promoters are available for recombinant protein production by *Y. lipolytica*. This organism can produce between 1-2 g.l⁻¹ of alkaline extracellular protease (AEP) which is encoded by the *XPR2* gene (Tobe *et al.*, 1976, Ogrydziak and Scharf, 1982; Barth and Gaillardin, 1997, Madzak *et al.*, 2004). *XPR2* (alkaline extracellular protease) gene has a strong constitutive promoter with a complex regulation mechanism. It is only active at a pH above 6 and requires high levels of peptone for induction (Madzak *et al.*, 2004). The *XPR2* promoter was analyzed and an upstream activating sequence (UAS1) region was found not to be significantly affected by environmental conditions such as specifically defined medium requirements and pH (Blanchin-Roland *et al.*, 1994; Madzak *et al.*, 1999). This UAS1 fragment was used to design a synthetic promoter called hp4d by placing four copies of the UAS1 fragment in tandem, upstream from a *LEU2* (β -isopropylmalate dehydrogenase) promoter (Madzak *et al.*, 1995; Madzak *et al.*, 2000). The hp4d promoter's regulation mechanism is independent of pH, carbon, nitrogen or peptone levels in medium and is able to drive strong expression levels of proteins (Madzak *et al.*, 1995; Madzak *et al.*, 2000). According to literature the hp4d promoter is quasi-constitutive and its regulation is growth-phase dependent and it is switched on during the early stationary growth phase (Madzak *et al.*, 2004; Madzak *et al.*, 2000).

A number of proteins have been produced by the Council for Scientific and Industrial Research (CSIR) using *Y. lipolytica* as a host organism. These include the enzymes, epoxide hydrolase from *Rhodotorula araucariae* (Maharajh *et al.*, 2008), endo- β -1, 4-mannanase from *Aspergillus aculeatus* (Roth *et al.*, 2009), the Human Immunodeficiency Virus (HIV) fusion inhibitor, Enfuvirtide (unpublished data) and a galactose oxidase M1 enzyme, a camelid antibody fragment and a trypsin inhibitor by Hofmeyer *et al.*, (2014). The last four products were fused to the native Lip2 (endogenous lipase) gene and expressed as fusion precursor peptides. Extracellular Lip2, a native lipase enzyme, was shown to reach high levels in the medium when secreted by *Y. lipolytica* (Pignede *et al.*, 2000a; Pignede *et al.*, 2000b; Fickers *et al.*, 2005; Hofmeyer *et al.*, 2014). The secretion signal used for the pre-pro Lip2 (Pignede *et al.*, 2000a; Pignede *et al.*, 2000b) allows direct secretion into the culture medium. Hofmeyer *et al.* (2014) expressed several recombinant proteins using Lip2 as a fusion partner. All of the proteins were produced under regulation of the hp4d promoter.

The CSIR is developing a process for the recombinant production of pharmaceutical peptides using *Y. lipolytica* as a host. In order to optimise conditions for heterologous protein production by *Y. lipolytica*, the production using the hp4d promoter needed to be optimised. The production of Lip2, one of the most highly secreted enzymes by *Y. lipolytica*, under regulation of the hp4d promoter was previously elucidated in continuous fermentation and found to be linked to growth rate with high production yields occurring at growth rates lower than 0.045 h^{-1} (van Zyl, 2013). However this does not explain if growth rate has an effect on the regulation of the hp4d promoter (transcription) or at the level of cellular metabolism where lower growth rates would allow metabolism to shift from biomass production to product formation.

In this study, the *Y. lipolytica* Po1f host strain was genetically engineered to produce the exenatide peptide (39 amino acids) as a fusion to the Lip2 gene under the regulation of the hp4d promoter. Exenatide (Byetta[®]) was the drug chosen due to the expensive processes associated with its production (Chapter 3). A cell bank of the production strain (to ensure strain stability) was generated and the cell bank was validated by measuring total recombinant protein production and lipase activity (used as a reporter enzyme) in shake flask cultivation and batch fermentation. This cell bank was used to determine the effect of growth rate on transcription levels of the gene under regulation of the hp4d promoter. The level of transcription was quantified by using a sandwich hybridisation assay which has been described as “ideal” for quantifying mRNA in bacterial and yeast cells during fermentation (Rautio *et al.*, 2003). The hybridisation solution for this assay and cell breakage was optimised and validated to ensure reproducibility. *Y. lipolytica* was cultivated in continuous fermentation at different growth rates using carbon limitation as the growth regulator and the level of transcription and product formation was determined at the different growth rates. The data obtained from the continuous fermentation was validated in fed batch fermentations by controlling the feed rate to maintain the desired growth rates during production of Lip2:Exe fused product.

1.2 Hypothesis of Study

The production of a pharmaceutical peptide precursor under regulation of hp4d promoter, by *Yarrowia lipolytica*, is growth rate dependent and is regulated at the level of transcription.

In order to prove this hypothesis, the following objectives were met:

Evaluation of a *Yarrowia lipolytica* construct expressing a pharmaceutical peptide precursor under regulation of the hp4d promoter:

- Cell banking of *Y. lipolytica* (YIEx-gly) expressing the fused Lip2:Exe precursor
- Growth curve study and validation of the cell bank for production of the fused Lip2:Exe precursor
- Batch fermentation of *Y. lipolytica*

Optimisation of methodology to monitor mRNA transcription by the hp4d promoter:

- Generation of mRNA oligonucleotide probes for sandwich hybridization assay
- Generation of standards for mRNA sandwich hybridization assay
- Optimization of mRNA sandwich hybridization method
- Optimization of cell breakage
- Shake flask study to evaluate the mRNA sandwich hybridization assay for repeatability

Determining the effect of growth rate on the regulation of the hp4d promoter by monitoring mRNA transcription

- Determination of carbon to nitrogen ratio to achieve carbon limitation
- Evaluation of the effect of growth rate on regulation of the hp4d promoter in continuous fermentation
- Validation of the regulation of the hp4d promoter using a growth-rate limiting fed-batch fermentation strategy

1.3 Outline of thesis

Chapter 1: This chapter is the introduction to the thesis. It provides a background and discusses the rationale for the study. The hypothesis and objectives are also provided.

Chapter 2: This chapter gives a literature review of recombinant protein production. The different types of hosts and promoter systems are compared. An insight in *Y. lipolytica*, the different promoters available for heterologous gene expression and some of recombinant proteins produced thus far are highlighted.

Chapter 3: This chapter displays the evaluation of *Y. lipolytica* (YIEx-gly) expressing a pharmaceutical peptide precursor under regulation of the hp4d promoter.

Chapter 4: This chapter involves the optimisation of a methodology to monitor mRNA transcription under regulation of the hp4d promoter using *Y. lipolytica*.

Chapter 5: This chapter details the effect of growth rate on the regulation of the hp4d promoter by monitoring mRNA transcription. Subsequently a fed-batch strategy for the production fused Lip2:Exe is established.

Chapter 6: This chapter provides the conclusion and future recommendations for this study.

Chapter 7: This chapter gives all the references used throughout this thesis.

Chapter 2: Literature Review

2.1 Importance of peptide base therapeutics in the pharmaceutical Industry

Peptides play an important role in the pharmaceutical market. In comparison to proteins, they can be described as molecules consisting of less than 50 amino acids (Craik *et al.*, 2013). A gap (Figure 1) between molecules less than 500 Da and biologics of more than 5000 Da exist in the pharmaceutical industry and by the use peptides as therapeutics potentially offers to address this gap (Craik *et al.*, 2013). There are several peptides that form part of the top selling injectable therapeutics in the pharmaceutical industry such as for the treatment of multiple sclerosis, worth \$3 billion, the immunomodulator Copaxone or glatiramer acetate peptide consisting of 10 amino acids; for the treatment of prostate and breast cancer, the peptide Zoladex or goserelin, worth \$1.1 billion, consisting of 9 amino acids and for multiple myeloma, the peptide Velcade or bortezomib, a proteasome inhibitor, worth \$1.5 billion, consisting of 2 amino acids (Craik *et al.*, 2013). These are some of the many peptides used for the treatment of diseases.

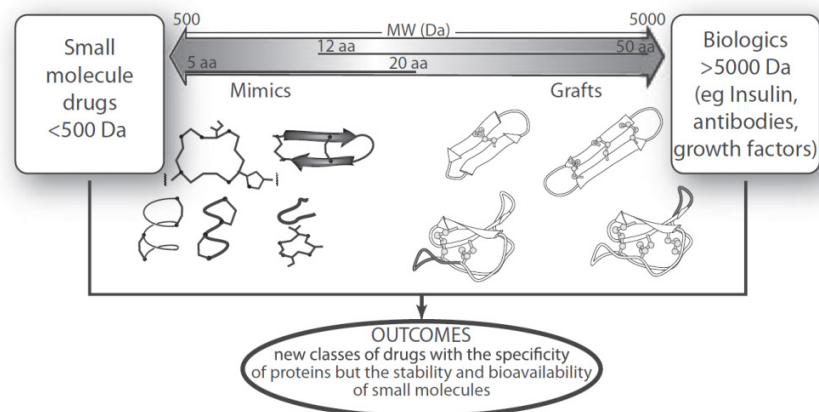


Figure 1: Gap present in the pharmaceutical industry potentially filled by peptides (reproduced from Craik *et al.*, 2013)

Several advantages as to why peptides are important to patients include high binding specificity for target sites, low levels of accumulation in tissues, high biological and chemical diversity, large range of targets and since peptides form part of proteins which are expressed by individuals, each therapeutic treatment can be modified to suit the needs of the patient (Craik *et al.*, 2013). However, one needs to consider the disadvantages as well. The oral bioavailability of peptides can be low, their metabolic stability can be described as low and depending on the peptide itself, solubility can be limited (Craik *et al.*, 2013). One of the biggest challenges that exist in the pharmaceutical industry is the high production costs associated with the manufacturing of these therapeutic peptides. The larger the peptides, the more amino acids are required for chemical synthesis resulting in additional production steps. This will be discussed further in the next section.

2.2 Chemical synthesis of peptides in the pharmaceutical industry

Over the last two centuries, numerous major developments in the pharmaceutical industry have taken place, from the discovery of the first protein vaccine produced for cow-pox, by Jenner in 1796, to by the first pharmaceutically produced protein insulin, by Banting and Best in 1922 (Demain and Vaishnav, 2009), to the discovery of the first antibiotic penicillin, by Alexander Flemming in 1928 (Brown, 2004; Martínez *et al.*, 2012). The pharmaceutical market is a booming sector with several new drugs frequently emerging. In 2008, it was estimated that the market was worth \$8.5 billion (Moorcroft, 2009). The production of peptides allow for high specificity for treatment of illness although the costly synthesis and bioavailability continues to be a problem (Russell, 2012). This high cost price tag is mainly due to the numerous chemical steps required during the manufacturing process using solid and liquid-phase chemistry (Figure 2). Synthesis of peptides is crucially dependent on the sequence, where complexity of solubility and racemisation problems arise (Thayer, 2011). The bigger the peptide, the more amino acids are required with the possibility of impurities occurring within the synthesised drug (Thayer, 2011).



Figure 2: Peptide synthesis using liquid phase chemistry (Russell, 2012)

On average, drugs like Enfuvirtide (Fuzeon[®]), a Human Immunodeficiency Virus (HIV) fusion inhibitor, requires 106 steps to manufacture (Bray, 2003). However, a cost effective alternative for manufacturing recombinant drugs exists, using heterologous gene expression. There are several host organisms available that are capable of producing large quantities of recombinant peptides and these will be discussed in detail.

2.3 Recombinant production of peptides in the pharmaceutical industry

Heterologous expression has provided an alternative to costly chemical synthesis of pharmaceutical peptides and enzymes. For several years, both prokaryotic and eukaryotic organisms have been used for production of recombinant therapeutic peptides and naturally available biopharmaceuticals and this has created massive interest in the pharmaceutical area (Faye and Gomord, 2010).

Production of enzymes found predominately in pathogenic or toxin-producing species could now be recombinantly expressed by different hosts making it easier and safer to obtain (Demain and Vaishnav, 2009). Some natural products cannot be made using chemical synthesis and it can be difficult to isolate them from natural sources hence recombinant protein expression would

serve as an ideal alternative for production (Naseby *et al.*, 2009). Insulin was the first recombinantly produced protein to enter the pharmaceutical market and was expressed by *E. coli* during the early 1980's and approved by the American Food and Drug Administration (Ferrer-Miralles *et al.*, 2009; Martínez *et al.*, 2012). Recombinant DNA technology has therefore had an effect on the pharmaceutical and enzyme industry making it possible for the recombinant production of pharmaceutical peptides, vaccines and enzymes using microbial fermentation (Falch 1991).

In 2002, over 155 vaccines and pharmaceutical proteins were approved and developed by biopharmaceutical companies (Demain and Vaishnav, 2009). In 2007, 25% of the pharmaceutical market comprised of recombinant therapeutic drugs (Redwan, 2007). In 2010, sales of biopharmaceuticals exceeded 100 billion dollars (Goodman, 2009). By 2014 (Figure 3), it was estimated that the market would be worth approximately 169 billion dollars (Goodman 2009).

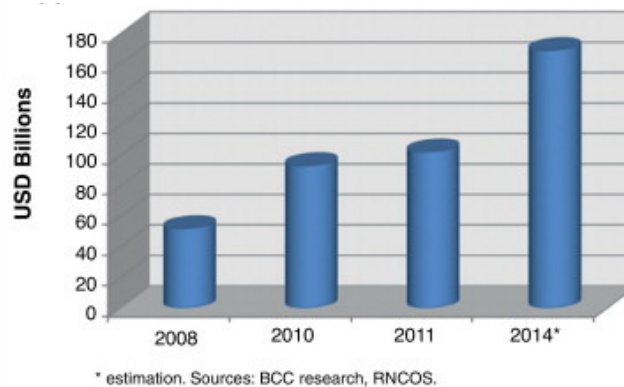


Figure 3: Global market for recombinant protein drugs (reproduced from Goodman, 2009, Martínez *et al.*, 2012)

There are several expression systems (Figure 4) that are currently being used for recombinant protein production and these include bacterial, plant, mammalian and yeast hosts (Faye and Gomord, 2010). Several factors need to be considered before choosing an expression host including cell growth characteristics of the organism, the expression levels, secretion location

(intracellular, extracellular or both), posttranslational modifications and the characteristics of the recombinant protein of interest (Goeddel, 1990; Hodgson, 1993; Makrides, 1996).

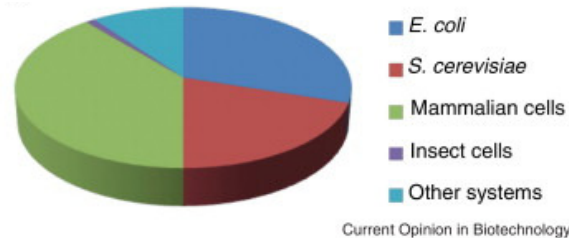


Figure 4: Percentage of protein-based recombinant pharmaceuticals, produced by different expression systems (reproduced from Ferrer-Miralles *et al.*, 2009)

There is no 'universal' expression system that can be used to achieve high recombinant protein yields and as the demand for more complex therapeutic peptides or proteins grow, the search for new expression systems for recombinant production continues (Verma *et al.*, 1998; Faye and Gomord, 2010). Bacterial host systems are the easiest to manipulate and have been used for expression of smaller proteins whereas mammalian cells and yeast systems are used for expression of more complex proteins i.e. post-translational modification requirements such as disulphide bond formation or glycosylation (Demain and Vaishnav, 2009).

2.4 Bacterial expression systems

Bacterial systems are a common host of choice due to easy manipulation and offer a cheaper option for producing the large amounts of protein necessary for industrial, research and commercial use (Georgiou and Valax, 1996). *Escherichia coli* (*E. coli*) is a gram negative bacterium and is the most exploited expression system used for recombinant protein production (Ferrer-Miralles *et al.*, 2009).

The main reasons are: *E. coli* has the ability to achieve 100 g.l⁻¹ biomass (dry cell weight) using high cell density culture systems (fermentation) for the production of recombinant proteins (Hannig and Makrides, 1998), several molecular tools available for genetic manipulation, its

genomes and metabolic pathways are annotated, capable of achieving high cell density, ability to grow fast and up to 80 % of its dry cell weight can consist of the protein of interest (Panda, 2003; Tripathi *et al.*, 2009; Porro *et al.*, 2011; Martínez *et al.*, 2012). There are a large number of cloning vectors and strains available for heterologous protein production, in particular *E. coli* expression hosts BL21 and K12, (Terpe, 2006) and only minimal amounts of foreign DNA is required for transformation (Verma *et al.*, 1998). In comparison to mammalian cells, *E. coli* grows faster allowing purification; analysis and marketing of the recombinant protein in shorter period of time (Verma *et al.*, 1998).

There are several advantages and disadvantages when using *E. coli* as a recombinant expression system and these are summarized in Figure 5.

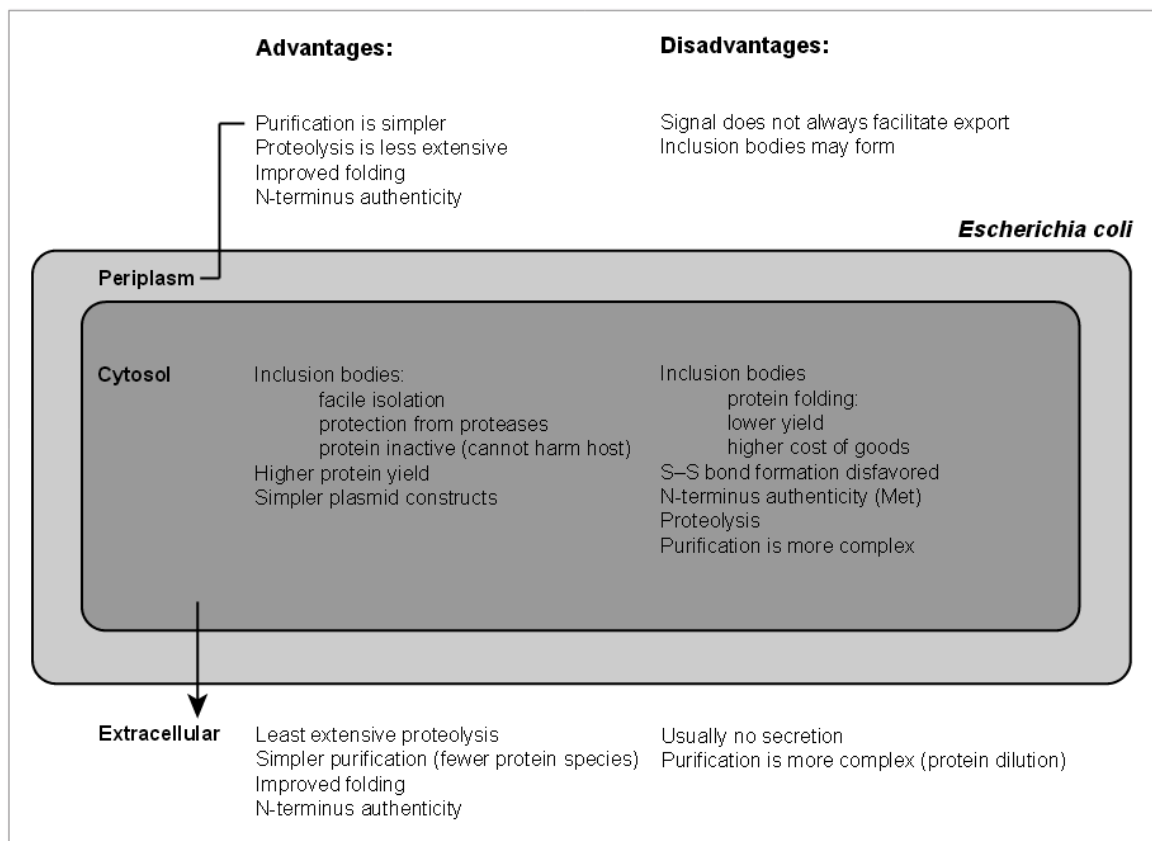


Figure 5: Advantages and disadvantages during recombinant protein production by *E. coli* (reproduced from Hanning and Makrides, 1998)

One of the common drawbacks is, bacterial systems often produce low levels of recombinant protein. Other limitations include: large proteins are produced in an insoluble form, proteins cannot be released into the culture medium due to the lack of a secretion mechanism, limited capacity for facilitating extensive disulphide bond formation, inability to fold proteins into their native state resulting in protein degradation or inclusion body formation and its inability to perform complex post-translational modifications (Georgiou and Vakax, 1996; Makrides, 1996; Hanning and Makrides, 1998, Jenkins, 2007; Gräslund *et al.*, 2008; Ferrer-Miralles *et al.*, 2009). One of the stumbling blocks when using *E. coli* as an expression system is the accumulation of endotoxins (also known as lipopolysaccharide-LPS) which are harmful to mammals (Terpe, 2006). In order for the proteins to become endotoxin-free, a second purification is required (Petsch and Anspach, 2000, Terpe, 2006). Another problem is the misfolding of mammalian proteins due to prosthetic groups, disulfide bonds and multiple subunits (Georgiou and Valax, 1996). This is one of the reasons why antibodies lacking glycosylation, are not recognised by mammals (Jenkins and Curling, 1994). Protein degradation is also another problem due to the presence of proteases present in the outer and inner membranes, the cytoplasm and the periplasm (Goldberg and Goff, 1986; Baneyx and Georgiou, 1992; Goldberg, 1992; Gottesman and Maurizi, 1992; Maurizi, 1992; Makrides, 1996).

E. coli is used for the production of products such as hormones, interferons and interleukins (Ferrer-Miralles *et al.*, 2009). These proteins produced by *E. coli* are important in the treatment of several disorders (metabolic, nutritional and endocrine) and infectious diseases (Ferrer-Miralles *et al.*, 2009). According to results reported by Roman *et al.* (1995), more than 20 mg.ml⁻¹ of active rat neuronal nitric oxide synthase (nNOS) was produced by *E. coli* as opposed to less than 1 mg.ml⁻¹ by recombinant human kidney cells. *E. coli* was also used, along with the fungus *Aspergillus niger*, to recombinantly produce mammalian chymosin and this was approved in USA (Cowan, 1996). In comparison to natural calf chymosin, the price of the recombinant produced chymosin decreased by half (Cowan, 1996).

There are other bacterial expression hosts that are commonly used for heterologous protein production such as the gram positive *Bacillus* species. There are several pharmaceutical peptides that have been expressed by *Bacillus* strains (Table 1). One of the advantages of using *Bacillus* strains as opposed to *E. coli* stains is that they have the ability to naturally

secrete proteins into the extracellular medium and the outer membrane does not contain endotoxins (Terpe, 2006). Several proteins that have been expressed are enzymes, hormones and antibodies. Udaka and Yamagata (1993) managed to express 3000 mg.l⁻¹ of α -amylase from *B. stearothermophilus* using *B. brevis* as an expression system (Terpe, 2006).

Others strains include *B. subtilis* used by Olmos-Soto and Contreras-Flores (2003) to produce 1000 mg.l⁻¹ of Proinsulin and *B. megaterium* used to produce 362 U.g⁻¹ of Dextranucrase from *Leuconostoc mesenteroides* (Malten *et al.*, 2005; Terpe, 2006). The use of bacteria as expression systems play a vital role in recombinant protein production especially ones that don't require glycosylation but it ultimately depends on the properties of the protein of interest (Verma *et al.*, 1998).

Table 1: List of some the proteins heterologously produced by *Bacilli* strains (adapted from Terpe, 2006).

Bacillus strain	Recombinant protein	Reference
<i>B. brevis</i>	Epidermal growth hormone (human)	Yamagata <i>et al.</i> , 1989
	α -amylase (human)	Konishi <i>et al.</i> , 1990
	Cholera toxin B	Ichikawa <i>et al.</i> , 1993
	α -amylase (<i>Bacillus stearothermophilus</i>)	Udaka and Yamagata, 1993
	Pepsinogen (swine)	Udaka and Yamagata, 1993
	Epidermal growth hormone (mouse)	Wang <i>et al.</i> , 1993
	Mouse/human chimeric Fab'	Inoue <i>et al.</i> , 1997
	Interleukin-2 (human)	Takimura <i>et al.</i> , 1997
	Protein disulfide isomerase	Kajino <i>et al.</i> , 1999
	Gelatin	Kajino <i>et al.</i> , 2000
	Interleukin-6 (human)	Shiga <i>et al.</i> , 2000
	Cellulase	Kashima and Udaka, 2004
<i>B. subtilis</i>	α -amylase (<i>Bacillus amyloliquefaciens</i>)	Palva, 1982
	Interferon- α 2 (human)	Palva <i>et al.</i> , 1983
	Lipase A	Lesuisse <i>et al.</i> , 1993
	Epidermal growth hormone (human)	Lam <i>et al.</i> , 1998
	Staphylokinase	Ye <i>et al.</i> , 1999
	Penicillin G acylase	Yang <i>et al.</i> , 2001
	PHA depolymerase A (<i>Paucimonas lemoignei</i>)	Braaz <i>et al.</i> , 2002
	ScFv	Wu <i>et al.</i> , 2002
	Streptavidin	Wu and Wong, 2002
	Thioredoxin (<i>Alicyclobacillus acidocaldarius</i>)	Anna <i>et al.</i> , 2003
	Proinsulin	Olmos-Soto and Contreras-Flores, 2003
<i>B. megaterium</i>	Dextranucrase (<i>Leuconostoc mesenteroides</i>)	Malten <i>et al.</i> , 2005
	Toxin A (<i>Clostridium difficile</i>)	Burger <i>et al.</i> , 2003

2.5 Plant expression systems

During the past two decades, plant based expression systems have been used for heterologous protein production. The first production of an antibody using a plant based expression system occurred in 1989 and the first recombinant hormone produced was human growth hormone using the tobacco plant (Hiatt *et al.*, 1989; Staub *et al.*, 2000). Several therapeutic drugs, recombinantly expressed in plants, are currently in clinical trials. Two in particular: IgA used for tooth decay (CaroRx™- from Planet Biotechnology Inc, Ma *et al.*, 1998, Ma *et al.*, 2005) and a human intrinsic factor used for the treatment of vitamin B12 deficiency (Cobento Biotech AS) has been approved for consumption by humans (Faye and Gormord, 2010). Ventria Bioscience (<http://www.ventria.com>) has been genetically modifying field grown rice for the heterologous production of human lactoferrin and human lysozyme (Zavaleta *et al.*, 2007; Huang *et al.*, 2008; Faye and Gormord, 2010).

SemBioSys Genetics Inc ([http:// www.sembiosys.com/](http://www.sembiosys.com/)) had one of its products, recombinant insulin produced in safflower plant seeds, enter phase III of clinical trials (Boothe *et al.*, 2010). The American Food and Drug Administration (FDA) have approved vaccines, made by plants, used in the treatment of non-Hodgkin's lymphoma (McCormick *et al.*, 2008). Plant cells have proven to be alternative systems for biopharmaceutical production including the approved vaccine by the USDA Center for Veterinary Biologics produced in tobacco suspension-cultured cells by a company called Dow Agrosience for chickens against the Newcastle disease virus and genetically engineered carrot cells for the production of human glucocerebrosidase against Gaucher's disease (Faye and Gormord, 2010; Shaaltiel *et al.*, 2007).

Plants have the advantages of low cost cultivation, lack of human pathogens and high cell mass production (Ferrer-Miralles *et al.*, 2009) however using plant expression systems can also be time-consuming as they grow slowly. Although plant expression systems can produce recombinant peptides at a lower cost, free from bacterial contaminants and human viruses, many pharmaceutical industries are reluctant to use them (Faye and Gormord, 2010). One of the major problems encountered by plant expression systems is that its post-translational modification mechanisms sometimes produces recombinant proteins that have an adverse

immune response in mammals (Ferrer-Miralles *et al.*, 2009). Mammalian expression systems are commonly used for expression of mammalian proteins that require a higher level of post-translational modification.

2.6 Mammalian expression systems

Mammalian cells are most commonly used for producing recombinant mammalian glycosylated proteins (Demain and Vaishnav, 2009). Genetically Modified Animals are commonly used to secrete recombinant proteins in their milk, blood or urine (Demain and Vaishnav., 2009). Currently only ATryn has been approved using transgenic animals and this was secreted in goat's milk. (Ferrer-Miralles *et al.*, 2009). Mammalian cell systems, CHO (Chinese hamster ovary cells) and hybridoma cells, are most similar to human cells therefore allowing recombinant expression of correctly folded and glycosylated proteins which are readily recognised by mammalian cells (Verma *et al.*, 1998, Martínez *et al.*, 2012). CHO cells grow in medium that is chemically defined, serum and protein free (Bleckwenn and Shiloach, 2004). Post translational modification are similar to human cells when using CHO cells as expression systems but it was established that glycosylation patterns differed between different batches of the same recombinant protein (Yuen *et al.*, 2003; Ferrer-Miralles *et al.*, 2009). The FDA approved two products: Xyntha and Recothrom produced using CHO cells (Ferrer-Miralles *et al.*, 2009). Other expression systems include human cell lines which have been used to express approved therapeutic proteins such as Dynepoerithropoietin, Replagal-alfa-galactosidase A and elapraserirundonate-2-sulfatase (Ferrer-Miralles *et al.*, 2009).

However, one needs to consider the disadvantages when using mammalian expressing systems for recombinant gene protein production. These include the high cultivation costs associated with growth medium such as costly growth factors and the high risk of contamination, limited secretion capacity and low protein yields (Demain and Vaishnav, 2009; Martínez *et al.*, 2012). Yeast expression systems offer the advantage of producing glycosylated proteins using minimal, inexpensive medium.

2.7 Yeast expression systems

Several yeast expression systems have acquired GRAS status by the FDA as opposed to several prokaryotic organisms that may contain endotoxins and mammalian cells with the possibility of viral DNA contamination (Domínguez *et al.*, 1998). They offer numerous advantages for the expression of complex proteins in that they retain the easiness of bacterial manipulation and growth characteristics (Madzak *et al.*, 2004). Being unicellular, they also “possess an eukaryotic subcellular organisation able to perform the post-translational processing of complex proteins” (Madzak *et al.*, 2004). In comparison to *E. coli*, yeast expression systems have the capability to secrete correctly folded proteins (Verma *et al.*, 1998). Yeast expression systems also have the ability to reach high cell densities in bioreactors and are faster and cheaper to manipulate as opposed to animal and plant expression hosts (Ferrer-Miralles *et al.*, 2009; Martínez *et al.*, 2012).

Yeast cells are less sensitive to contamination due to their cell walls being resistant to shear stress during protein production and have the ability to grow in media that is not costly as compared to mammalian cells (Verma *et al.* 1998; De Pourcq., 2010; Martínez *et al.*, 2012). During fermentation, protein production can reach more than 1 g.l⁻¹ in a matter of days as compared to other expression systems (Gerngross., 2004). The production of hirudin, a thrombin inhibitor was compared using different expression systems (Table 2) by Demain and Vaishnav (2009). Yeast showed the highest production of the recombinant protein.

Table 2: Comparison of hirudin production using different expression systems (adapted from Demain and Vaishnav, 2009).

Recombinant hosts	Organism	mg.l ⁻¹
BHK cells	Mammalian	0.05
Insect cells	Insect	0.40
<i>Streptomyces lividans</i>	Bacterial	0.25-0.5
<i>Escherichia coli</i>	Bacterial	200-300
<i>Saccharomyces cerevisiae</i>	Yeast	40-500
<i>Hansenula polymorpha</i>	Yeast	1500
<i>Pichia pastoris</i>	Yeast	1500

Saccharomyces cerevisiae is the most commonly used yeast for heterologous protein production in the past. Vast amounts of knowledge are widely available for this host. It has been used as an expression system for several proteins such as 0.09 g.l⁻¹ of human serum albumin (Okabayashi *et al.*, 1991), 1 g.l⁻¹ of Tetanus toxin fragment C (Romanos *et al.*, 1991) and 9 g.l⁻¹ of glucose oxidase from *Aspergillus niger* (Park *et al.*, 2000; Adrio and Demain, 2010) to name a few.

S. cerevisiae, however, has several limitations: “low product yield, poor plasmid stability, difficulties in scaling-up production, hyperglycosylation, and low secretion capacities” (Madzak *et al.*, 2004). Due to these limitations of *S. cerevisiae*, alternative yeast systems have been found and these include: the methylotrophs, *Pichia pastoris* and *Hansenula polymorpha*; the dairy yeast, *Kluyveromyces lactis*; the amylolytic yeast, *Schwanniomyces occidentalis* and the alkaline utilizer, *Yarrowia lipolytica* (Müller *et al.*, 1998).

P. pastoris is another commonly used yeast expression system that has been exploited over the past few decades with high level recombinant production (Cereghino and Gregg., 1999, reviewed by Cereghino and Cregg, 2000). It was developed by the Phillips Petroleum Company for the expression of single cell proteins with the potential to achieve more than 130 g.l⁻¹ of dry cell weight in a continuous culture and has the ability to grow on methanol as an only source of energy (Ogata *et al.*, 1969; Wegner, 1990; Domínguez *et al.*, 1998). *P. pastoris* has been used in the production of 10 g.l⁻¹ of human serum albumin (Kobayashi *et al.*, 2000), 12 g.l⁻¹ of Tetanus toxin fragment C (Clare *et al.*, 1991) and 14.8 g.l⁻¹ of Gelatin (Werten *et al.*, 1999) to name a few. As an expression system, its production yields are much higher than *S. cerevisiae* when expressing the same proteins (111 fold higher for HSA and 12 fold higher for Tetanus toxin fragment C).

Another expression host, that has been described as one of the most attractive non conventional yeast host strains for recombinant protein expression is *Yarrowia lipolytica* (Müller *et al.*, 1998). During the mid-1960s, the production for single-cell proteins emerged. *Y. lipolytica* was able to arouse a strong industrial interest because of its capability to grow on *n*-paraffins.

This substrate could be used as an only carbon source and was very cheap and plentiful during this period. (Barth and Gaillardin., 1997).

2.8 *Yarrowia lipolytica*

Y. lipolytica ascomycetous yeast was previous classified as *Candida*, *Endomycopsis* and *Saccharomyces lipolytica* (Barth and Gaillardin., 1997). It was first discovered by Wickerham in 1945 after isolation from a jar that contained fibre tailings from a corn processing plant which was found to form hyphal elements attached to asci when a certain type of medium was used (Barth and Gaillardin, 1997). It has been used in many industrial applications many of which have been classified as safe by the FDA. These include the production of citric acid, peach flavour and peptides (Nicaud *et al.*, 2002).

This dimorphic yeast has the capacity to secrete several recombinant proteins efficiently with several molecular tools available for post-translational modification (Madzak *et al.*, 2004; Pignede *et al.*, 1998; Lopes *et al.*, 2008). *Y. lipolytica* has been classified as non pathogenic (Holzschu *et al.*, 1979) with the ability to naturally secrete several enzymes including lipases, RNAses, proteases and esterases (Barth and Gaillardin., 1997). In comparison to other well-known yeasts such as *S. cerevisiae* and *Schizosaccharomyces pombe*, *Y. lipolytica* is able to utilize several substrates: alkanes, fatty acids, glucose and organic acids (Barth and Gaillardin, 1997). In the past, *Y. lipolytica* was used to produce high amounts of organic acids by growing on *n*-paraffins (Tsugawa *et al.*, 1969). This allowed for upscaling of products with the accumulation of large amounts of data for fermentation processes. (Barth and Gaillardin., 1997). This organism can produce between 1-2 g.l⁻¹ alkaline extracellular protease (AEP) when grown on YPD medium (Tobe *et al.*, 1976, Ogrydziak and Scharf, 1982; Barth and Gaillardin, 1997).

Lip2 is a native enzyme expressed by *Y. lipolytica* at high concentrations (Pignede *et al.*, 2000a; Pignede *et al.*; 2000b; Fickers *et al.*, 2005) and hence was used as a fusion partner for

expression of a galactose oxidase M1 enzyme, a camelid antibody fragment and a trypsin inhibitor (Hofmeyer *et al.*, 2013).

There are several *Y. lipolytica* strains available for heterologous gene expression. These include the Pold strain deleted for an alkaline extracellular protease by Le Dall *et al.*, (1994) with the ability to utilise the carbon source sucrose (Nicaud *et al.*, 1989) and the Po1f, Po1g and Po1h strains (Madzak *et al.*, 2000; Madzak, 2003) deleted for both extracellular proteases (acid and alkaline). Pof and Polh strains were used for integration of yeast expression cassettes from auto-cloning vectors (Madzak *et al.*, 2000; Madzak., 2003). Po1g and Po1h retained one auxotrophic marker with Po1g containing an extra pBR322 docking integration platform (Madzak *et al.*, 2000; Madzak., 2003). The most commonly used selection markers are LEU2 and URA3 (Le Dall *et al.*, 1994; Madzak *et al.*, 2004).

There are several plasmids that exist and are designed specific for host strains with selection markers. The pINA1296 plasmid was designed by Madzak *et al.*, (2000) for integration at the pBR322 docking platform of the Polg strain of *Y. lipolytica*. The pINA1297 vector was designed as a “zeta based auto-cloning multi copy vector” for integration into the Po1f and Po1h strains (Nicaud *et al.*, 2002; Madzak *et al.*, 2004). The integrative pKOV410 plasmid is commonly used by the CSIR (Bulani *et al.*, 2012; Hofmeyer *et al.*, 2014) (which was kindly donated by the University of the Free State) was used for the purpose of this study (Figure 6).

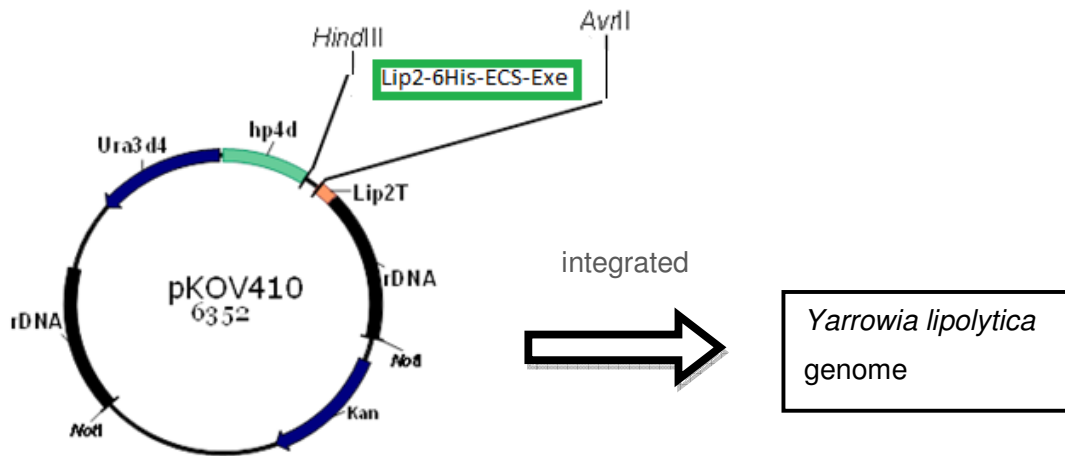


Figure 6: The pKOV410 plasmid containing the Lip2-Exe fusion gene was randomly integrated into the genome of *Y. lipolytica*.

This plasmid was transformed to contain expression cassette: which consisted of a lipase gene (Lip2), 6 x histidine (His) tag, an enterokinase cleavage site (ECS) and the exenatide (Exe) peptide (39 amino acids) with the addition of a glycine residue to the C-terminal end (necessary for downstream processing – amidation) (Figure 6). The plasmid contained the sequence that targets the rDNA location of the strain YIEx-gly and a LEU2 auxotrophic marker.

Apart from choosing the most appropriate plasmid for gene expression, finding the correct promoter for optimal expression is crucial. According to Müller *et al.* (1998), a promoter that is tightly regulated allows one to separate the growth and expression stages hence allowing protein expression which can inhibit cell growth. When choosing the appropriate promoter for expression, the following needs to be considered: it must be strong, capable of producing between 10- 30% of a recombinant protein in excess of the total protein produced by the organism, it should show a low level of basal transcription during growth of the organism especially for toxic proteins and induction should be cost-effective and simple (Hannig and Makrides, 1998). There are a variety of promoters available for heterologous gene expression by *Y. lipolytica* and these are listed in Table 3.

Table 3: Promoters available for *Y. lipolytica* and their characteristics (reproduced from Madzak *et al.*, 2004).

Promoters (source)	Characteristics
<u>pLEU2</u> (β -isopropylmalate dehydrogenase)	Inducible by leucine precursor
<u>pXPR2</u> (alkaline extracellular protease)	Inducible by peptones
<u>pPOX2</u> , <u>pPOT1</u> (respectively, acyl-CoA oxidase 2, 3-oxo-acyl-CoA thiolase)	Inducible by fatty acids and derivatives, and alkanes
<u>pICL1</u> (isocitrate lyase)	Inducible by fatty acids and derivatives, alkanes, ethanol and acetate
<u>pPOX1</u> , <u>pPOX5</u> (acyl-CoA oxidases 1 and 5)	Weakly inducible by alkanes
<u>pG3P</u> (glycerol-3-phosphate dehydrogenase)	Inducible by glycerol
<u>pMTP</u> (bidirectional: metallothioneins 1 and 2)	Inducible by metallic salts
<u>hp4d</u> (hybrid promoter derived from pXPR2)	Growth-phase-dependent
<u>pTEF</u> , <u>pRPS7</u> (respectively, translation elongation factor-1 α , ribosomal protein S7)	Constitutive

The XPR2 promoter is the most commonly used promoter for recombinant protein production by *Y. lipolytica*. It is only active at pH's above 6 in medium that doesn't contain carbon and nitrogen and induction requires high levels of peptone (Ogrydziak *et al.*, 1977; Ogrydziak and Scharf, 1982, Franke *et al.*, 1988). The RPS7 and TEF promoters were isolated by Müller *et al.* (1998) and have been described as constitutive and strong. The downfall of having a constitutive promoter is that early protein production could affect the growth of the organism (Madzak *et al.*, 2004). Other promoters that were isolated by Madzak *et al.* (2004) include the POX promoters (acyl-CoA oxidases POX1, POX2 and the POX5), the POT1 promoter (3-oxo-acyl-CoA thiolase) and ICL1 (isocitrate lyase). These promoters were studied against the XPR2, the hp4d and the G3P (glycerol-3-phosphate dehydrogenase) promoters by monitoring their regulatory mechanism during growth of *Y. lipolytica* (Juretzek *et al.*, 2000; Madzak *et al.*, 2004). The outcomes yield that POT1 and POX2 was repressed by the carbon sources glycerol and glucose and induced by alkanes and fatty acids, ICL1 was induced by acetate, alkanes, fatty acids and ethanol although not completely repressed by glycerol and glucose (Juretzek *et al.*, 2000; Madzak *et al.*, 2004).

The XPR2 promoter was critically analysed by Blanchin-Roland *et al.*, 1994 and Madzak *et al.*, 1999). It was found that an upstream activating sequence, designated as UAS1, was not affected significantly by environmental conditions. This sequence was used to design a recombinant promoter called hp4d that was not affected by medium composition such as

nitrogen and carbon sources or pH or peptones (Madzak *et al.*, 1995; Madzak *et al.*, 2000). It was said to drive a strong gene expression and its regulation to be growth phase dependent starting at early stationary phase (Madzak *et al.*, 2000; Nicaud *et al.*, 2002). This promoter has been used for the recombinant production of several proteins such as epoxide hydrolase from *Rhodotorula araucariae*, endo- β -1, 4-mannanase from *Aspergillus aculeatus*, a galactose oxidase M1 enzyme, a camelid antibody fragment and a trypsin inhibitor (Maharajh *et al.*, 2008; Roth *et al.*, 2009, Hofmeyer *et al.*, 2014; Madzak *et al.*, 2004).

2.9 Concluding Remarks

The CSIR is developing a process for the recombinant production of pharmaceutical peptides in *Y. lipolytica*. The production of lipase (Lip2), under regulation of the hp4d promoter was previously elucidated in continuous fermentation and found to be linked to growth rate with high production yields occurring at growth rates lower than 0.045 h^{-1} (van Zyl, 2013). However it is unclear if this regulation is at the level of transcription or cellular metabolism. Therefore to achieve maximum production of pharmaceutical peptides and proteins, the regulation of the hp4d promoter needed to be established. A *Y. lipolytica* strain expressing a fused Lipase and Exenatide (Lip2:Exe) precursor, under regulation of the hp4d promoter, was generated (Chapter 3). The growth and production profiles will therefore be established and compared to literature in Chapter 3. An optimised mRNA transcription methodology will be developed and validated in Chapter 4 followed by the evaluation of the effect of growth rate on the recombinant production of fused Lip2:Exe, under regulation of the hp4d promoter, by *Y. lipolytica* in Chapter 5.

Chapter 3: Evaluation of *Yarrowia lipolytica* (YIEx-gly) expressing a pharmaceutical peptide precursor under regulation of the hp4d promoter

3.1 Introduction

Exenatide (Byetta[®]) is a type 2 diabetic drug that was discovered by Dr John Eng in the early nineties (Mueller, 2007). He isolated a compound from the saliva of the Gila monster after he noticed that the endangered lizard's poison stimulated the body's production of insulin, a hormone that helps cells decrease blood glucose levels (Somers, 2005). The compound also prevented the blood-glucose levels from dropping dangerously low and stopped it from spiking hence preventing damage to the eyes, liver and kidneys (Somers, 2005). It was later licensed by Amylin Pharmaceuticals and marketed as the diabetic drug Byetta[®] and therefore is used as an alternative to insulin for the treatment of type 2 diabetes (Mueller, 2007; Kyriacou and Ahmed, 2010). The cost of treatment using this drug is very expensive. On average, it costs approximately \$ 1800 for 5 µg and \$ 2200 for the 10 µg dosages annually per patient per year (Bond, 2006). The main reason for the high treatment costs is due to several steps required during the manufacturing process (chemical synthesis) of the drug. A potential cost-effective alternative using recombinant protein expression exists and with *Y. lipolytica* as a host.

A *Y. lipolytica* Po1f strain, expressing the fused Lip2:Exe precursor, was generated by Dr Ramagoma¹ and Dr Bulani² from the Biosciences Department at the CSIR (Table 4) and kindly donated for the purpose of this study. The integration plasmid pKOV410 (kindly donated by the University of the Free State) was used which contained the sequence that targets the rDNA location of the strain YIEx-gly, the expression cassette: which consisted of a lipase gene (Lip2), 6 x histidine (His) tag, an enterokinase cleavage site (ECS) and the exenatide (Exe) peptide (39 amino acids) with the addition of a glycine residue to the C-terminal end (necessary for downstream processing – amidation) (Figure 7) and a LEU2 auxotrophic marker.

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Table 4: Genotypes of *Y. lipolytica* strain containing the Lip2:Exe fusion peptide.

Strain	Characteristics	Reference
<i>Y. lipolytica</i> Po1f	Δ MatA, Δ leu2-270, Δ ura3-302, Δ xpr2-322, Δ axp-2	(Madzak <i>et al.</i> , 2000)
<i>Y. lipolytica</i> YIEx-gly	Δ MatA, Δ leu2-270, Δ xpr2-322, Δ axp-2, Lip2:Exe	

**Figure 7:** The Lip2:Exe expression cassette integrated into the genome of *Y. lipolytica*.

3.2 Materials and Methods

3.2.1 Cell banking of *Y. lipolytica* (YIEx-gly) expressing the fused Lip2:Exe precursor

Ultra Yield (2.5 l, Biosilta®, United Kingdom) flasks (n = 5), containing sterile (autoclaved at 121 °C for 20 min) YPD (100 ml) medium [1 % yeast extract (m.v⁻¹, Merck, Germany), 1 % peptone (m.v⁻¹, Merck, Germany) and 2 % glucose (m.v⁻¹, Merck Millipore, Germany), pH 6.4], were inoculated by transferring a single colony of *Y. lipolytica* (YIEx-gly) from a plate counting agar (PCA) plate (stored at 4 °C for 1 week). These plates containing the YIEx-Gly strain were donated by Dr Bulani and Dr Ramagoma. The flasks were incubated on an orbital shaker (180 rpm) at 28 °C and aseptically sampled (2 ml) every 2 h. The growth of the organism was followed by measuring the optical density (OD) at 660 nm using a Beckman Coulter DU800 spectrophotometer (Beckman Coulter, USA). When mid-exponential phase (OD: 4–7) was reached, all flasks were microscopically checked for monoculture and pooled together followed

by cryopreservation of organism by mixing cold sterile glycerol (4 °C, 50 % m.v⁻¹) with culture broth at a ratio of 1:1 followed by aseptically dispensing 2 ml aliquots into sterile cryo-vials (2 ml). The cryo-vials were thereafter placed in Nalgene cryo-containers containing 250 ml Isopropyl Alcohol (IPA) and stored at -80 °C. After a period of 48 h, the cryo-vials were transferred to storage cryo-boxes and stored at -80 °C. This was followed by growth curves and validation studies of the cell bank.

3.2.2 Growth curve study and validation of the cell bank for production of the fused Lip2:Exe precursor

Ultra yield (2.5 l) flasks (Biosilta®, United Kingdom) containing sterilised (autoclaved at 121 °C for 20 min) YPD (700 ml, pH 6.4) medium were inoculated with cryo-vials (one cryo-vial per flask) from the cell bank (n = 5). The flasks were incubated on an orbital shaker (180 rpm) at 28 °C and growth profiles were established by aseptically removing culture broth every 2 h and measuring OD_{660nm}, pH, biomass production (section 3.2.4.1) and residual glucose concentration (Accutrend®, Germany) for the first 26 h. The growth rate (Equation 1) and doubling time (Equation 2) for *Y. lipolytica* were established using the following formulas:

$$\text{Growth rate } (\mu) = 2.303 \times (\log_{10} \text{OD}_2 - \log_{10} \text{OD}_1) / (\text{Time}_2 - \text{Time}_1) \quad \text{Equation 1}$$

$$\text{Doubling time } (t_D) = \ln 2 / \mu \quad \text{Equation 2}$$

Following the growth curve study, the cell bank was validated for production of the fused Lip2:Exe precursor by *Y. lipolytica* (YIEx-gly). Ultra yield (2.5 l) flasks containing sterilised (autoclaved at 121 °C for 20 min) YPD (700 ml, pH 6.4) medium were inoculated using YIEx-gly containing cryo-vials (one cryo-vial per flask) from the cell bank (n = 5). The flasks were incubated on an orbital shaker (180 rpm) at 28 °C. Samples (10 ml) of culture broth were aseptically removed from each flask, at regular intervals, to measure pH, residual glucose concentration, biomass production (section 3.2.4.1) and OD_{660nm} followed by centrifugation of 2 ml samples (n = 3) at 13000 x g for 10 min. The supernatant was dispensed into sterile Eppendorf tubes and stored at -20 °C for quantification of total extracellular protein and lipase production (sections 3.2.4.2 and 3.2.4.3 respectively).

3.2.3 Batch fermentation of *Y. lipolytica*

Upon validation of the cell bank using a flask trial, batch fermentation was used to scale-up production of the fused Lip2:Exe precursor. Labfors 3 fermenters (Infors HT, Switzerland) with a 1.7 l working volume ($n = 4$), were initially pressure tested for any leaks followed by calibration of pH and DO (Dissolved Oxygen) probes. YPD medium (1.2 l, pH 6.8) was prepared in each fermenter and autoclaved at 121 °C for 45 min. Once the temperature of the medium had reached 25 °C, each fermenter was inoculated with a 2 ml cryo-vial, containing *Y. lipolytica* (YIEx-gly) expressing the fused Lip2:Exe precursor using a 2 ml cyro-vial from the cell bank. A sterile syringe was used to inject the inoculums via the septated port. The temperature was controlled at 28 °C and the aeration was set to 1 slpm (standard liters per minute). The starting agitation was set at 300 rpm and ramped to 1000 rpm in order to maintain the pO_2 (dissolved oxygen) at 30 % saturation. Samples (20 ml) of culture broth were aseptically removed, at regular intervals, to measure pH, residual glucose concentration, biomass production (section 3.2.4.1) and OD_{660nm} . Triplicate samples (2 ml) were centrifuged at 13000 x g for 10 min and the supernatant was dispensed into sterile Eppendorf tubes and stored at -20 °C for quantification of total extracellular protein and lipase production (sections 3.2.4.2 and 3.2.4.3 respectively). The supernatant was also used to observe extracellular protein production by running SDS PAGE gel electrophoresis (section 3.2.4.4). The fermentation culture broth was microscopically checked on a daily basis to ensure monoculture status.

3.2.4 Analysis used for study

3.2.4.1 Biomass production determination

Biomass (Dry Cell Weight) production was quantified by centrifuging 2 ml samples at 13000 x g for 10 min in pre-weighed (Weight 1) Eppendorf tubes (2 ml). The supernatant was discarded and the pellet left to dry at 110 °C until constant weight was reached (Weight 2). Each tube was weighed and the biomass calculated using Equation 3.

$$\text{Biomass (g.l}^{-1}\text{)} = (\text{Weight 2} - \text{Weight 1}) \times 500$$

Equation 3

3.2.4.2 Protein concentration determination assay

The Bradford (Bradford, 1976) assay method uses the dye-binding principle, giving off a colour change, depending on the concentration of total extracellular protein. A BSA (Bovine Serum Albumin; Roche, Germany) standard curve was generated prior to quantification at a concentration range of 0.0 - 0.5 mg.ml⁻¹.

Bio-Rad (Bio-RadTM, USA) protein assay reagent (200 µl) was added to 10 µl of sample (n = 4) in a 96 well flat-bottom microtiter plate (samples were diluted to fit within the standard curve). The plate was left shaking (200 rpm) for 5 min at room temperature to ensure efficient binding of the dye to the protein. The end point reaction was quantified at 595 nm (BioTek PowerWaveTM HT, USA) and read against a BSA standard curve in order to determine the total extracellular protein concentration of each sample. All assays done were done in quadruplicate.

3.2.4.3 Lipase kinetic assay

Lipase (Lip2) was used as a reporter enzyme to give an indication of the amount of fused Lip2:Exe produced. The lipase assay (Vorderwülbecke *et al.*, 1992) measured the release of p-nitrophenol during cleavage of the substrate p-nitrophenylpalmitate by the Lip2 enzyme. Solutions A and B were prepared according to Table 5.

Table 5: Lipase Assay components used to quantify the amount of fused Lip2:Exe produced.

Solution	Component	Concentration
A	p-Nitrophenylpalmitate	3.0 mg.ml ⁻¹
	Propan-2-ol	1.0 ml.ml ⁻¹
B	Tris-HCl buffer (50 mM, pH 8.0)	1.0 ml.ml ⁻¹
	Na-deoxycholate	4.4 mg.ml ⁻¹
	Gum arabic	1.1 mg.ml ⁻¹

Solution A was added drop-wise to solution B at a ratio of 1:9 whilst stirring (200 rpm, 37°C) using a heating-stirrer plate. The reaction was initiated by the addition of 240 µl of the combined solution (37°C) to 10 µl of enzyme sample (supernatant) in a 96 well flat-bottom microtiter plate. All assays done were done in quadruplicate. The release of p-nitrophenol was monitored at 410 nm (37°C) using a microtiter plate reader (BioTek PowerWave™ HT, USA). The activity of the enzyme was calculated as U.ml⁻¹ using Equation 4:

$$U.ml^{-1} = (V/v \times \epsilon \times d) \times A/min \quad \text{Equation 4}$$

Where by:

V = final volume,

v = sample volume,

ε = Extinction coefficient of p-nitrophenol at 410 nm (pH 8.0)

$$= 15 (1 \times nmol^{-1} \times cm^{-1} = ml \times \mu mol^{-1} \times cm^{-1}),$$

d = light path of cuvette,

A.min⁻¹ = change of absorbance per minute.

The U.ml⁻¹ was converted to nKat.ml⁻¹ by multiplying with a constant of 16.8.

3.2.4.4 SDS PAGE gel electrophoresis to monitor extracellular protein production

In order to observe extracellular protein production, SDS PAGE gel electrophoresis was used (Laemmli., 1970). Supernatant (10 µl) was combined with 10 µl of sample buffer (62.5 mM Tris-HCl, pH 6.8, 20 % Glycerol, 2 % SDS, 5% β-Mercaptoethanol) followed by 10 min incubation at 95°C, on a heating blocking. The tubes were briefly centrifuged for 5 sec to ensure even mixing of the sample and buffer. The samples (10 µl) were loaded onto a 12% SDS-PAGE gel [separating gel consisted of: 30 % Bis/Acrylamide (Sigma-Aldrich, USA), 1.5 M Tris- HCl, pH 8.8, 10 % (w/v) SDS, 10 % (w/v) Ammonium Persulfate (Sigma-Aldrich, USA) and TEMED (Sigma-Aldrich, USA); stacking gel consisted of: 30 % Bis/Acrylamide (Sigma-Aldrich, USA), 0.5 M Tris- HCl, pH 6.8, 10 % (w/v) SDS, 10 % (w/v) Ammonium Persulfate (Sigma-Aldrich, USA) and TEMED (Sigma-Aldrich, USA)] and electrophoresed (running buffer: 25 mM Tris, 192 mM glycine and 0.1 % (w/v) SDS, pH 8.3) through the stacking gel at 100 V for 20 min followed

by 200 V for 30 min for the separating gel. The molecular weight marker that was used as a reference is the Spectra™ Multicolor Broad Range Protein Ladder (Thermo Scientific USA). The SDS gel was stained using the Colloidal Coomassie Blue Staining method (Kang *et al.*, 2002; Neuhoff *et al.*, 1988).

3.3 Results and Discussion

A cell bank was generated for *Y. lipolytica* (YIEx-gly) expressing the fused Lip2:Exe precursor. The glycerol stocks were checked for a monoculture followed by the evaluation of growth curve profiles and validation of recombinant production of the fused Lip2:Exe precursor.

3.3.1 *Y. lipolytica* (YIEx-gly) growth profile characteristics and recombinant production of the fused Lip2:Exe precursor

The cell bank for *Y. lipolytica* (YIEx-gly) was generated and confirmation of a monoculture present in the glycerol stocks was microscopically checked (Figure 8) and then streaked (10 μ l) onto PCA plates. Growth was followed by measuring the OD_{660nm} and biomass (section 3.2.4.1) for 26 h (Figure 9A).

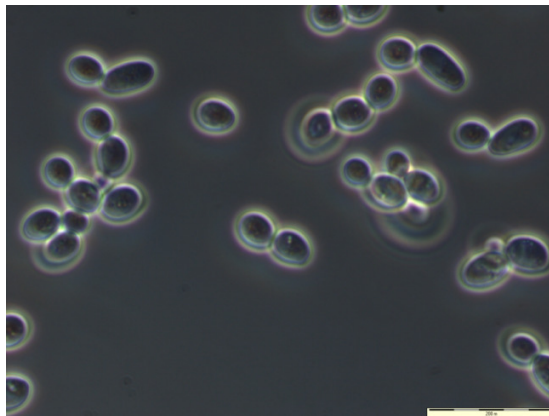


Figure 8: Confirmation of monoseptic culture. The glycerol stocks were visualised, at 100 x magnification, for the presence of *Y. lipolytica* cells using a light microscope.

The maximum growth rate of *Y. lipolytica* (YIEx-gly), as determined by the OD_{660nm} (Figure 8A), was 0.35 h^{-1} with a doubling time of 2.89 h. This occurs during the exponential phase of growth in the presence of an excess of nutrients. After 20 h, the growth rate of the organism started to decrease, a change from exponential to early stationary phase indicative of a metabolic shift. It was observed that the pH of the medium and residual glucose concentration started decreasing during the exponential phase (Figure 9B). The drop in pH is most probably a result of the accumulation of acids since *Y. lipolytica* is known to produce organic acids during metabolic overflow (Forster *et al.*, 2007). These secreted acids include organic acids such as the TCA cycle intermediates; isocitric acid, α -ketoglutaric acid and pyruvic acid (Coelho *et al.*, 2010). In the past *Y. lipolytica* was also used for the production of citric acid (Rywińska *et al.*, 2010).

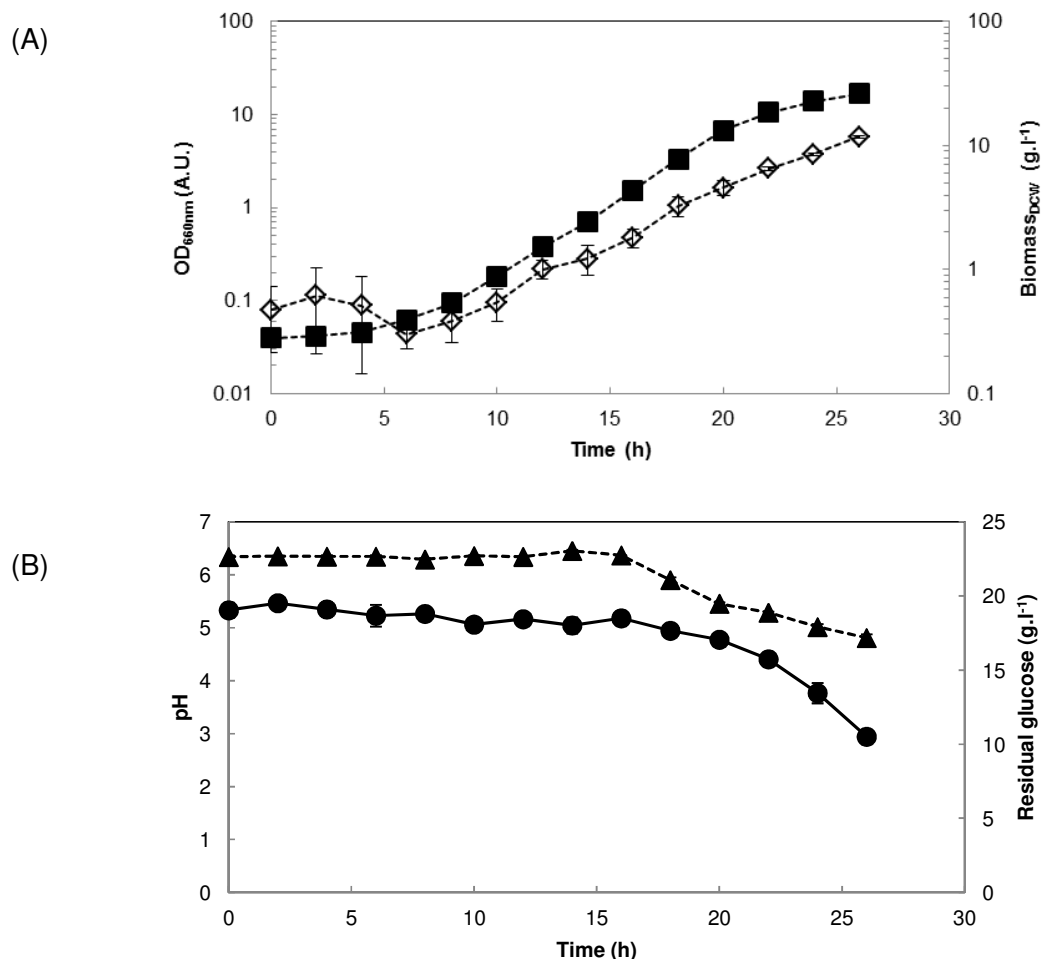


Figure 9: Growth of *Y. lipolytica* (YIEx-gly) in 2.5 l (n=5) flasks. A: Optical density (■) and biomass (◇) production profiles and B: pH (▲) and residual glucose concentration (●).

The cell bank of *Y. lipolytica* (YIEx-gly) was validated in flasks for the production of the fused Lip2:Exe precursor peptide (Figure 10). A maximum OD_{660nm} of 27(± 0.17) was reached after 40 h at a growth rate of 0.014 h⁻¹ (Figure 10A). The organism reached stationary phase after 40 h and this correlates to data achieved by Hofmeyer *et al.* (2014) during the production of a galactose oxidase M1 enzyme, a camelid antibody fragment and a trypsin inhibitor as fusion peptides to Lip2 by *Y. lipolytica*. The glucose was utilised at a rate of 0.48 g.l⁻¹.h⁻¹ for the first 40 h. The pH dropped from 6.3(± 0.03) to 4.1(± 0.03) at 40 h and increased to 7.7(± 0.02) at 50 h.

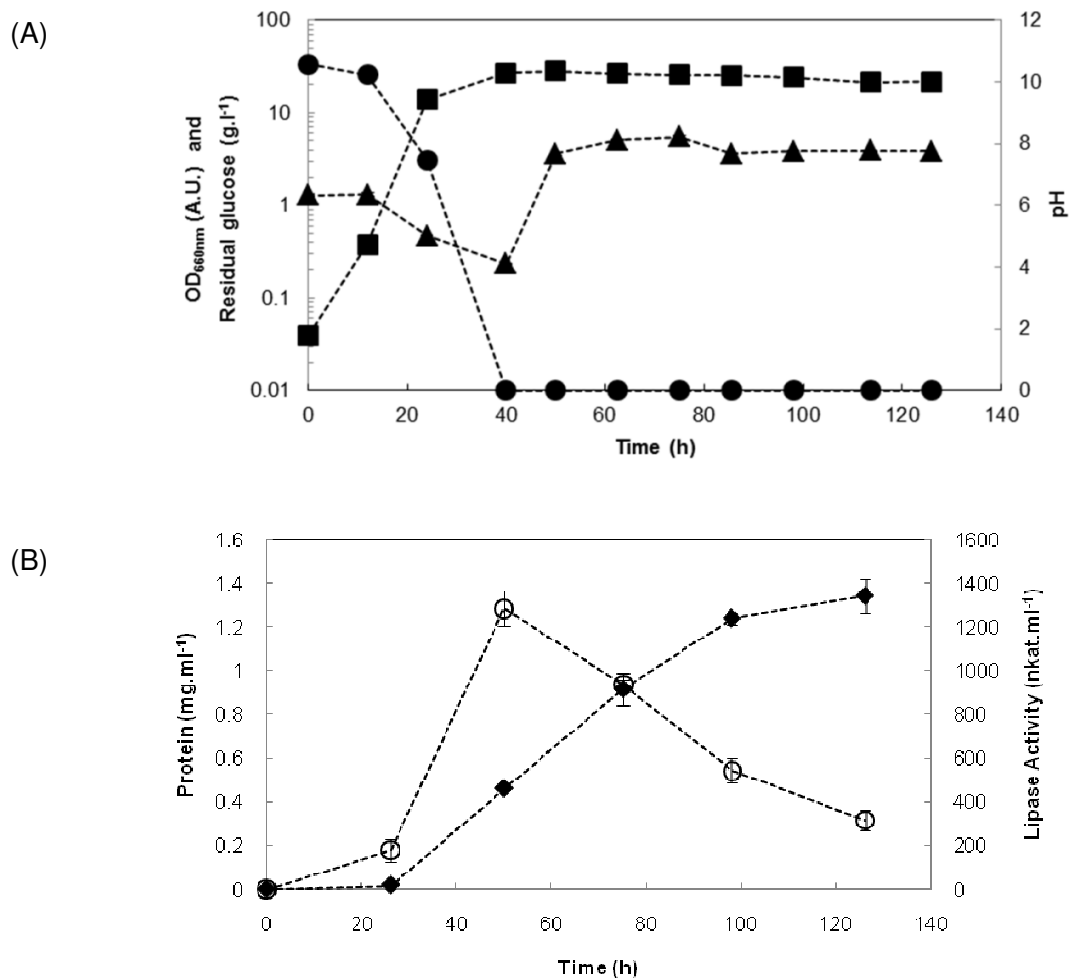


Figure 10: Validation of cell banked *Y. lipolytica* (YIEx-gly) for the recombinant production of extracellular lipase in Ultra Yield Flasks (n=5, 2.5 l). A: Optical density (■), pH (▲) and Residual glucose concentration (●) profiles of the medium during growth, B: Total extracellular protein (◆) and volumetric lipase production (○).

When using glucose as a substrate, the decrease in pH is due to the production of organic acids (such as citric acid) and the increase in pH due to proteolysis and the reutilization of organic acids after glucose depletion (Revah and Lebeault., 1989; Rane and Sims., 1996; Papanikolaou *et al.*, 2002). Lipase was detected after 24 h (Figure 10B). A maximum lipase activity of $1287(\pm 81.40 \text{ nKat.ml}^{-1})$ was observed at 50 h, 10 h after glucose depletion, followed by a rapid decrease to $319(\pm 46.30) \text{ nKat.ml}^{-1}$ at 126 h.

The total extracellular protein concentration increased from $0.916(\pm 0.08) \text{ g.l}^{-1}$ at 75 h to $1.34(\pm 0.08) \text{ g.l}^{-1}$ at 126 h. This was 7.7 fold higher than Hofmeyer *et al.* (2014) who achieved up to 174 mg.l^{-1} of fused protein during shake flask studies. Following the validation of the cell bank using a flask trial, batch fermentation was used to further upscale the production of the fused Lip2:Exe precursor peptide.

3.3.2 Batch fermentation of *Y. lipolytica*

Batch fermentation was used to validate the cell bank for production of the fused Lip2:Exe precursor at a 1.2 l scale. The maximum growth rate during the exponential phase was 0.346 h^{-1} with a doubling time of 2.89 h. The maximum $\text{OD}_{660\text{nm}}$ of 26.1 and biomass of $18.33(\pm 0.54) \text{ g.l}^{-1}$ was reached after 38 h when glucose in the medium was depleted (Figure 11A). The maximum volumetric lipase activity of $14042.32(\pm 1008.95) \text{ nkat.ml}^{-1}$ was reached after glucose depletion with an overall productivity of $379.52 \text{ nkat.ml}^{-1}.\text{h}^{-1}$. A 10.91 fold higher production in lipase activity was observed in batch fermentation as opposed to flask studies (Figure 11B). Although the lipase productivity decreased after 37 h (explained in Figure 12), protein production remained constant and reached a maximum of 0.34 mg.l^{-1} after 47 h. However, it is unclear if Lip2:Exe transcription, under regulation of the hp4d promoter, started at the beginning of the exponential growth phase and was only translated at early stationary phase or if Lip2:Exe transcription only started at the early stationary phase.

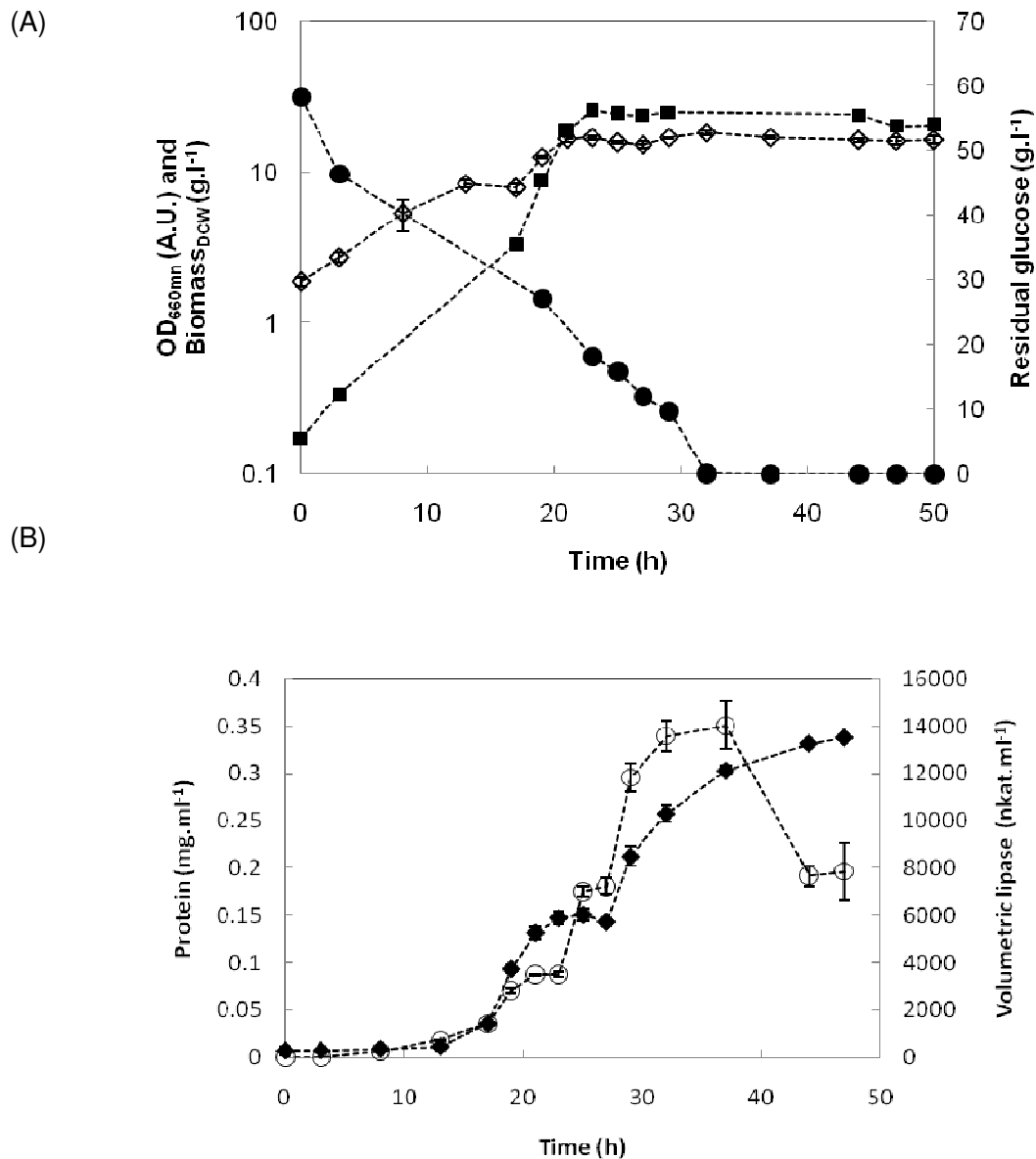


Figure 11: Validation of the production of extracellular fused Lip2:Exe precursor peptide by *Y. lipolytica* in batch fermentation. A: Optical density (■), Biomass (◇) and Residual glucose (●), B: Total extracellular protein (◆) and volumetric lipase activity (○).

SDS PAGE gel was used to monitor the production of the total extracellular protein (Figure 11). The Lip2:Exe fusion peptide (42 kDa) was the majority of the total extracellular protein produced. The production of Lip2:Exe stopped after 41 h followed by degradation of the peptide resulting in a decrease in lipase activity but not a decrease in extracellular protein

concentration as observed in Figures 10B, 11B and 12. Similar lipase profiles have been observed by several authors (Lopes et al., 2008; Alonso *et al.*, 2005; Domínguez *et al.*, 2003; Corzo and Revah., 1999).



Figure 12: Total extracellular protein production profile by *Y. lipolytica* (YIEx-gly).

3.4 Concluding Remarks

The *Y. lipolytica* strain expressing the fused Lip2:Exe precursor was cell banked and validated for the production of the recombinant protein using flask and batch fermentation studies. It was observed that production, under regulation of the hp4d promoter, only started in early stationary phase. In order to fully understand the regulatory mechanism of the hp4d promoter, the transcription of Lip2:Exe gene fusion needed to be quantified during growth of *Y. lipolytica*. Before this could be done, a methodology to monitor transcripts needed to be optimised and validated for reproducibility. This will be undertaken in Chapter 4 prior to the determination of the regulation of the hp4d promoter in Chapter 5.

Chapter 4: Optimisation of a mRNA sandwich hybridisation methodology for Lip2:Exe transcript quantification

4.1 Introduction

Transcription of the gene is the first step in the expression of a product and this is initiated by a promoter. The regulation of a promoter can be due to several factors such as growth rate, growth phase and the presence of substrates (eg. fatty acids, peptones, amino acids, etc.). By measuring the amount of transcripts (mRNA) of a particular gene, one can determine the regulatory mechanism of a promoter. This therefore will aid in determining the regulation of the hp4d promoter by quantifying the amount of Lip2:Exe mRNA transcripts produced during growth of *Y.lipolytica* YIEx-Gly.

The evolution of transcription monitoring methodologies and development has increased overtime. Many assays have provided the high-throughput monitoring of the mRNA transcripts which are based on high specificity and sensitivity (Bustin, 2002). There are several methodologies that are currently available to quantify mRNA transcripts under regulation of a promoter such as northern hybridization and reverse transcription (RT) PCR. The disadvantages of using these protocols are that northern hybridization: is time consuming, displays low sensitivity, its quantification potential is limited and RT-PCR requires RNA of high quality which does not contain any DNA contaminants and therefore can be costly (Thieme *et al.*, 2008).

A fast, reliable methodology was described by Rautio *et al.* (2003) specific for the quantification of mRNA transcripts produced during fermentation. In Figure 13, the principles of the assay are highlighted using a modified schematic diagram. The sandwich hybridisation assay depends on four oligonucleotide probes (the two helper, capture and detection) bind to the target mRNA strand. The capture (biotin-labeled) probe binds to the strepavidin magnetic beads, forming a stable support for the interaction between the anti-DIG (Digoxigenin)-alkaline phosphatase FAB fragments and the detection (DIG-labelled) probe. An AttoPhos[®] (Promega, Germany) substrate (BBTP: 2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole phosphate) is added and this causes a reaction to occur. BBTP is cleaved by the alkaline phosphatase yielding inorganic phosphate and BBT (2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole) which gives off a yellow fluorescence colour. This fluorescence intensity is used to quantify the amount of transcripts produced using

a generated standard curve of known concentrations. (Rautio *et al.*, 2003; Resina *et al.*, 2007; Thieme *et al.*, 2008). Before this method could be used, the conditions for the hybridisation solution [1] and breakage of *Y. lipolytica* (YlEx-gly) cells, prior to quantification of transcripts, needed to be optimised. This methodology was then validated for reproducibility using a flask trial.

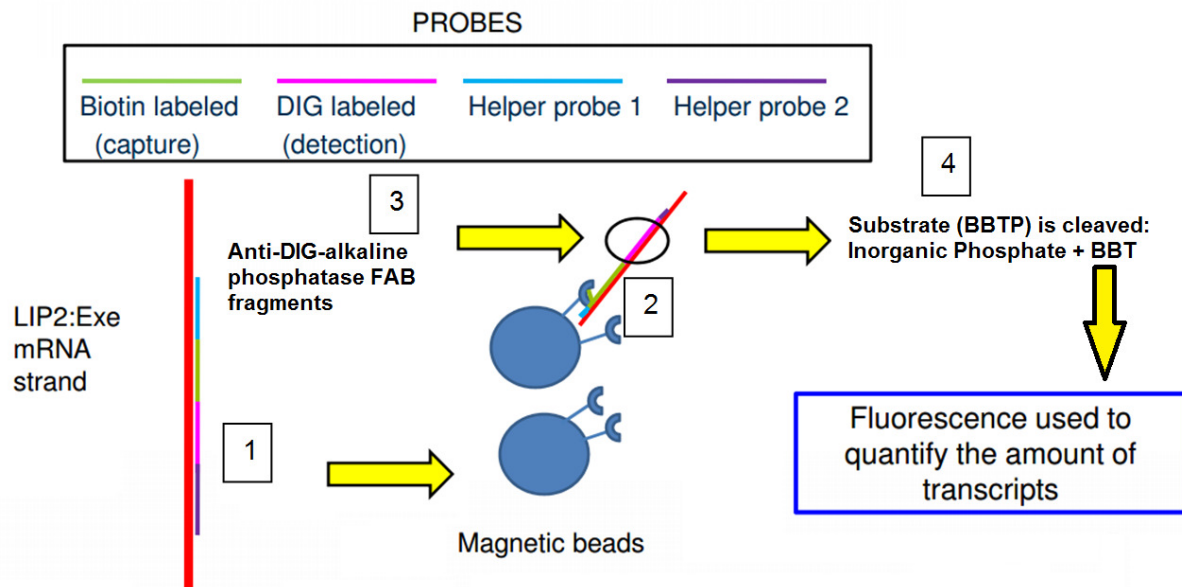


Figure 13: Modified schematic diagram showing mRNA sandwich hybridisation assay (adapted from Rautio *et al.*, 2003). [1]: Binding of probes to the mRNA strand using a hybridisation solution, [2]: The Lip2:Exe strand-probe complex [1] binds to the streptavidin magnetic beads providing a structural support, [3]: The anti-DIG-alkaline phosphatase binds to the DIG labelled probe, [4]: The added substrate (BBTP) is cleaved by the conjugated phosphatase yielding an inorganic phosphate and BBT which gives off a yellow fluorescent colour that is used to quantify the amount of transcripts (Lip2:Exe) in the sample.

1.25 mg.ml⁻¹ BSA, pH 6.6), 4 µl of a 25 mM CoCl₂ solution, 1 µl of a 1 mM DIG-dUTP solution, 1 µl of a 10 mM dATP solution and 1 µl of a 400 U.µl⁻¹ Terminal transferase solution (prepared in 60 mM K-phosphate; pH 7.2), 150 mM KCl, 1 mM 2-Mercaptoethanol, 0.5 % (v.v⁻¹) Triton X- 100 and 50 % (v.v⁻¹) glycerol) was added. The tube was mixed and centrifuged for 5 sec followed by incubation for 15 min at 37°C. The reaction was placed on ice and stopped by the addition of 2 µl 0.2 M EDTA (pH 8). The labelled probe was dispensed into sterile Eppendorf tubes and stored at -80°C. The biotin probe was labeled by Inqaba Biotec.

4.2.2 Generation of standards for mRNA sandwich hybridization assay

4.2.2.1 Generation of DNA standard using PCR

Primers were designed (Table 7) to generate copies of a portion of the Lip2:Exe peptide (vector kindly donated by Dr. S.I. Bulani³) using a technique called polymerase chain reaction (PCR). The T7 Exe Forward primer was designed to include the T7 promoter sequence (in blue) that was necessary for downstream transcription of DNA to RNA using the MAXIscript[®] Kit (Life Technologies, USA) which uses a T7 polymerase. The KAPA 2G PCR kit (Kapa Biosystems, South Africa) was used to generate copies of the DNA fragment during PCR (Table 8). Using a Thermocycler (Labnet International Inc, USA), all PCR amplifications started with an initial denaturing step for 30 sec at 95°C followed by 35 cycles of denaturing for 30 sec at 95°C, annealing for 30 sec at 50°C and extension for 45 sec at 72°C. The last step involved elongation at 72°C for 2 min.

Table 7: Primers used for PCR reaction of Lip2:Exe DNA fragment

Primer	Sequence
T7 Exe Forward	5' TAATACGACTCACTATAGGGAGATGGCCCCTGATCCACCCTCC 3'
Exe Reverse	5' TGGTGCGCCAGAAGAGGGTCC 3'

³Dr Siyavuya Ishmael Bulani, CSIR Biosciences, P. O. Box 395, Pretoria, South Africa

Table 8: PCR reaction to generate standards for mRNA hybridisation assay.

Solution	Volume (μl)
Lip2:Exe plasmid template ($49 \text{ ng} \cdot \mu\text{l}^{-1}$)	2.5
dNTPs (10 mM)	0.5
Exe- Reverse primer (10 μM)	1.5
T7-Forward primer (10 μM)	1.5
Buffer A (5 x)	5
2G Fast enzyme ($5 \text{ U} \cdot \mu\text{l}^{-1}$)	0.1
Distilled water	Made up volume to 25 μl

4.2.2.2 Detection of PCR product using Agarose Gel Electrophoresis and Quantification using the Nanodrop

The PCR product was observed on a 1 % agarose gel stained with $0.5 \mu\text{g} \cdot \text{ml}^{-1}$ ethidium bromide. The samples were mixed with 4 μl loading buffer [10 % (v.v⁻¹) glycerol, 0.09 % (w.v⁻¹) Bromophenol Blue, 0.09 % (w.v⁻¹) Xylene cyanol ff, 10 mM Tris pH 7.6 and 10 mM EDTA]. The marker (5 μl , Thermo Scientific (USA) O'GeneRuler 1 kb DNA Ladder) was used to establish if the 297 bp PCR fragment was present. Agarose gel electrophoresis was run at 120 V (labnet power station 300 plus, USA) in 1 x TAE buffer (40 mM Tris base, 40 mM glacial acetic acid, 1 mM EDTA, pH 8.0). The gel was visualized using UV. The PCR product was excised from the gel followed by clean-up using the Zymoclean™ gel DNA recovery kit, as per manufacturer's protocol. The excised gel was placed into a sterile Eppendorf tube. ADB buffer was added to the gel fragment at a ratio of 3:1 and incubated at 55°C for 10 min. The solution was added to a Zymo-spin™ column and placed onto a collection tube (2 ml). This was centrifuged for 5-10 sec at 13000 x g. The collection tube content was discarded. Wash buffer (200 μl) was added to the spin column and centrifuged for 10 sec. The collection tube content was discarded once again and an additional 200 μl of wash buffer was added and centrifuged at 13000 x g for 30 sec. The column was transferred to a sterile 1.5 ml Eppendorf tube. TE buffer (10 μl , 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.) was added to the column and centrifuged at 60 sec to elute the DNA that

was bound to the matrix. The eluted product was quantified using a Nanodrop UV-Vis Spectrophotometer (Thermo Scientific, USA).

The purified PCR product was then transcribed into mRNA using the MAXIscript® Kit, as per manufacturing protocol. Briefly, the PCR product ($\pm 1 \mu\text{g}$) was added to a sterile Eppendorf tube, followed by $2 \mu\text{l}$ 10 x transcription buffer, $1 \mu\text{l}$ 10 mM ATP, $1 \mu\text{l}$ 10 mM CTP, $1 \mu\text{l}$ 10 mM GTP, $1 \mu\text{l}$ 10 mM UTP and $2 \mu\text{l}$ Enzyme mix. The reaction mixture was made up to $20 \mu\text{l}$ with nuclease-free water. The tube was gently mixed and briefly centrifuged to collect the mixture at the bottom. Turbo DNase ($1 \mu\text{l}$) was added to the reaction and mixed well followed by incubation at 37°C for 15 min. The reaction was stopped by the addition of 0.5 M EDTA. An aliquot ($5 \mu\text{l}$) was run on a formaldehyde agarose gel (section 4.2.2.3) and the remaining solution was subjected to ethanol precipitation to remove any unbound nucleotides (section 4.2.2.4). The RNA product was quantified using the Nanodrop UV-Vis Spectrophotometer (Thermo Scientific, USA) and aliquots of 100 fmol stocks were stored at -80°C .

4.2.2.3 Detection of RNA standard using Formaldehyde Agarose Gel Electrophoresis

An aliquot ($5 \mu\text{l}$) of the transcribed RNA was run on a 1.2 % (v.v^{-1}) formaldehyde agarose (FA) gel. Agarose (12 g.l^{-1}) was mixed with 10 x FA gel buffer (200 mM 3-[N-morpholino]propanesulfonic acid (MOPs) (free acid), 50 mM sodium acetate, 10 mM EDTA, pH 7.0 using sodium hydroxide). The buffer was made in DEPC-treated water which is RNase free. This water was prepared by adding DEPC (Diethylpyrocarbonate, 1 ml) to 1 l dH_2O (distilled water) and incubated at room temperature for 1 h followed by autoclaving for 20 min at 121°C . The agarose solution was heated to melt the agarose and cooled to 65°C using a water bath. For a 100 ml solution, 1.8 ml of 37 % (v.v^{-1}) formaldehyde and $1 \mu\text{l}$ of ethidium bromide (10 mg.ml^{-1}) were added. The solution was mixed by gently swirling for 5 sec and then poured into a gel casting support. Once the gel was set, it was equilibrated with 1 x FA gel running buffer (100 ml 10x FA gel buffer, 20 ml 37% (v.v^{-1}) formaldehyde, 880 ml DEPC-treated water) for 30 min. The samples were mixed with 5x loading buffer ($16 \mu\text{l}$ saturated aqueous

bromophenol blue solution, 80 μl 500 mM EDTA (pH 8), 720 μl 37% (v.v⁻¹) formaldehyde, 2 ml 100 % (v.v⁻¹) glycerol, 3.1 ml formamide, 4 ml 10 x FA gel buffer and DEPC-treated water to a final volume of 10 ml) at a ratio of 4:1. The samples were mixed by vortexing for 5 sec and incubated at 65°C for 5 min. This was then transferred onto ice for 5 min. It was then loaded onto the equilibrated FA gel along with a marker (RiboRuler High Range RNA ladder, Thermo Scientific, USA). The gel was run in 1x FA gel running buffer at 40 V for 2 h.

4.2.2.4 Ethanol precipitation of RNA to remove unbound nucleotides and quantification using the Nanodrop

DEPC-treated water (30 μl) was added to the transcription reaction (20 μl) to bring the final volume to 50 μl in an Eppendorf tube. In order to facilitate precipitation, 5 μl of 5 M Ammonium acetate was added. The contents were then vortexed for 5 sec to ensure even distribution. Ethanol (100 %, v.v⁻¹) was added to the tube to a final volume of 165 μl . The solution was chilled at -20°C for 30 min. The tube was centrifuged at 13000 X g for 15 min at 4°C. The supernatant was carefully discarded and the pellet washed with 4°C 70 % (v.v⁻¹) ethanol. The tube was centrifuged briefly (13000 X g for 5 min). The supernatant was discarded and the tube left to air dry in a laminar flow for 1 h to remove the excess ethanol. The RNA was quantified using the NanoDrop UV-Vis Spectrophotometer (Thermo Scientific, USA).

4.2.3 Optimisation of mRNA sandwich hybridization method

The mRNA sandwich hybridization assay was optimised by evaluating three different hybridization solutions (Table 9). Standards (0, 10, 20, 40 and 60 fmol diluted from a 100 fmol stock) were used as samples during the optimization. Solutions A, B and C (86 μl per sample) were prepared fresh in a reaction tube. DIG-tail labeled detection probe (1 μl), biotin-labeled capture probe (1 μl), helper 1 probe (1 μl) and helper 2 probe (1 μl) was added for each reaction into the reaction tube. Each hybridization solution was tested with 20 μg or 50 μg of magnetic beads.

Each solution (90 μ l containing the hybridization solution and probes) was added to a well ($n = 3$) of a 96 well clear reaction plate (flat - bottom). The plate was incubated using a thermomixer (Eppendorf Thermomixer® comfort, Germany) at 60°C for 5 min (650 rpm). The sample (10 μ l) was added into each well containing the hybridization solution. The plate was placed in the thermomixer for 40 min at 650 rpm, 60°C (temperature was determined using T_m values of probes to prevent non-specific binding - see table 6). Magnetic beads [Streptavidin *MagneSphere*® Paramagnetic Particles (Promega, USA)] were prepared by adding the volume of beads required (20 μ g or 50 μ g per reaction well) in an Eppendorf tube. The tube was placed onto a *Magnosphere*® Magnetic Separation Stand (Promega, USA) to allow the removal of the supernatant. The supernatant was discarded and 5 x SSC (Saline-Sodium Citrate buffer) buffer (diluted from a 20 x stock using DEPC treated water: 3 M NaCl and 0.3 M sodium citrate, pH 7.0) was added. The tube was vortexed for 5 sec and placed on the separation stand. This was repeated twice and the magnetic bead solution finally made-up to the original volume using 5 x SSC. Streptavidin Magnetic beads (20 or 50 μ l) were added into each well containing the hybridization solution and sample. The target RNA molecules were immobilized onto the streptavidin magnetic beads by incubating in the thermomixer for 30 min at 60°C (650 rpm). After immobilization the wells were washed by placing the reaction plate onto a *MagnaBot*® 96 Magnetic Separation Device (Promega, USA) and allowing the beads to be collected for 1 min. The liquid was removed from the wells and 150 μ l of washing solution (1 x SSC, 0.01 % (m.v⁻¹) SDS and 0.1 % (v.v⁻¹) TWEEN20) was added. The plate was incubated in the thermomixer for 2 min at 25°C (650 rpm). This washing step was repeated three times.

Diluted anti-DIG Alkaline Phosphatase Fab-fragments (100 μ l) was added to each well. The plate was incubated in the thermomixer at 25°C at 750 rpm for 5 sec and then decreased to 450 rpm for 30 min. The reaction plate was again placed onto a *MagnaBot*® 96 Magnetic Separation Device for 1 min and the washing step was repeated for three times as described above. After the 3rd washing step, both the liquid and beads were transferred to a new reaction plate to avoid signal from the anti-DIG Alkaline Phosphatase that was bound to walls. The new plate was incubated for 2 min at 25°C with 750 rpm shaking. The plate was again placed onto a *MagnaBot*® 96 Magnetic Separation Device and the liquid removed followed by washing of the beads once more. The substrate [100 μ l, AttoPhos® fluorescent substrate (Promega, USA)] was added into each well and incubated in the thermomixer at 37°C at 1100 rpm for 5 sec and decreased to 750 rpm for 20 min. The plate was placed onto the *MagnaBot*® 96 Magnetic

Separation Device for 1 min and 90 μl of the liquid (not beads) was transferred to a black reaction plate (96 well, flat bottom) for fluorescence measurement. The fluorescence signals were measured with a Victor 2 Fluorescence reader (Perkin Elmer Wallac, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Table 9: Components of hybridization solution used to optimise mRNA assay.

Solution	Components per well (90 μl)	Reference
A	25 μl 20x SSC buffer, 2 μl 10% (m.v ⁻¹) SDS (Sigma-Aldrich, USA) , 15 μl 20 % (m.v ⁻¹) Dextran sulphate (Sigma-Aldrich, USA), 2 μl 50x Denhardt's reagent (Life Technologies, USA) and 42 μl DEPC-H ₂ O	Rautio <i>et al.</i> , 2006; 2007
B	25 μl 20x SSC, 5 μl 10% (m.v ⁻¹) SDS, 20 μl 20 % (m.v ⁻¹) Dextran sulphate, 2 μl 50x Denhardt's reagent, 20 μl Formamide and 14 μl DEPC-H ₂ O	Rautio., 2003
C	25 μl 20x SSC, 20 μl Formamide, 15 μl 20 % (m.v ⁻¹) Dextran sulphate, 2 μl 10 % (v.v ⁻¹) TWEEN20, 1 μl 100x Denhardt's reagent, 10 μl 10x blocking reagent, 13 μl DEPC-H ₂ O.	Thieme <i>et al.</i> , 2008

4.2.4 Optimisation of cell breakage

4.2.4.1 Harvesting of cells

In order to harvest the same amount of cells, an OD versus cell count relationship had to be determined. Cells were harvested from a flask and their OD at 660 nm was determined using the spectrophotometer. Serial dilutions were made and 100 μl of sample spread onto PCA plates. The OD:cell count ratio was used to ensure a constant number of cells was harvested every time. An Eppendorf tube, containing the constant number of cells, was centrifuged at 13000 x g at 4°C (Sigma Laborzentrifugen 1K15). The supernatant was discarded and the pellet washed with 1 ml DEPC-treated water. The suspension was centrifuged again at 13000 x g at 4°C. The supernatant discarded and the pellet was snap-frozen using liquid nitrogen (200 ml) and stored at -80°C.

4.2.4.2 Different cell breakage buffers

Different buffers were used to optimise cell breakage. Once the pellet was defrosted, 500 μl of each buffer (Table 10) was added to the Eppendorf tube. The contents of each Eppendorf tube, containing the different buffers, were transferred to a homogenizer tube containing acid washed glass beads (0.5g, 425 - 600 μM , section 3.2.4.3). This suspension was disrupted for 2 min at 6.0 $\text{m}\cdot\text{s}^{-1}$ (MP tm FastPrep (R)-24 homogenizer) and then centrifuged at 13000 x g for 10 min at 4°C. The supernatant (400 μl) was aliquoted into four (100 μl) sterile Eppendorf tubes (2 ml) and frozen using liquid nitrogen. This was stored at -80°C for the mRNA sandwich hybridisation assay.

Table 10: Different buffers evaluated for cell breakage.

Buffer	Components
Lysis buffer	5x SSC buffer, 4% SDS and 1 $\mu\text{l}.\text{ml}^{-1}$ RNA guard RNase inhibitor
TEN buffer	200 mM Tris-HCl buffer (pH 8), 2 mM EDTA, 200 mM NaCl and 1 $\mu\text{l}.\text{ml}^{-1}$ RNA guard
Y-PER ((Thermo Scientific, USA)) and TEN buffer combination	Y-PER (250 μl) was added to the pellet and the tube was incubated at 25°C, 650 rpm using the Eppendorf Thermomixer® comfort (Germany) for 30 min. TEN buffer (250 μl) was added to suspension.

4.2.4.3 Acid washing of Glass beads

Glass beads (425-600 μM) used for cell breakage, were prepared by adding 100 g of glass beads in a 250 ml Schott bottle. HCl (100 ml, 5.8 M, v.v⁻¹) was added to the bottle until the beads were covered. The content of the bottle were incubated at room temperature (25°C) for 1 h. The HCl was carefully removed and sterile 160 ml of dH₂O was added. The bottle was swirled for 10 sec to ensure the beads were mixed. Once the beads sank to the bottom, the water was carefully removed. This wash was repeated 10 times. The beads were autoclaved for 20 min at 121°C and left in the oven at 50°C to dry overnight.

4.2.5 Shake flask study to evaluate the mRNA sandwich hybridization assay

Ultra yield flasks (2.5 l) containing 700 ml YPD medium, pH 6.8, were prepared and autoclaved at 121 °C for 20 min. Each flask was inoculated using a single cryo-vial from the cell bank. The temperature was maintained at 28 °C and the flasks were left shaking at 180 rpm. Growth and mRNA transcription were followed by taking samples (10 ml) at regular time intervals. Growth was measured by determining the optical density at 660 nm.

Based on the optical density, a constant amount of cells were centrifuged at 13000 x g for 10 min at 4 °C (section 4.2.4.1). Upon harvesting, cells were broken using the Y-PER-TEN Buffer method (section 4.2.4.2). The frozen supernatant was then defrosted and hybridization solution C (refer to Table 9) using 20 µl of beads (based on optimised studies), was used for the mRNA assay (section 4.2.3). A transcription and growth (OD_{660nm}) profile was hence established.

4.3 Results and Discussion

4.3.1 Generation of standards for mRNA sandwich hybridization assay

mRNA transcripts of known concentrations needed to be generated for a standard curve. PCR was used to upscale the quantity of the Lip2:Exe fragment. After PCR, agarose gel electrophoresis (Figure 15) was used for detection of the product followed by quantification using the Nanodrop.

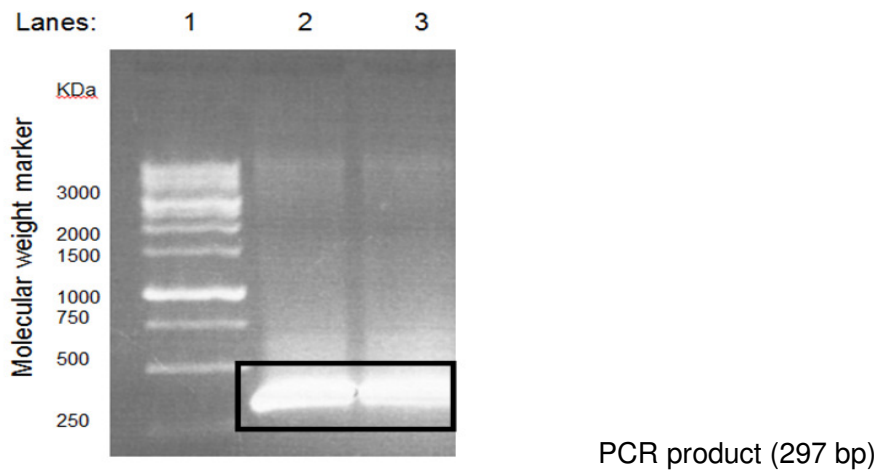


Figure 15: Detection of PCR product using agarose gel electrophoresis. Agarose gel indicating the 297 bp fragment of the Lip2:Exe fragment attached to a T7 promoter produced during PCR.

After quantification using the nanodrop, the PCR product was transcribed into RNA using the MAXIscript® Kit. An aliquot of the reaction was run onto a formaldehyde agarose gel for detection of the 297 bp Lip-Exe fragment (Figure 16). The components of the transcription reaction were precipitated with ethanol to remove any unbound nucleotides and the pure RNA quantified using the Nanodrop. This RNA sample could now be used as a standard to quantify Lip2:Exe transcripts with the sandwich hybridization assay.

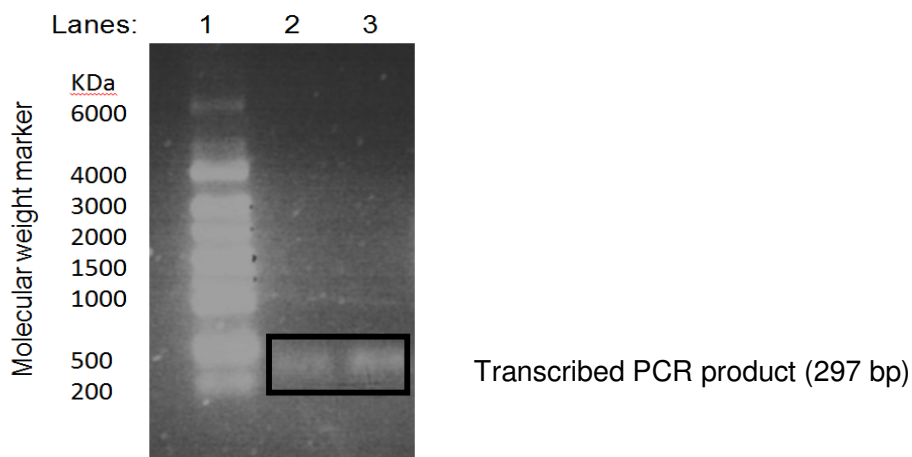


Figure 16: Detection of transcribed PCR product using formaldehyde agarose gel electrophoresis. Formaldehyde agarose gel indicating the 297 bp fragment of the transcribed Lip2:Exe fragment.

4.3.2 Optimization of mRNA sandwich hybridization method

Probes were designed for the mRNA sandwich hybridization assay (Table 6). The biotin-labelled probe was used for capturing the Lip2:Exe mRNA transcripts onto the streptavidin magnetic beads and the DIG-labelled probe used for detection. Anti-DIG Alkaline Phosphatase Fab fragments were then used to quantify the amount of mRNA transcripts generated under the regulation of the hp4d promoter. It has been shown that the use of helper probes assist in binding of the DIG and biotin-labelled probes (Barken *et al.*, 2004). Following the generation of the mRNA Lip2:Exe fragments of known concentration, this was used as a standard to evaluate three sandwich hybridization assays methodologies for repeatability (Figure 17). In addition, two streptavidin magnetic bead concentrations (20 and 50 μg per sample) were tested to define maximum binding capacity (Figure 17).

The same standards of mRNA Lip2:Exe fragments (0, 10, 20, 40 and 60 fmol diluted from a 100 fmol stock) were used for each protocol. The binding capacity of the 50 μg beads using protocol A was not linear with a R^2 value of 0.846 (Figure 17A). The linearity of the 20 μg magnetic beads was higher with an R^2 of 0.9672. Protocol B (Figure 17B) showed a $\pm 50\%$ difference (in fluorescence intensity between the two bead concentrations. R^2 values were 0.9989 and 0.9639 for the 50 μg and 20 μg respectively. Protocol C (Figure 17C) showed almost similar fluorescence intensities for both concentrations of magnetic beads. The 20 μg beads reached their maximum binding capacity at 40 fmol whereas the binding capacity of the 50 μg beads was linear up to 60 fmol, the highest concentration tested. Protocol C, at a streptavidin magnetic bead concentration of 20 μg , was therefore chosen for the mRNA sandwich hybridisation assay. The reason being that the binding capacity in terms of the components of the assay was similar for both bead concentrations therefore it was more cost effective to chose the 20 μg over the 50 μg bead concentration.

4.3.3 Optimization of cell breakage

Once the sandwich hybridisation assay was optimised using the Lip2:Exe mRNA standard, the repeatability of cell breakages of samples, taken at various time points, was tested. Firstly, an OD: cell ratio was determined and used as a constant to ensure that the same number of cells are harvested for every sample. The determined OD constant was 4, equalling 1.2×10^8 cells.ml⁻¹. The first buffer tested (Figure 18) was the lysis buffer (Table 10).

The standard deviation between flasks using the lysis buffer was high indicating inefficient cell breakage and results were not repeatable (Figure 18). It was also noticed that white precipitate formed after beads were used to break the cells. This interfered with the contents of the samples as observed by the high standard deviations for each sample. This buffer was therefore not used further. Y-PER, an enzyme that degrades the cell walls of yeast, TEN buffer and a Y-PER-TEN buffer combination were tested in combination with mechanical cell breakage (Figure 19). All three solutions showed repeatable cell breakage. Y-PER showed repeatability but the fluorescence intensity was low indicating the solution lacked components necessary for an optimal sandwich hybridisation assay. A combination of Y-PER and TEN buffer yielded the highest fluorescence intensity and was therefore selected for cell breakage.

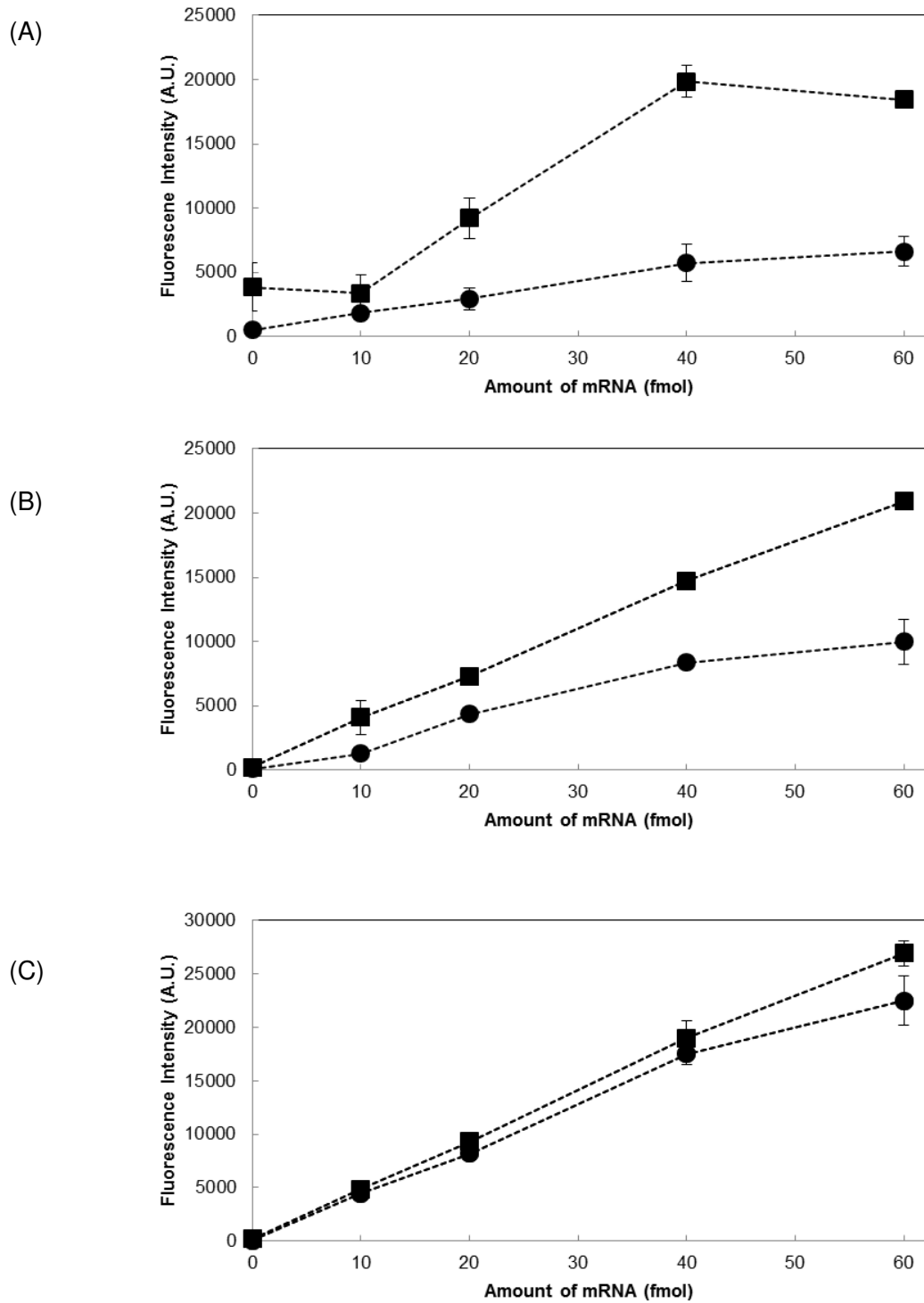


Figure 17: Comparison of three different protocols using different hybridisation solutions and two different magnetic bead concentrations 50 µg (■) and 20 µg (●) concentrations used to optimise the mRNA sandwich hybridisation assay. A: Protocol A, B: Protocol B, C: Protocol C.

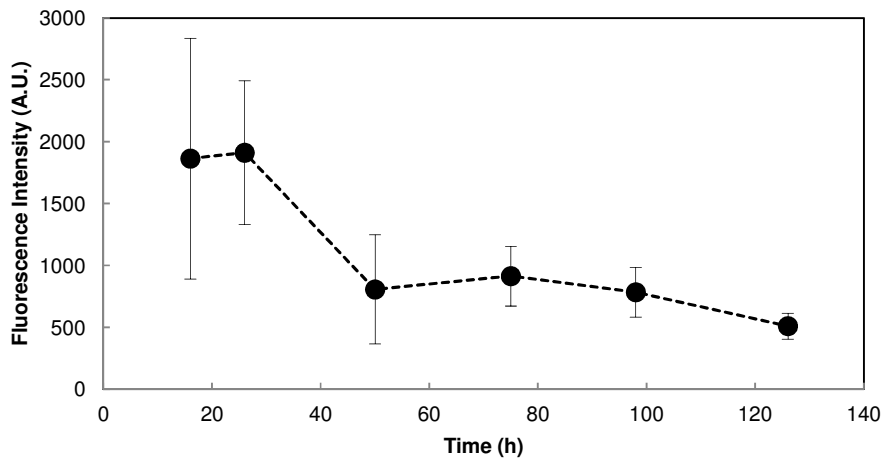


Figure 18: Variation of Fluorescence intensity after cell breakage of *Y. lipolytica* cells during cultivation in Ultra yield (2.5 l) flasks.

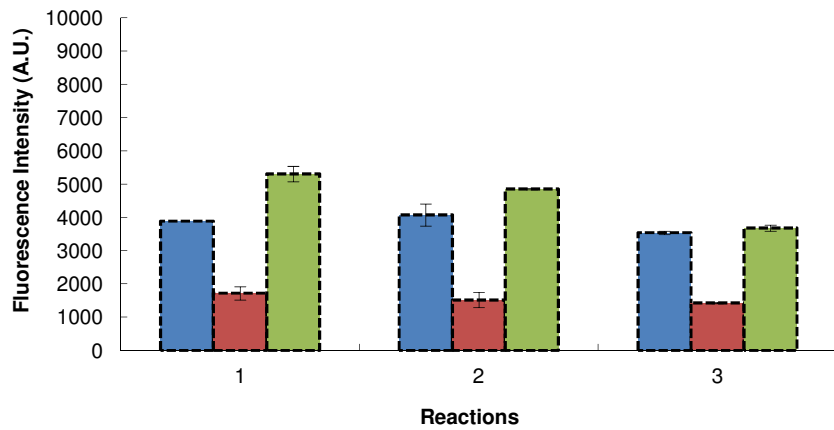


Figure 19: Replicates of a sample using different cell breakage solutions. Y-PER (red), TEN buffer (blue) and Y-PER-TEN buffer combination (green) was compared for repeatability and fluorescence intensity.

4.3.4 Shake flask study to evaluate the mRNA sandwich hybridization assay

After optimisation of the assay and cell breakage, a flask trial was used to validate the assay for repeatable mRNA transcriptional trends of the hp4d promoter (Figure 20). The growth of the organism ($n = 3$) was also measured at OD_{660nm} (Figure 20). The assay was reproducible (each sample was done in quadruplicate) with different flasks showing similar results (as observed by the error bars). It can be seen that the production of mRNA (Figure 20) took place throughout the growth of *Y. lipolytica* (Figure 20). This shows that the hp4d promoter is quasi-constitutive. The amount of Lip2:Exe mRNA transcripts increased during the exponential phase and as the organism switched from exponential to stationary phase, the amount of mRNA transcripts, started to decrease. A possible explanation could be that during exponential phase, metabolism of the organism is high and hence a spike is observed due to freely available amino acids present in the medium for transcription. Once metabolism started slowing down, the amount of Lip2:Exe mRNA transcripts started, under regulation of the hp4d promoter, to decline. However, when compared to Figure 9B, extracellular lipase and protein production started once the organism reached early stationary phase. According to Fickers *et al.* (2004) and Fickers *et al.* (2011), during the exponential growth phase of *Y. lipolytica*, extracellular lipase is associated with the cell wall and is only released into the culture broth at the end of the exponential growth phase. This would explain why the lipase activity is only observed during early stationary phase once metabolism slows down and transcription of the Lip2:Exe mRNA, under regulation of the hp4d promoter is observed during exponential growth phase.

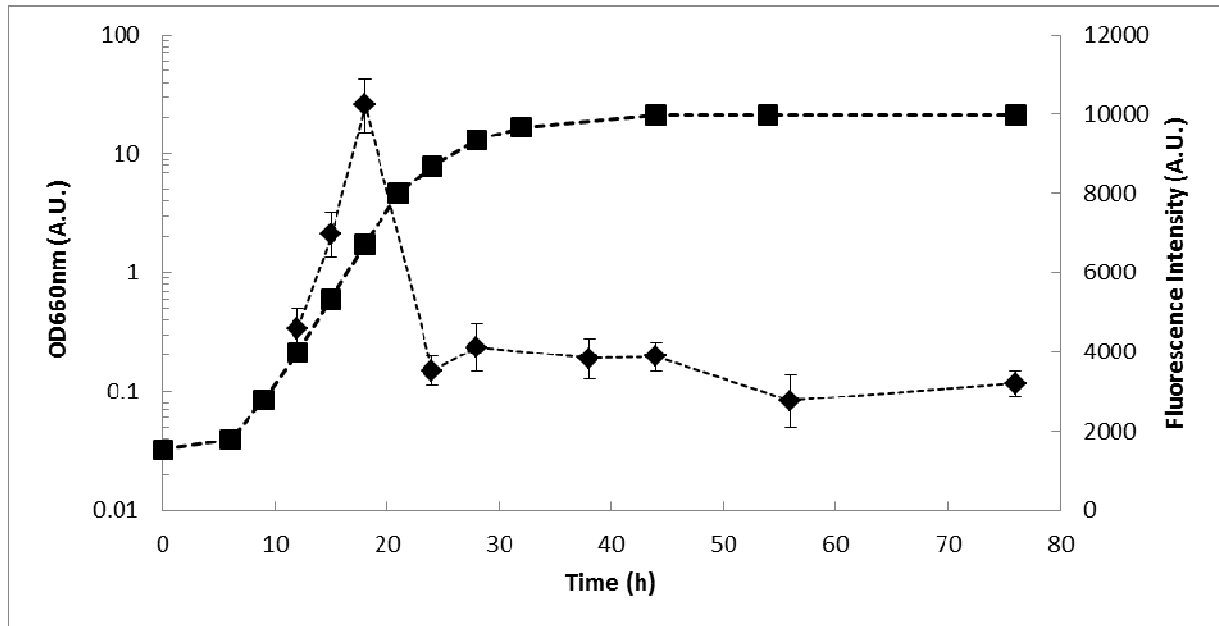


Figure 20: Growth profile (■) and validation of mRNA sandwich hybridisation assay protocol (◆) during shake flask production of Lip2:Exe fusion by *Y. lipolytica*. Flasks showed the same repeatable trend for time profiles during growth of *Y. lipolytica* expressing fused Lip2-Exe.

4.4 Concluding Remarks

It was important to monitor mRNA transcription of the Lip2:Exe gene in order to understand the regulation of the hp4d promoter. Measuring the activity of lipase, although, gave an indication of the amount of the mRNA transcripts translated, it did have several limitations (activity decrease due to degradation). It also did not explain if recombinant production was at the level of transcription which would be due to the promoter or at the level of translation which would be due to metabolism. Therefore a methodology was developed to quantify the amount of LIP2:Exe mRNA transcripts produced during the growth of *Y. lipolytica* under regulation of the hp4d promoter. The methodology was optimized for cell breakage and for the hybridization assay followed by validation using a flask study. Compared to other methodologies, the assay was not time-consuming as opposed to northern hybridization and it did not require RNA free from DNA contaminants as opposed to RT-PCR which made it more cost efficient (Thieme *et al.*, 2008). The results obtained from the flask trial showed that the hp4d promoter is quasi-constitutive. In order to evaluate effect of growth under regulation of the hp4d promoter during fed-batch

cultivation, continuous fermentation will be used to control growth rate and the amount of transcripts will be quantified (Chapter 5).

Chapter 5: Determining the effect of growth rate on the regulation of the hp4d promoter by monitoring mRNA transcription

5.1 Introduction

Yarrowia lipolytica is used as an expression host for the production of heterologous enzymes. A *Y. lipolytica* (YIEx-gly) strain with the potential to express the exenatide peptide was developed. Exenatide is a therapeutic drug used as an alternative to insulin for the treatment of type 2 diabetes. The Lip2 expression cassette which consisted of the Lip2 gene, 6 X Histidine tag, an enterokinase cleavage site, the exenatide peptide and a glycine amino acid (Figure 6) was integrated into the genome of *Y. lipolytica* (YIEx-gly). This was expressed under the regulation of the synthetic quasi-constitutive hp4d promoter (Chapter 3). The CSIR is developing a process for the recombinant production of pharmaceuticals peptides in *Y. lipolytica*. According to literature, the hp4d promoter was said to be growth phase dependent hence it is switched on during early stationary growth phase of *Y. lipolytica* (Madzak *et al.*, 2000; Nicaud *et al.*, 2002). However, the production of lipase, under regulation of the hp4d promoter was elucidated in continuous fermentation and found to be linked to growth rate with high product production occurring at growth rates lower than 0.045 h^{-1} (van Zyl, 2013). During optimisation of a methodology to monitor mRNA transcripts under regulation of the hp4d promoter, it was observed that transcripts were produced throughout the exponential and stationary growth phases of *Y. lipolytica* during batch cultivation (Chapter 4) and therefore could not be growth phase dependent. It is, however, unclear if this regulation is at the level of transcription or cellular metabolism and how mRNA transcription would be affected during fed-batch fermentation cultivations under controlled growth conditions. In order to determine the effect of growth rate on transcription levels of the gene under regulation of the hp4d promoter, *Y. lipolytica* will be cultivated in continuous fermentation at different growth rates using carbon limitation as the growth regulator. Subsequently total extracellular protein production using fed-batch fermentation will be compared at different growth rates to validate the results obtained during continuous fermentation.

5.2 Materials and Methods

5.2.1 Determination of carbon to nitrogen ratio to achieve carbon limitation

Erlenmeyer flasks ($n = 3$), containing sterile medium (autoclaved at 121°C for 20 min) of 20 g.l^{-1} yeast extract and different glucose concentrations (Table 11), were each inoculated with cells of *Y. lipolytica* YIEx-gly from a 2 ml cryo-vial from the cell bank. The flasks were incubated at 28°C and 180 rpm on a shaking incubator. After 18 h (late exponential growth phase as determined by growth curve study) samples were taken hourly until constant OD (660 nm) was reached. The residual glucose was measured for each sample. Once stationary phase was reached (constant OD for three successive samples), 2 ml of the broth was centrifuged at $13000 \times g$ for 10 min in pre-weighed Eppendorf tubes. The supernatant was decanted and the pellet left to dry in an oven at 50°C . The Eppendorf tubes were weighed and the biomass determined for each sample. The biomass yield on glucose utilized was determined by measuring the amount of biomass per glucose utilized (g.l^{-1} of biomass/ g.l^{-1} of glucose). The ratio of carbon to nitrogen was determined for a carbon limitation medium required for continuous fermentation.

Table 11: Media composition for determining glucose limitation

Glucose (g.l^{-1})	Yeast Extract (g.l^{-1})
0	20
5	20
10	20
15	20
20	20
30	20
40	20

5.2.2 Evaluation of the effect of different growth rates on the production of fused Lip2:Exe by *Y. lipolytica* during continuous fermentation

A 2 l continuous fermenter (Infors HT, Switzerland) containing sterilise (autoclaved for 45 min at 121 °C) 1.2 l modified CSIRman medium (20 g.l⁻¹ yeast extract, 20 g.l⁻¹ glucose and 1 ml.l⁻¹ antifoam, pH 6.8) was aseptically inoculated via the septated port with cells of *Y. lipolytica* YIEx-gly from a 2 ml cryo-vial from the cell bank using a sterile syringe. The pH was controlled at 6.8 with 25% NH₄OH (m.v⁻¹) or 25% H₂SO₄ (m.v⁻¹). The temperature was controlled at 28 °C, aeration at 2 slpms and agitation at 1000 rpm. In order to determine when mid-exponential phase was reached, OD_{660nm} was measured by taking 2 ml samples. The feed (20 g.l⁻¹ yeast extract and 20 g.l⁻¹ glucose) was then started. The feed was controlled to maintain growth rates ranging from 0.012 – 0.049 h⁻¹. Monoculture was checked microscopically on a daily basis. Once steady state was reached, for each growth rate, as indicated by constant protein production (Chapter 3, section 3.2.4.2) and OD_{660nm} for three retention times, a sample (10 ml) was taken for analysis. Biomass (Chapter 3, section 3.2.4.1), OD, enzyme activity (Chapter 3, section 3.2.4.3), extracellular protein concentration (Chapter 3, section 3.2.4.2), residual glucose concentration and mRNA transcription profiles (Chapter 4, sections 4.2.4.1, 4.2.4.2 and 4.2.3) were established. To validate the results obtained during continuous fermentation, the production of total extracellular protein by fed-batch fermentations, at different growth rates, was compared.

5.2.3 Evaluation of different feed rates to maintain the growth rate during Fed-batch fermentation of *Y. lipolytica* under regulation of the hp4d promoter

5.2.3.1 Inoculum for fed-batch fermentation

Fernbach flasks (2 L) containing 700 ml sterile YPD medium (autoclaved for 20 min at 121 °C), were inoculated with cells of *Y. lipolytica* YIEx-gly from a 2 ml cryo-vial from the cell bank

followed by incubation on an orbital shaker at 28 °C and 180 rpm. Once mid-exponential phase (OD_{660nm} : 1-3; Age: 16-18 h) was reached, the contents of these flasks were used to inoculate the fermenters.

5.2.3.2 Fed-batch fermentation

Biostat C fermenters (15 l, Germany) containing 6.5 l of modified CSIRman medium (30 g.l⁻¹ glucose, 30 g.l⁻¹ yeast extract and 1 ml.l⁻¹ antifoam) were sterilised at 121 °C for 45 min. The pH was automatically controlled at pH 6.8 with 25 % NH₄OH (v.v⁻¹) or 20 % H₂SO₄ (v.v⁻¹). After inoculation of each fermenter, agitation started at 500 rpm and was ramped up hourly (100 rpm intervals) to maintain the pO₂ at 30 % saturation. The temperature was controlled at 28 °C and airflow at 2 slpms. Once residual glucose was depleted, the feed (200 g.l⁻¹ glucose and 200 g.l⁻¹ yeast extract) was started and fed at a rate to maintain the growth rates of choice (0.019- 0.049 h⁻¹). Samples (20 ml) of culture broth were aseptically removed, at regular intervals, to measure pH, residual glucose concentration, biomass production (Chapter 3, section 3.2.4.1) and OD_{660nm} . Triplicate (2 ml) samples were centrifuged at 13000 x g for 10 min. The supernatant was dispensed into sterile Eppendorf tubes and stored at -20 °C for quantification of total extracellular protein (Chapter 3, section 3.2.4.2). Monoculture status was checked daily, microscopically.

5.3 Results and Discussion

5.3.1 Determination of carbon to nitrogen ratio to achieve carbon limitation

The study was done to determine the ratio of yeast extract to glucose to ensure carbon limitation necessary for continuous fermentation. At initial charge glucose concentrations higher than 20 g.l⁻¹, the glucose concentration was not yet depleted at the start of stationary growth indicating that nitrogen had become the growth limiting nutrient in the medium. In batch cultures,

where both nitrogen and carbon are present in medium, nitrogen is consumed first as soon as growth starts (Beopoulos *et al.*, 2009). In order to prevent citric acid production, the ratio of carbon to nitrogen consumption is essential (Beopoulos *et al.*, 2009). The biomass yield on glucose utilized decreased, as the residual glucose in the medium increased to a concentration of 20 g.l⁻¹ (Figure 21). At higher glucose concentrations (30 to 40 g.l⁻¹) the yield was constant at 1.6 g.g⁻¹ (± 0.2). It can be observed from the yield of biomass on glucose utilised, at 10 g.l⁻¹ glucose becomes the limiting factor. It was determined to ensure a glucose limited medium 20 g.l⁻¹ of glucose will be used therefore the ratio of glucose to yeast extract should be 1.1.

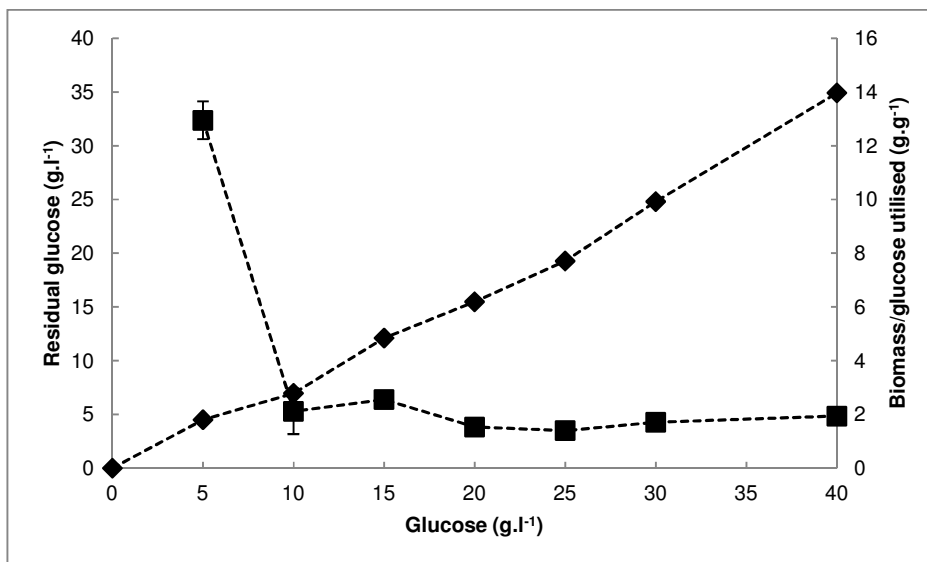


Figure 21: The effect of different glucose concentrations on the growth of *Y. lipolytica* (YIEx-gly) in shake flasks at 20 g.l⁻¹ yeast extract concentration. Residual glucose concentration at the end of exponential growth (◆) and the yield of *Y. lipolytica* biomass on glucose utilised (■).

5.3.2 The effect of different growth rates on the production of fused Lip2:Exe by *Y. lipolytica* during continuous fermentation

Continuous fermentation was used to observe the effect of growth rate on the regulation of the hp4d promoter during the production of fused Lip2:Exe under carbon limitation. Glucose limitation was used to maintain different growth rates between 0.01 and 0.05 h⁻¹ by changing the dilution rate of the continuous fermentation. Small samples were taken after each residence

time and the OD and protein concentrations measured to determine steady state at the desired dilution rate. Once steady state was confirmed by three constant values, the fermenters were sampled and analysed for biomass, lipase and protein production before changing the dilution rate (Figure 22). Biomass remained constant at $13.16(\pm 1.12)$ g.l⁻¹ and the residual glucose was below detection levels at the dilution rates evaluated (Figure 22A). The lipase activity (used as a reporter enzyme) and total extracellular protein gave an indication of the amount of fused Lip2:Exe produced (Figure 22B). Similar trends were observed for extracellular lipase activity and protein production. Volumetric lipase production increased from 2734.26 (± 100.50) to 3314.14 (± 283.79) nKat.ml⁻¹ when the growth rate increased from 0.01 to 0.02 h⁻¹. When the growth rate was increased above 0.02 h⁻¹ to 0.03 h⁻¹ the lipase activity decreased 3.7 fold to 900.65 (± 16.15) nKat.ml⁻¹ followed by a further 2.3 fold decrease to 398.90 (± 16.92) nKat.ml⁻¹ at a growth rate of 0.05 h⁻¹. Total protein also followed the same trend as the lipase activity. An increase from 0.502 (± 0.015) to 0.554 (± 0.008) mg.ml⁻¹ was observed when the growth rate was increased from 0.01 to 0.02 h⁻¹ followed by a 1.3 fold decrease of 0.433 (± 0.003) mg.ml⁻¹ at a growth rate of 0.03 h⁻¹. When the growth rate was increased from 0.02 to 0.05 h⁻¹ the lipase activity decreased 8.3 fold and the extracellular total protein concentration decrease 1.6 fold. The highest lipase and total extracellular protein concentration was produced at a growth rate of 0.02 h⁻¹.

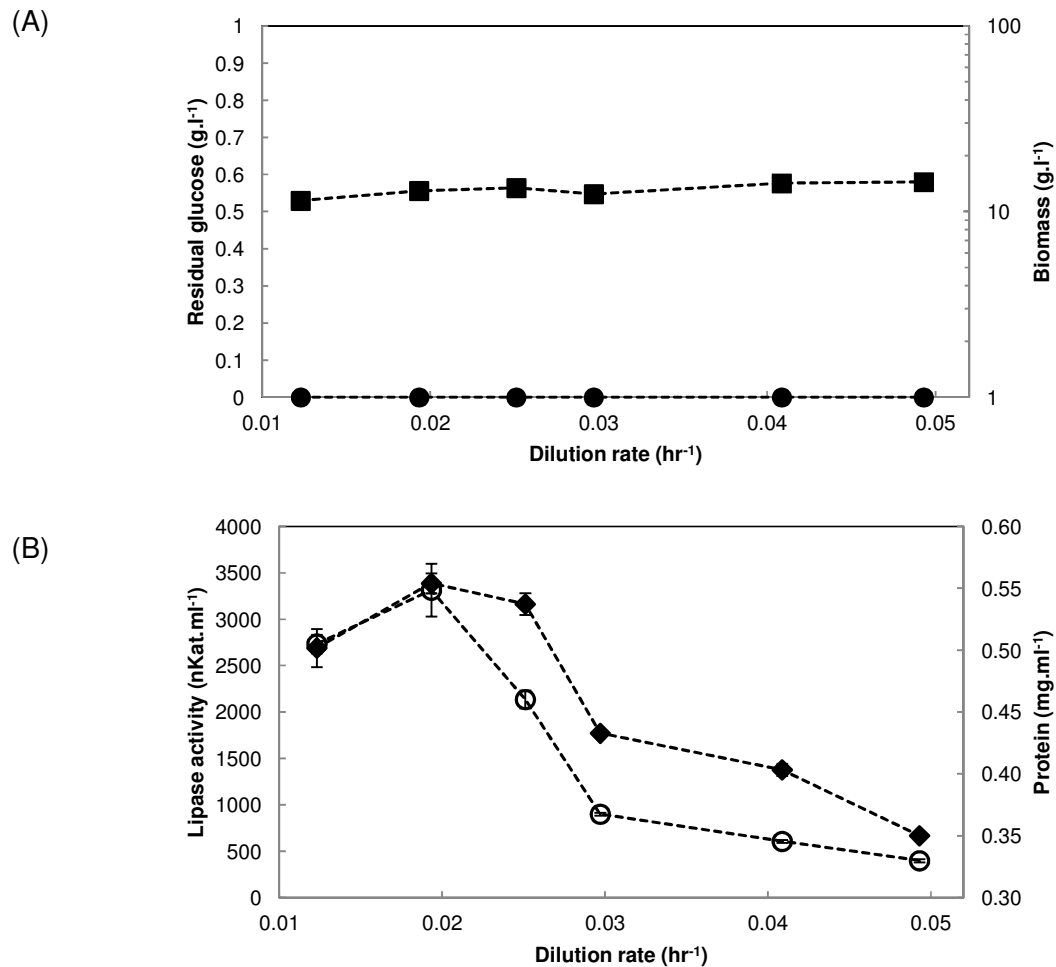


Figure 22: The effect of growth rate on biomass and Lip2: Exe production by *Y lipolytica* (YIEx-gly) grown in continuous fermentation. A: biomass (■) and residual glucose concentration (●). B: Lipase activity (○) and protein concentration (◆)

To determine if the increase in total protein concentration was due to increased production of protein under the hp4d promoter the volumetric lipase productivity was compared to specific lipase activity at different growth rates (Figure 23A). The specific lipase activity increased from 5448.53 to 5980.76 nKat.mg⁻¹_{protein} when the growth rate was increased from 0.01 to 0.02 h⁻¹ followed by a 5.3 fold decrease in specific activity to 1138.43 nKat.mg⁻¹_{protein} at a growth rate of 0.05 h⁻¹. Using the optimised conditions for the mRNA sandwich hybridisation assay, the amount of mRNA transcripts per gram of biomass was measured during different growth rates

and this was compared to volumetric lipase productivity (Figure 23B). The amount of mRNA transcripts.g⁻¹ increased from 10385.04 to 14217.98 fmol.g⁻¹ when the growth rate was increased from 0.01 to 0.02 h⁻¹ followed by a rapid 14.5 fold decrease to 980.16 fmol.g⁻¹ at a growth rate of 0.03 h⁻¹. A further increase in growth rate to 0.05 h⁻¹ did not have an effect on the level of transcription of the hp4d promoter.

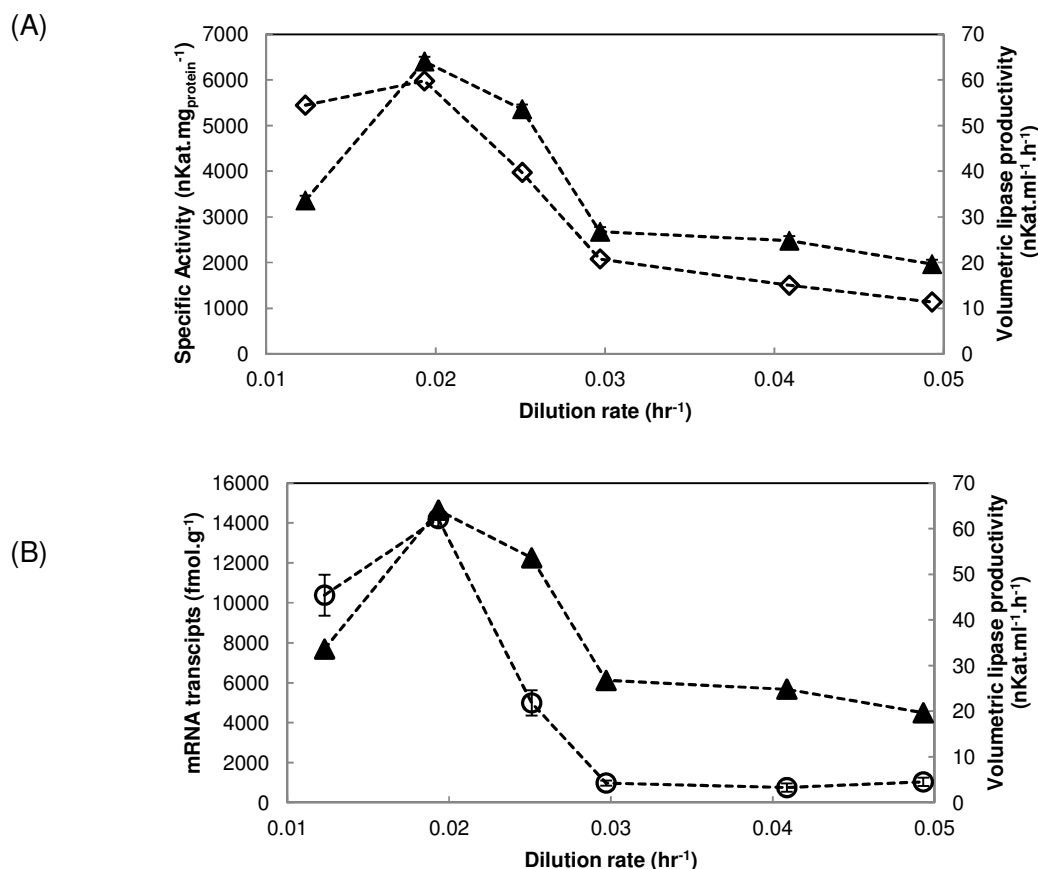


Figure 23: The effect of growth rate (dilution) on transcription and translation during production of Lip2:Exe fusion by *Y. lipolytica* (YIEx-gly) under regulation of the hp4d promoter. A: Specific lipase activity (◇) and lipase productivity (▲). B: mRNA transcripts per gram of biomass (○) and lipase productivity (▲).

Volumetric lipase productivity followed the same trend as transcription. The amount of transcripts produced under the regulation of the hp4d promoter decreased at growth rates above 0.02 h⁻¹. The optimum growth rate for production of the Lip2:Exe fusion peptide under regulation of the hp4d promoter was 0.02 h⁻¹ and in order to validate these results, total

extracellular protein production was quantified at different growth rates during fed-batch fermentation.

5.3.3 Evaluation of different feed rates to maintain the growth rate during Fed-batch fermentation of *Y. lipolytica* under regulation of the hp4d promoter

A fed-batch fermentation strategy was used to confirm that production of fused Lip2:Exe under regulation of the hp4d promoter is growth rate dependent. Four different growth rates between 0.02 to 0.05 h⁻¹, were compared by controlling the glucose feed (Figure 24). During the batch phase of the fermentation the growth, as determined by optical density and initial charge glucose consumption followed the same trends for all fermentations. After depletion of the initial charge glucose the glucose feed rate was used to maintain the growth rate of *Y. lipolytica* at the desired rates.

Protein production started at the end of the exponential growth phase i.e. when the growth rate of the organism decreased, (Figure 24). When the production of the fused Lip2:Exe precursor, during fed-batch and continuous fermentation, were compared, both showed the same trends at different growth rates (Figure 26). As the growth rate increases, extracellular protein production decreased.

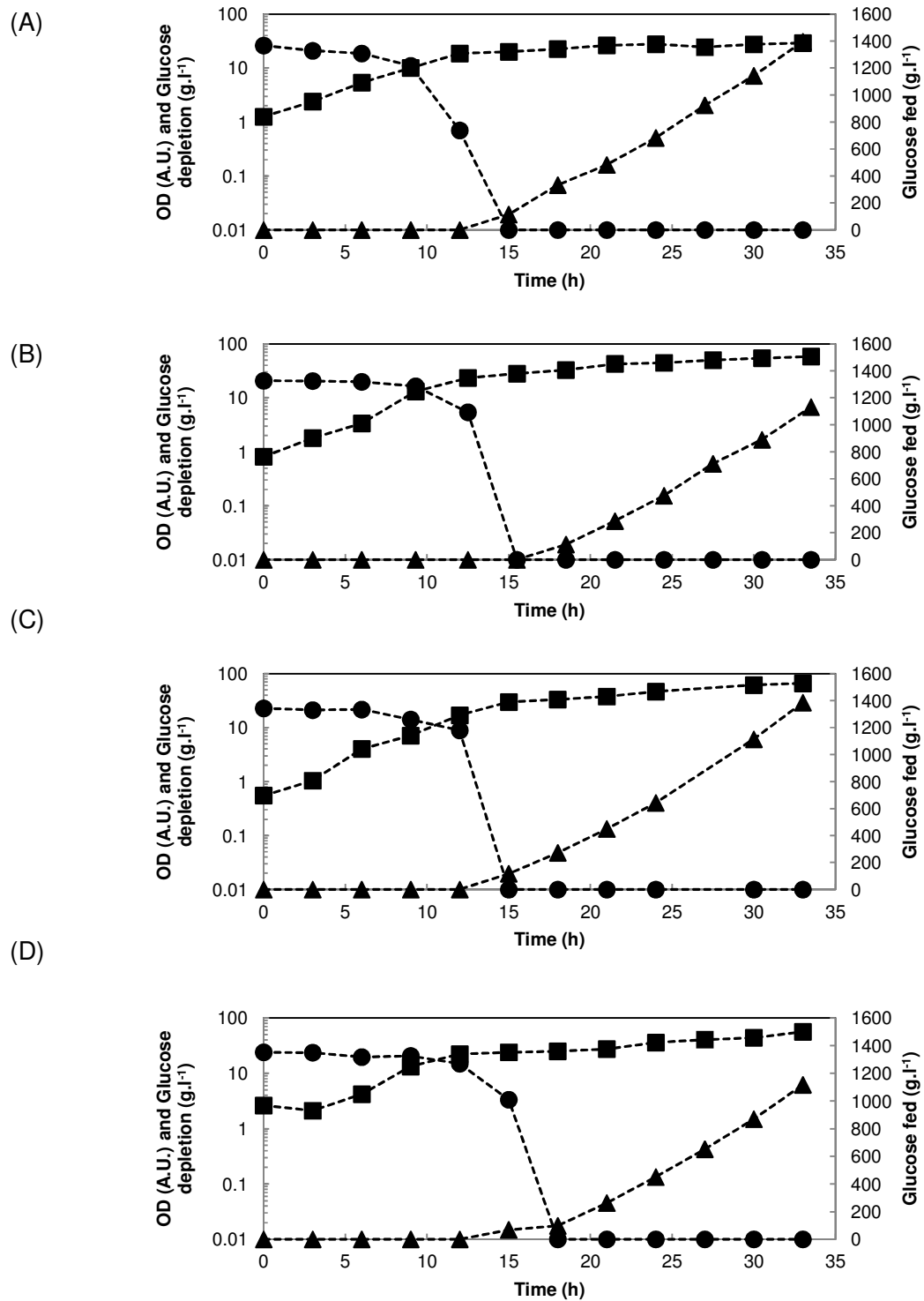


Figure 24: Fed-batch fermentation of *Y. lipolytica* (YIEx-gly) expressing fused Lip2:Exe at different growth rates. Optical density (■), residual glucose (●) and glucose feed (▲) were compared at different growth rates: A: 0.019; B: 0.029, C: 0.046 and D: 0.049 h⁻¹.

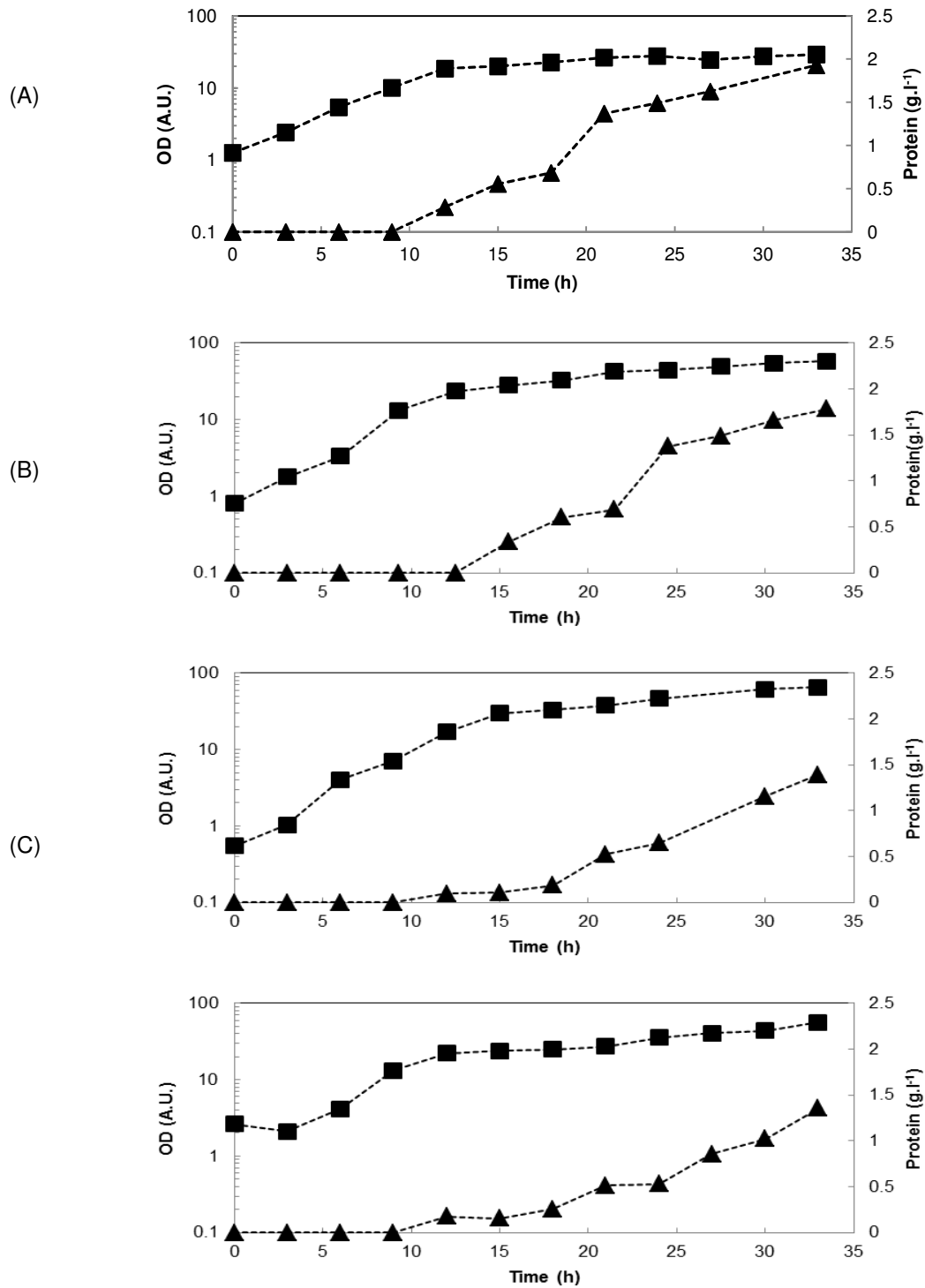


Figure 25: Total extracellular protein production and optical density during fed-batch fermentation at different growth rates. Optical density (■) and total extracellular protein production (▲) by *Y. lipolytica* (YIEx-gly) expressing fused Lip2:Exe during fed-batch fermentation at different growth rates. A: 0.019; B: 0.029, C: 0.046 and D: 0.049 h⁻¹.

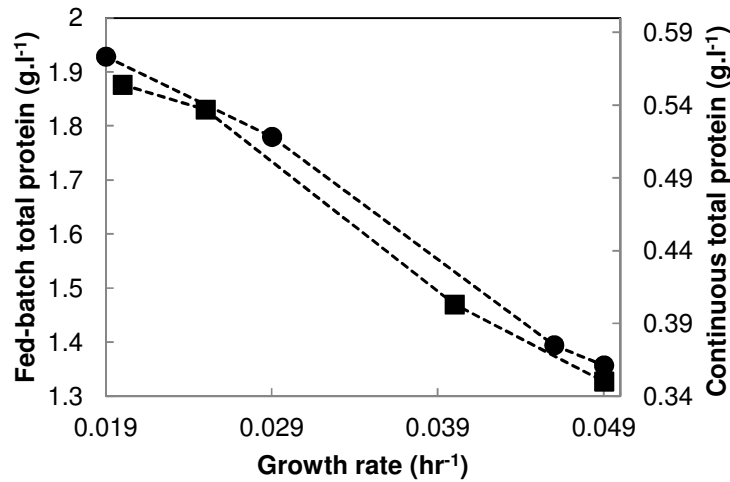


Figure 26: Comparison of extracellular protein production of *Y. lipolytica* during Fed-Batch and Continuous fermentation. Production of fused Lip2:Exe by fed-batch fermentation (●) and continuous fermentation (■) were compared at different growth rates: 0.02 to 0.05 h⁻¹.

The fed-batch fermentation therefore confirmed the data obtained in continuous fermentation that the production of proteins under regulation of the hp4d promoter is growth rate dependent as reported by van Zyl (2013). By decreasing the growth rate of the organism, a 60 % increase in total extracellular protein production took place. Growth rate has shown to have an effect on the recombinant production of proteins in several yeast expression systems. This was observed in *Saccharomyces cerevisiae* during the heterologous production of human insulin precursor and α -amylase (Liu *et al.*, 2013). Similar results were achieved by Madzak *et al.* (2005) during the production of a *Pycnoporus cinnabarinus lac 1* gene by *Yarrowia lipolytica* under regulation of the hp4d promoter with the highest protein production taking place at the lower growth rate (Madzak *et al.*, 2005).

Growth rate has also shown to have an effect on secretion of human serum albumin in *Pichia Pastoris*. In a glucose-limited chemostat, production was measured during growth rates of 0.015- 0.15 h⁻¹ of *P. pastoris*. At high growth rates, several transcription regulation factors showed to be down regulated resulting in a decrease in protein production. Maximum production was observed at growth rates from 0.05 0 0.075 h⁻¹. (Rebnegger *et al.*, 2014).

Although transcription levels showed a brief increase during exponential growth (Figure 20), during batch cultivations, it was not sustained whereas growth rate had a sustainable effect on the level of transcription (Figure 22 and 23) during continuous and fed-batch fermentations. The absence of extracellular lipase despite the increased level of transcription observed during mid exponential growth, during batch cultivations, can be explained by the fact that lipase secretion is triggered under carbon starvation or when the easily utilised amino-acids are depleted (Barth, 2013). The data obtained during continuous and fed-batch fermentations as observed in Figures 22, 23, 25 and 26, showed the hp4d promoter is growth rate dependent. This corresponds to the data achieved by van Zyl (2013). Compared to flask trial studies, the optimised fed-batch strategy yielded a 3.29 fold increase in total extracellular protein production.

5.4 Concluding Remarks

The regulation of the hp4d promoter in *Y. lipolytica* (YIEx-gly) was elucidated by using continuous fermentation to evaluate the effect of different growth rates on the regulation of the hp4d promoter. Transcription levels and extracellular lipase activity was measured and it was found that there was a direct correlation between the two and the growth rate. Maximum total extracellular recombinant protein production, lipase activity and mRNA transcript levels were observed at a growth rate of 0.02 h^{-1} . These levels dropped sharply with increase in growth rate indicating that the synthetic hp4d promoter is growth rate dependent. This information was used to develop an optimised fed batch strategy for the production of the fused Lip2:Exe precursor.

Chapter 6: Conclusion and Future Recommendations

6.1 Conclusion

A *Y. lipolytica* strain expressing the fused Lip2:Exe precursor was generated and cell banked. Recombinant protein production was validated in flask and batch fermentation (Chapter 3). It was observed that production by *Y. lipolytica*, under regulation of the hp4d promoter, took place during early stationary phase. In order to optimise conditions for the production, the regulation of the promoter needed to be established. A methodology to monitor fused Lip2:Exe mRNA transcripts was optimised (Chapter 4) and validated for reproducibility. It was observed that mRNA transcripts, under regulation of the hp4d promoter, were produced throughout the exponential and stationary phase during growth of *Y. lipolytica* (YIEx-gly) during batch cultivations indicating hp4d is quasi-constitutive. Continuous fermentation was used to regulate the growth rate of *Y. lipolytica*, using glucose as a limiting factor (Chapter 5). The mRNA transcripts, extracellular lipase activity and total protein production was monitored and all followed the same trends. Maximum production, under regulation of the hp4d promoter, took place at a growth rate of 0.02 h^{-1} . This was confirmed by running several fed-batch fermentations and quantifying the amount extracellular protein at different growth rates. A 60 % increase in recombinant production was observed by growing *Y. lipolytica* (YIEx-gly) at a rate of 0.02 h^{-1} as compared to other growth rates. It was therefore established that the synthetic hp4d promoter is growth rate dependent.

6.2 Future Recommendations and research outputs

Ideally, this hypothesis should be tested using different *Y. lipolytica* strains and different reporter genes. During batch cultivation, the production of total protein should also be evaluated intracellularly, at the level of translation especially when using the Lip2 enzyme. Different peptides should be tested as fusions to the Lip2 gene to evaluate if size has an effect on recombinant production under regulation of the hp4d promoter.

The developed fed-batch fermentation strategy for the production of heterologous proteins under regulation of the hp4d promoter by *Y. lipolytica* (YIEx-gly) has the potential to be used for the production of pharmaceutical peptides such as exenatide. This could lead to a more affordable treatment for type two diabetic patients that cannot afford the more expensive chemically produced drugs. This technology also has the potential to establish a recombinant production industry in South Africa and contribute to the South African bio-economy.

Chapter 7: References

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