Investigation of methylotrophic yeasts expression systems for the production of self-assembling proteins with biological applications

By

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Declaration

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Declaration by the candidate:

With regards to the entity of the dissertation, my contributions were as follows:

Nature of contribution	Extent of contribution
Planning, experimental work and preparation of dissertation draft	90%

The following co-authors have contributed to the dissertation:

Name	E-mail address	Nature of contribution	Extent of contribution
Dr JJ Smith	[redacted]	Construction of pHIPX4–ChiΔH- L2, pBLHIS– ChiΔH-L2, pHIP4X-VP6 and pBLHIS-VP6 plasmid vectors	8%
WA Prins		Expression of rotavirus VP6 in *E. coli* shake-flask cultures	2%
Prof M Rautenbach		Intellectual input as collaborator	Non-quantifiable
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The undersigned hereby confirm that:

1. The declaration above accurately reflects the nature and extent of the contributions of the candidate and co-authors.

2. No other co-authors, other than those specified, have contributed to the completion of this manuscript.

3. Potential conflicts of interest have been revealed to all interested parties.

Summary

Functional biological proteins are gaining interest with potential application in medicine and biotechnology. The methylotrophic yeasts, especially *Pichia pastoris* and *Hansenula polymorpha*, have been successfully used for the production of heterologous proteins with high industrial and medicinal importance. The aim of this study was to explore the production of self-assembly proteins in methylotrophic yeasts. The specific proteins investigated were the Human Papilloma virus (HPV) and Rotavirus (RV) proteins that self-assemble into virus-like particles (VLPs), and the α-chain proteins that assembles into procollagen.

Cervical cancer is ranked the fourth most common cancer in women worldwide. Human Papilloma virus types 16 and 18 account for 70% of all cervical cancers worldwide. Despite the availability of two commercial prophylactic vaccines, it is unaffordable for most women in developing countries. We compared the optimized expression of monomers of the unique HPV type 16 L1/L2 chimeric protein (SAF) in two yeast strains of *P. pastoris*, KM71 (Mut^S) and GS115 (Mut^+), together with *H. polymorpha* NCYC 495, to determine the preferred host in bioreactors. SAF was uniquely created by replacing the h4 helix of the HPV type 16 capsid L1 protein with a 13-amino acid peptide from the L2 protein. Two different methanol-feeding strategies in fed-batch cultures of *P. pastoris* Mut^S were evaluated: a predetermined feed rate versus feeding based on the oxygen consumption by maintaining constant dissolved oxygen levels (DO stat). All cultures showed a significant increase in biomass concentrations when methanol was fed using the DO stat method. In *P. pastoris*, the SAF concentrations were higher in the Mut^S strains than in the Mut^+ strains. Our results showed the maximum concentration SAF expressed in both yeasts reported to date with *H. polymorpha* the best producer of all the yeasts evaluated. Further, accurate quantification of SAF was obtained through direct comparison with known concentrations of purified HPV16 L1. Quantification is usually inferred from ELISA results using standards not always compatible to the L1 monomer.

Previous research in insect cells showed that the HPV16 L1/L2 chimeric protein self-assembled in capsomeres or capsomer aggregates during expression in insect cells. With the human codon-optimized chimeric gene, occational T=7 VLPs were visible in the insect cells. Similarly, when SAF was expressed in both methylotrophic yeasts, the majority of heterologous protein was observed as capsomeres (10 nm in diameter) with the occasional T=1 VLPs (25-30 nm in diameter)

assembled. This is the first report showing the formation of T=1 VLPs in *H. polymorhpa*. SAF proteins displaying L2 epitopes offer simultaneously high titres of L1 specific neutralizing antibodies, as well as cross-neutralizing antibodies against L2.

Gastroenteritis is one of the leading causes of deaths in children under the age of five years worldwide and caused by RV. We expressed a RV VP6 protein, derived from a prevalent South African RV strain (G9P), intracellularly in *Escherichia coli*, *P. pastoris* and *H. polymorpha*. Despite producing the lowest biomass levels of all the expression systems in shake flasks, the highest VP6 concentration was obtained with *E. coli*. In the controlled environment of bioreactors, all three expression systems attained higher cell densities and increased growth-associated VP6 production, in comparison to shakeflasks. Unlike in shake flask expressions, *H. polymorpha* outperformed both *P. pastoris* and *E. coli* during bioreactor cultivation. In contrast to yeast expressions, bacterial expressed VP6 protein was found to be insoluble upon analysis. This is the first report of VP6 expressed in methylotrophic yeast and holds the promise for the inexpensive production of VP6 as a possible vaccine candidate, booster dose or drug delivery mechanism.

Collagen is the main structural protein of various animal connective tissues and also has the natural ability to self-assemble; therefor, it poses similar expression challenges than VLPs. Treatment of burn victims can benefit from combining a collagen α-chain with an antimicrobial peptide (AMP) in wound dressings. The collagen can aid in wound healing and the AMP in fighting infectious agents present. This collagen-AMP fusion protein was extracellularly produced by *H. polymorpha*. Presence of a correctly-sized single band on tricine-SDS PAGE gels revealed the successful expression of the putative fusion protein. Proteomic analysis of this protein species only identified part of this fusion. This is the first evidence of the possible successful expression of a recombinant collagen-AMP fusion protein in yeast.

This study suggests that *H. polymorpha* is the preferred host, among the host cells tested, for the production of self-assembly proteins, such as the protein-components of virus-like particles and structural fusion proteins, such as collagen-AMP.

Opsomming

Funksionele biologiese proteïene met potensiële toepassing in medisyne en biotegnologie wek al hoe meer belangstelling. Metilotrofiese giste, veral *Pichia pastoris* en *Hansenula polymorpha*, word suksesvol aangewend vir die vervaardiging van heteroloë proteïene met hoë industriële en medisinale belang. Die doel van hierdie studie was om die produksie van self-monterende proteïene in metilotrofiese giste te ondersoek. Die spesifieke proteïene wat ondersoek is, was die Menslike Papilloomvirus (MPV) en Rotavirus (RV) proteïene wat self-monteer in virusagtige partikels (VAPs), asook die α-kettingproteïene in prokollageen.

Servikale kanker is die vierde mees algemene kanker by vroue wêreldwyd. Menslike Papilloomvirus tipes 16 en 18, verteenwoordig 70% van alle servikale kankers wêreldwyd. Ten spyte van die beskikbaarheid van twee kommersiële voorkomende entstowwe, is dit vir die meeste vroue in ontwikkelende lande onbekostigbaar. Ons het die geoptimiseerde uitdrukking van die unieke MPV tipe 16 L1 / L2 chimeriese proteïen (SAF) monomere in twee gisstamme van *P. pastoris*, KM71 (Mut^S) en GS115 (Mut^+), vergelyk tesame met *H. polymorpha* NCYC 495, om die voorkeurgasheer in bioreaktors te bepaal. SAF was uniek geskep deur die h4-helix van die HPV tipe 16 kapsied L1 proteïen met 'n 13-aminosuurpeptied van die L2 proteïen te vervang. Twee verskillende metanolvoedingstrategieë in *P. pastoris* Mut^S kulture is geëvalueer: 'n voorafbepaalde voertempo versus voeding gebaseer op die suurstofverbruik deur konstante opgeloste suurstofvlakke (OS) te handhaaf. Alle kulture het 'n beduidende toename in biomassa getoon toe metanol met behulp van die OS metode gevoer is. Die SAF konsentrasies was hoër in *P. pastoris* Mut^S-kulture as in Mut^+-kulture. Ons resultate toon dat albei giste die maksimum konsentrasie SAF wat gerapporteer is, oortref het met *H. polymorpha* wat die hoogste vlakke het van al die giste wat geêvalueer was. Verder is akkurate kwantifisering van SAF verkry deur direkte vergelyking met bekende konsentrasies van suiwer HPV16 L1. Kwantifisering word gewoonlik afgelei uit ELISA resultate deur gebruik te maak van standaarde wat nie altyd met L1 monomere ooreenstem nie.

In vorige studies in insekselle het hierdie HPV16 L1 / L2 chimeriese proteïen self gemonteer om kapsomere of kapsomeer-aggregate te vorm tydens uitdrukking. Met die menskodon-geoptimiseerde chimeriese geen was T = 7 VAPs soms sigbaar in insekselle. Soortgelyk, toe SAF in beide metilotrofiese giste uitgedruk is, was die meerderheid heteroloë proteïen as kapsomere (10 nm in deursnee) waargeneem met hier en daar ‘n T = 1 VAP (25-30 nm in deursnee). Dit is die eerste verslag wat die vorming van T = 1 VAPs in *H. polymorhpa* demonstreer. SAF proteïene wat L2-

epitope vertoon, bied gelyktydig hoë titers van L1-spesifieke neutraliserende teenliggame, sowel as kruis-neutraliserende teenliggame teen L2.

Gastro-enteritis is wêreldwyd een van die grootste oorsake van sterftes by kinders onder die ouderdom van vyf jaar en word deur RV veroorsaak. Ons het 'n RV VP6 proteïen, gebasseer op 'n Suid-Afrikaanse RV stam (G9P), intrasellulêr in *Escherichia coli*, *P. pastoris* en *H. polymorpha* uitgedruk. Ten spyte van *E. coli* se lae biomassavlakke in skudflesse, het hierdie uitdrukkingsisteem die hoogste VP6 konsentrasie gelewer. In vergelyking met skudflesse, het al drie uitdrukkingstelsels in die beheerde omgewing van bioreaktors tipiese hoër seldigtheid en groeiverwante VP6 produksie gelewer. Anders as in skudflesuitdrukkings, het *H. polymorpha* beter as beide *P. pastoris* en *E. coli* in bioreaktor kulture presteer. In teenstelling met gisuitdrukkings, was bakteriële uitgedrukte VP6 proteïene onoplosbaar. Dit is die eerste verslag van VP6 wat in metilotrofiese giste uitgedruk is en toon belofte vir bekostigbare VP6 produksie wat as moontlike entstofkandidaat kan dien, of as aanvullende vaksien of die moontlike draer van sekere teikenmedisyne.

Kollageen is die hoof strukturele proteïen van verskeie diere bindweefsels en het ook die natuurlike vermoë om self te monteer; dus stel dit soortgelyke uitdagings as VAPs om uitgedruk te word. Behandeling van brandslagoffers kan baat vind by die kombinasie van 'n kollageen-α-ketting met 'n antimikrobiese peptied (AMP) in wondbedekkings. Die kollageen kan help met wondgenesing en die AMP om aansteeklike patogene te beveg. Hierdie kollageen-AMP-fusie-proteïen is ekstrasellulêr in *H. polymorpha* vervaardig. Teenwoordigheid van die korrekte grootte proteïenband op trisien-SDS PAGE-jels het die suksesvolle uitdrukking van die vermeende fusie-proteïen bevestig. Proteomiese analise van hierdie proteïenspesie het slegs ‘n gedeelte van die fusie proteïen geïdentifiseer. Nietemin, dit is die eerste bewys van die moontlike suksesvolle uitdrukking van 'n rekombinante kollageen-AMP-fusie-proteïen in gis.

Hierdie studie dui daarop dat *H. polymorpha* die voorkeurgasheer is vir die produksie van self-monterende proteïene, soos die proteïen-komponente van virusagtige deeltjies, asook strukturele fusieproteïene, soos kollageen-AMP.

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List of Abbreviations and Acronyms

α-MF	alpha-mating factor
AMPs	antimicrobial peptides
AOX	alcohol oxidase
DHAS	dihydroxyacetone synthase
DO	dissolved oxygen
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EPI	Expanded Program on Immunization
ER	endoplasmic reticulum
FBP	fructose-1,6-bisphosphate
FMD	formate dehydrogenase
GAVI	Global Alliance for Vaccines and Immunisation
GSH	glutathione
HbsAg	Heptitis B surface antigen gene
HEPES	hydrozyethyl piperazineethanesulfonic acid
HIS	histidine
HPLC	high performance liquid chromatography
HPV	Human Papillomavirus
H. polymorpha	*Hansenula polymorpha*
I	integral
LB	Luria Bertani
MOX	methanol oxidase
MUT^-	methanol utilization negative
MUT^+	methanol utilization plus
MUT^S	methanol utilization slow
O_2	oxygen

PAP smear	Papanicolaou smear
P. pastoris	*Pichia pastoris*
P	proportional
RV	Rotavirus
S. cerevisiae	*Saccharomyces cerevisiae*
UPR	unfolded protein response
VLPs	virus-like particles
WHO	World Health Organization
SASBMB	South African Society for Biochemistry and Molecular Biology
Xu5P	xylulose-5-phosphate

Outputs of this PhD study

Poster presentations

- **Bredell H**, Smith JJ, Van Zyl WH, Görgens JF. *Hansenula polymorpha* as the preferred yeast expression system for the production of Human papilloma virus type 16 L1/L2 chimeric proteins as a possible vaccine candidate. SASBMB 2014 Conference, Goudini, South Africa

Peer-reviewed articles

- **Bredell H**, Smith JJ, Prins WA, Gorgens JF,Van Zyl WH (2016) Expression of rotavirus VP6 protein: a comparison amongst *Escherichia coli*, *Pichia pastoris* and *Hansenula polymorpha*. *FEMS Yeast Research*, 16. doi: 10.1093/femsyr/fow001

- **Bredell H**, Smith JJ, Görgens JF, Van Zyl WH (2018) Expression of unique chimeric human papilloma virus type 16 (HPV-16) L1/L2 proteins in *Pichia pastoris* and *Hansenula polymorpha*, *Yeast*, Apr 30. doi: 10.1002/yea.3318

Contribution to an article:

- Smith J, Burke A, **Bredell H**, Van Zyl W, Görgens J (2012) Comparing cytosolic expression to peroxisomal targeting of the chimeric L1/L2 (ChiΔH-L2) gene from human papillomavirus type 16 in the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha*. *Yeast* 29: 385–393. doi:10.1002/yea.2917

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Chapter 1

General introduction

In developing countries such as South Africa, approximately 83% of the population of 50 million do not have health insurance and rely primarily on the public health system when challenged with health issues (Blecher *et al.,* 2012). Worldwide cervical cancer is the second most prevalent cancer amongst women aged between 15 and 44 years with the highest rate amongst black women (Mqoqi *et al.,* 2004; Bruni *et al.,* 2017). Although routine screening using cervical cytology significantly reduced the number of new cases of invasive carcinoma, limited resources and capacity could not sustain frequent screening and did not significantly impact on mortality statistics in South Africa (Sankaranarayanan *et al.,* 2001; South African HPV Advisory Board 2011). Development of two prophylactic vaccines, Cervarix (GlaxoSmithKline, 2011) and Gardasil (Merck and Co. 2011) targeted against the Human Papillomavirus (HPV), the etiological agent in cervical cancer (Muñoz *et al.,* 2006), offer great potential for primary prevention, but until recently was only available in the private sector (South African HPV Advisory Board, 2011). To obtain maximum effectiveness, these vaccines need to be administered prior the onset of sexual activity during which the virus can be transmitted. Modelling studies estimated that vaccination of 70% of girls aged 9-12 years against HPV 16 and 18 with Gardasil will reduce the lifetime risk of cervical cancer with 43%. This percentage will increase to 61% with 100% coverage of schoolgirls in that age group (Sinanovic *et al.,* 2009). The World Health Organization (WHO) has recommended that routine HPV vaccination be included in the national immunisation programme, making it available to the public sector as well. This resulted in the launch of the government's HPV vaccine campaign in 2014 where girls between 9-12 years old were vaccinated with two doses, six months apart (Richter, 2015).

In developing countries, diarrhoea is a major contributor to the high rates of childhood mortality, estimated at 1.34 million deaths each year (Black *et al.,* 2010). Rotavirus (RV) is the single most common cause of severe diarrhoea in children under 5 years of age, constituting about 527 000 deaths annually (World Health Organization, 2010). In South Africa alone, diarrhoea causes more than 10 000 deaths a year, of which almost half is due to rotavirus infection (Ngcobo, 2009). Despite the availability of two licensed rotavirus vaccines, Rotarix® and Rotateq™, rotavirus remained the most common cause of severe diarrhoea due to the absence of targeted vaccination (Wang *et al.,* 2013). During August 2009, the World Health Organization made a global

recommendation to include the rotavirus vaccine (at birth, 6 and 14 weeks) into the routine Expanded Program on Immunization (EPI) of all infants in South Africa (World Health Organization, 2009). South Africa is the first country in Africa to introduce the RV vaccine in its EPI. A follow-up vaccine efficacy study on RV vaccine observed 73% protection against severe rotavirus gastroenteritis among African infants during the first year of life (Madhi *et al.*, 2010).

The cost of vaccines has become a major burden in most countries. In 2009, the vaccine cost of a fully immunised child was estimated at R1 338 in the public sector and R4 103 in the private sector, with a 10-fold increase forecasted in the next 10 years (Ngcobo and Cameron, 2010). An HPV vaccine dose costs R542 each (between R700 and R1000 in the private sector) and after three doses, which provides for 98%-100% effectiveness in preventing the virus (Paavonen *et al.*, 2009; The FUTURE II Study Group, 2007), the total cost could be between R1 500 and R1 620. Also, the South African government increased its budget allocation from approximately R200 million to R1.25 billion per annum to implement the addition of the rotavirus vaccine (Madhi *et al.*, 2014). South Africa remains the only country in the World Health Organization Africa region that does not qualify for donor support from the Global Alliance for Vaccines and Immunisation (GAVI) (Eastern and Southern Africa EPI Managers Meeting, 2016). Hence, South Africa is liable to finance its own immunization programmes.

Another health challenge in developing countries is the high mortality rate due to thermal injuries. Collagen is widely used in the management of severe burn wounds as it enhances wound healing. However, animal collagen, the most abundant protein in mammals, used as substitute in wound dressings can be allergenic to humans. Commercialized wound dressings are very expensive and not freely available to most people in developing countries. In addition, bacterial resistance against conventional antibiotics poses an increasing threat. One possible solution to antimicrobial resistance might be antimicrobial peptides (AMPs), which form part of the innate immunity and possess antimicrobial activity against bacteria, viruses, fungi and parasites. In order to produce affordable wound-dressings, large quantities of both human collagen and AMP are needed. A more cost-effective production method would be to express recombinant collagen-AMP fusions.

Structural proteins, such as HPV, RV and collagen, which self-assemble into either VLPs or structured fibrils, have shown increasing potential as modern vaccine substitutes or medical applications for treating burn wounds. In the light of high production costs and the demand for high expression quantities, there is a great urge to consider cheaper options to manufacture high yield

self-assembling protein components. Downstream processing of recombinantly expressed proteins can result in significant losses to the final yield. For this reason, large enough quantities of the desired protein are needed prior to purification. A minimum of 1 g protein per litre culture broth is required for cost-effective manufacturing of vaccines (Melinda Scanlen, The Biovac Institute, personal communication). High-yielding production will enable us to push the boundaries of expression and determine whether it is also possible to reach high enough yields of the product to be cost-effective. A broad spectrum of expression systems is available, but the methylotrophic yeasts is an established system for the production of heterologous proteins, particularly biopharmaceuticals and industrial enzymes.

1.1 Aim of the study

In this study, we investigated the methylotrophic yeast expression system for the production of self-assembling proteins with biological applications. Both *Pichia pastoris (P. pastoris)* and *Hansenula polymorpha (H. polymorpha)* have successful track records as cost-effective production hosts of various industrial and pharmaceutical proteins, including vaccines. All of this attests to the favourable characteristics of these two yeast species, such as their ability to reach cell densities of several grams per litre in defined media during fermentation, subsequently expressing high levels of recombinant protein (Stratton *et al.*, 1998). Despite the availability of current vaccines in the fight against cervical cancer and gastroenteritis, developing countries have limited access to these vaccines due to the high costs involved. Also, South Africa has included both the HPV as well as Rotavirus vaccines recently in its Expanded Programme on Immunization (EPI). It is quite evident that there is an urgent need to supply these vaccines in large scale. This significant vaccine demand places a huge financial burden on the country's health system. There is therefore a need to explore the possibility of a more cost-effective expression system to produce large quantities of these vaccine proteins. Also, inclusion of the cross-neutalizing L2 epitope into the HPV L1 protein could add further value to the current HPV vaccine.

The methylotrophic yeasts were also considered for the production of an unique collagen-AMP fusion protein, which can be applied to wound-dressings used for the effective treatment of burn wounds. In developing countries such as South Africa, the fatality rate due to burns in children is seven times higher than in developed countries as a the result of candles and kerosene stoves falling over in overcrowded houses (World Health Organization, 2017). Treating severe burn

wounds require expensive wound dressings and hospitalization is often needed to minimize morbidity and mortality due to infection. Bacterial resistance against antibiotics hamper conventional treatment. We designed an antimicrobial peptide (AMP) linked to a human α-1(type I) collagen fusion protein of which the collagen part can self-assemble. The envisaged outcome would be a product that can be applied to existing wound dressings. This will then aid in wound healing as well as serve as a prophylactic against possible wound pathogens.

1.2 Specific objectives of the study

1.2.1 To evaluate the potential to achieve high-yielding production systems for monomers of the unique HPV type 16 L1/L2 chimeric protein (SAF) in two yeast strains of *P. pastoris*, GS115 (Mut^+) and KM71 (Mut^S), and compare it to the production thereof in *H. polymorpha*.

1.2.1.1 Two different methanol feeding strategies to induce protein expression were compared in fed-batch cultures of *P. pastoris*_KM71 strain, due to the high sensitivity of this strain to methanol under- or over-feeding. In the first strategy, methanol was supplied at a predetermined exponential rate according to the *Pichia* Fermentation Protocol (Invitrogen Life Technologies, 2002). The second strategy was based on the oxygen consumption of the microorganism, where methanol feeding was controlled by the dissolved oxygen level (DO) in the culture. Cultures were done in 3 L bioreactors.

1.2.1.2 The preferred method of methanol feeding was applied in 10 L bioreactors to compare the *P. pastoris*_GS115 (Mut^+) strain with the *P. pastoris*_KM71 (Mut^S) strain.

1.2.1.3 The best performing *P. pastoris* strain was then compared to *H. polymorpha* for the production of SAF in 1.3 L bioreactors.

1.2.2 To semi-purify the SAF protein and visualize the possible formation of virus-like particles (VLPs).

1.2.3 To evaluate the expression of rotavirus VP6 protein in *Escherichia coli (E. coli), P. pastoris* and *H. polymorpha.*

1.2.3.1 Firstly, the expression of RV VP6 in *E. coli, P. pastoris* and *H. polymorpha* was compared in shake flasks.

1.2.3.2 Subsequently, the expression of RV VP6 in *E. coli, P. pastoris*_GS115 and *H. polymorpha* was compared in 1.3 L bioreactors.

1.2.4 To design, synthesize and produce fused collagen-AMP cassette in at least one of the methylotrophic yeasts.

1.3 Implications of the study

The main focus of this study was to develop high-yielding expression of self-assembling proteins in methylotrophic yeasts. Different methanol feeding strategies and yeast strains were compared, also relative to *E. coli*, in order to optimize the expression of these biological proteins. High yields of sub-unit vaccine proteins can alleviate the vaccine burden in South Africa, while the proposed collagen-AMP fusion protein can significantly contribute to burn-wound treatment.

The addition of a 13 amino acid L2 peptide to the chimeric HPV-16 L1/L2 protein can elicit cross-neutralizing antibodies and could therefore be included in HPV VLP-based vaccines (Kawana *et al.*, 1998; Kawana *et al.*, 1999; Kawana *et al.*, 2001a; Kawana *et al.*, 2001b). In addition, the production of a cheaper alternative for the treatment of burn wounds can save the lives of many victims who cannot afford the expensive treatment during serious burn injuries.

Knowledge gained during the optimization of these fermentation processes led to a better understanding of the different hosts investigated and how the expression of different self-assembling proteins was affected.

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Chapter 2

Literature overview

This section will highlight the use of strains of the methylotrophic yeasts *P. pastoris* and *H. polymorph*a as expression systems for viral structural proteins used as vaccine subunits, and all the factors contributing to optimize the protein yield. The current status and additional demands for both HPV and RV vaccines will be discussed. Also, a novel approach using collagen and an antimicrobial peptide used in burn wound dressings will be considered. Both viral proteins as well as collagen can self-assemble into well-ordered structures, but can face degradation in the host if not folded properly.

2.1 Heterologous gene expression in yeast

Yeast has gained widespread interest as one of the expressing systems of choice for the production of commercially valuable proteins. It shares the molecular, genetic and biochemical features of higher eukaryotes, and is ideal for large-scale industrial fermentation. Initially, baker's yeast, *Saccharomyces cerevisiae*, was used successfully as host for heterologous gene expression, but the system has various limitations, such as *S. cerevisiae* tends to hyperglycosylate recombinant proteins, which might hinder protein folding and subsequently protein activity (Tang *et al.*, 2016), it has a limited variety of carbon sources to use during its metabolism that restricts the fermentation process (Gellissen, Strasser and Suckow, 2005) as well as lower cell density growth and lower secreted protein yield than *Pichia pastoris* (Tran *et al.*, 2017). In addition, glycosylated proteins secreted by both *P. pastoris* and *H. polymorpha* do not have the terminal α-1,3-oligosaccharides linkages added by *S. cerevisiae* and is subsequently less allergenic than hyperglycosylated recombinant proteins expressed in *S. cerevisiae*. The hyper-antigenic nature of these proteins makes them unsuitable for therapeutic use (Gellissen *et al*., 2005; Trimble *et al.*, 1991).

As a result of these limitations of *S. cerevisiae*, methylotrophic yeasts such as *P. pastoris* and *H. polymorpha*, have been successfully exploited to offer cost-effective expression of various pharmaceuticals, including vaccines, antibodies fragments, hormones, cytokines and matrix proteins (Table 2.1). They both are able to utilize methanol as sole energy and carbon source during their metabolism. Methylotrophic yeasts pose several advantages as the ideal expression system, such as that expression of recombinant proteins is under tightly regulated and highly productive promoters

(*AOX* in *P. pastoris* and *MOX* in *H. polymorpha*) (Cregg *et al.*, 1989 ; Van Dijk *et al.*, 2000). In addition, *P. pastoris* can utilize a broad range of carbon sources (Gellissen *et al.*, 2005), can reach high-cell densities of 150 $g.l^{-1}$ dry cell weight in bioreactors (Cereghino and Cregg, 1999), relatively low levels of endogenous protein are secreted into the medium (Cereghino and Cregg, 2000), increased expression levels are achieved, up to 100 times more than that of *E. coli* (Faber, Harder and Veenhuis, 1995), and tend not to hyperglycosylate recombinant proteins (Sudbery, 1996). Unlike bacterial expression systems, methylotrophic yeasts have the ability to perform many of the post-translational modifications usually performed in higher eukaryotes, e.g. correct folding, disulfide bond formation, O- and N-linked glycosylation, processing of signal sequences and certain types of lipid addition (Cereghino and Cregg, 2000; Macauley-Patrick *et al.*, 2005). The latter makes methylotrophic yeasts an useful alternative to the bacterial expression of eukaryotic proteins that requires post-translational modifications or disulfide bridges (Cereghino *et al.*, 2002).

Hansenula polymorpha is also known as *Pichia angusta*. The former genus *Hansenula* was renamed *Ogataea* but since it is still not widespread in use, more investigators prefer to use the old name *Hansenula (*Stoyanov and Lahtchev, 2016). *H. polymorpha* is more thermo-tolerant than *P. pastoris* (37°C versus 30°C) and capable of faster high-yield fermentation on simple, defined media, making this yeast the preferred choice for industrial applications (Hollenberg and Gellissen, 1997). The higher growth temperature may be favourable for the production of mammalian (including human) proteins. In addition, this expression system allows a better cooling management and reduces the risk of contaminations in large scale fermentations (Van Dijk *et al.*, 2000). Although these two yeasts genera are phenotypically identical, only *H. polymorpha* is able to assimilate nitrogen (Mack *et al.*, 2009).

Table 2.1. Examples of foreign proteins produced in *P. pastoris* and *H. polymorpha.*

Protein expressed	**Expression level**	**Reference**
P. pastoris		
Bacterial:		
Clostridium botulinum serotype F heavy chain fragment C	205 $mg.kg^{-1}$ of cells	(Johnson *et al.*, 2003)
Escherichia coli AppA	117±1 $U.mL^{-1}$	(Stahl, Wilson and Lei, 2003)
Escherichia coli phytase	6.4 $g.L^{-1}$	(Chen *et al.*, 2004)
Fungal:		
Aspergillus oryzae tannase	72 $mg.L^{-1}$	(Zhong *et al.*,2004)
Candida parapsilosis lipase/acyltransferase	5.8 $g.L^{-1}$	(Brunel *et al.*, 2004)
Trametes versicolor cellobiose dehydrogenase (CDH)	225 $U.mL^{-1}$	(Stapleton *et al.*, 2004)
Invertebrates:		
Boophilus microplus BM95 antigen	550.0 $mg.L^{-1}$	(Boue *et al.*, 2004)
Pandalus borealis (shrimp) cathepsin L	60.0 $mg.L^{-1}$	(Aoki, Ahsan and Watabe, 2003)
Vertebrates (non-human):		
Bovine enterokinase light chain (EKL)	350.0 $mg.L^{-1}$	(Peng *et al.*, 2004)
Ovine interferon-τ (r-oIFN-τ)	391.7 $mg.L^{-1}$	(Sinha *et al.*, 2003)
Mouse endostatin	133.33 $mg.L^{-1}$	(Trinh, Phue and Shiloach, 2003)
Spider silk fusion protein		(Jansson *et al.*, 2016)
Undecapeptide P_{11}-2 β-sheet tapes		(Moers *et al*., 2010)
Human:		
Granulocyte-macrophage colony-stimulating factor	180.0 $mg.L^{-1}$	(Wu *et al.*, 2003)
Serine protease inhibitor SERpin C1-INhibitor	180.0 $mg.L^{-1}$	(Bos *et al.*, 2003)6
Angiostatin	108.0 $mg.L^{-1}$	(Xie *et al.*, 2003)
Human type I collagen	0.5 $g.L^{-1}$	(Nokelainen *et al*., 2001)
Human type I/II/III collagen	200-600 $mg.L^{-1}$	(Myllyharju *et al*., 2009)
Plants:		
Gsp (Panax ginseng C, medicinal peptide)	800.0 $mg.L^{-1}$	(Yan, Chen and Li, 2003)
Rice α-amylase (Amy 1A/3D)	340.0 $mg.L^{-1}$	(Lee *et al.*, 2003)
Amaryllidaceae snowdrop agglutinin	80.0 $mg.L^{-1}$	(Baumgartner *et al.*, 2003)

Viruses:		
Measles virus nucleoprotein (MeN)	29% of total protein	(Slibinskas *et al.*, 2004)
HPV type 16 L1 VLPs	13.8 g.mL^{-1}	(Bazan *et al.*, 2009)
HPV type 16/18/33 L1 VLPs		(Coimbra *et al.*, 2011)
HPV type 58 L1 VLPs	20 mg.L^{-1}	(Jiang *et al.*, 2011)
HPV type 16 L1 VLPs	104.8 µg.L^{-1}	(Kotzé *et al.*, 2011)
HPV type 16/18 L1 VLPs	9.5/6.4 mg.L^{-1}	(Rao *et al.*, 2011)
HPV type 16 L1/L2 chimeric protein	1.43mg.L^{-1}	(Smith *et al.*, 2012)
HPV type 16 L1 VLPs	5.35U.mL^{-1}	(Zhao *et al.*, 2015)
Rotavirus VP2, VP4 VP6 and VP7 VLPs		(Zhang and Lou, 2011)
Coxsackievirus A16 VLPs		(Feng *et al.*, 2016)
Norovirus VLPs	100 mg.L^{-1}	(Tomé-Amat *et al.*, 2014)
Dengue virus type 1 glycoprotein VLPs		(Poddar *et al.*, 2016)
Dengue virus type 2 envelope VLPs		(Mani *et al.*, 2013)
Hepatitis B surface antigen VLPs		(Lünsdorf *et al*., 2011)
Tick-borne encephalitis virus VLPs		(Yun *et al*., 2014)
Heterocapsa circularisquama RNA virus VLPs	2.5 g.L^{-1}	(Wu *et al*., 2012)

Protein expressed	**Expression level**	**Reference**
H. polymorpha		
Bacterial:		
β-Lactamase (*E. coli*)		(Ryan, *et al*., 1989)
β-Galactosidase (*E. coli*)		(Brito *et al.*, 1999)
Fungal:		
Glucoamylase (*Schwanniomyces*)	1.4 g.L^{-1}	(Gellissen *et al.*, 1991)
Glucose oxidase (*Aspergillus*)	2.25 g.L^{-1}	(Hodgkins *et al.*, 1993)
Phytase (*Aspergillus*)	13.5 g.L^{-1}	(Mayer *et al.*, 1999)
Plants:		
Seed storage protein		(Buckholz and Gleeson, 1991)
Malate dehydrogenase (water melon)		(Faber *et al.*, 1994)

Phytochrome (oat)	0.0013 $g.L^{-1}$	(Mozley, Remberg and Gärtner, 1997)
Viruses		
HPV type 16 L1 protein	0.72$mg.L^{-1}$	(Smith *et al.*, 2012)
HPV Type 52 L1 VLPs		(Liu *et al.*, 2015)
HPV Type 16 L1 protein	78.6 $mg.L^{-1}$	(Li *et a*l., 2009)
Animals:		
Hirudin (Hirudo)	>1 $g.L^{-1}$	(Weydemann *et al.*, 1995)
Aprotinin (Bovine)	0.3 $g.L^{-1}$	(Zurek *et al.*, 1996)
Green fluorescent protein (jellyfish)		(Amuel, Gellissen and Suckow, 2000)
Human:		
Hepatitis B surface antigen		(Shen *et al.*, 1989)
Hemoglobin		(Gilbert *et al.*, 1994)
Human serum albumin	0.46 $g.L^{-1}$	(Cox *et al.*, 2000)
Heat shock protein gp96	150$mg.mL^{-1}$	(Li, *et al.*, 2011)
Hydroxylated gelatin/ mouse α1(I) collagen		(De Bruin *et al.*, 2002)

2. 1.1 Alcohol oxidase

Methylotrophic yeasts share a compartmentalized methanol-metabolic pathway that harbours high abundance key enzymes, such as alcohol/methanol oxidase (AOX/MOX), formate dehydrogenase (FMD) and dihydroxyacetone synthase (DHAS) during growth on methanol (Fig. 2.1). Their synthesis is regulated at transcriptional level and gene expression is subjected to a carbon-source-dependent repression/derepression/induction mechanism inherent of the specific promoter. These promoters are repressed, derepressed or induced in the presence of glucose, glycerol or methanol, respectively. Heterologous gene expression under the control of these promoters can be regulated by adding different carbon source to the growth medium (Gellissen *et al.*, 2005).

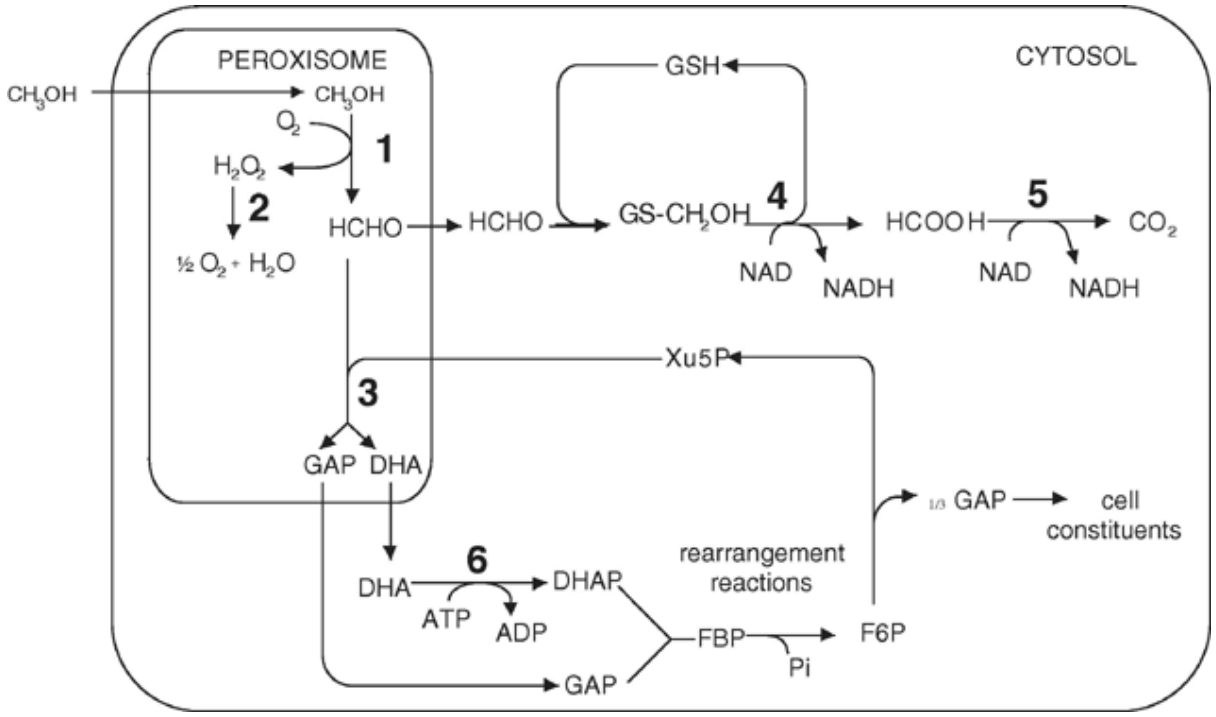

Figure 2.1. Methanol metabolism pathway in methylotrophic yeasts. Enzymes: (1) alcohol oxidase, (2) catalase, (3) dihydroxyacetone synthase, (4) formaldehyde dehydrogenase, (5) formate dehydrogenase, (6) dihydroxyacetone kinase (Gellissen *et al.*, 2005).

Protein expression in *H. polymorpha* can also be induced by glycerol in the growth medium that derepresses the *MOX* promoter in the same way that methanol does. This added feature offers a safer alternative to using methanol to induce protein expression due to the dangers associated with the storage of large quantities of methanol (Hollenberg and Gellissen, 1997).

The most noticable enzyme in the methanol-utilization pathway is AOX, which catalyzes the first step in this pathway. This enzyme is undetectable in cells cultured on media containing glucose, glycerol or ethanol. Protein synthesis is greatly enhanced and the protein can constitute levels up to 30% of the total soluble protein in cells fed with methanol at growth limiting rates in bioreactor cultures (Couderc and Baratti, 1980). Two genes in *P. pastoris* code for the AOX protein, *AOX I* and *AOX II*. The *AOX I* gene is responsible for 85% of the AOX activity, whereas AOX II provides the other 15% (Veenhuis, Van Dijken and Harder, 1983; Ellis, 1985). Therefore, it is not surprising that the *AOX I* gene has been isolated and the cloned version of the *AOX I* promoter is used to drive heterologous gene expression in transformation vectors (Tschopp *et al.*, 1987).

2.1.2 Genotypes and phenotypes of *P. pastoris* and *H. polymorpha*

Most of the *P. pastoris* host strains are auxotrophic, which allow for the selection of expression vectors containing the respective genes for complementation and serve as appropriate selectable marker gene upon transformation (Sreekrishna *et al.,* 1997). Wild-type strain X-33 has both *AOX* genes fully functional and its phenotype is designated Mut^+, **m**ethanol **ut**ilization plus. A derived Mut^+ *P. pastoris* host strain, GS115, has a mutation in the histidinol dehydrogenase gene (genotype *his4*), which prevents them from synthesizing histidine (phenotype His^-Mut^+). Oxygen supply is a major concern in Mut^+ cultures where high cell densities are obtained, since the bioreactor is unable to sustain the metabolic oxygen supply when air is used for sparging, thus requiring the enrichment of air feed with oxygen (Cos *et al.,* 2006). More oxygen is required for the oxidation of methanol than for glycerol and therefore, considerable more heat is generated during methanol induction than during glycerol feeding. Rapid and efficient cooling is required as excessive heat in the culture vessel may affect the productivity and quality of the recombinant proteins (Jungo, Marison and von Stockar, 2007). Additionally, large scale production of this phenotype requires the storage of large quantities of methanol, which constitutes a potential fire hazard

The parent *P. pastoris* KM71 strain has a mutation in the arginosuccinatelyase gene, which prevents the strain from growing in the absence of arginine. Wild-type *ARG4* gene was used to disrupt the *AOX I* creating a genotype *arg4 aoxI::ARG4* (Balamurugan, Reddy and Suryanarayana, 2007) Since this strain must rely on the weaker *AOX II* gene for methanol metabolism, it grows slowly on this carbon source and produce low levels of biomass (Harder and Veenhuis, 1989); it therefore has the **m**ethanol **ut**ilization **s**low phenotype (Mut^SArg^+) (Inan and Meagher, 2001). It requires less oxygen supply due to slower growth, which reduces the rate of heat generation, but also has less requirement for heat elimination. In order to increase cell density and process productivity, a multi-carbon substrate in addition to methanol is recommended. Mixed feeding occasionally resulted in enhanced protein expression (Katakura *et al.*, 1998), while Brierley *et al.* (1990) reported lower productivity than when methanol was fed alone. Growth is therefore supplemented with alternative carbon sources such as sorbitol, mannitol, trehalose or alanine (Inan and Meagher, 2001; Sreekrishna *et al.*, 1997). In addition, host strains with both *AOX* genes being disrupted, are unable to grow on methanol, and has the **m**ethanol **ut**ilizing minus phenotype (Mut^-) (Cregg *et al.,* 1989).

Of the three *H. polymorpha* parental strains, NCYC495 grows poorly in methanol-containing media and therefore does not have the strong methanol pathway-derived promoters. This strain, previously called *Hansenula angusta*, was obtained from spoiled concentrated orange juice in Florida (Wickerham, 1951). However, this strain has been improved by crossing the original NCYC495 with CBS4732, giving rise to a methanol-utilizing strain, also called NCYC495 (Titorenko *et al.*, 1993).

2.1.3 Multiple gene intergration versus single integration

One of the preferred methods to generate genetically stable yeast transformants, is the integration of the expression cassette into the chromosome (genomic DNA) of the yeast (Sreekrishna *et al.*, 1997). Integration into the chromosome occurs via homologous recombination when the yeast vector contains regions that are homologous to regions in the yeast genome. Integration happens by means of gene insertion from a single crossover point at either the AOXI/MOX promoter fragment, the *aoxI::ARG4* gene or in the marker *his4*gene, or gene replacement mediated through double cross-over events (Romanos, 1995) (Fig. 2.2, a-b). Transformants generated via gene replacement arise from a double-crossover event between the *AOXI* promoter and *3'AOXI* region of the vector and the genome. Double crossover events result in the complete demolishing of the *AOXI* coding region, alias gene replacement (Cregg *et al.*, 1985) (Fig. 2.2, c). These transformants are generally single copy transformants and are genetically more stable (Romanos, Scorer and Clare, 1992; Clare *et al.*, 1991). Insertion events at specific loci are more likely than replacement events, with frequencies between 50-80% of HIS^+ transformants. Before integration, vector DNA is linearized with a restriction enzyme that cuts only once within the *AOX*1 promoter region or marker gene (e.g. *HIS4*). This linearized plasmid is then used to transform a specific auxotrophic mutant (Cregg *et al.*, 1985).

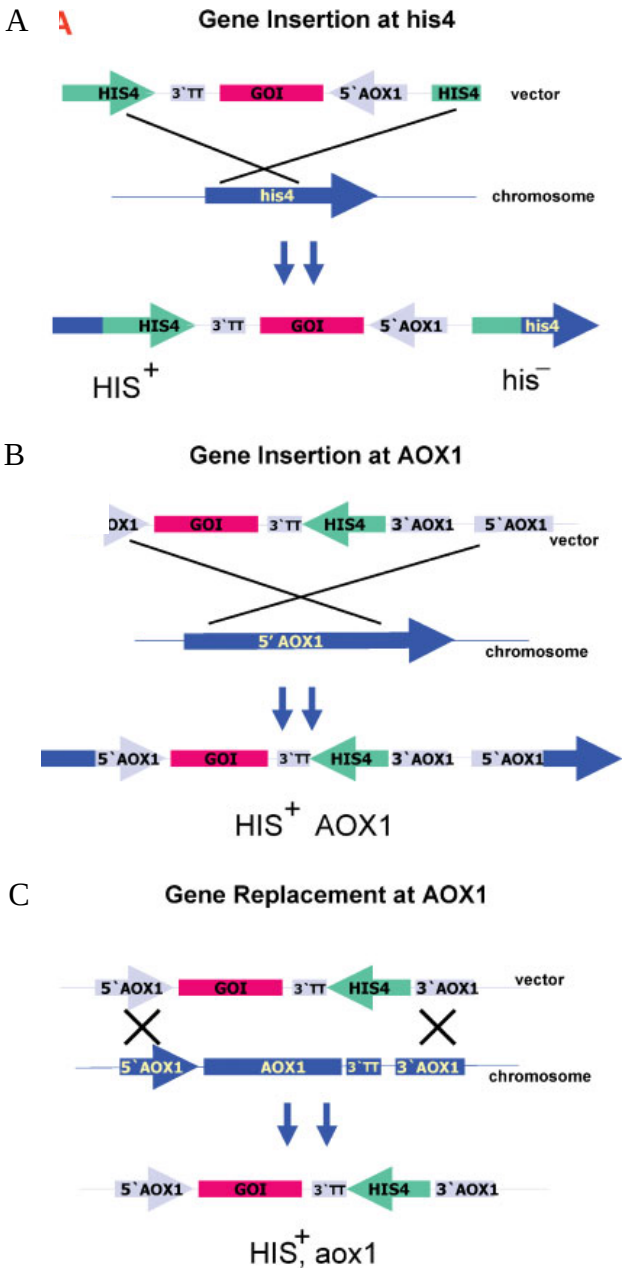

Figure 2.2. Integration into the *P. pastoris* genome, either by (A, B) gene insertion or (C) gene replacement (Daly and Hearn, 2005).

Yeast host strains containing multiple integrated copies of the expression cassette have been shown to yield more heterologous protein than single-copy strains (Clare *et al.*, 1991; Thill *et al.*, 1990). Multiple integration at a single locus do occur spontaneously, but with a low frequency of between 1-10% of all selected His^+ transformants. Various authors have reported on the proportional relationship between an increase in copy number and increase in heterologous protein production. Transformants containing up to four copies of the Heptitis B surface antigen gene (*HbsAg)* were

found to increase the yield of HbsAg four-fold (Vassileva *et al.*, 2001). Expression of the same protein in *H. polymorpha* correlated with the gene dosage of the intergrated DNA (Gellissen *et al.*, 1992). Similarly, a 10-fold increase in copy number of the β-galactosidase gene also resulted in a 22-fold increase of the enzyme (Sunga and Cregg, 2004). Multi-copies of the inserted vector can be detected using dot-blot analysis, PCR, Southern blot analysis and differential hybridization. In contrast, *P. pastoris* transformants mostly consists of single-copy integrants (Balamurugan, Reddy and Suryanarayana, 2007).

When hybridizing host DNA with the *AOX*1 promoter sequence, it should be possible to visualize two bands, one representing the endogenous *AOX* gene and the other representing the transformed and integrated vector. Theoretically, with every insertion of the gene into the genome, the intensity of the band representing the vector should increase due to an increase in the number of copies present (Klabunde *et al*., 2007). Another approach to construct multi-copy expression in *P. pastoris*, is to transform vectors harbouring multiple head-to-tail copies of an expression (Fig. 2.3). The advantage of this approach is that the precise number of expression cassettes is known as it can be determined via DNA sequencing (Brierley, 1998).

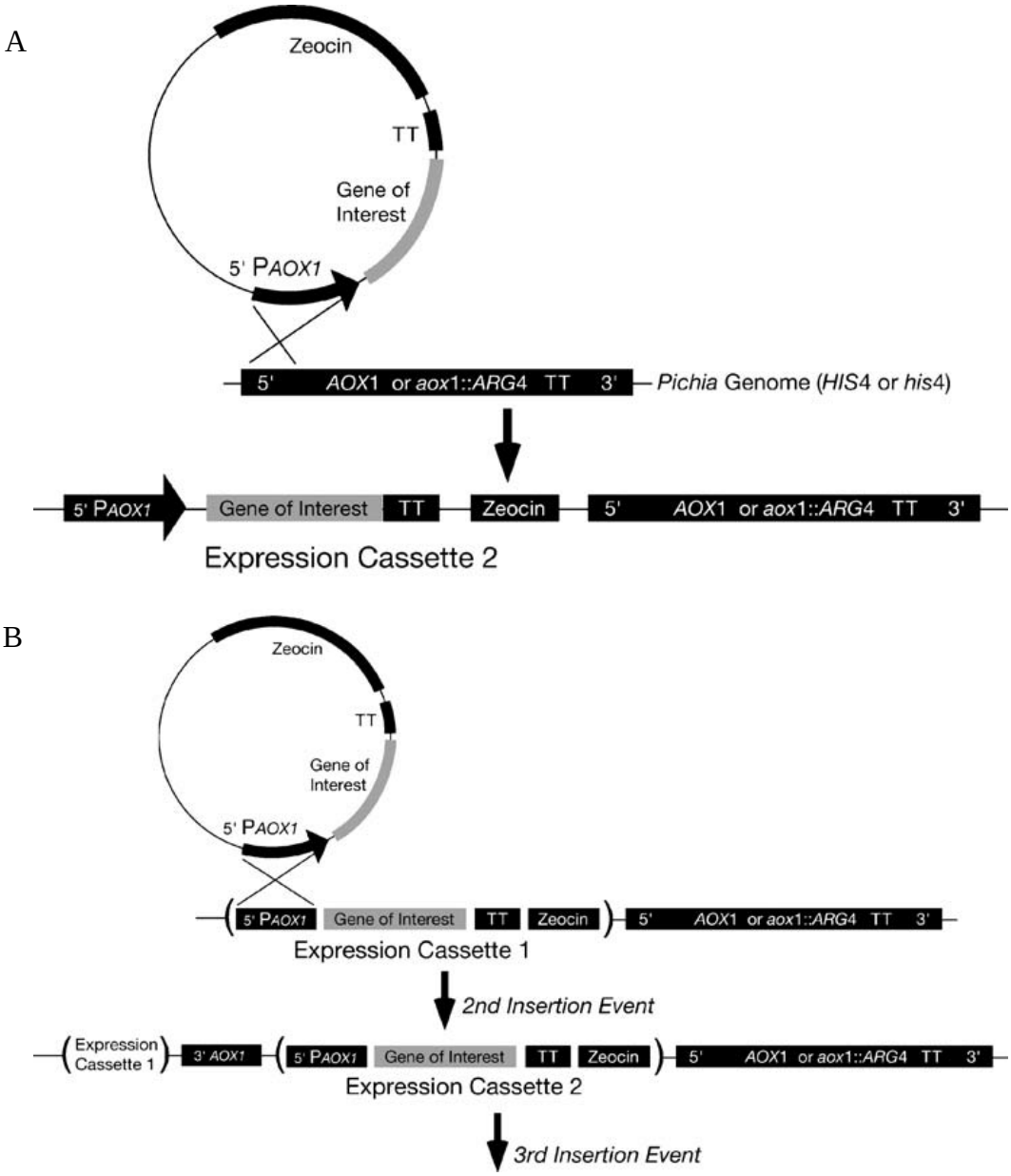

Figure 2.3. Map showing the integration of heterologous genes into the genome of *P. pastoris.* (A) Single copy integration versus (B) multiple copy integration (Li *et al.,* 2007).

In *H. polymorpha*, transformation vectors harbouring a *HARSI* (*Hansenula ARSI*) sequence as a replication signal can be randomly inserted into the genome resulting in recombinant strains harbouring multiple tandemly repeated copies of the expression cassette integrated into the target locus. The HARSI element allows autonomous replication with copy numbers as high as 40 (Roggenkamp *et al.* 1986), 60 (Gellissen *et al.*, 2005) and even 100 copies per cell (Hollenberg and Gellissen, 1997; Klabunde *et al.,* 2007). Integration of these multiple copies into the *H. polymorpha* genome takes place via non-homologous recombination, which is different from *P. pastoris* where

recombination is homologous (Gellissen *et al*., 1992). These DNA integrations of the initially episomal vector is then present in a "head-to-tail" arrangement (Gellissen *et al*., 2005). It is possible to estimate the copy number in *H. polymorpha* by observing the colony sizes on selective agar plates. Larger colonies tend to have higher copy numbers when compared to smaller and medium-sized colonies. Popov and co-workers (1997) showed when cells with more than one integrated vector and auxotrophic marker are grown under selective conditions, these cells are able to divide faster and thus form bigger colonies than cells containing only one copy of the vector. Colony size can thus be used as preliminary screening for varying copy numbers (Popov, Buztler and Bruggemann, 1997).

On the contrary, very high copy numbers may have a negative effect on transcription and translation levels, reducing the yield of secreted proteins (Inan *et al.*, 2006). A possible explanation may be that the increased copy number coding for an increased protein yield may block the secretory pathway (Wittrup *et al.*, 1994). The unfolded protein response (UPR) is triggered in response to the accumulation of unfolded protein. In order to restore endoplasmic reticulum (ER) homeostasis, UPR activation upregulates a number of other genes, which ultimately leads to the transport of unfolded or misfolded proteins to the cytosol for proteasome degradation. Post-translational folding within ER, membrane translocation, signal sequence processing and disulfide bond formation are all regarded as rate-limiting factors (Hohenblum *et al.*, 2004). Overloading these post-translational processes could result in bottle-necks during protein expression. A common strategy to overcome such bottle-necks entails the overexpression of folding helper proteins or the transcriptional regulator, *HAC1*, of the UPR pathway genes. Unlike in *S. cerevisiae*, this *HAC1* gene is constitutively expressed in *P. pastoris,* resulting in the high titers of proteins secreted in this organism (Guerfa *et al.*, 2010). However, it has been reported that overexpression of folding helper proteins sometimes may either have no effect or reduce protein secretion (van der Heide *et al.*, 2002). When proteins are naturally expressed intracellularly, there is no stress on the secretory pathway and no negative effect on the UPR (Aw and Polizzi, 2013).

The linear correlation between gene dosage and protein productivity is also strain dependent. The growth rate of Mut^+ *P. pastoris* strains were significantly impaired when the gene copy number of porcine insulin precursor (PIP) exceeded 12 copies (Zhu *et al.*, 2009). Cos *et al.* (2005) showed a similar effect was not observed in Mut^S strains where single copy and multiple copies of the *Rhizopus oryzae* lipase (ROL) gene had comparable effects on growth rates.

2.1.4 Intracellular versus extracellular protein expression

Whether a recombinant protein is destined intra- or extracellularly, is depended on the presence of (native) secretion sequences. Where proteins need to be directed to the secretory pathway, a special secretion signal sequence is required. The most commonly used signal sequence is the *S. cerevisiae* alpha-mating factor pre-pro peptide (MFα1) (Cereghino and Cregg, 2000). This peptide comprises of a 19 amino acid signal pre-sequence followed by a 60 amino acid pro-sequence. The protein of interest is fused to the N-terminal pre-sequence, which directs the protein to the secretory pathway. An endopeptidase site separates the expressed protein from the pre-sequence, allowing the endoplasmic reticulum (ER) peptidase to cleave (Raemaekers *et al.,* 1999). It is thought that the pro-sequence play a determining role in transferring the protein from the ER to the Golgi compartments (Julius *et al.,* 1984). During translation, the signal sequence and the pro-region are cleaved off by the peptidase and the yeast's Kex2 protease, respectively. This results in the release of the mature protein into the culture supernatant (Raemaekers *et al.,* 1999).

Foreign secreted proteins can easily be isolated from the culture medium by removal of the yeast cells through centrifugation. An added advantage is that methylotrophic yeasts secrete very low levels of endogenous protein into the growth medium, which reduce the downstream purification process of secreted proteins (Barr, Hopkins and Sreekrishna, 1992). As a result, up to 90% of the total extracellular protein in *H. polymorpha* cultures comprises of secreted heterologous protein (Van Dijk *et al.,* 2000).

Pichia pastoris, unlike prokaryotic expression systems, has the ability to perform many of the post-translational modifications usually performed in higher eukaryotes, such as correct folding, disulphide bond formation, *O*- and *N*- linked glycosylation and the processing of signal sequences. Due to its folding requirements, the option of secretion is usually reserved for foreign proteins that are normally secreted by their native hosts (Cereghino and Cregg, 2000). When a protein in its native form is not secreted and it is forced through the secretory pathway of the expression system, it may be altered by post-translational modifications, such as adding a secretion signal sequence, which may influence its functionality (Cereghino and Cregg, 2000). Moreover, folding and disulphide bond formation of certain recombinant proteins is often a slow, rate-limiting process (Hohenblum *et al.,* 2004). It is more desirable to use Mut^S strains for expression of these proteins as the protein induction rate is slower, it allows for proper folding of the expressed protein and disulphide bond formation (Romanos, 1995).

Recombinant proteins expressed intracellularly represent less than 1% of the total intracellular proteins, and thus the purification of such proteins is more complicated and cumbersome than extracellular expressed proteins (Rees *et al.,* 1999). Also, Mut^S strains are useful for the expression of intracellular proteins, because lower levels of alcohol oxidase inside the yeast cells allow a higher level of heterologous protein production (Romanos, 1995).

Due to the reducing environment of the cytoplasm, prokaryotic systems such as *E. coli* have been generally unsuccessful in disulphide bond formation and glycosylation. Thus, foreign proteins are usually insoluble in *E. coli*, whereas *P. pastoris* has been used successfully to produce soluble proteins with multiple disulphide-bonds (White, Kempi and Komives, 1994).

2.1.5 Scale-up of high cell density growth in bioreactor cultures

Small-scale expressions performed in shake flasks are in general the first level of expressions to be employed in order to select the best producing transformant. Production of high levels of heterologous proteins during shake flask expression is difficult due to the limited volume and the inability to accurately control factors such as culture induction, oxygen transfer, pH and substrate utilization. Even at rotations of 200 per minute in baffled flasks, conditions offer limited aeration, resulting in protein levels about 10-fold lower compared to that obtained in bioreactors (Romanos, 1995).

P. pastoris strains are grown in bioreactor cultures using a three-stage process: a) a batch stage containing a repressing carbon source such as glycerol to accumulate biomass; b) the second fed-batch phase during which the culture is fed with glycerol at a growth-limiting rate to further maximise the biomass and to prepare (derepress) the cells for induction; and c) the third stage (induction) when the culture is adapting to and utilising methanol, and recombinant proteins are synthesised (Cereghino *et al.,* 2002). Brierley *et al.* (1990a, 1990b) recommended a maximum glycerol concentrations of 6% as higher concentrations can inhibit cell growth (Sing *et al.,* 2008). The first two phases are similar for both Mut^+ and Mut^S phenotypes. The third phase, also known as the induction phase or methanol fed-batch phase, differs depending on yeast phenotype. Since the methanol growth rates are different between the between Mut^+ and Mut^S phenotypes, the methanol consumption rates are also different. The Mut^+ phenotype has a faster growth rate on methanol compared to the Mut^S yeast phenotype, which translates to a faster methanol consumption rate and a faster production rate of heterologous protein.

2.1.6 Induction with methanol

Monitoring and control of methanol concentrations are crucial during the induction of recombinant proteins, because the productivity of the bioprocess is directly related to this parameter. Strains of *P. pastoris* can assimilate methanol, but they cannot tolerate high methanol concentrations. Formaldehyde and hydrogen peroxide (oxidized products of methanol by the alcohol oxidase) accumulate inside the cell and are toxic (Couderc and Baratti, 1980; Cregg and Madden, 1988; van der Klei, Bystrykh and Harder, 1990). This cytotoxic effect can reduce cell viability and the subsequent expression of recombinant proteins. Bioreactor cultures can yield up to 6 times more biomass and 3.5 times more expressed protein compared to shake flasks cultures (Zhang *et al.*, 2002). Optimal concentrations of 0.5-1.0% (2-3.65 $g.L^{-1}$) methanol (Cunha *et al.*, 2004; Schenk, Marison and von Stockar, 2007) are used in *P. pastoris* cultures to maximize protein production. Various studies contradict the accumulated methanol concentrations at which it is inhibitory to cell growth and can lead to decreased protein production (Romanos, 1995; Vassileva *et al.*, 2001). Generally, methanol concentrations exceeding 3.65% in the culture medium have been reported to negatively affect cell growth (Zhang *et al.*, 2000). However, an increase in methanol concentrations from 0.5 to 5% showed a 3-fold increase in the production of carboxypeptidase A2 in Mut^+ transformants (Reverter *et al.*, 1998). In the case of Mut^S strains, low levels of methanol may not be enough to initiate transcription (Cereghino and Cregg, 2000). Since Mut^S strains metabolize methanol poorly, the possible accumulation of excess methanol in the culture medium needs to be avoided. According to Invitrogen's *Pichia* Fermentation Process Guidelines (Invitrogen Life Technologies, 2002), methanol concentrations more than 0.3% should be completely avoided in Mut^S strains. Transcription levels of the *AOX1* promoter can be 3-5 times greater in cells that are fed with methanol at growth-limiting rates, compared to cells grown in excess methanol (Cereghino and Cregg, 2000). Therefore, it is important to determine the optimum methanol levels for each specific protein to be expressed. The lack of constant methanol supply in shake flasks is yet another limiting factor in these small-scale fermentations. Methanol metabolism requires oxygen and the expression of heterologous proteins is negatively affected by oxygen deprivation. Only bioreactors allow for the monitoring and control of all the parameters, such as pH, aeration and carbon source, simultaneously.

Various methods exist for the monitoring of methanol levels in the culture media, either directly using gas chromatography, or high performance liquid chromatography (HPLC) for off-line analysis. Botht involve the processing of samples before analysis, which can be very costly and

running the risk of allowing the methanol to evaporate before determining the concentration (Schenk, Marison and von Stockar, 2007). On-line methanol sensors analyse the methanol concentrations in the bioreactor exhaust gas and adjust the feed rate accordingly (Potvin, Ahmad and Zhang, 2012), which may be essential for maintaining optimum levels of unmetabolized methanol. Alternatively, methanol levels can be controlled indirectly by the measurement and control of the dissolved oxygen (DO). Because the level of the DO in the culture is directly related to the rate of methanol consumption by the yeast, due to the highly oxidative nature of this process: An increase in DO levels therefore indicates that the rate of methanol accumulation has slowed down, typically due to insufficient methanol concentrations, requiring the (automatic) addition of more methanol (Li *et al.*, 2007). Another strategy for controlling the methanol feeding rate is to adjust it manually to the specific growth rate of the yeast during the induction phase.

2.1.7 Medium components

Components for the Fermentation Basal Salts medium with PTM_1 Trace Salts (Invitrogen Life Technologies, 2002) are listed in Table 2.2 and 2.3, respectively. Since the mineral medium components for fermentation (glycerol, methanol, biotin, salts and trace elements) are all economical and well-defined, *P. pastoris* is ideal for large-scale production of heterologous proteins but also safe for human use. The relatively low pH and the presence of methanol make this medium less likely to become contaminated by other microorganisms (Cereghino and Cregg, 2000). Despite the versatility of this medium, the most frequent problem is the precipitation of one or more of the salts at pH levels above 5. Upon closer investigation, our research group found that it was indeed the ferrous sulfatehepta-hydrate that precipitates at pH 5. As a result, the actual remaining salt concentration is difficult to assess and result in an unbalanced nutrient supply and the turbidity complicates the absorbance reading of the culture sample. Brady *et al*. (2001) showed that salt precipitation can be overcome by reducing the salt concentration to one-quarter of the original recipe (Invitrogen Life Technologies, 2002), without any negative effect on growth rate and biomass yield (Brady *et al.*, 2001). Alternatively, Oehler *et al.* (1998) presented a medium in which the phosphoric acid and potassium hydroxide is replaced with 25.0 g sodium hexametaphosphate and 9.0 g ammonium sulphate (Oehler, Lesnicki and Galleno, 1998). Ammonium hydroxide (28%) is added to control the pH at the desired level, but also act as a nitrogen source.

Table 2.2. Defined medium composition for *P. pastoris* and *H. polymorpha* fermentations.

Component	Quantity
Phosphoric acid, 85%	26.7 ml
Calcium sulphate	0.93 g
Potassium sulfate	18.2 g
Magnesium sulphate-7H_2O	14.9 g
Potassium hydroxide	4.13 g
Glycerol	40.0 g
Water	to 1 liter

Table 2.3. Defined PTM_1 Trace Salt components for *P. pastoris* and *H. polymorpha* fermentations.

Component	Quantity
Cupric sulfate-5H_2O	6.0 g
Sodium iodide	0.08 g
Manganese sulfate-H_2O	3.0 g
Sodium molybdate-2H_2O	0.2 g
Boric Acid	0.02 g
Cobalt chloride	0.5 g
Zinc chloride	20.0 g
Ferrous sulfate-7H_2O	65.0 g
Biotin	0.2 g
Sulfuric acid	5.0 ml
Water	to 1 liter

2.1.8 Temperature and pH

Other important process parameters that affect the yield and activity of recombinant proteins, are culture temperature and pH. Higher temperature fermentations may result in the exposure of protein hydrophobic surfaces during peptide folding, which favour hydrophobic interactions and predispose proteins to aggregation. These aggregated proteins are more susceptible to intracellular proteolytic degradation (Lee, Choi and Yu, 1990). Lower growth temperatures result in reduced protein synthesis which allow proteins to fold properly. This can be advantageous to self-assembly proteins that could be seen as aggregated proteins by the yeast's degradation system. Low-temperature expression reduces proteolytic degradation of secreted proteins and is suitable for aggregation-prone proteins, but the fermentation period is longer compare to higher fermentation temperatures (Li *et al.*, 2007).

Controlling the medium pH during the fermentation process is critical for cell growth, protein formation and protein stability. It is recommended that *P. pastoris* is cultured at a pH of 5.0 to 6.0 (Invitrogen Life Technologies, 2002). When the presence of inhibiting proteases are to be avoided, lowering the pH to 3.0 or below (Brierley, 1998).

2.1.9 Dissolved oxygen control

Methylotrophic yeast requires oxygen to metabolize glycerol and methanol. Sufficient oxygen supply is therefore indispesable for *P. pastoris* cell growth and heterologous protein expression (Harder and Veenhuis, 1989). As the cells metabolise the available carbon source, the oxygen is consumed and the dissolved oxygen (DO) will drop. Once the carbon source such as glycerol and methanol are depleted, the DO will rise rapidly and the methanol-feeding rate should be increased accordingly to maintain cell metabolism. Thus, methanol feeding is closely related to the DO level (Li et al., 2007).

In the absence of a methanol sensor, the DO is a good indicator of cell growth, carbon availability and general state of the yeast culture. Methanol concentration is not given as a precise value but rather indicated by the DO levels. Therefore, the exact methanol level can probably be either under or exceeding the limiting value. A constant (DO) level of 30-35% is optimum for most *P. pastoris* fermentations (Li *et al.,* 2007). Excessive high DO levels are cytotoxic and reduce cell viability (D'anjou and Daugulis, 2001). DO can be controlled using the agitation rate of the bioreactors' impellers and aeration through air or oxygen (O_2) sparging, while it is also very sensitive to the medium addition strategy. In addition, bioreactors also have metal baffles to improve aeration (Srivastava, 2008).

Dissolved oxygen is sensed by a DO electrode and controlled by a cascade effect between agitation rate (200-1000 rpm) and aeration rate (1 volume of oxygen per volume of fermentation culture per minute, 1 vvm), which is maintained by the proportional (P) and integral (I) controller. Glycerol/methanol feeding is carried out in a fed-batch mode based on an open-loop control strategy, the DO stat method, also referred to as 'demand-feed'. The feeding rate is adjusted according to a control algorithm that attempts to control the level of the dissolved oxygen in the bioreactor. This entails the controlled addition of glycerol/methanol depending on the DO level. As the yeast metabolises actively, glycerol/methanol becomes depleted and the DO level begins to rise. Once the DO >30%, the glycerol feed is switched on. In the presence of glycerol, the metabolic pathway becomes activated and the DO level begins to decrease. When the DO < 30%, the glycerol

feed is switched off. The speed of agitation increased and O_2 supply is automatically supplied to the system after the agitation speed reached its maximum, causing the DO tension to increase again. Once the DO >30%, the glycerol is switched on again (Valero, 2013).

Indirect methods such as DO levels in bioreactors can mask the actual reason why DO increases. Increased DO levels could be attributed to the lack of methanol in the bioreactor, but also when inhibitory methanol levels are reached in case of overfeeding. Subsequently, the culture will be inhibited, yeasts cells will die and the DO will increase. This can be incorrectly interpreted by the control system as a methanol "shortage" resulting in more feeding, and subsequent higher methanol accumulation in the bioreactor (Cos *et al.*, 2006). Despite the fact that the DO stat is used widely, neither the methanol concentration nor the growth rate is kept constant by this system. Open loop systems are also easy to implement, but show no response to possible perturbations of the system. Heterologous protein production, particular in *P. pastoris*, is characterized by a complex and non-linear dynamics, making this control strategy inadequate for precise methanol control (Cos *et al.*, 2006).

2.1.10 Foam control

Due to the high agitaion and aeration rate during yeast fermentation, together with the nature of the culture medium, foam is generated at the surface of the culture medium. Excess foam may cause denaturation of the secreted proteins, reduce the headspace in the bioreacter and even complicates gas exchange at the surface (Invitrogen Life Technologies, 2002). Hence, excess foam needs to be eliminated by adding a non-silicone reagent to the culture medium (Antifoam 204, SIGMA).

2.1.11 Codon usage

The production of heterologous proteins comprises many complex transcription and translation processes, which may exert stress on the host cells at different levels. Gene expression is regulated by transcription, as the level of the steady-state mRNA is indicative of the final yield of foreign protein. Introducing changes in the mRNA sequence may affect the overall stability of mRNA and result in degradation (Rai and Padh, 2001). Furthermore, translation efficiency is highly dependent on the preferred codon usage of the host organisms. Highly expressed genes show a strong bias towards a subset of codons. Rare codons may result in limited aminoacyl-tRNAs and increased mistranslation, followed by lower yields of the desired protein (Bennetzen and Hall, 1982). *Pichia pastoris* shows a preference for GC-rich codons. Due to its high AT content, the expression of HPV

L1 genes is negatively affected when expressed in this yeast (Kotzé *et al.*, 2011; Li *et al.*, 2003; Sinclair and Choy, 2002).

2.1.12 Production of viral proteins in yeast

The expression of structural proteins poses additional challenges to recombinant yeasts, some of which may have deleterious effects. In order to maintain their functionally-active conformation, structural proteins need to fold properly after translation within the lumen of the endoplasmic reticulum (ER). The structural folding programming is strongly contained in the primary polypeptide structure (Gething and Sambrook, 1992). Misfolded proteins can be retained in the ER, causing intracellular accumulation. This bottleneck might interfere with the secretion process of host proteins (Romanos, Scorer and Clare, 1992). Folding, disulphide bond formation (Hohenblum *et al.*, 2004) and gene copy number (Clare *et al.*, 1991) are all considered to be possible rate-limiting steps in the secretion of foreign proteins from yeast cells. *P. pastoris* has been very successful in producing proteins that contain a high number of disulphide-bonds (White, Kempi and Komives, 1994). Inter-capsomeric disulfide bonds, formed between conserved cysteines, are crucial for virus-like particle (VLP) formation (Sapp *et al.*, 1998). However, disulphide bond formation and glycosylation are not favoured when secreted proteins are expressed intracellularly. This can result in unstable and inactive products, followed by degradation (Rai and Padh, 2001).

Increasing the copy number of the expression cassettes could have both positive and negative effects on recombinant protein expression (Thill *et al.*, 1990). Rodriguez-Limas *et al.* (2011) showed that a single plasmid expressing three rotavirus genes had several advantages over three individual plasmids expressing separate rotavirus genes. Increased gene dosage can cause a significant metabolic stress on the host cell due to limited carbon and nitrogen resources, leading to reduced growth rate and gene expression (Rodríguez-Limas *et al.*, 2011).

In addition to correct folding, proper assembly and extracellular transport are required during multimeric protein complex formation. VLPs are good examples of monomeric proteins that self-assemble into multimeric structures mimicing native virions. Viral VLPs comprise of different building blocks, representing either one monomer or combinations of various different monomers (Grgacic and Anderson, 2006). The efficiency of self-assembly of human papilloma VLPs can be sensitive to changes in even a single amino acid base (Kirnbauer *et al.*, 1993). Human papillomavirus (HPV) L1 VLP formation, for example, requires inter-capsomeric hydrophobic

interactions; folding of the monomeric L1 proteins therefore needs to expose these hydrophobic areas (Chen *et al.*, 2000).

In case of rotavirus (RV), the minimum requirement for VLP formation is the co-expression of structural proteins VP2 and VP6. Since VP6 is present at higher quantities than VP2 in the viral particle, different kinetics apply for the synthesis of each structural protein. The production of appropriate amounts of each structural component is vital for efficient VLP production and avoiding accumulation of waste monomers (Vieira *et al.*, 2005). Aggregation of monomeres/capsomeres competes with VLP formation and is a major cause of protein instability (Ding *et al.*, 2010). Also, advances in VLP technology via *in vitro* manipulation of buffers have shown to increase the stability of VLP by improving native disulphide bond formation (Mach *et al.*, 2006).

2.2 Cervical cancer and current Human papillomavirus vaccines

Cervical cancer is characterised by abnormal growth of cells in the cervix and have the ability to spread to other parts of the body. Early stages have no symptoms, therefore, regular screening is important to identify the condition. It is the fourth most common cancer in women worldwide after breast, colorectal and lung cancers (Ferlay *et al.*, 2012), with the highest incidence in black females in developing countries, such as South Africa (Kay *et al.*, 2003). Although South African women in the public sector are entitled to three free Papanicolaou (Pap) smears, at the age of 30, 40 and 50 years, it is difficult to implement, especially in rural areas where access to health care is limited. This screening regime is predicted to reduce the risk of cervical cancer by 67% (Marcus, 2008). The lack of screening programmes resulted in higher incidence rates of cervical cancer in lower-resource countries of sub-Saharan Africa. Almost 70% of the global cervical cancer burden falls in developing countries (Ferlay *et al.*, 2012) (Fig. 2.4). It has been reported that the incidence rate of cervical cancer in Zimbabwe is up to 14 times higher than in non-African countries (Marcus, 2008).

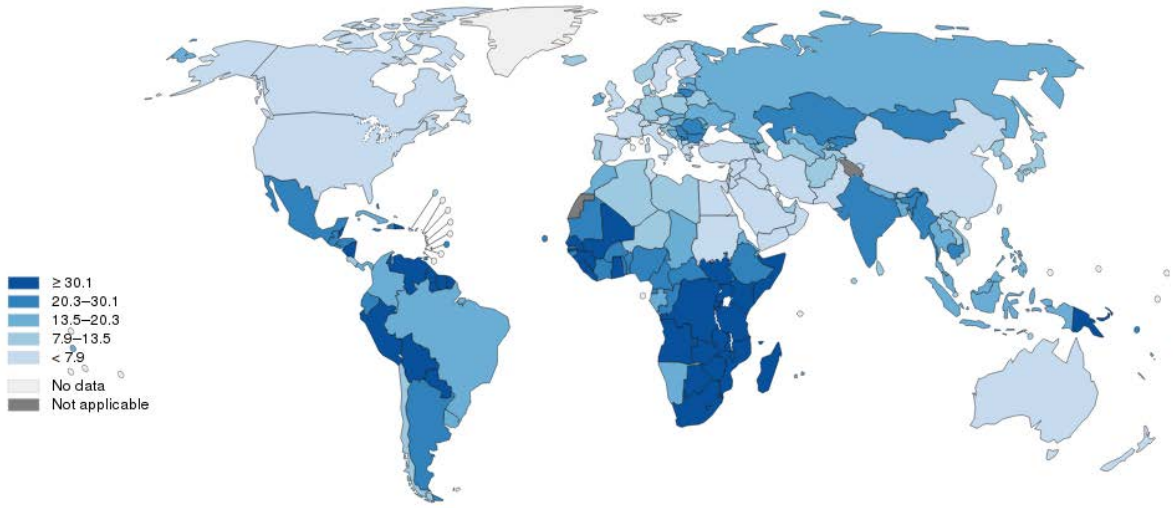

Figure 2.4. Geographic distribution of the world age-standardized incidence rate of cervical cancer worldwide in 2012 (Ferlay *et al.*, 2012). Intensity of colour bar reflects incidence rate.

Almost 100% of all invasive cervical cancers contain human papillomavirus (HPV) (Walboomers *et al.* 1999), identifying HPV type 16, 18 and a few others as major risk factors (Muñoz *et al.*, 1992; Bosch *et al.*, 1992). Even on home ground, Denny *et al.* (2014) reported evidence of HPV type 16 in more than 50% of cervical cancer biopsies from South African women with invasive cervical cancer (Denny *et al.*, 2014). Other HPV types , such as HPV types 35 and 45 are also now emerging and becoming amongst the most common types in sub-Saharan Africa (Clifford *et al.*, 2005; Allan *et al.*, 2008; Denny *et al.*, 2014).

The two HPV vaccines currently registered in South Africa are both VLP vaccines: Merck's Gardasil and GlaxoSmithKline's Cervarix. VLPs or pseudovirions are formed by the self-assembly of structural proteins L1 alone or the combination of L1 and L2, but lack any genetic material making them ideal immunogens ,without the risk of being infectious (Grgacic and Anderson, 2006). Gardasil comprises of HPV type 6, 11, 16 and 18 L1 recombinant proteins expressed in *Saccharomyces cerevisiae* (Shi *et al.*, 2007). Cervarix consists of HPV type 16 and 18 L1 proteins made in the recombinant baculovirus expression system (Campo and Roden, 2010; Deschuyteneer *et al.*, 2010). These two vaccines are targeted at the HPV types causing cervical cancer and have the potential to prevent this disease by 70%, but unfortunately these vaccines are very expensive (Marcus, 2008). In addition, the South African government extended its national vaccination programme in 2014 by administering the HPV vaccine to school girls between the age of 9 -12 years old in two doses, six months apart. This will escalate the cost of subsidised vaccinations dramatically, hence the quest for cheaper manufacturing costs.

2.2.1 Structure of the Human papillomavirus

The virion (capsid) has a non-enveloped icosahedral structure, 55 nm in diameter, made up of 72 pentameric capsomeres (Fig. 2.5). The HPV capsid consists of two proteins, the major structural L1 protein and the minor one, L2. L2 is present at a ratio of 1 to every 30 L1 proteins (Kirnbauer *et al.*, 1993). HPV contains a viral genome of almost 8 000 bp of double-stranded circular DNA and is divided in three parts: two thirds code for early proteins E1 to E7, and the remainder for the structural proteins (Fig. 2.6) (Garcea and Chen, 2007). The L1 protein alone can self-assemble into either capsomeres (pentamers of L1) or VLPs (72 capsomeres) (Kirnbauer *et al.*, 1992). The i*n vitro* self-assembly process is accomodated by a combination of low pH and high salt concentrations (Liu *et al.*, 2007; Mach *et al.*, 2006).

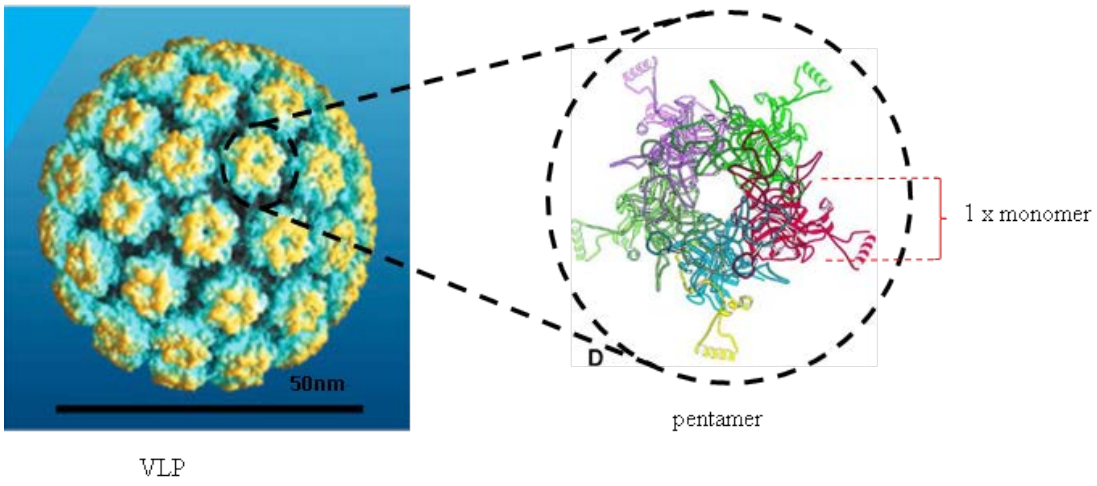

Figure 2.5. Atomic model of the HPV16 VLP, consisting of 72 pentamers with each pentamer containing 5 monomers (Zhao *et al.*, 2012 ; Chen *et al.*, 2000).

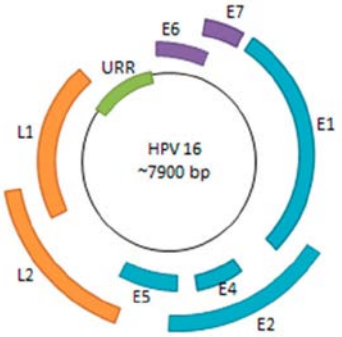

Figure 2.6. Genome organization of Human papillomavirus type 16 (Shanmugasundaram and You, 2017).

VLP formation requires inter-capsomeric interactions involving helices 2, 3 and 4 near the carboxy-terminus of each L1 monomer. These helices project laterally and outwards where helix 4 from a L1 monomer with in a capsomer forms hydrophobic interactions with helices 3 and 4 from a L1 monomer of an adjacent capsomer. (Fig. 2.7) (Chen *et al.,* 2000). Finnen *et al.* (2003) described a similar hydrophobic region near the carboxy terminus of L2 that specifically interacted with L1. The strong interaction indicates it as the principal domain responsible for anchoring L2 within the papillomavirus virion (Finnen *et al.*, 2003). Yet, L2 is not necessary for VLP assembly.

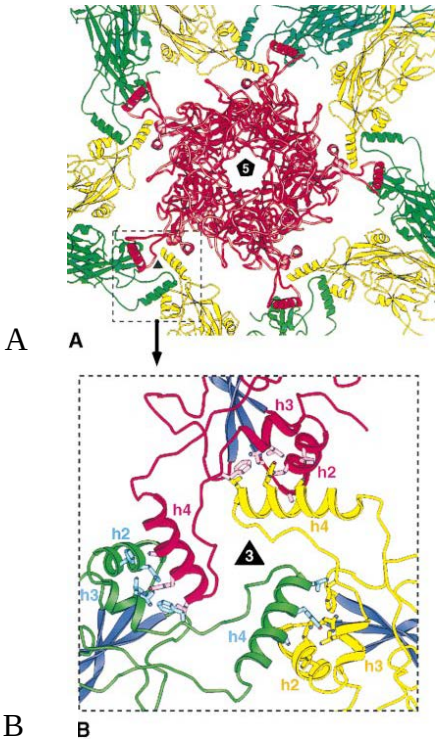

Figure 2.7. (A) Diagram showing the interactions amongst different pentamers.All subunits in the central pentamer are red; two monomers in each adjacent pentamer are shown in yellow and green, respectively. The helix 4 from each monomer projects laterally. (B) and interacts with helices 2 and 3 of a monomer in an adjacent pentamer (Chen *et al.*, 2000).

2.2.2 HPV immunogenicity

Thönes and Müller (2007) showed that different assembly forms of HPV type 16 L1, including capsomeres and VLPs, induce humoral and cellular immune responses after oral vaccination of mice (Thönes and Müller, 2007). Due to the amino acid sequence conservation of the L2 protein among different HPV types, L2 shows broad-spectrum cross-reactivity among different HPV genotypes and therefore great promise as a broadly neutralizing prophylactic vaccine (Yang *et al.*, 2003). The anti-L1 immune response, in turn, is more type-specific. Despite the cross-reactivity, the

titres of neutralizing antibody against L2 are rather low compared to those produced against L1 (Roden *et al.*, 2000). One option to overcome this problem is to include the L2-neutralizing epitope on the surfaces of VLPs, thus increasing the titers of neutralizing antibodies approximately 10-fold (Slupetzky *et al.*, 2007).

Gambhira *et al*. (2007) has found that antibodies raised against L2 also protect against low-risk HPV types and cutaneous types, which makes it even more useful in a broadly-acting HPV vaccine. A region of L2 has been identified that shows the broadest cross-neutralizing activity. This domain, which is situated between amino acids 17 and 36, is recognized by a monoclonal antibody (RG-1) and is implicated in binding to a putative HPV secondary receptor (Palmer *et al.*, 2009).

A study done by Buck and co-workers (2008) has shown that the HPV capsid can accommodate up to 72 molecules of L2. This contradicts other studies where only 12 molecules of L2 are found within the viral capsid (Roden *et al*., 1996; Volpers *et al*., 1994; Finnen *et al*., 2003). These studies have implications for the development of broadly antigenic HPV VLP vaccines as the variability in the number of L2 proteins present within the particle may affect its immunogenicity. L1 and L2 have been revealed to interact with each other through strong hydrophobic interactions involving 44 amino acids at the carboxy terminus of L2. This region is very proline-rich, which is characteristic of protein-protein interactions and would allow L2 to bend in a way that would allow a short region to protrude from the L1 pentamer (Finnen *et al*., 2003; Slupetzky *et al*., 2007). The N-terminal part of L2, which is recognized by neutralizing antibodies, extends into the external environment. Despite the broadly antigenic properties of L2, neutralizing antibodies mainly target L1 and it is thus the most important component of an HPV VLP vaccine (Kieback and Muller, 2006). High titers of neutralizing antibodies are induced when L1 and L2 are co-expressed and VLP assembly takes place. Levels of antibodies are comparable to those elicited by L1 alone, however, the addition of L2 to VLPs is advantageous because it increases the VLP yield (Schiller and Lowy, 1996; Wakabayashi *et al*., 2002). When expressing the structural proteins in an expression system for VLP production, it is important to keep in mind that codon usage in humans and the chosen expression system may differ greatly, which in turn may lead to reduced yields of VLPs. Codon optimization of the structural genes may ensure that expression takes place more efficiently and at high levels (Leder *et al*., 2001; Zhao *et al*., 2003).

Up-regulation of the immune response is partly due to the role played by dendritic cells (DC) in capturing antigens and migrating to the lymphoid tissues where they initiate and modify

particular cellular immune responses. De Witte *et al*. (2007) investigated the interaction between HPV L1 VLPs and DCs because the activation of DCs by HPV L1 VLPs is critical in initiating a complete immune response. It was established that HPV L1 VLPs do interact with and activate DCs, which lead to up-regulation of immune co-stimulatory molecules as well as the production of cytokines IL-6, IL-8, IL-10 and IL-12 (De Witte *et al*., 2007). These results are important as previous studies have shown that incomplete activation of DCs results in the development of antigen-specific tolerance, rather than the activation of an immune response (Harper *et al.*, 2006). Zhang and co-workers (2009) have shown that VLPs are able to induce B-cell proliferation and differentiation. B-cells form part of the lymphocyte population and play a central role in the humoral immune response. These immune cells are known to be activated by antigens with structured, repetitive configurations such as the capsids of most viruses, which lead to effective cross-linking with the B-cell surface. This in turn leads to the production of high-affinity antibodies that are extremely specific and long-lasting memory cells, which are advantageous when using VLP vaccines (Zhang *et al*., 2009).

2.2.3 Expression of HPV L1, L2 or chimeric L1/L2 proteins

HPV L1 VLPs have been produced in various expression systems, such as bacteria, e.g. *E. coli* (Chen *et al.*, 2001) or *Lactococcus lactis* (Cortes-Perez *et al.*, 2009), yeast such as *S. cerevisiae* (Woo *et al.*, 2008), *P. pastoris* (Zhao *et al.*, 2015; Bazan *et al.*, 2009; Kotzé *et al.*, 2011) and *H. polymorpha* (Li *et al.*, 2009), baculovirus-infected insect cells (Le Cann *et al.*, 1994), transgenic plants (Biemelt *et al.*, 2003) and mammalian cells (McLean *et al.*, 1990). L1/L2 VLP requires the co-expression of both L1 and L2, either as two separate genes on one plasmid or individual genes on separate plasmids. L1 and L2 VLPs have also been produced in human epithelial cells (Cianciarullo *et al.*, 2010) and insect cells (Kirnbauer *et al.*, 1993; Zhou *et al.*, 1991). Co-expression of L1 and L2 resulted in a four-fold increase in VLP yield (Kirnbauer *et al.*, 1993).

Furthermore, chimeric L1/L2 VLPs, comprising the L1 VLP displaying a common-neutralizing L2 epitope in various regions of the L1 protein, have been expressed in insect cells (Varsani *et al.*, 2003; McGrath *et al.*, 2013) and more recently in the yeasts *P. pastoris* and *H. polymorpha* (Smith *et al.*, 2012). One of these chimeric constructs was created by replacing the h4 helix of the L1 protein with a 13-amino acid peptide from the L2 protein (Varsani *et al.*, 2003). This construct was human codon-optimized and resulted in the formation of mainly capsomeres in insect cells, but the occasional T=7 VLPs (~50 nm in size) were seen (McGrath *et al.*, 2013). Similarly,

formation of VLPs in the baculovirus system was prohibited when the h4 helix of L1 was replaced with human respiratory syncytial virus (RSV) epitopes, resulting in capsomeres (Murata *et al.*, 2009). The highest concentration of HPV-16 L1 protein obtained from various expression sytems, is reported to be in the *P. pastoris* KM strain (14.2 $mg.L^{-1}$) (Bazan *et al.*, 2009) and in *H. polymorpha* (78.6 $mg.L^{-1}$) (Li *et al.*, 2009), all obtained in shake-flasks experiments.

2.3. Rotavirus infection and current vaccines

Gastroenteritis, causing severe diarrhea, is one of the leading worldwide causes of illness and deaths in children under the age of five years (Parashar *et al.*, 2006). Rotavirus (RV) infection accounts for one third of diarrhea-associated hospitalizations and more than 500 000 deaths annually (Parashar *et al.*, 1998; WHO 2007). Approximately, 85% of those deaths occur in South Asia and sub-Saharan Africa (Parashar *et al.*, 2009) (Fig. 2.8). There is no cure for gastroenteritis other than to treat the dehydration, and can therefore be especially harmful for infants and young children. Prevention with rotavirus vaccination is the best way to protect children from RV disease. Currently, there are two RV vaccines available in both the public and private sector: RotaTeq by Merck (Blue Bell, PA), given at three doses (2, 4 and 6 months) and Rotarix by GlaxoSmithKline Biologicals (Rixensart, Belgium), given at two doses (2 and 4 months). The latter vaccine is a live attenuated RV serotype G1P[8], the most globally common serotype, while Rotateq is produced through reassortment of VP7 proteins for serotypes G1-G4 strains and VP4 protein for the P[8] genotype (Gray, 2011).

These two vaccines can prevent up to 34% and 96% of severe diarrhea in the developing world and developed world, respectively (Soares-Weiser *et al.*, 2012). Differences in vaccine efficacy highlights the importance of including emerging region-specific genotypes in future vaccines. However, both vaccines are still more expensive than most other childhood vaccines included in the WHO's Expanded Programme on Immunization (Madsen *et al.*, 2012).

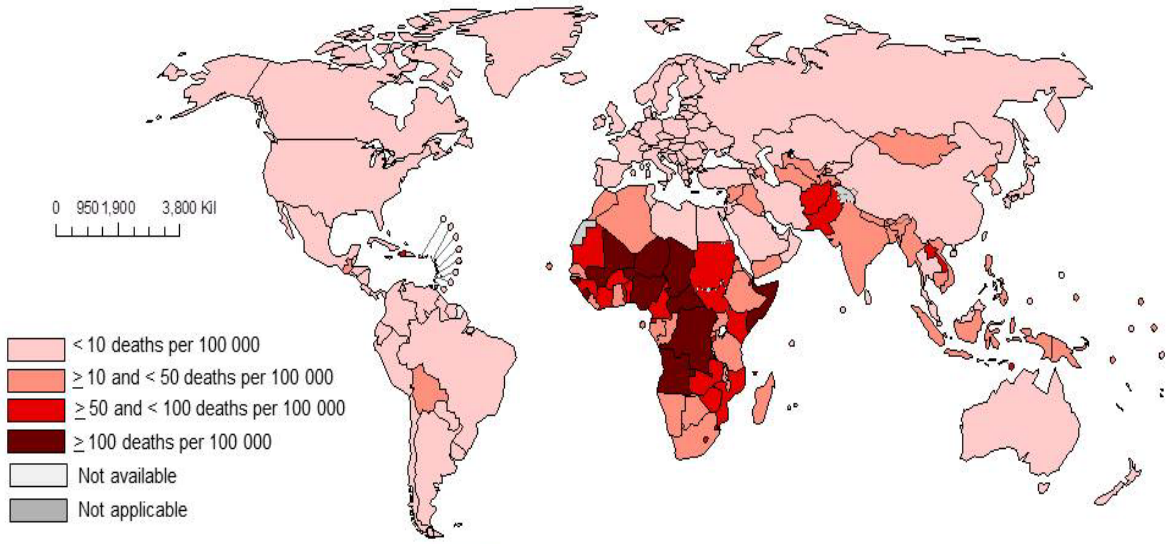

Figure 2.8. World map showing the rotavirus mortality in children younger than five by the end of 2013. (World Health Organization, 2016)

Due to the positive outcome of vaccine trials in South Africa (Madhi *et al.,* 2010) and elsewhere (Gray, 2011), as well as funding opportunities (GAVI-Alliance), the rotavirus vaccine has been included in the South African Expanded Programme on Immunization (EPI). Despite being a highly cost-effective means of securing public health, future vaccination in low-income, high-burden countries still have a large financial impact. Furthermore, current RV vaccines, constituted of live attenuated viruses, still have the risk of adverse effects, although these risks are considered to be very low. Recently, there has been an increase in the detection of RV strains other than the G1-G4, resulting in current RV vaccines having a low efficacy in developing countries (Page *et al.*, 2009). Lower efficacy seen in developing countries might also be the result of poor nutrition, co-infections with other enteric pathogens or co-administration with other live-attenuated oral vaccines such as oral poliovirus vaccines. Also, high concentrations anti-rotavirus antibodies in breast milk might neutralize live attenuated virus vaccines in a breast feeding community (Gray, 2011). Therefore, there is an urgent need to develop new vaccines with the intention to offer it at lower cost than the current approved vaccines.

2.3.1 Structure of rotaviruses

Rotaviruses are unenveloped viruses with an icosahedral triple-layered capsid of 75 nm in diameter (Iturriza-Gomara, Kang and Gray 2004) (Fig. 2.9). They are members of the Reoviridae family and show a noticeable tropism for the intestinal epithelium. The capsid consists of three layers: (1) the outer capsid, composed of 180 VP4 and 780 VP7 proteins; (2) the inner capsid, composed of 780 VP6 protein arranged in 260 trimers; and (3) the inner capsid composed of 120 nucleic acid proteins, VP2. These three layers are surrounding a core consisting of 11 distinct segments of double stranded (ds)RNA, which codes for two structural and six non-structural proteins (Parashar *et al.*, 1998) (Fig. 2.10). Although VP6 and VP7 are present in equal quantities in the RV particles, VP6 provides the biggest molecular mass. Due to its high abundance, the majority of antibodies generated in an infected individual is directed against VP6. The VP6 protein is highly conserved with less than 13% amino acid divergence between any two mammalian group A rotavirus strains (Tang *et al.*, 1997)

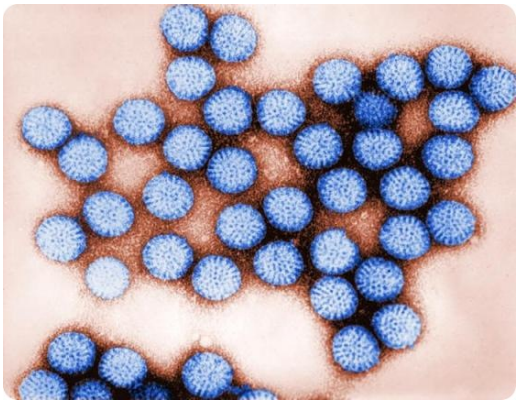

Figure 2.9. Transmission electron micrograph of rotavirus particles (Centre for Disease control and Prevention, 2016).

An intrinsic characteristic of VP6 is the oligomeric formation, in particular trimer structures, as seen from electron microscopic imaging (Gorziglia *et al.*, 1985). Ready and Sabara (1987) reported that the formation of VLPs requires the co-expression of VP2, without which protein VP6 alone can only form spherical or tubular aggregates (Ready and Sabara, 1987). Depending on the pH, ionic strength and divalent cation concentration (Ca^{2+} and Zn^{2+}), VP6 trimers can spontaneously self-assemble into morphological subunits, such as spherical (at pH 4) or tubular multimeric

structures (at pH 5-9) (Lepault *et al.*, 2001; Estes *et al.*, 1987). Assembly of aggregates is also inhibited by high concentrations of divalent cations of 100 mM and more (Ready and Sabara, 1987). Contradictory to this, O'Brian *et al.* (2000) reported that VP6 can also form icosahedral or VLPs when expressed in plants, resembling the morphology of dually expressed VP2 and VP6 VLPs as observed in the insect cell-baculovirus expression system (O'Brian *et al.*, 2000).

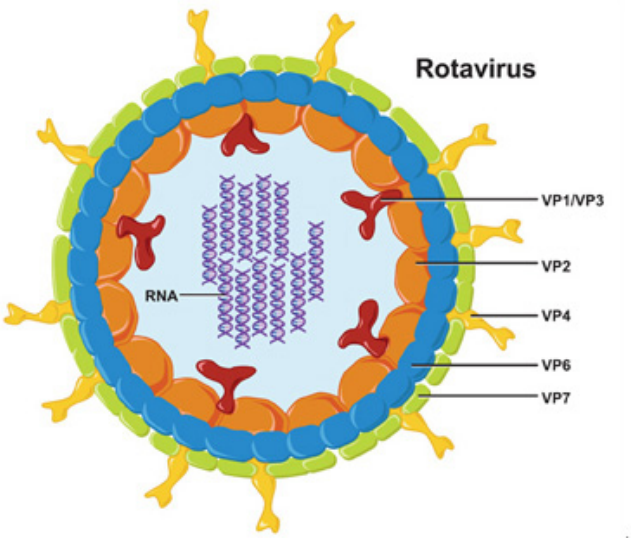

Figure 2.10. Triple-layered structure of rotavirus showing the localization of the 6 different structural proteins (VP1,VP2,VP3,VP4,VP6, and VP7 (UNC, 2014).

2.3.2 VP6 as a vaccine candidate

Structural proteins VP4 and VP7, present in the outer layer of triple-layered particles, are the only proteins able to elicit neutralizing antibodies. Despite this, mouse model studies showed that triple-layer particles provide the same protection as double-layered particles, indicating neutralizing antibodies are not required for protection (Svensson *et al.*, 1987). Irrespective of how many chimeric VP6 protein dosages were administered, protection for up to 1 year against rotavirus shedding was obtained (Choi *et al.*, 1999). Vaccination with VP6 via the oral, intranasal and intrarectal route was almost equally effective, suggesting the mucosal route is the preferred route to deliver antigens to the immune system (Choi *et al.*, 2002; McNeal *et al.*, 2007). VP6 is not only the most immunogenic protein, but due to its low divergence between different strains, the VP6 vaccine from any mammalian rotavirus may provide protection against all.

An alternative to VP6 vaccines is VLPs of different protein compositions. Co-expression of VP2 and VP6 results in the production of double-layered VLPs (2/6-VLP), whereas co-expression of VP2, VP6 and VP7, with or without VP4, results in the production of triple-layered VLPs (2/6/7-VLP or 2/4/6/7-VLP) (Crawford *et al.*, 1994; Labbé *et al.*, 1991; Agnello *et al.* 2006). Studies done in mice confirmed that VLPs composed of VP2 and VP6 provided better protection when administered intrarectally compared to intranasally (Agnello *et al.*, 2006), and intranasally better than orally (O'Neal *et al.*, 1997). VLPs administered mucosally offer a promising, safe, non-replicating vaccine alternative for rotavirus infection. Immunoglobulin A (IgA) levels correlate with protection from severe disease in humans (Jiang *et al.*, 2010; Franco, Angel and Greenber, 2006; Offit, 2001), prevent primary infection in mice as well as resolve chronic infection in mice (Burns *et al.*, 1996; S. Chen *et al.*, 1997). Epitopes present on VP6 are also recognized by both cytotoxic T-lymphocytes as well as T-helper cells, indicating a possible role for VP6 in stimulating a cell-mediated immune response (Franco *et al.*, 1994; Dharakul *et al.*, 1991; Bruce *et al.*, 1994). Despite the safety and stability of VLPs, commercialization of a VLP vaccine requires efficient quantities to minimise costs.

In addition to its application as a vaccine candidate, VP6 has also been applied as a drug delivery tool (Redmond *et al.*, 1991; Zhao *et al.*, 2011). Frenchick *et al.* (1992) demonstrated that peptides coupled to VP6 spheres elicited a greater humoral response than the peptides alone, and that the spheres were not neutralized by anti-rotavirus antibodies (Redmond *et al.*, 1991; Frenchick *et al.*, 1992). Due to their natural tropism and non-replicative properties, RV VLPs are promising candidates to deliver anti-inflammatories to the intestine using the same mechanism used by RV to infect intestinal cells (Cortes-Perez *et al.*, 2010). RV VLPs, especially VP6 multimeric structures, have been expressed in a variety of different heterologous systems. Mammalian cells (Gonzdlez and Affranchino, 1995), insect cell/baculovirus systems (IC-BVS) (Crawford *et al.*, 1994; Bertolotti-Ciarlet *et al.*, 2003), prokaryote *E. coli* (Choi *et al.*, 2004*;* Aijaz and Rao, 1996), the eukaryotic yeast *S. cerevisiae* (Rodríguez-Limas *et al.*, 2011; Zhao *et al.*, 2011), and more recently baculovirus-silkworm (Yao *et al.*, 2012) have been explored. RV VLPs were also positively identified in the yeast *P. pastoris* after intracellular co-expression of VP2, VP4 VP6 and VP7 (Zhang and Lou, 2011). No other attempt to express RV proteins in any other yeast strain have been reported ever since.

2.4 Burn wounds: treating with antimicrobial peptides and collagen

The skin acts as a first line of defence for the human body and a protective barrier against the environment and its invasive microorganisms. Loss of the integrity of large portions of the skin as a result of a burn injury may lead to major disability or even death. It is estimated that more than a million burn injuries occur annually in Africa with almost 20% of hospital admissions that are due to burns. Three quarters of all deaths in patients with severe burns, covering more than 40% of the total body surface area (TBSA), are currently related to sepsis from burn wound infection or other related complications (Baker, Miller and Trunkey, 1979; Bang *et al.*, 2002).

In South Africa, thermal injuries are the most common external cause of death for children under the age of 4 years and the third most common cause under the age of 18 (Rode, Berg and Rogers, 2011). Over 80% of fatal burn injuries are said to occur in informal settlements where two-thirds of the burns are due to dangerous and inappropriate energy sources (UNISA, 2016). Poverty, mass illiteracy, migration to urban areas with the development of slum areas and shanty towns are some of the factors that have led to an increase in burns during the past decade (Oluwasanmi, 1996).

The skin is equipped with an array of immune mediators, capable of recruiting inflammatory cells to enable neutralization and clearance of foreign invaders from the site of infection. Significant thermal injuries induce a state of immunosuppression, such as neutrophil dysfunction (Grogan, 1976), which predisposes burn patients to infectious complications and the emergence of antimicrobial drug-resistant microbes. Large amounts of necrotic tissue and cell debris present in the burn wound provides good culture medium for bacteria. Microbial invasion and subsequent doubling times of 20 minutes or less are often much more rapid than even the innate immune response, including the recruitment of neutrophils (Izadpanah and Gallo, 2005). Invading bacteria are powerful activators of the complement system, which in turn activate the neutrophils. Upon immune activation, neutrophil elastases are released and will hydrolyse proteins, such as those present in bacteria (Hasslen *et al.*, 1992).

2.4.1 Antimicrobial peptides (AMPs)

Since the discovery of antimicrobial peptides (AMPs) during the early 1980's (Steiner *et al.*, 1981), they have been recognized as excellent candidates for novel therapeutic agents and complements conventional antibiotic therapy. Bacterial resistance against conventional antibiotics is becoming an increasing problem worldwide, but microbial resistance against AMPs is quite rare (Peschel and

Sahl, 2006). These small cationic, amphipathic peptides constitute the innate immune defence mechanism and are found in a wide range of eukaryotic organisms, including humans, plants and insects (Reddy, Yedery and Aranha, 2004; Rinaldi, 2002; Wegner, 1990; Zasloff, 2002). Based on their three-dimensional structure, AMPs are broadly classified into 5 groups: (i) α-helical; (ii) cysteine rich; (iii) β-sheet; (iv) AMPs rich in regular amino acids; and (v) AMPs with rare modified amino acids (Reddy, Yedery and Aranha, 2004).

In humans, three different AMP families have been identified as part of the mammalian innate immune response: defensins, cathelicidins and histatins (Bals, 2000). AMPs are present in all human body areas exposed to microbes such as the skin and mucous membranes, and even some blood cell types including neutrophils, eosinophils and platelets. They are always produced as cocktails, either constitutively or induced by inflammation or injury, and act multifunctional (Wiesner and Vilcinskas, 2010). In addition to killing bacteria during wound healing, AMPs also stimulate the complex host repair mechanism during wound healing, comprising of cell proliferation and the migration of growth factors (Gallo *et al.,* 1994). Neutrophil elastases, secreted by immune activated neutrophils, activate cathelicidins present in neutrophils (Scocchi *et al.,* 1992). Cole *et al*. (2001) has reported that the inhibition of neutrophil elastases in porcine skin wounds prevents cathelicidin activation and impair clearance of bacteria during wound healing (Cole *et al.*. 2001).

Cathelicidins are synthesized as pre-pro-peptides and characterized by the conserved amino-terminal sequence of the pro-peptide, and a variable carboxy-terminal domain containing the antimicrobial activities (Zasloff, 2002). During activation, the cathelicidins pro-peptide must be proteolytically cleaved from the C-terminal peptide to unleash the microbicidal activity (Selsted *et al.,* 1992) (Fig. 2.11). Based on amino acid sequences, mature cathelicidin peptides can be organized into three groups (Fig. 2.12): group I – linear; *a*-helical peptides without cysteines; group II – peptides with an even number of cysteines linked by disulphide bridges, eg. protegrin; and group III – peptides with an unusually high proportion of one or two amino acids (Bals and Wilson, 2003). Protegrins, an AMP isolated from porcine leukocytes, are 18 amino acids long, rich in cysteines with two intramolecular disulphide bonds and *β*-sheet structures. Due to its ability to sustain increased concentrations of salt (Steiner *et al.,* 1981), protegrin is used as a broad-spectrum antibiotic against both Gram-positive and Gram-negative bacteria. Another member of the cathelicidin family is indolicidin, a 13-residue peptide isolated from bovine neutrophils with an unusual high number of tryptophans. Because of its small size and potent antimicrobial activity

against Gram-positive and Gram-negative bacteria (Sharp *et al.*, 1988), indolicidin has also been considered as a possible therapeutic agent

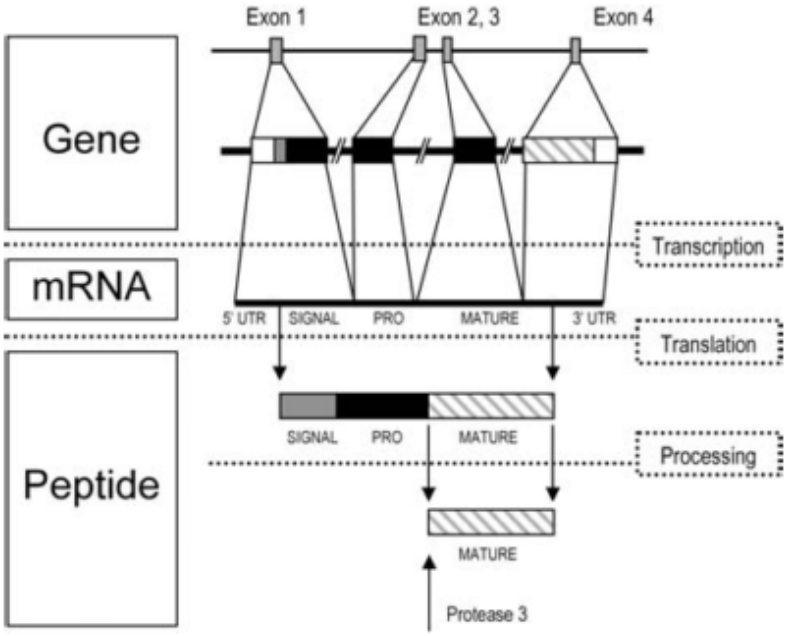

Figure 2.11 Structure of the gene and peptide of LL-37 as a prototypical example for the α-helical human cathelicidin. The C terminus (3' UTR) represents the antimicrobial part of the molecule. The gene is represented schematically with the following individual components: White box, 5' untranslated region (5'UTR); grey box, signal sequence; black box, pro-sequence; shaded box, mature peptide; white box, 3' UTR (Bals and Wilson, 2003).

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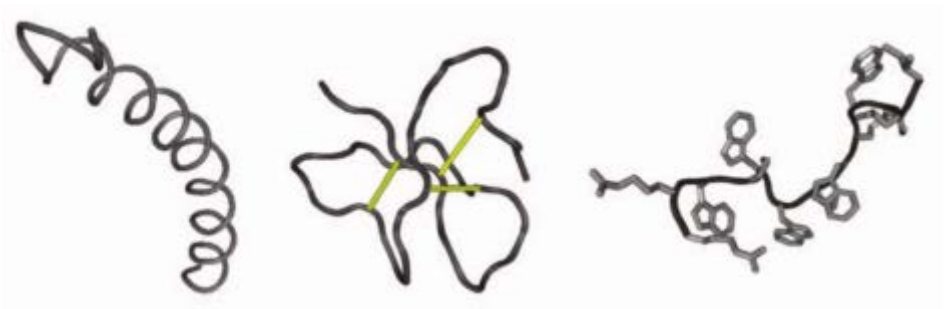

Figure 2.12 Examples of the three basic types of AMPs. a) The α-helical human cathelicidin LL-37, b) the human β-defensin hBD-2 stabilised by three intramolecular disulfide bonds indicated in yellow and c) the tryptophan-rich bovine indolicidin (Wiesner and Vilcinskas, 2010).

2.4.2 Mode of action

AMPs kill cells by disrupting the host's membrane, inhibit the synthesis of cellular proteins, DNA and RNA, or interacting with certain intracellular targets (Bahar and Ren, 2013). In case other intracellular sites being are targeted, an initial contact between the host's membrane and AMP is required for the antimicrobial activities of AMPs (He *et al.*, 1996). Therefore, the main action involves their ability to cause cell membrane damage. This is done through the electrostatic interaction between the positively charged amino acid residue of the AMPs and the negatively charged host's cell membrane. The hydrophobic part of AMPs assist to insert the AMP molecule into the host's cell membrane (Madani *et al.*, 2011). Several models have been proposed for how AMPs can insert into the cellular membrane leading to ion channels, transmembrane pores or extensive membrane rupture (Brogden, 2005). Three of the major models are described below (Fig. 2.13):

The carpet-like model. The peptides orientate themselves parallel to the cell membrane and cover a small area of the membrane. The AMPs form peptide-lipid aggregates and disrupts the lipid bilayer to form pores in the membrane (Chang *et al.*, 2008).

The Barrel-Stave model. The attached peptides insert themselves perpendicularly into the membrane bilayer so that the hydrophobic regions of the peptides align with the lipid core of the membrane and the hydrophilic regions form the interior of the pore (Rosado *et al.*, 2008).

The Toroidal model. This model differs from the Barrel-Stave model in that the attached peptides aggregate and cause the lipid monolayer to bend continuously through the pore. Thereby, the pores are lined with both the inserted AMPs and the lipid head groups (Yang *et al.*, 2001).

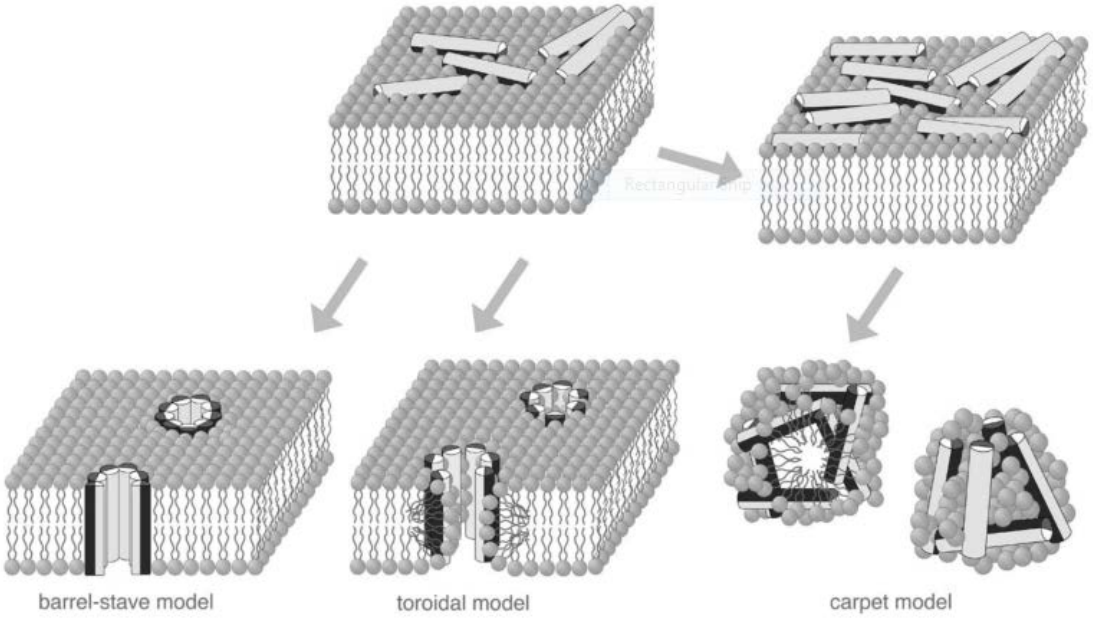

Figure 2.13. Schematic representation of the three major models explaining how cationic amphipathic AMPs insert into lipid bilayers and lead to membrane disruption. Barrel-Stave model. AMP molecules insert themselves into the membrane perpendicularly. Carpet model. Small areas of the membrane are coated with AMP molecules with hydrophobic sides facing inward leaving pores behind in the membrane. The toroidal model resembles the Barrel-stave model, but AMPs are always in contact with phospholipid head groups of the membrane. Hydrophilic and lipophilic parts of the AMPs are indicated in light grey and black respectively (Wiesner and Vilcinskas, 2010).

2.4.3 Collagen

Collagen is the major component of wound connective tissue. An outstanding feature of collagen is their ability to self-assemble (Fessler, 1974). During the wound healing process, fibroblasts continue to generate collagen, which assemble into fibres, becomes cross-linked and are finally organized into bundles (Robson, Burns and Phillips 1994). Collagen consist of three polypeptide chains, known as alpha peptides, each possessing the conformation of a left-handed helix. Each polypeptide chain (procollagen) has a repetitive amino acid sequence of glycine-proline-X- or glycine-X-hydroxyproline where “X” represents any amino acid. Three left-handed helixes are twisted around each other in a right handed triple helix. A prerequisite for triple helix formation is glycine (Gly) in every third position as the side chain of glycine, an H atom, is the only one that can fit into the crowded center of a three-stranded helix. That is also the reason why the rings of the proline (Pro) and hydroxyproline (Hyp) must point outward. This triple helical structure is referred to as tropocollagen and is stabilized by numerous hydrogen bonds between the Hyp and hydroxylysine

(Hyk) residues. Many tropocollagens aggregate together to form a fibril, and many fibrils then form a fibre (Fig. 2.14) (Campbell, 1995).

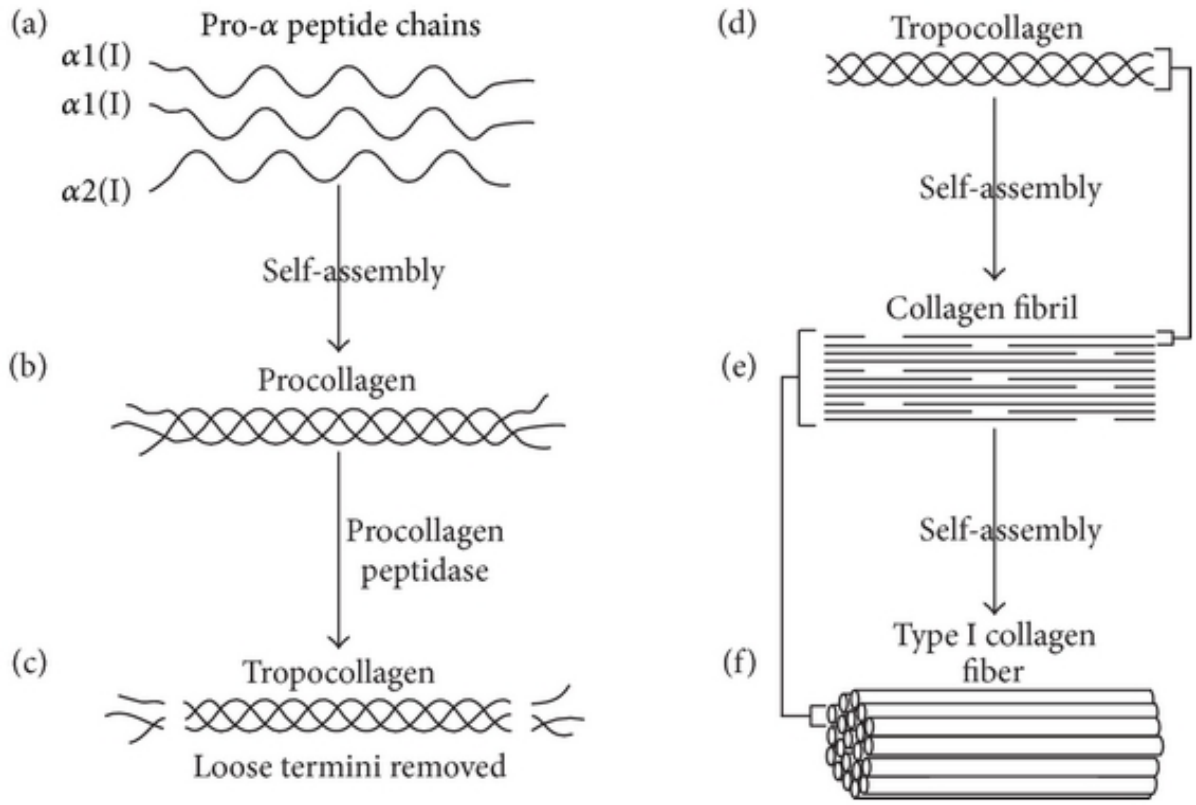

Figure 2.14. Synthesis of collagen. Three polypeptides coil to form tropocollagen. Many tropocollagens then bind together to form a fibril, and many of these then form a fibre *(*Kruger *et al.*, 2013)

Different types of collagen occur in different places throughout the human body. Collagen type 1 is the most abundant collagen and is also synthesized in response to injury. This heterotrimer comprises two identical α1(I) chains and one α2(I) chains (Campbell, 1995) (Fig. 2.15).

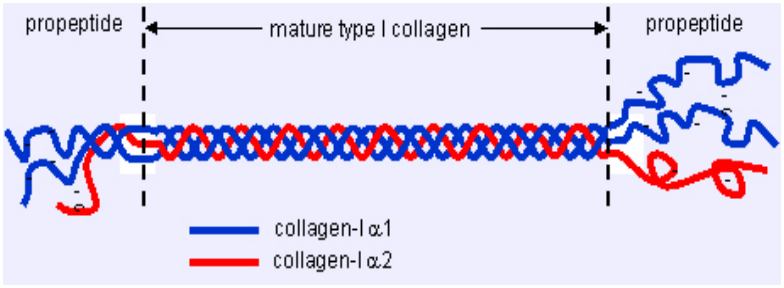

Figure 2.15. Collagen type 1 consisting of 2 identical α1(I) chains and one α2(I) chain (National Center for Biotechnology Information, 2017).

Collagen has been the major ingredient in a wide range of wound dressings and burn wound creams (Ruszczak and Schwartz, 1999). Previously, collagen was isolated from animals, but this process was quite expensive. Recombinant expression systems allowed for the production of large quantities of collagen at a fraction of the cost. Recombinant collagen has the advantage in that it does not elicit an immune response in humans as in the case of animal-derived collagen (Nokelainen *et al.*, 2001). Significant effort has been made to express human collagen in recombinant systems, such as bacterial cells, yeast, insect cells and plants (Ruggiero *et al.*, 2000; Myllyharju, 2009; An, Kaplan and Brodsky, 2014). However, the requirement for post-translational proline hydroxylation has been a major obstacle in achieving high production levels, as the responsible enzyme, prolyl 4-hydroxylase, is either insufficient or lacking. The 4-hydroxyproline residues are essential for the formation of stable triple helices and therefore, the recombinant collagen polypeptide chains produced in these systems will remain in the form of a non-triple-helical, non-functional protein. Therefore, the co-expression of prolyl 4-hydroxylase together with the α1 and α2 chains of type I procollagen ensures the successful expression of human type I collagen in yeast (Nokelainen *et al.*, 2001).

Thermal trauma to the skin requires intensive treatment of the wounds involving expensive wound dressings (Olawoye, Osinupebi and Ayoade, 2013). In most cases of third-degree burns, skin grafting is necessary. Caring for burn injured patients can be a financial burden and may not be available to most people in developing countries. Therefore, there is a great need for alternative remedies THAT can aid in the effective treatment of burn wounds in developing countries.

2.5 Concluding remarks

Both viral proteins as well as collagen represent structural proteins with the ability to aggregate into higher order structures. HPV L1 protein alone and also RV VP6, with the addition of VP2, can self-

assemble into VLPs (Kirnbauer *et al.*, 1992; Gorziglia *et al.*, 1985). The latter resembles the original viral capsid capable of mimicking an immune response similar to that against native viruses (Roy *et al*., 2008). Both HPV L1 and RV VP6 are highly immunogenic due to their abundant presence in the original VLP structure (Kirnbauer *et al.*, 1993; Tang *et al.*, 1997), which explainS their inclusion in current HPV and RV vaccines. High-yield expression systems are needed for the production of these protein subunits and methylotrophic yeasts qualify as highly efficient producers. Methylotrophic yeasts are established protein expression hosts that have rapid growth rates, use inexpenisve media and well-established fermentation technology. The expression of HPV16 L1 monomers in methylotrophic yeast systems has been limited to *P. pastoris*, mainly in shake-flask cultures. However, in our study the production was expanded to a human codon optimized chimeric HPV16 L1/L2 protein, called SAF, in *H. polymorha*. As discussed, *H. polymorpha* is more thermo-tolerant, has faster high-yield fermentation and is ideal for the production of mammalian proteins such as collagen (Hollenberg and Gellissen, 1997).

Although triple-layered rotavirus-like particles, consisting of VP2, VP6 and VP7 proteins, have succesfully been expressed in *S. cerevisiae*, the yields were very low. Expression of RV VP6 was never explored in any other yeast, including the methylotrophic yeasts *P. pastoris* and *H. polymorpha.*

Despite several attemps to express HPV L1 or RV VP6 proteins in methylotrophic yeasts, none of them yielded high enough concentrations to be cost-effective. These low yields prompted us to scale-up expression of these self-assembling proteins in the controlled environment of bioreactors in order to increase production levels. Scaled-up fermentations pose additional challenges as the yeasts were cultured on glycerol to increase biomass and protein production was induced using methanol. Depending on which *P. pastoris* strain was used, methanol feeding must be adjusted during feb-batch cultures to maintain optimum levels of methanol and prevent the toxic accumulation thereof. Very few studies address the impact of different feeding strategies on methylotrophic yeasts as well as the impact of different yeast strains on the production of HPV L1 and RV VP6 monomers.

High concentrations of these viral subunit proteins result in the self-assembly into VLPs. One of the objectives was to semi-purify the HPV16 SAF proteins and visualize any potential secondary structures using microscopy. SAF was previously expressed in a baculovirus recombinant

system. Despite the low expression levels, SAF proteins mostly assembled into capsomer and the occasional VLPs, 50 nm in size, have been seen (McGrath *et al.*, 2013).

Similarly, the three polypeptide strands (alpha peptides) of collagen are intertwined together into a right-handed triple helix (Hollenberg and Gellissen, 1997). The latter self-assembles to form a fibril that ultimately assembles in order to form a fibre. Various studies reported on the successful expression of collagen in *P. pastoris* before, but we have adapted the expression of a collagen α1(I) chain fused with an antimicrobial peptide, which can aid in burn wound dressings. This novel approach has never been explored in any expression system before.

In the next chapters we report on the investigation of the methylotrophic yeasts *P. pastoris* and *H. polymorpha* for the expression of viral proteins, HPV SAF and RV VP6, as well as a novel collagen-antimicrobial fusion peptide.

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Chapter 3

Expression of unique chimeric human papilloma virus type 16 (HPV-16) L1/L2 proteins in *Pichia pastoris* and *Hansenula polymorpha*

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Abstract

Cervical cancer is ranked the fourth most common cancer in women worldwide. Despite two commercially available prophylactic vaccines, it is unaffordable for most women in developing countries. We compared the optimized expression of monomers of the unique HPV type 16 L1/L2 chimeric protein (SAF) in two yeast strains of *Pichia pastoris*, KM71 (Mut^S) and GS115 (Mut^+), with *Hansenula polymorpha* NCYC 495, to determine the preferred host in bioreactors. SAF was uniquely created by replacing the h4 helix of the HPV-16 capsid L1 protein with a L2 peptide. Two different methanol feeding strategies in fed-batch cultures of *P. pastoris* Mut^S were evaluated: a predetermined feed rate versus feeding based on the oxygen consumption by maintaining constant dissolved oxygen levels (DO stat). All cultures showed a significant increase in biomass when methanol was fed using the DO stat method. In *P. pastoris,* the SAF concentrations were higher in the Mut^S strains than in the Mut^+ strains. *Hansenula polymorpha* produced the highest level of SAF at 132.10 $mg.L^{-1}$ culture, while *P. pastoris* Mut^S only produced 23.61 $mg.L^{-1}$. The *H. polymorpha* strain showed greater potential for the expression of HPV-16 L1/L2 chimeric proteins despite the track record of *P. pastoris* as a high-level producer of heterologous proteins.

Introduction

Cervical cancer ranks as the fourth most frequent cancer among women in the World with an estimated 527 000 new cases diagnosed and 265 000 cervical cancer-related deaths in 2012 (Bruni *et al.*, 2016b). In South Africa, cervical cancer is the second leading cause of female cancer and the most prevalent cancer in women between the ages of 15-44 years (Bruni *et al.*, 2016a). Vaccination against HPV infection has proven to be most advantageous in the prevention of cervical cancer. Today, vaccinations performed using any one of the two commercial subunit-based HPV vaccines, Cervarix® or Gardasil®, provide almost 100% efficacy (White, 2014). Prior to 2014, the availability and subsequent dissemination of HPV vaccines in South Africa was constrained primarily due to the high costs associated with vaccination regimes in developing countries. HPV vaccination in the South Africa costs between USD 38-57 per dose, and with three doses needed to be effective, it is unaffordable for most women and place an enormous burden on developing countries' economy (Minister Aaron Motsoaledi, 2015). In 2014, the South African National Department of Health implemented a school-based only HPV vaccination program (two-dose vaccination with Cervarix®) for girls 9 years and older in public schools (Richter *et al.*, 2014; Richter, 2015). Despite these limited free vaccination programs, there is still a need for cheaper alternative vaccines. In addition, their effectiveness could also be hampered by the regional HPV type-specific prevalence. Human papilloma virus types 16 and 18 account for about 70% of all cervical cancers worldwide (Muñoz *et al.*, 2004; Schiffman *et al.*, 2007), whereas types 31, 33, 35, 45, 52 and 58 account for an additional 20% (Clifford *et al.*, 2006). Other HPV types are also emerging in Africa with type 35 becoming as prevalent as type 16 (Clifford *et al.*, 2005; Allan *et al.*, 2008). There is, therefore, an urgent need to develop local production capacity in the developing world for HPV vaccines targeted at future emerging types.

Current HPV vaccines consist of VLPs that are morphologically indistinguishable from native virions. They are not infectious as they do not contain any genetic material and therefore serve as excellent vaccine candidates (Chackerian, 2007). Recombinant expression of the HPV type 16 major capsid protein, L1, results in the spontaneous self-assembly of dimers, to which free L1 monomers subsequently associate until a closed icosahedral VLP is formed that resembles native virions (Kirnbauer *et al.*, 1992; Ding *et al.*, 2010). Although the minor capsid protein, L2, is a prerequisite for infectious virions, it is not required for VLP formation (Fahey *et al.*, 2009). HPV L1 VLPs have been produced in various expression systems, such as bacteria, e.g. *Escherichia coli*

(Chen *et al.*, 2001), yeast such as *Saccharomyces cerevisiae* (Woo *et al.*, 2008), *Pichia pastoris* (Bazan *et al.*, 2009; Coimbra *et al.*, 2011; Jiang *et al.*, 2011; Kotzé *et al.*, 2011; Rao *et al.*, 2011; Smith *et al.*, 2012; Zhao *et al.*, 2015), and *Hansenula polymorpha* (Li *et al.*, 2009), baculovirus-infected insect cells (Le Cann *et al.*, 1994), transgenic plants (Biemelt *et al.*, 2003), and mammalian cells (McLean *et al.*, 1990). VLPs consisting of both L1 and L2 have been produced in human epithelial cells (Cianciarullo *et al.*, 2010) and similarly bovine papillomavirus type 4 (BPV-4) VLPs in insect cells (Kirnbauer *et al*., 1996). Furthermore, VLPs of various chimeric L1/L2 permutations, such as the chimeric L1/L2 SAF protein, were expressed in insect cells (Varsani *et al.*, 2003; McGrath *et al.*, 2013) and more recently in the yeasts *P. pastoris* and *H. polymorpha* (Smith *et al.*, 2012). The chimeric SAF construct (~55 kDa) was created by replacing the h4 helix of the L1 protein with a 13-amino acid peptide from the L2 protein, which is a common-neutralizing epitope for HPV-6 and HPV-16 (Kawana *et al.*, 1998). Neutralizing antibodies, predominantly targeted against L1, play a preventative role in papilloma viral infections. Breitburd *et al.* (1995) determined that high levels of prophylactic antibodies are primarily elicited by L1, whereas L2 only evokes a low level of prophylaxis, yet the antibodies are more cross-neutralizing (Roden *et al.*, 2000; Kawana *et al.*, 2003; Rubio *et al.*, 2009; Schiller *et al.*, 2014). Replacing the h4 helix of L1 with the L2 peptide in the SAF protein resulted in improved immunogenicity and cross-neutralization during mice immunization following successful capsomer formation in insect cells (Varsani *et al.*, 2003; Rubio *et al.*, 2009; McGrath *et al.*, 2013).

Although HPV structural proteins have been readily expressed in many platforms, methylotrophic yeasts are regarded as prime candidates for the production of pharmaceutical-based products. These yeasts are best known for having strong methanol-inducible promoters of their alcohol oxidase genes (*AOX1* and *AOX2*) in *P. pastoris* (Cregg *et al.*, 1989) and methanol oxidase gene (*MOX*) in *H. polymorpha* (Ledeboer *et al.*, 1985). Strong promoters and well-established fermentation protocols using inexpensive defined media allow for the scale-up expression of recombinant proteins in these yeasts from shake-flasks to bioreactors. Transcription levels of the *AOX1* promoter gene is 3-5 times greater and the oxidase protein can constitute up to 30% of the total soluble proteins when these yeasts are cultured at growth-limiting rates in bioreactors (Couderc *et al.*, 1980; Higgins et *al.*, 1998). In addition, methylotrophic yeasts secrete relatively low levels of endogenous proteins into the medium, resulting in less cumbersome purification of target proteins (Cereghino *et al.*, 2000). Unlike bacterial expression systems, methylotrophic yeast has the ability to perform many of the post-translational modifications usually performed in higher eukaryotes, e.g.

correct folding, disulfide bond formation, O- and N-linked glycosylation, processing of signal sequences and certain types of lipid addition (Cereghino *et al.*, 2000; Cereghino *et al.*, 2002; Macauley-Patrick *et al.*, 2005). VLP formation requires trimerization of the L1 proteins by disulfide bridges between conserved cysteins in the protein (Sapp *et al.*, 1998).

In this study, we combined the unique HPV type 16 L1/L2 chimeric SAF protein with high expression in methylotrophic yeasts. We evaluated the intracellular production of SAF monomers in both *P. pastoris* and *H. polymorpha*. First, we optimized the feeding strategy in *P. pastoris*_KM71 (Mut^S) fed-batch cultures by supplying methanol either at a predetermined exponential rate or one based on the oxygen consumption of the microorganism. The ideal feeding strategy was then applied in 10 L bioreactors to compare the *P. pastoris*_GS115 (*(*Mut^+) strain with the *P. pastoris*_KM71 (Mut^S) strain. The preferred *P. pastoris* strain was then compared to *H. polymorpha* for SAF production. This study is one of a very few studies addressing different feeding strategies of methylotrophic yeasts, as well as comparing different yeast strains for the production of HPV16 L1 viral proteins. This study is also the first to compare the production of the unique HPV16 L1/L2 SAF chimeric protein in the yeasts *P. pastoris* and *H. polymorpha* in bioreactors, with higher production levels than reported previously in both systems.

Materials and Methods

Construction of plasmids, strains and yeast transformation

The chimeric L1/L2 sequence, termed ChiΔF-L2, was kindly provided by Ed Rybicki (Molecular and Cell Biology Department, University of Cape Town). The h4 helix (corresponding to amino acid position 414-426) of the HPV16 L1 protein was replaced with amino acid 108-120 of the HPV16 L2 protein (McGrath *et al.*, 2013; Varsani *et al.*, 2003). The resulting ChiΔF-L2 gene (called *SAF*) sequence was codon-optimized for both yeasts and commercially synthesized by GeneArt (Invitrogen, Regensburg, Germany) as part of a plasmid, pGA15-ChiΔH-L2. Restriction enzymes *Bam*HI and *Hind*III were used to directional subclone the ChiΔF-L2 gene into the *H. polymorpha* plasmid pHIPX4–HNBESX (*leu2*, kan^R) (Gietl *et al.*, 1994), and *Eco*RI and *Xho*I into the *P. pastoris* plasmid pBLHIS–IX *(his4*, amp^R*)* (Cereghino *et al.*, 2001). *Escherichia coli* DH5α cells were used as the host strain for propagation of recombinant DNA. Following transformation of competent *E. coli* cells, colonies were maintained on antibiotic-specific LB agar plates. Putative recombinant colonies were selected by PCR amplification using primers pHIPX4-F and pHIPX4-R (Smith *et al.*, 2012) specific for plasmid pHIPX4–HNBESX, and primers 5'AOX and 3'AOX

(Invitrogen, California, USA) for plasmid pBLHIS–IX. Isolation of an amplicon of 1626 bp using agarose gel electrophoresis followed by nucleotide sequencing confirmed the presence of the *SAF* gene. Restriction enzyme digests, using the same enzymes as for cloning, were used to verify the presence of the *SAF* expression cassettes in the integration plasmids. Transformation of linearized plasmids into *H. polymorpha* NCYC 495 (Jan Kiel, University of Groningen, The Netherlands) and *P. pastoris*_KM71 (Invitrogen, California, USA) were carried out by electroporation as previously described by Smith *et al.* (2012). Putative recombinant yeast colonies were screened using yeast colony PCR and appropriate primers.

Bioreactor fermentations

Pre-cultures were prepared by inoculating a sub-cultured recombinant colony, previously confirmed by yeast colony PCR, from YPD (1% [$g.L^{-1}$] yeast extract, 2% [$g.L^{-1}$] tryptone, 2% [$g.L^{-1}$] glucose) agar plates into buffered glycerol complex medium (BMGY; 10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 100 mM KH_2PO_4, pH 6.0, 13.4 $g.L^{-1}$ YNB, 4×10^{-4} $g.L^{-1}$ biotin, 10 $g.L^{-1}$ glycerol) in baffled shake flasks. Following overnight incubation at 30°C for *P. pastoris* and 37°C for *H. polymorpha* at 250 rpm, cultures were diluted to an optical density (A_{600}) of 0.1 in fresh BMGY medium. Subsequently, these seed-cultures were again grown overnight at the same conditions to an A_{600} of between 2 and 6, before diluting 10 times into fresh BMGY medium in bioreactors (New Brunswick Scientific, Edison, NJ). Initially 3 L bioreactors were used, but when different feeding strategies were accessed, fermentation volumes were increased to 10 L. Once methanol feeding was adjusted to accommodate the addition of micro volumes, the 1.3 L bioreactors were used to compare *P. pastoris* versus *H. polymorpha* cultures.

Culture conditions for both yeasts were set up according the Invitrogen *Pichia* Fermentation protocol (California, USA), consisting of fermentation basalt salt medium, supplemented with 4% glycerol and 4.35 ml PTM_1 trace salts.L^{-1} basalt salt medium. Culture conditions were conducted as follows: 30°C for *P. pastoris* and 37°C for *H. polymorpha*, pH 5.0 using 28% ammonium hydroxide (NH_4OH) as a hydroxide donor and a nitrogen source, aeration rate of 1.0 volume of oxygen per volume of fermentation culture per minute (vvm) and dissolved oxygen (DO) of 30%. According to Li *et al.* (2007) most *Pichia pastoris'* fermentations use a DO of 30-35%, but different proteins need different optimal levels. Since Invitrogen's *Pichia* Fermentation Process Guidelines (Invitrogen Life Technologies, 2002) suggest a DO > 20%, we decided to use 30% as a constant to measure different culture variables such as different feeding strategies and different yeast strains. Dissolved oxygen

was sensed by a Mettler Toledo DO electrode (Ohio, USA) and controlled by a programmable cascade whereby the agitation range (200-1000 rpm) and aeration rate (1 vvm) were maintained by the proportional (P) and integral (I) controller. Upon glycerol depletion in the BMGY medium at the end of the glycerol batch phase (24 h post-inoculation), as indicated by a spike in the DO levels (DO > 30%), a 50% glycerol feed, supplemented with 12 ml PTM_1 trace salts.L^{-1} was initiated. Glycerol feeding was carried out in a fed-batch mode based on the DO stat method, also referred to as 'demand-feed', where glycerol addition was dictated by the DO concentration. As the yeast metabolises actively, glycerol becomes depleted and the DO concentration starts to increase. Once the DO > 30%, the glycerol feed is activated and the presence of glycerol in the medium lowers the DO concentration as the glycerol is metabolised. Conversely, when the DO < 30%, the glycerol feed is stopped. DO of 30% is maintained first by increased or decreased agitation, depending on the whether the DO is below or above 30%. If agitation has reached its maximum and cannot increase the DO further, aeration is supplemented with pure O_2. Subsequently, the DO concentration increases and once it reaches > 30%, the glycerol feed is switched on again. Wet cell weights (WCW) of 180-220 g.L^{-1} indicated the end of the glycerol fed-batch phase. The glycerol feed was terminated and replaced with 100% methanol (48 h post-inoculation), supplemented with 12 ml PTM_1 trace salts.L^{-1} of methanol. The same DO stat method was followed for six consecutive days or until the WCW started to decrease (see Appendix A).

Alternatively, methanol feeding was administered using the predetermined controlled feed rate, as indicated by the *Pichia* Fermentation protocol (Invitrogen, California, USA): 1 mL.h^{-1}.L^{-1} for the first 2 h, then increased gradually to 3 ml.h^{-1}.L^{-1}. This feeding strategy was compared with the DO stat method.

During 10 L fermentations glycerol and methanol consumption was monitored by placing the reservoirs on a balance and changes in mass were recorded. However, during the 1.3 L and 3 L fermentations, volumes added to the bioreactors were so little that reservoirs were only weighed at the end of the fermentation to gain cumulative volumes added. For each fermentation variable, such as different *P. pastoris* strains, different methanol feeding strategies as well as *P. pastoris* versus *H. polymorpha*, comparative fermentations were carried out in triplicate.

Analytical methods

Cell growth

Five milliliter samples of the cultures were collected at the end of the glycerol batch (24 hours post-inoculation) and glycerol fed-batch phase (48 hours post-inoculation), as well as every day during the methanol fed-batch phase (72, 96, 120, 144, 168 and 192 hours post-inoculation). Culture samples were monitored for cell growth using A_{600}, WCW and dry cell weights (DCW). Three 1 ml culture aliquots were centrifuged at 14 000 rpm for 5 min, and used to determine the WCW. After drying the cell pellets overnight at 60°C dry heat, the DCW was determined. In addition, cumulative culture volumes, glycerol volumes, methanol volumes, and NH_4OH were measured at each time point. The remainder of the collected samples were frozen as cell pellets at -80°C for the quantification of the expressed protein.

Crude cell extracts

Crude extracts were prepared from 60 mg of both *P. pastoris* and *H. polymorpha* cells, as previously described (Smith *et al.*, 2012). Cell pellets were resuspended in 1.2 M sorbitol, 100 mM EDTA and 14 mM β-mercaptoethanol, followed by centrifugation at 13 000 rpm for 5 minutes. Following centrifugation, the supernatant was discarded and the cell pellets resuspended in 400 µl breaking buffer containing 50 mM NaH_2PO_4, pH 7.4, 1 mM EDTA, 5% v/v glycerol, Protease Inhibitor Cocktail (Sigma-Aldrich) at a concentration of 1 µl protease inhibitor cocktail for every 20 mg wet weight cells, and equal volume of glass beads measured by displacement. Cells were subjected to 20 cycles of vigorous vortexing for 30 sec followed by incubation on ice for 30 sec. After the final cycle, 400 µl 2 × SDS PAGE loading dye (125 mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 0.01% bromophenol blue; 5 mM 2-mercaptoethanol) was added before incubating at 95°C for 5 min.

Protein quantification

Serial dilutions of the crude cell extracts were loaded onto a 10% polyacrylamide gel. Purified HPV16 L1 protein (50 kDa) in the form of virus-like particles (Deschuyteneer *et al.*, 2010) was used as a standard for protein concentration determination during densitometry. Appropriate dilutions of the standard were loaded in parallel on the same gel to serve as a standard curve. Proteins were separated after which the proteins were transferred onto nitrocellulose membranes (BioTrace™ NT, PALL) using the Semi Phor blotting apparatus (Hoefer Scientific Instruments) at 43 mA per minigel for 75 minutes (Khyse-Andersen, 1984). According to Smith *et al*. (2012), membranes were blocked overnight in 5% $g.L^{-1}$ skimmed milk powder (Sigma) in 1 × TBST (10mM Tris, pH 8.0, 125 mM NaCl, 0.1% v/v Tween 20). Subsequently, the membranes were incubated for 2 h in blocking

solution containing 15 ng.L^{-1} anti-HPV-16 L1 monoclonal antibody CAMVIR-1 (Santa Cruz Biotechnology, Dallas, Texas). After washing with 1×TBST, the membranes were incubated in blocking solution with 80 ng.mL^{-1} alkaline phosphatase conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Dallas, Texas). Detection was carried out using NBT/BCIP detection reagents (Fermentas, ThermoFisher, Massachusetts, USA). The area and intensity of bands were quantified using the software ImageJ (Abramoff *et al.*, 2004) using the methodology outlined by Miller (2007). To verify the mean protein quantity, SDS-PAGE followed by Western Blot analysis was performed in triplicate for each time point.

Gas chromatography-flame ionization detector (GC-FID)

Fifty-milliliter culture fluid samples were taken daily post-induction and subjected to centrifugation to separate the supernatant fluid (SNF) from the yeast cells. One milliliter diethyl ether was used to extract 5 µl SNF with internal standard, 4-methyl-2-pentanol (100 µl of 0.5 mg.mL^{-1} soaking solution) in solution (12% alcohol spirit and 2.5 g.L^{-1} tartaric acid, pH 3.5), by placing the ether/SNF mixture in an ultrasonic bath for 5 minutes. The SNF/ether mixture was then centrifuged at 4000 rpm for 3 min, and the ether layer was removed. Any residual traces of water were removed by adding $NaSO_4$. This diethyl ether extracts of culture samples were analysed for their methanol content using the Model 8610C gas chromatograph (SRI Instruments, USA), equipped with a 60 m DB-FFAP column (id 0.32 mm, 0.5 µm f.t), on-column injector and FID set to 200°C; H_2 flow of 30 mL.min^{-1} and air flow of 350 mL.min^{-1}. The GC was also equipped with an internal air compressor and hydrogen generator. Nitrogen was used as carrier gas with pressure control and a flow set to 30 mL.min-1. Sample runs were performed through manual injections of 3 µL sample using a 5 µl Hamilton syringe. The syringe was thoroughly washed with ethyl acetate between injections to avoid cross-contamination. Each injection was repeated three times. A temperature gradient was implemented after injection with oven, column and injector temperatures all initially set at 33°C, held for 17 min and then elevated to 240°C, and held for a final 5 min. Peak Simple software version 2.8 (SRI Instruments, USA) was used for data analysis and GC control.

Results

Investigating different feeding strategies for *P. pastoris* Mut^S strains

Initially, the different methanol feeding strategies for the expression of HPV-16 L1/L2 chimeric protein (ChiΔF-L2, termed SAF) in *P. pastoris* was investigated in 3 L bioreactors using the KM71

(Mut^S) strain. The glycerol fed-batch fermentations were carried out using the DO stat method to ensure aerobic conditions for biomass generation, followed by inducing heterologous gene expression by replacing glycerol with methanol. Sixty milligrams of yeast cells were collected at 24-hour intervals starting 72 h post-inoculation, and analyzed by Western Blot and densitometry for SAF production. Both the SAF concentration and biomass in these *P. pastoris* cultures (DCW) decreased over time (Fig. 3.1A). Cumulative glycerol and methanol volumes, added to the bioreactor, were only measured at the end of each fermentation (Table 3.1). There was an increase in bioreactor volume at the end of all three fermentations. Residual methanol in the culture fluid was only measured on the last day of induction (192 hurs post-inoculation) and found to be less than 0.045 g methanol per liter culture.

Table 3.1 Changes in 3 L bioreactor volumes after glycerol and methanol addition in *P. pastoris*_KM71 (Mut^S) cultures using a predetermined methanol feeding rate.

	Reactor Starting volume (mL)	Inoculum added (mL)	Gly added (mL)	MeOH added (mL)	Cumulative samples taken (ml)	Reactor End volume (mL)
Fermentation 1	1200	40	110	405	60	1650
Fermentation 2	1200	40	115	410	60	1690
Fermentation 3	1200	40	150	400	60	1710

The same *P. pastoris* strain (KM71, Mut^S) was methanol-fed using the dissolved oxygen (DO) stat method where the yeast culture's rate of oxygen consumption dictated the rate at which the carbon source was administered. This resulted in a 2- to 3-fold increase ($p<0.005$) in *P. pastoris* biomass during the course of methanol induction using the DO stat method, compared to a predetermined feed rate (average range: 91.12 - 108.94 g DCW.L^{-1} versus 57.51 - 36.54 g DCW.L^{-1}) (Fig. 3.1B). Once the biomass in all the cultures reached a WCW of 180-220 g.L^{-1}, the methanol feed was initiated; hence why DCW of all cultures was similar up until 48 hours post-inoculation. Due to the impact of different methanol feeding, the biomass started to divert from 48 hours onwards post-inoculation.

Despite elevated levels of biomass compared to that observed during predetermined feeding, the biomass and SAF productivity did not increase significantly over time (Fig. 3.1C). Since *P.*

pastoris Mut^S strains, such as the KM71 strain, metabolize methanol at a much slower rate than the Mut$^+$ strains, the constant level of biomass of *P. pastoris*_KM71 might be due to methanol toxicity. Adding methanol to the bioreactor occurred through an inlet in the roof of the vessel. In case of smaller culture volumes, the demand for methanol is lower and controlling the addition of micro volumes methanol is mechanically more challenging than in larger bioreactors. The methanol feeding strategy using the DO stat method could have resulted in overfeeding and subsequently into the accumulation of methanol. It is known that methanol concentrations higher than 3.65 g.L^{-1} in the culture broth can inhibit cell growth (Zhang *et al.*, 2000b).

If residual methanol levels were increasing, larger fermenter volumes, such as 10 L, could accommodate the dilution of excess methanol more efficiently. This urged us to investigate the expression of SAF in larger culture volumes such as 10 L bioreactors.

Effect of the DO stat control strategy on biomass accumulation, methanol consumption and specific SAF production values of *P. pastoris*_GS115 (Mut$^+$) versus *P. pastoris*_KM71 (Mut^S) strains in larger fermentation volumes

The *P. pastoris*_GS115 (Mut$^+$) strain containing the chimeric L1/L2 gene (SAF) was expressed in 10 L cultures under similar controlled fermentation conditions than the *P. pastoris*_KM71 SAF strain. The DO stat method was used to administered methanol in both cultures. As expected, *P. pastoris*_GS115 (Mut$^+$) had a faster growth rate than *P. pastoris*_KM71 (Mut^S). This was evident from the difference in biomass values from the start of culturing onwards (24 hour onwards post-inoculation) (Fig. 3.2A). Mut^S and Mut$^+$ reached maximum biomass densities of 246.37 and 334.46 g. L^{-1} wet cell weight, respectively (75.50 g.L^{-1} and 118.40 g.L^{-1} dry cell weight). The methanol feeding rate in the DO stat cultures was 10% higher in *P. pastoris*_GS115 (Mut$^+$) than in *P. pastoris*_KM71 (Mut^S) (Fig. 3.2B) resulting in 1.84 L cumulative methanol addition for GS115 compared to 1.81 L for KM71. After initiating the methanol feed, the methanol consumption increased in both cultures (Fig. 3.2C). Gas chromatography was performed to determine if residual methanol was present in the cultures at toxic concentrations. Samples taken during methanol induction contained less than 0.045 g methanol per liter culture for both strains (data not shown).

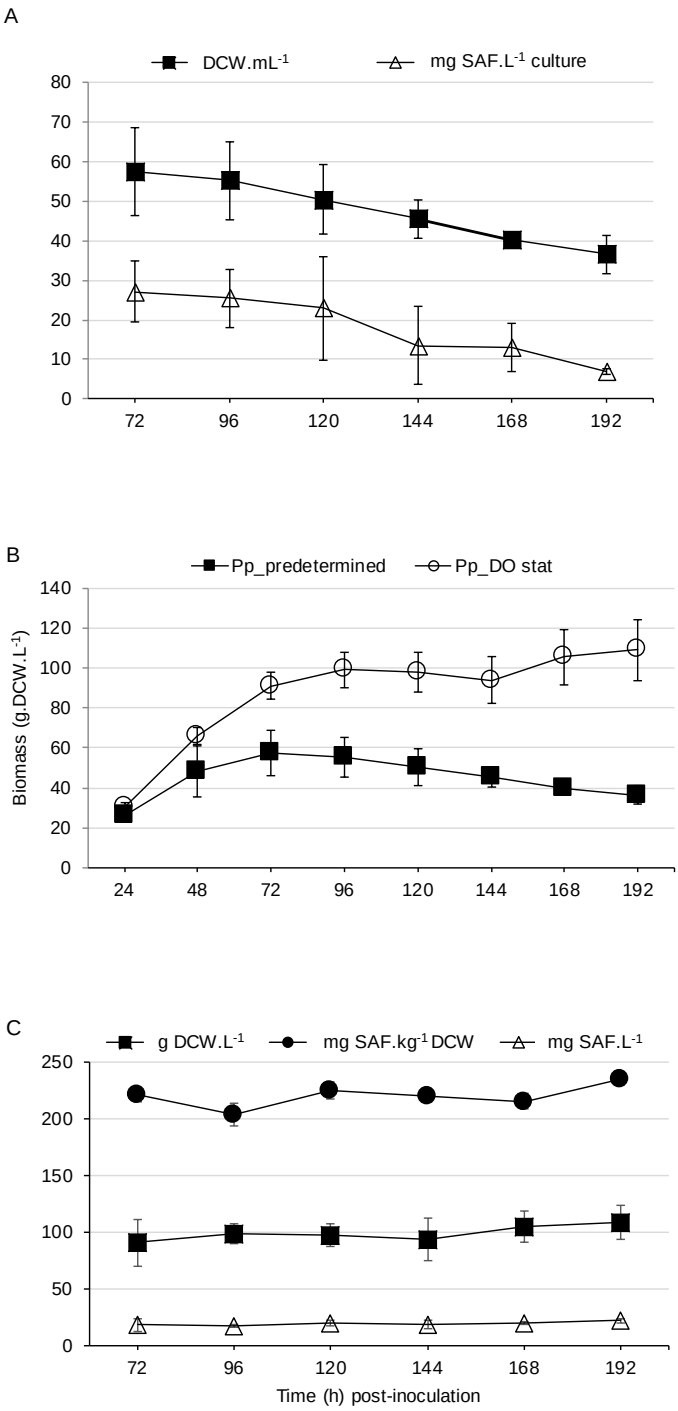

Fig. 3.1 *P. pastoris*_KM71 (Mut^S) cultures induced at 72 h (3 days) post-inoculation using methanol. (A) Biomass (g *P. pastoris* $DCW.L^{-1}$) and SAF concentration (mg $SAF.L^{-1}$ *P. pastoris*) during a predetermined feed rate using the Invitrogen's protocol. (B) Comparing biomass levels in response to different methanol feeding strategies: a predetermined versus the DO stat feed rate which is related to the metabolic state of the culture. Increased biomass during DO stat feeding was significantly higher than during the predetermined feeding rate ($p<0.05$). (C) SAF concentration (mg $SAF.L^{-1}$ *P. pastoris*), yield $_{(P/X)}$ (mg $SAF.g^{-1}$ *P. pastoris* DCW) and biomass concentration (g *P. pastoris* $DCW.L^{-1}$) during the DO stat feed rate for methanol induction in 3 L bioreactors. Each culturing was performed in triplicate to ensure reproducibility. Error bars represent standard deviations from the mean of triplicate samples.

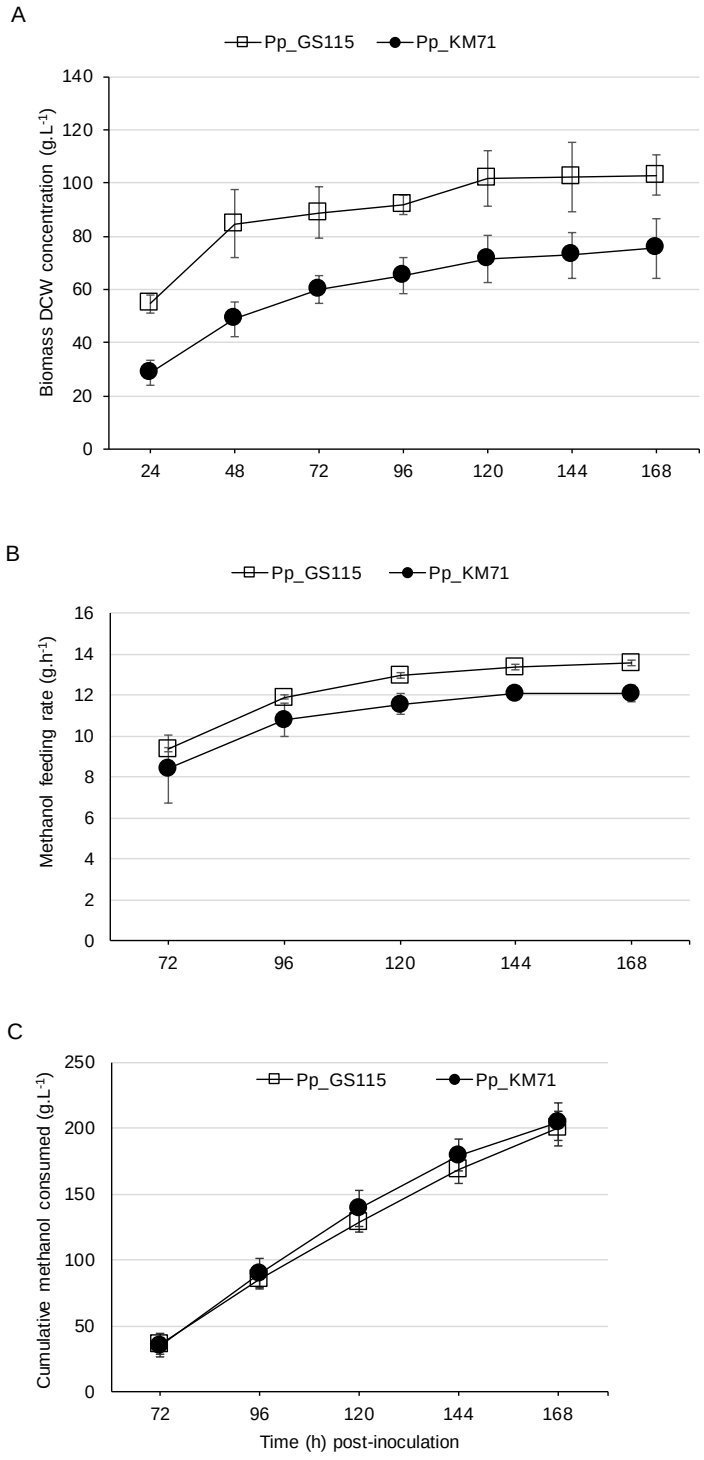

Fig. 3.2 *P. pastoris*_GS115 (Mut^+) versus *P. pastoris*_KM71 (Mut^S) cultures during the methanol induction phase, 72 h (3 days) post-inoculation, in 10 L reactor vessels. (A) Biomass dry cell weight concentrations. (B) Methanol feeding rates. (C) Cumulative methanol concentration consumed. Each culturing was performed in triplicate to ensure reproducibility. Error bars represent standard deviations from the mean of triplicate samples.

The SAF concentration, biomass concentration and SAF yield ($Y_{P/X}$) per biomass were significantly higher in *P. pastoris*_KM71 than in *P. pastoris*_GS115 ($p<0.05$) (Table 3.2). However, the SAF concentration was 2-fold higher in the 10 L cultures of *P. pastoris*_KM71 than in the 1.3 L-cultures (48.96 versus 23.61 mg.L^{-1}) (Table 3.2, 3.3). These results confirmed that the *P. pastoris*_KM71 strain was the preferred option to compare SAF the expression profiles with that of *H. polymorpha*.

Table 3.2 Mean concentrations and yields of *P. pastoris* Mut^S versus Mut$^+$ cultures in 10 L bioreactors

Strain	**SAF concentration ($p<0.05$)**	**Biomass concentration ($p<0.05$)**	**Yield $_{(P/X)}$ ($p<0.05$)**	**Yield $_{(P/S)}$**
	mg SAF.L culture $^{-1}$	g DCW.L^{-1}	mg SAF.kg DCW^{-1}	mg SAF.g methanol^{-1}
*P. pastoris*_KM (Mut^S)	48.97±1.83	75.50±5.29	734.76±28.29	0.61±6.15
*P. pastoris*_GS115 (Mut$^+$)	25.70±1.49	118.40±8.04	273.27±13.7	0.45±4.93

Table 3.3 Mean productivity parameters of scaled-up fermentations (1.3 L) in bioreactors for the production of SAF in *P. pastoris* versus *H. polymorpha* cultures

	***P. pastoris*_ KM (Mut^S)**	***H. polymorpha***
Volumetric productivity (mg SAF.L^{-1}.h^{-1})*	0.20±0.01	0.61±0.07
Specific productivity (mg SAF.g DCW^{-1}.h^{-1})*	2.33±0.3	6.78±1.5
$Yield_{P/S}$ (mg SAF.g^{-1} methanol)	0.10±0.07	0.32±0.16
$Yield_{P/X}$ (mg SAF.kg^{-1} DCW)*	257.87±74.59	1353.10±373.84
$Yield_{X/S}$ (g DCW.g^{-1} methanol)	0.18±0.01	0.35±0.12
SAF concentration(mg SAF.L^{-1} culture)*	23.61±11.78	132.10±35.94

*p <0.05

Expression of SAF in *P. pastoris* (Mut^S) related to *H. polymorpha*

Another methylotrophic yeast strain, *H. polymorpha* NCYC 495, was investigated for the expression of SAF in 1.3 L bioreactors. Various factors, such as multimeric integrants and higher growth temperature (37-43°C), make this strain ideal for industrial applications (Gellissen *et al.*,

2000; Gellissen, 2002). Methanol fed-batch fermentations were carried out using the DO stat method for 120 h. The addition of methanol was optimized using a modified inlet in the roof of the bioreactor to allow small volume increments and prevent the potential accumulation of excess methanol. The volumetric and specific productivity for each of these strains are shown in Table 3.3. *H. polymorpha* produced significantly higher levels of SAF proteins than *P. pastoris*_KM71 (Mut^S) ($p<0.05$). Although not significant, *H. polymorpha* produced more biomass per substrate methanol, but significantly higher SAF per biomass than *P. pastoris*. Both the specific and volumetric productivity rates of SAF were 3-fold higher in *H. polymorpha* than in *P. pastoris*_KM71 (Mut^S) ($p<0.05$). Since the methanol inlet in the KM71 bioreactor was modified, the methanol level was measured to verify the absence of residual methanol.

Western Blot analysis showed the presence of bands representing proteins smaller than the SAF protein. This phenomenon is more evident in *H. polymorpha* cell extracts than in those from *P. pastoris* (Fig.3.3). Since both examples of *H. polymorpha* and *P. pastoris* had similar concentrations (13.01 $mg.L^{-1}$ versus 18.80 $mg.L^{-1}$, respectively), the smaller protein species evident in the *H. polymorpha* cultures were not due to a difference in the amount of protein loaded onto SDS-PAGE.

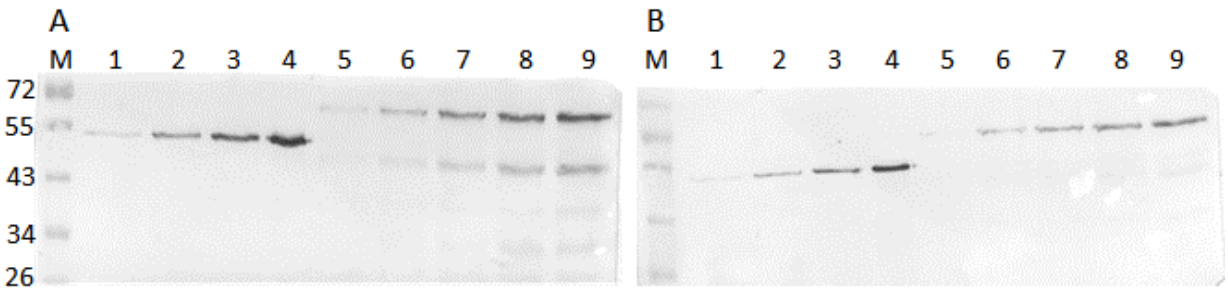

Fig. 3.3 Western Blot analysis of SAF proteins (~55 kDa) expressed in (A) *H. polymorpha* and (B) *P. pastoris*. Crude cell lysates were prepared from 60 mg of yeast cells and aliquots of the lysates were loaded for quantification by densitometry. M: PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, USA); Lane 1: 3 ng; lane 2: 7 ng; lane 3: 12 ng; lane 4: 20 ng HPV16 L1 (~50 kDa); lane 6: 0.5µl; lane 7: 1 µl; lane 8: 2 µl; lane 9: 3 µl; lane 10: 5 µl SAF crude cell lysate.

Statistical analysis

The one-way analysis of variance (ANOVA) was used in combination with the Bonferroni *post hoc* test to determine any statistical differences amongst the means of the three recombinant systems in

terms of various expression parameters. Comparisons were also compiled between different feeding strategies. For all comparisons, $p < 0.05$ was considered statistically significant.

Discussion

Human papillomavirus infections are amongst the most threatening sexually transmitted diseases in the world as it is almost entirely associated with cervical cancer. A breakthrough in cervical cancer prevention is to vaccinate against HPV infection using prophylactic vaccines. Developing cheaper vaccines could reduce the cost associated with the prevention of this cancer in developing countries. This could be more feasible with the cost-effective expression of viral proteins.

Previously our group compared the cytosolic expression of the same unique SAF protein in both *P. pastoris*_KM71 and *H. polymorpha*. Expression was only performed in shake flask cultures for 72 hours and yields were compared with the expression in peroxisomes of *H. polymorpha*. The levels of cytosolic SAF in *P. pastoris* was higher than those obtained in *H. polymorpha.* Directing the production to *H. polymorpha* peroxisomes resulted in very low expression of SAF (Smith *et al.*, 2012).

In this study, we focused on the expression of the same SAF protein in both methylotrophic yeasts, but under controlled conditions in bioreactors. We first evaluated two different feeding strategies to optimize expression of HPV L1/L2 chimeric proteins in two *P. pastoris* strains, KM71 (Mut^S) and GS115 (Mut^+), by evaluating a predetermined feeding rate versus the DO stat method. Trinh and co-workers (2003) compared a predetermined exponential methanol feeding rate with two different methods based on the metabolism of the yeast *P. pastoris*_GS115 *(*Mut^+); one method responded to the methanol consumption as measured by an on-line methanol sensor and the other method to the oxygen consumption. The predetermined exponential methanol feeding rate proved to be the most efficient at controlling the methanol feed rate as the specific production per biomass and per methanol was higher (Trinh *et al.*, 2003). In another study, the predetermined feeding rate was also the preferred feeding method in *P. pastoris* Mut^S cultures (Kupcsulik *et al.*, 2001). Minning *et al.* (2001) investigated different strategies for maintaining the methanol concentration of both *P. pastoris*_GS115 *(*Mut^+) and KM17 (Mut^S) strains. One is based on the monitoring of methanol concentration with gas chromatography (GC) versus adjusting the feed rate according the DO concentration in the cultures (Minning *et al.*, 2001). Their results showed that monitoring the methanol concentration by means of GC resulted in a 2.5-fold increase in productivity. They argued that the use of DO measurements to control methanol concentration was not optimum as methanol

was not measured directly, but rather indicated indirectly by the level of DO present. Therefore, the methanol concentration may not be maintained precisely and probably exceed the limiting value when the dissolved oxygen was increasing. This may pose a problem for methanol-sensitive strains such as *P. pastoris*_KM71. Slower growth rates of these *P. pastoris* strains can cause the methanol to accumulate in the bioreactor to toxic levels (Cregg *et al.*, 1989), especially in smaller culture volumes where the lower flow rates of methanol addition are inherently more difficult to control accurately. In contrast, Kotzé *et al.* (2011) showed that DO controlled feeding produced both higher volumetric production of HPV type 16 L1 protein and biomass in *P. pastoris* Mut^+ (X-33) cultivations compared to a predetermined feeding option. Our current results confirmed that the DO stat control was also the preferred feeding strategy for Mut^S cultures (Fig 3.1B). Since residual methanol was too low during the preferred feeding, the decrease in biomass and expressed SAF could not be attributed to methanol toxicity (Fig. 3.1A). Considering the increase in culture volume at the end of the fermentation period, it is evident that the decrease in SAF concentration was attributed to a dilution effect of the culture (Table 3.1). According to Chung (2000), the DO stat method is only applicable to cells growing under a single carbon source. In our study, methanol was the sole carbon source during the induction of the recombinant HPV L1/L2 chimeric proteins.

Secondly, the effect of the DO stat methanol feeding on biomass, methanol consumption and SAF production was assessed in *P. pastoris* Mut^+ versus Mut^S strains. Expression of recombinant proteins in methylotrophic yeast was induced in the presence of methanol, which also acts as a carbon source. Therefore, methanol concentration determines both the physiological condition of the cells as well as the production rate of recombinant proteins (Guarna *et al.*, 1997). In addition to the wild-type *P. pastoris* strain (GS115, Mut^+), which have both functional *AOX* genes, the KM71 strain (Mut^S) has a defective *AOX1* gene and relies solely on the *AOX2* gene to produce AOX. The *AOX2* is weaker and therefore produces lower levels of the oxidase, accounting for only 15% of the total AOX activity of the cell. In general, Mut^S strains have a slower growth rate and lower productivity than Mut^+ strains (Romanos *et al.*, 1992; Chiruvolu *et al.*, 1997; Van den Burg *et al.*, 2001; Kim *et al.*, 2009). Hence, it also consumes methanol at a slower rate (Cregg *et al.*, 1988). Thus, the Mut^S strains require much less methanol for induction, making it advantageous for large-scale fermentations where large quantities of methanol is considered a fire hazard (Krainer *et al.*, 2012). Slower growth rates are not only preferable for proteins where the folding is rate-limited to ensure correct folding, but also during the initial dimer formation in VLPs (Romanos, 1995; Ding *et al.*, 2010). However, the slower growth rate of Mut^S strains can cause the methanol to accumulate in

the fermentation culture to levels that are toxic to the yeast cells (Cregg *et al.*, 1989), especially in smaller culture volumes. Due to its higher metabolic rate, *P. pastoris* Mut^+ strains result in higher productivity and faster accumulation of methanol oxidation by-products (formaldehyde and hydrogen peroxide) inside the cell (Hazeu *et al.*, 1983; Brierley *et al.*, 1990; Chiruvolu *et al.*, 1997). Excess of these by-products can lead to cellular stress and ultimately to cell death (Couderc *et al.*, 1980; Cregg *et al.*, 1988; Kern *et al.*, 2007; Van der Klei *et al.*, 1990; Zhang *et al.*, 2000a). As the biomass increases, the methanol consumption rate also increases. Maintaining optimum methanol concentrations (preferably at 4 $g.L^{-1}$) is critical during the successful product formation in *P. pastoris* fermentation as lower methanol levels also reduce the productivity by triggering the proteolytic degradation of the recombinant proteins (Kupcsulik *et al.*, 2001).

We have adopted a closed-loop proportional-integral (PI) controller to maintain a constant DO of 30%. Higher biomass concentration and methanol feeding rate in *P. pastoris* Mut^+ cultures confirmed that *P. pastoris* Mut^+ strains had faster growth rates, associated with a higher rate of methanol consumption, than *P. pastoris* Mut^S strains (Fig. 3.2A and B, respectively). However, the SAF protein concentration, biomass concentration and protein yield per biomass were almost double that of the Mut^+ strain, making Mut^S strains attractive hosts for the production of recombinant proteins (Table 3.2). The methanol consumption rate differed by 10% between the two *P. pastoris* strains (Fig. 3.2C), demonstrated by the different cumulative volumes of methanol added to each 10 L bioreactor (1.81 L versus 1.84 L for KM71 and GS115, respectively). Residual methanol levels of less than 0.045 $g.L^{-1}$ were measured at the end of both cultures (data not shown), indicating the absence of methanol accumulation. Reduced biomass levels in Mut^S cultures could therefore not be attributed to accumulated methanol levels in the bioreactors. Several other studies support Mut^S strains to be superior in the production of some recombinant proteins (Pla *et al.*, 2006; Orman *et al.*, 2009). Krainer *et al.* (2012) recently confirmed that both volumetric and specific productivities were higher for the Mut^S strain than the Mut^+ strain. The substrate methanol in their Mut^S experiments was converted 7 times more efficiently into product than the Mut^+ strain (Krainer *et al.*, 2012).

The expression of SAF was scaled up to 10 L bioreactors using the *P. pastoris*_KM71 (Mut^S) strain in parallel with the Mut^+ *P. pastoris*_GS115 strain. Cultures can reach cell densities up to 150 g DCW.L^{-1} in the controlled environment of a bioreactor (Cereghino *et al.*, 1999; Cereghino *et al.*, 2000). The biomass yield of the Mut^S strain in 3 L bioreactor volumes was higher than that obtained in 10 L bioreactors. This phenomenon could be attributed to protein burden. Kafri *et al.* (2016) reported that protein overexpression can reduce growth rates. Highly abundant transcripts of the

recombinant protein compete for the limited resource of ribosomes present in the host, which is otherwise responsible for the translation of endogenous proteins. Both the SAF concentration as well as the SAF yield per biomass were higher in 10 L cultures. SAF concentrations were 2-fold higher in the 10 L cultures of *P. pastoris*_KM71 than in the 1.3 L-cultures, reaching a biomass concentration up to 75.50 and 118.40 g DCW.L^{-1} (for Mut^S and Mut^+, respectively). The SAF yield per biomass (734.76 mg.kg^{-1} DCW) was almost 3 times more in 10 L *P. pastoris*_KM71 cultures than in 1.3 L cultures (257.87 mg.kg^{-1} DCW). Our results also showed that the larger 10 L fermentation volumes tolerated the addition of methanol with greater ease than in the smaller culture volumes. A possible reason for the lack of biomass increase in the 3 L fermenters (Fig. 3.2B, C) could be a result of methanol toxicity as methanol was added as droplets from an inlet in the lid of the bioreactor. These droplets were diluted more efficiently in larger cultures volumes than in the smaller volumes.

The third objective of our study was to evaluate the expression of HPV type 16 L1/L2 chimeric proteins in *P. pastoris*_KM71 (Mut^S) versus *H. polymorhpa* in bioreactors. Previously our shake flask expressions showed *P. pastoris*_KM71 produced higher concentrations of SAF than *H. polymorpha*. Mack *et al.* (2009) compared the production of mammalian proteins in *H. polymorpha* with *P. pastoris* wild type X33 (Mut^+) in shake flasks and found that *P. pastoris* yielded more than 3-fold more protein than *H. polymorpha*. It could be that the amount of methanol added to batch cultures of *H. polymorpha* was inefficient to maintain maximum induction from the AOX promoter, compared to *P. pastoris.* This contradicts the results from our current study that showed *H. polymorpha* is superior in producing SAF proteins in bioreactors. As mentioned before, Mut^S strains have a slower growth rate and lower productivity than Mut^+ strains. In this controlled environment, the slow growth rate of Mut^S strains, such as KM71, contributed to the lower yield obtained in *P. pastoris* cultures than in *H. polymorpha*. Again, no residual methanol was measured in this KM71 culture. Therefore, the lower SAF yield in the KM71 culture was not attributed to methanol toxicity.

Southern Blot analysis revealed that two or more copies of the *SAF* expression cassette were integrated into the genome of the *H. polymorpha*_NCYC 495 strain. The copy number of the SAF expression cassette could not be verified in the *P. pastoris*_KM71 strain using a similar method. In addition, a PCR protocol was used to distinguish between different copy numbers depending on the size of the amplicons. The *P. pastoris*_KM71 strain harbored only one copy of the SAF cassette (data not shown). However, Aw *et al.* (2013) demonstrated that higher copy number does not necessarily result in higher productivity. Li *et al.* (2009) reported the first case of HPV type 16

expression in *H. polymorpha*, only reaching levels of 78.6 mg.L^{-1} in shake-flasks. Our results showed the highest concentration of expressed SAF protein reported to date in both yeasts, i.e. 23.61 mg.L^{-1} (48.96 mg.L^{-1} in 10 L) and 132.10 mg.L^{-1} for *P. pastoris*_KM71 and *H. polymorpha*, respectively. Previously, the highest concentration of HPV-16 L1 protein obtained from *P. pastoris*_KM71 in shake-flasks was 14.2 mg/L (Bazan *et al.*, 2009). Although the yield of biomass or SAF production per methanol substrate was not significantly different between *H. polymorpha* and *P. pastoris*_KM71 (Table 3.3), *H.* These species are possibly degradation products of L1, since they were not seen in total extracts of *P. pastoris* that did not show expression of the recombinant protein. 5-fold.

Our Western blot analysis revealed the presence of lower molecular weight protein species in the *H. polymorpha* crude cell lysates, but not in the *P. pastoris* KM71 crude cell lysates (Fig.3.3). Several other groups have seen this phenomenon after expressing HPV16 L1 in insect cells (Kirnbauer *et al.*, 1992), yeast (Bazan *et al.*, 2009; Jiang *et al.*, 2011; Deschuyteneer *et al.*, 2010) and bacteria (Aires *et al.*, 2006; Kelsall and Kulski, 1995; Chen *et al.*, 2001; Lai and Middelberg, 2002; Zhang *et al.*, 1998). The lower molecular weight proteins are most probably degradation products of the full-length L1 since they were not seen in total extracts of *H. polymorpha* before the induction of recombinant proteins (data not shown) (Bazan *et al.*, 2009; Zhang *et al.*, 1998). Smaller L1 fragments could also be formed due to premature termination of the L1 translation (Finnen *et al.*, 2003). The degradation of SAF proteins was more evident in *H. polymorpha* cultures performed at 37°C than in *P. pastoris* cultures incubated at 30°C (Fig. 3.3A). Mack *et al*. (2009) also showed less degradation of the recombinant protein in the *P. pastoris* Mut^{+} strain than in *H. polymorpha*. It is known that yeast cells are stressed by various factors during methanol metabolism, including change of carbon source, heat and toxic by-products. Cells of *H. polymorpha* metabolizes methanol at a faster rate than the Mut^{S} strain of *P. pastoris.* The combination of enhanced methanol utilization and higher fermentation temperature at 37ºC could have contributed to the higher proteolytic action in *H. polymorpha* (Inan *et al.*, 2006). Despite the observed degradation, *H. polymorpha* still produced higher yields of SAF per methanol substrate than *P. pastoris* as the degraded products were not quantified and added to the total recombinant protein yield.

Several groups have produced HPV-16 L1 in yeast in small-scaled shake-flasks as well as up-scaled bioreactors. Our study showed that the up-scale fermentation of *P. pastoris* and *H. polymorpha* in bioreactors provide substantial quantities of expressed protein. *H. polymorpha* proved to be an ideal system for the production of high yield recombinant proteins. The present data

also allowed us to investigate the DO stat method to control methanol feeding in both *P. pastoris* Mut^+ as well as Mut^S cultures. Increased production of HPV type 16 chimeric L1 proteins, such as in *H. polymorpha*, hold promise in developing countries as it can lead to the manufacturing of cheaper HPV vaccines.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interests

The authors declare that they have no conflict of interest.

Future work

Fermentations in bioreactor should be fitted with an on-line mentanol sensor. Measuring methanol concentrations, using GC-FID, during culturing is too time consuming and does not allow adjustments to be made to the system on time to prevent overfeeding. The initially screening of the optimum feeding method in *P. pastoris* Mut^S cultures must also include both the Mut^+ and *H. polymorpha* strains. It could be that the optimum feeding strategy for the one strain is not necessarily the preferred feeding method for the other. Subsequently, each yeast strain with its own preferred feeding method must then be compared to verity the best producing yeast strain.

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Chapter 4

Semi-purification of the SAF protein and visualization of secondary structures

Introduction

The human papillomavirus capsid is a non-enveloped icosahedral shell consisting of two structural proteins, L1 and L2, which self-assemble *in vivo* into either capsomeres (pentamer = 5 L1 monomers) or T=7 icosahedrons, (72 L1 pentamers = 360 L1 monomers) (Hagensee *et al.,* 1994). *In vivo,* the L1 protein alone is capable to self assemble into capsid-like structures referred to as virus-like particles (VLPs) (Kirnbauer *et al.,* 1992). Capsomeres can self-assemble into VLPs *in vitro* by removing reducing agents, lowering the pH and increasing the ionic concentrations (Liu *et al*., 2007; Mach *et al*., 2006).

When helix 4 of the HPV-16 L1 protein is either abolished (Bishop, Dasgupta and Chen 2007; Murata *et al.,* 2009; Schädlic *et al.,* 2009) or replaced with a HPV-16 L2 epitope (McGrath *et al.,* 2013; Varsani *et al.,* 2003), the formation of higher-order structure, such as T=7 or T=1 particles, is unexpected and capsomeres, 8-10 nm in diameter, or capsomer aggregates are most likely to form. If helices 2 and 3 are deleted, the formation of pentamers will be prohibited (Chen *et al.,* 2001). Failing to assemble into bigger structures are mainly due to the lack of hydrophobic interaction between relevant helices (Chen *et al.,* 2000). The chimeric SAF protein was constructed by replacing the h4 helix of the HPV-16 L1 protein with a 13-amino acid peptide from the L2 protein. When expressed in insect cells, SAF proteins predominantly self-assembled into capsomeres (McGrath *et al.,* 2013; Varsani *et al.,* 2003). McGrath *et al.* (2013) reported the occasional presence of T=7 VLPs, 50 nm in diameter, during microscope analysis, but capsomeres were found most abundantly (McGrath *et al.,* 2013).

Capsomeres are much less immunogenic than VLPs (Thönes *et al.,* 2008). Despite less immungenicity, immunization with capsomeres using various strategies including oral, intranasal and subcutaneous, have shown to still induce high titres of neutralizing antibodies and T-cell responses (Dell *et al.,* 2006; Fligge *et al.,* 2001; Ohlschlager *et al.,* 2003; Rose *et al.,* 1998; Thönes and Müller 2007; Yuan *et al.,* 2001). Schädlic *et al.* (2009) demonstrated that certain capsomeres can even exhibit immunogenicity similar to that of VLPs.

Previously, the chimeric SAF protein was expressed in insect cells and their ability to assemble into high-order structures was assessed (Varsani *et al.*, 2003; McGrath *et al.*, 2013). Only McGrath *et al.* (2013) showed the occasional formation of VLPs using the SAF protein. However, in this study, the methylotrophic yeasts, *P. pastoris* and *H. polymorpha*, were employed to express the SAF protein intracellularly (Bredell *et al.*, 2017, under review). Following expression, recombinant proteins were semi-purified from the yeast cell lysate and subjected to electronmicroscopy. Upon transmission electron microscopy (TEM), secondary structures of 25-30nm in diameter were observed.

Materials and Methods

Crude cell extracts

Crude extracts were prepared from both *P. pastoris* and *H. polymorpha* cells, as previously described (Chapter 3). Cell pellets were washed with 1.2 M sorbitol, 100 mM EDTA and 14 mM β-mercaptoethanol, and resuspended in breaking buffer containing 50 mM NaH_2PO4, pH 7.4, 1 mM EDTA, 5% v/v glycerol and Protease Inhibitor Cocktail (Sigma, 1 ml/20 mg wet weight cells). Equal volume of glass beads was added to the cell suspension and subjected to vigorous vortexing, followed by incubation on ice. This was repeated for 20 cycles and the lysates were cleared by centrifugation at 10 000 g for 10 min at 4°C.

Positive identification of SAF expression in cell lysates

An aliquot of clear cell lysate, mixed with an equal volume of 2 x loading dye (125 mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 0.01% bromophenol blue; 5 mM 2-mercaptoethanol), was heated at 95°C for 5 min before loading onto a 10% polyacrylamide gel. Proteins were separated and then transferred onto nitrocellulose membranes (BioTrace™ NT, PALL) as previously described (Smith *et al.*, 2012). Membranes were treated accordingly and incubated with an anti-HPV type 16 L1 monoclonal antibody CAMVIR-1 (Santa Cruz Biotechnology, Dallas, Texas). Following detection using alkaline phosphatase-labelled antibodies and NBT/BCIP detection reagents (Fermentas, ThermoFisher, Massachusetts, USA), the presence of a 55 kDa protein specie indicated the expression of SAF and were selected for protein purification. Unless stated otherwise, all blots were treated with anti-HPV- type 16 L1 and alkaline phosphatase antibodies. One hundred grams of wet yeast cells were used to prepare yeast cell lysate for further L1 protein purification.

Ammonium sulphate precipitation

SAF protein was recovered from the cleared lysates by ammonium sulphate (Sigma, USA) precipitation. Yeast cell lysates were adjusted to 20% saturated ammonium sulphate followed by stirring for 60 min at 4°C. Precipitated protein was pelleted at 13 000 x *g* for 20 min at 4°C. The protein pellets were resuspended in 50 µl distilled water (1 x PBS, pH 7.2 + 0.01% Tween 80) and the remaining supernatant was adjusted to a 10% increment in ammonium sulphate at 4°C, followed by stirring and centrifugation. The process was repeated until the lysates reached an ammonium sulphate concentration of 60% (Green and Hughes 1955). The resuspended pellets were subjected to SDS-PAGE analysis and Western blotting to verify the presence of SAF monomer proteins.

After ammonium sulphate precipitation, the resuspended pellets were dialyzed against 1 x PBS (pH 7.2) + 0.01% Tween 80 buffer with two changes of buffer in 24 h and subjected to size-exclusion chromatography.

Size-exclusion chromatography

Size-exclusion chromatography was performed with Sephacryl^{TM} S-1000 size column (GE Healthcare, US). The running buffer contains 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 0.01% Tween 80. Fractions were collected from the column at 0.16 ml/min (fractions were taken every 5.8 min, total fraction volume of 928 µl) and their absorbance was measured at 280 nm with a spectrophotometer. Fractions were analysed by SDS-PAGE, and Western blotting.

In addition, the nitrocellulose blots were also stained with Ponceau stain to verify the presence of any proteins bands on the blots. Membranes were incubated for one hour in staining solution (0.1% ($g.L^{-1}$) Ponceau S (Sigma, USA) in 5% (v/v) acetic acid) with gentle agitation, followed by rinsing the membrane in distilled water until the background is clear.

Cation-exchange chromatography

The fractions containing the SAF monomer proteins were collected from the size-exclusion chromatography and equilibrated at 4°C with binding buffer containing 20 mM Tris, pH 6.5, 0.1 M NaCl, 0.1 mM EDTA, 5% glycerol and 15 mM DTT. The equilibrated fractions were loaded onto a CM Sepharose Fast Flow column (GE Healthcare, US). The column was washed with five column volumes of wash buffer containing 0.6 M NaCl. The SAF monomer proteins were eluted with elution buffer containing 1.1 M NaCl.

Sucrose gradient centrifugation

As an alternative to chromatography, cleared yeast cell lysates were loaded onto a sucrose density gradient (50-70% g/L) in 1 x PBS, pH 7.2 and ultracentrifuged. The gradient is prepared by layering less dense sucrose solutions upon one another, starting with the 50% sucrose at the bottom of a polyallomer tube (Beckman, CA). The tubes were then centrifuged at 100 800 x *g* in a swinging bucket SW 50.1 type rotor (Beckman, CA) for 18 h at 4°C. Five hundred microliter fractions were collected through a fine piercing at the very bottom of the tube, and analysed by SDS-PAGE and Western blotting. Sucrose fractions containing the HPV type16 L1 protein were then dialysed against a disassembly buffer (PBS, pH 8.2, containing 0.166 M NaCl, 2 mM DTT and 2 mM EDTA, with 0.01% Tween 80), followed by a reassembly buffer (PBS, pH 7.0, containing 0.5 M NaCl) with 4 changes of buffer at 4°C.

Electron microscopy

Carbon coated copper grids were glow discharged using the Glow Discharge Unit to render the carbon surface hydrophilic. Samples were adsorbed to the grids for 5 min. Grids were washed twice in distilled water and negatively stained with 2% uranyl acetate for 1 min. Grids were blotted on filter paper to remove excess liquid and air-dried prior viewing. Alternatively, carbon coated grids were treated with the HPV type 16-conformation-specific antibody H16:V5 (donated by Ed Rybicki, University of Cape Town) at a 1:1000 dilution for 10 min. These antibodies captured only L1 pentamers and other higher-order aggregates including VLPs onto the grids (Christensen *et al.*, 1996; Varsani *et al.*, 2003]. Unbound antibody was first washed off by floating the grids on PBS droplets for 1 min before the samples were absorbed to the grids, washed with water and negatively stained. Air-dried grids were viewed and micrographs taken using a transmission electron microscope (Zeiss 912 Omega Cryo Eftem, USA) at 50 000 times magnification.

Results

Effect of different percentage ammonium sulphate on SAF precipitation

Following different percentage ammonium sulphate precipitation, protein pellets were analysed using SDS-PAGE and Western blotting. Blots revealed a prominent band correspondig to the correct-size (55 kDa) HPV16 SAF monomers. Faint lower and higher molecular weight protein species were also detected. The lower bands corresponded to degradation products and the higher bands represent possible oligomers.

Initially, ammonium sulphate was added add 20%, then 30%, 40%, 50% and 60% to precipitate the SAF monomer proteins. When the highest concentration SAF proteins was recovered at 40% and 50% (Fig 4.1 A), smaller increments of 5% were subsequently investigated and showed that 45% precipitated most of the SAF proteins (Fig 4.1B).

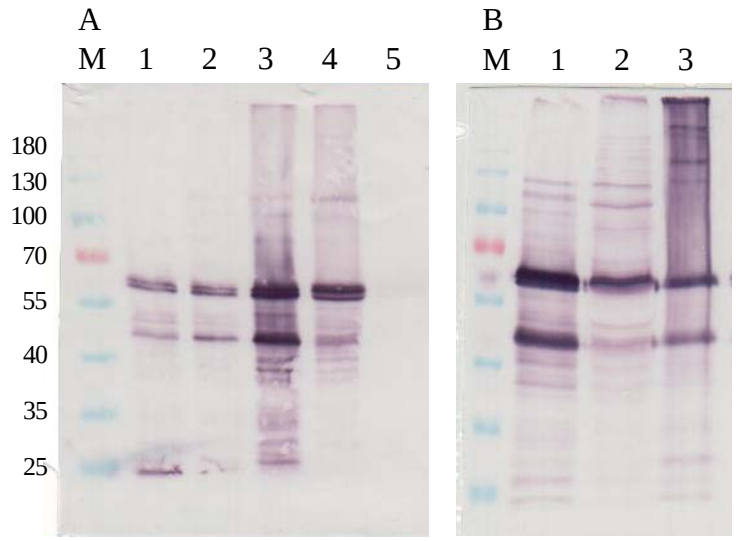

Figure 4.1. Western blots of precipitated SAF monomer proteins using different ammonium sulphate percentages. (A) M: PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, USA); lane 1: 20%; lane 2: 30%; lane 3: 40%; lane 4: 50%; lane 5: 60% ammonium sulphate, (B) M: Protein Ladder; lane 1: 45%; lane 2: 50%; lane 3: 40% ammonium sulphate.

Size-exclusion chromatography

After the 40% ammonium sulphate precipitation, the resuspended protein pellet was dialyzed before it was loaded onto a Sephacryl^{TM} S-1000 size column. All the fractions were examined at 280 nm (Fig. 4.2) and analysed by SDS-PAGE and Western blotting for the presence of SAF monomer proteins (Fig. 4.3A, B). Ponceau S-stained SDS-PAGE analysis indicated the presence of proteins only in fractions 10-21 with HPV16 SAF confirmed in all 12 fractions. However, only fractions 14-20, showing the absence of degradation products, were pooled and subjected for further purification using cation exchange chromatography.

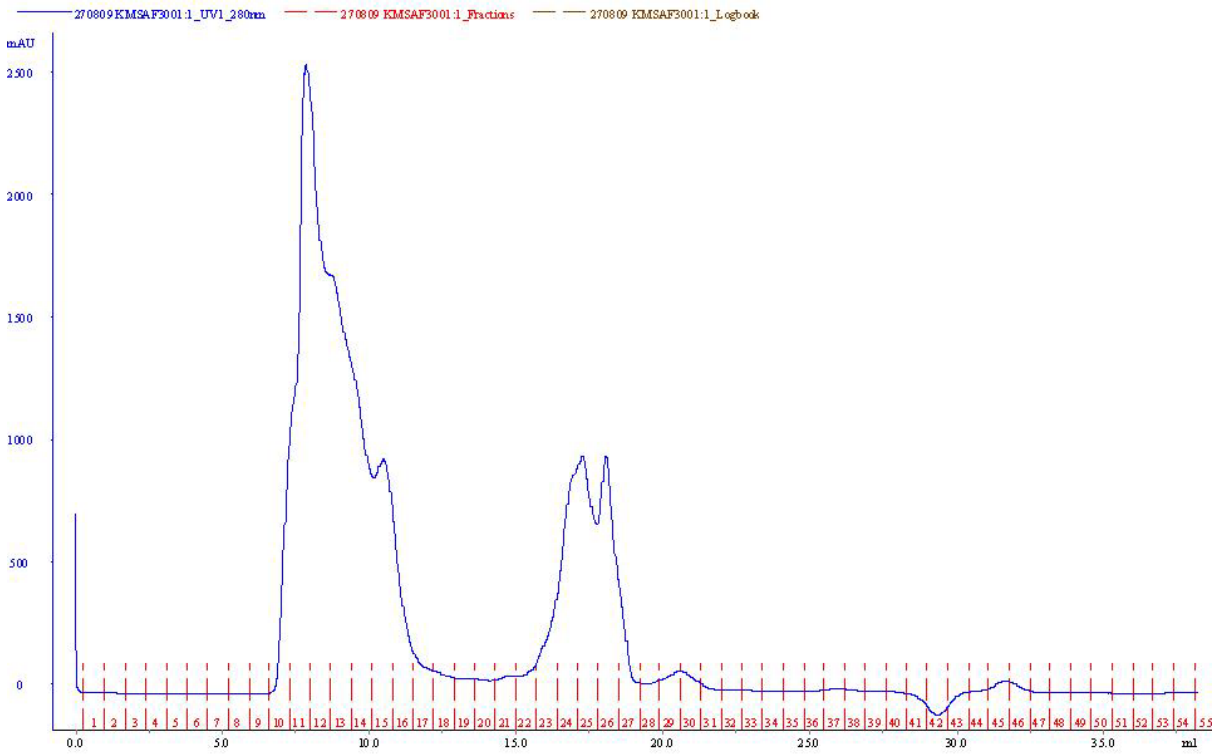

Figure 4.2. Size-exclusion chromatography curve indicating 55 intervals when fractions were taken.

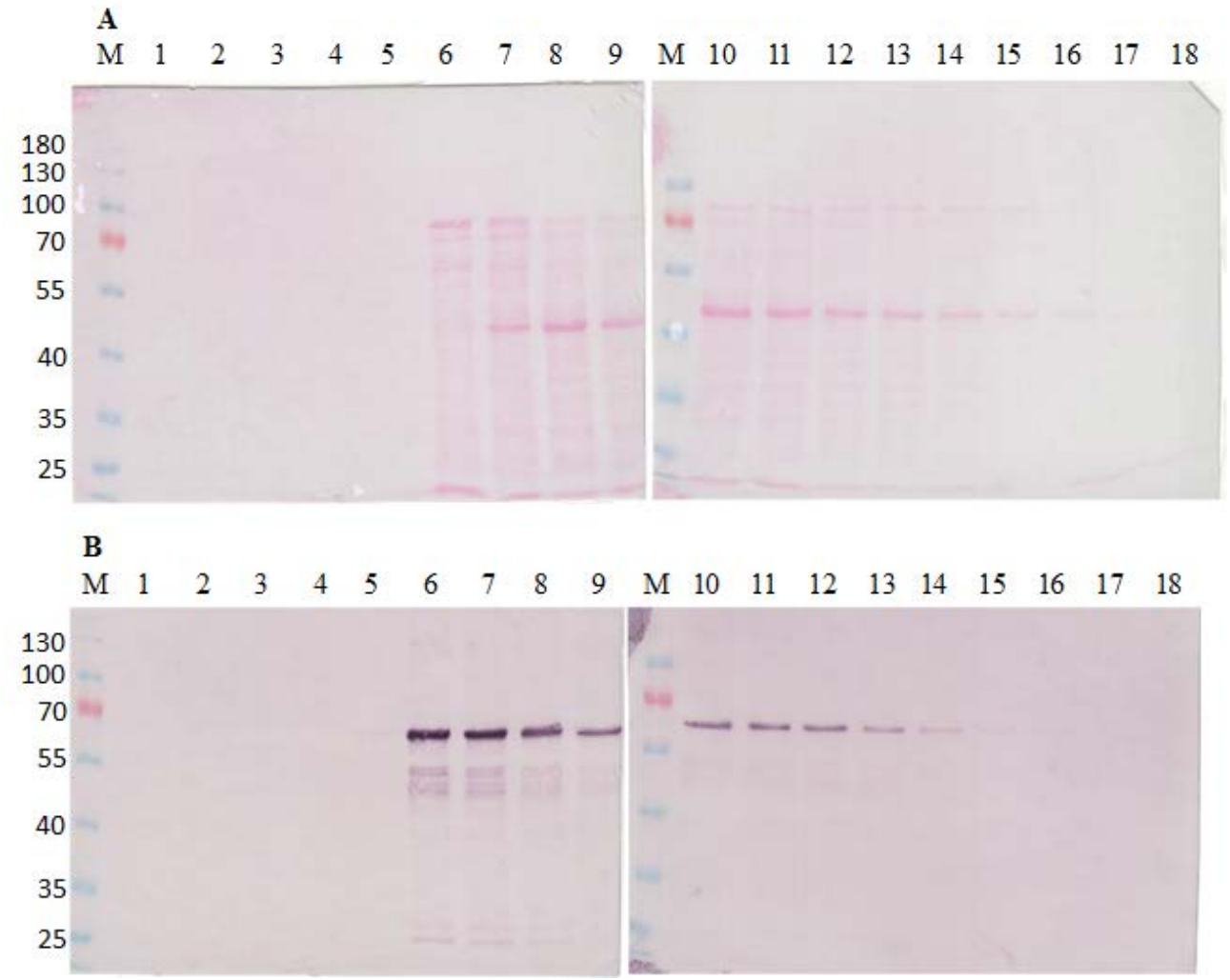

Figure 4.3. Size-exclusion chromatography fractions separated by SDS-PAGE followed by Western blotting. (A) Western blots showing all extracted proteins using Ponceau S staining. M: PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, USA); lane 1-9: fractions 5-13; lane 10-18: fractions 14-22. (B) Western blots showing only HPV-16 L1 proteins using anti-HPV-16 L1monoclonal and alkaline phosphatase-labelled antibodies. M: PageRuler™ Prestained Protein Ladder; lane 1-9: fractions 5-13; lane 10-17: fractions 14-22.

Cation-exchange chromatography

Fractions 14-20 from the size-exclusion chromatography (Fig 4.2) were pooled, equilibrated and fractionated using cation-exchange chromatography (Fig 4.4). The SAF monomer proteins were eluted with 1.1 mM NaCl and cation chromatography fractions 1-12 were analysed by SDS-PAGE, followed by Western blotting (Fig 4.5A, B). The SAF protein (~55 kDa) was successfully eluted in fractions 1-8 (Fig. 4.5A).

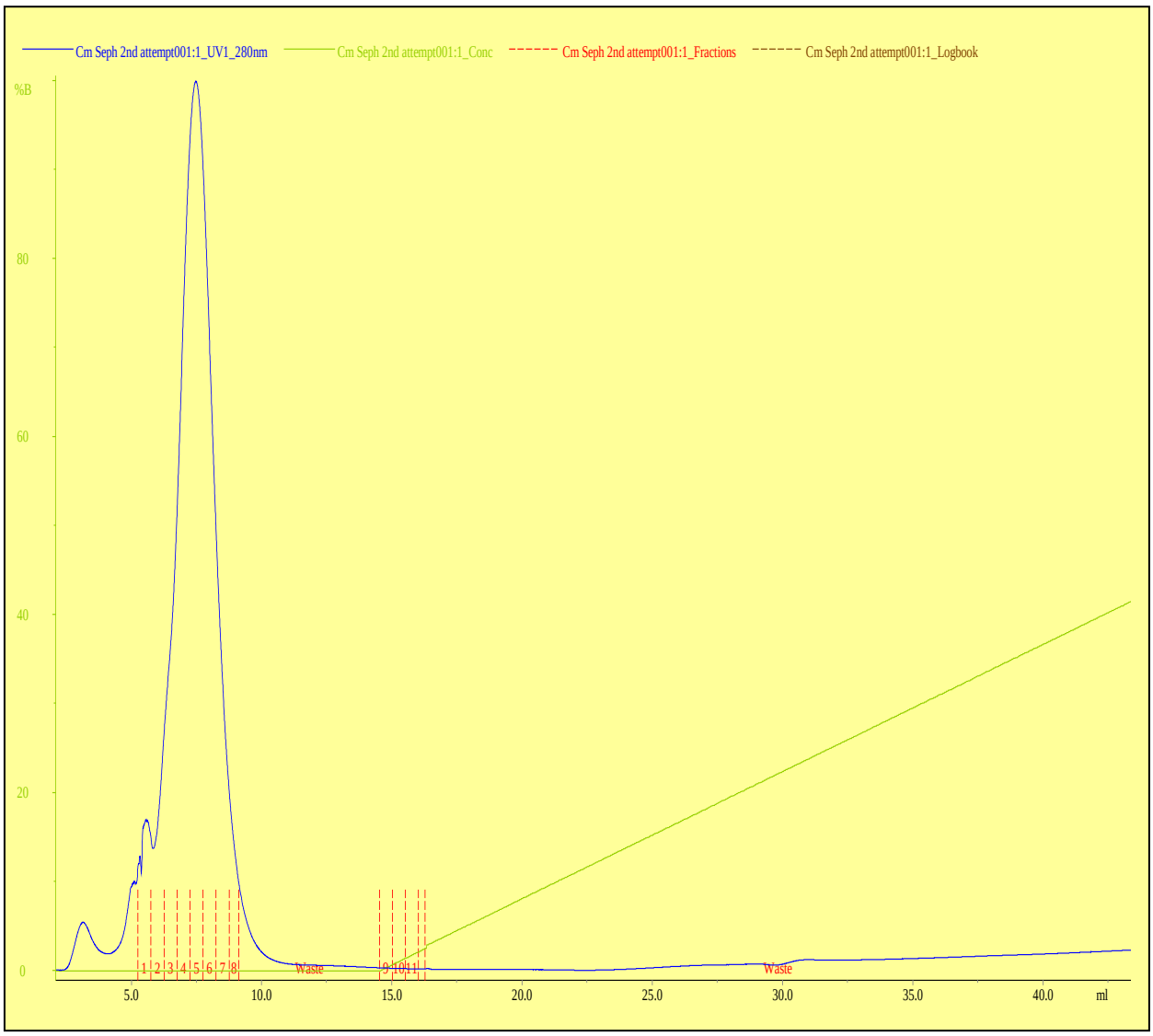

Figure 4.4. Cation-exchange chromatography curve indicating 12 intervals when fractions were taken.

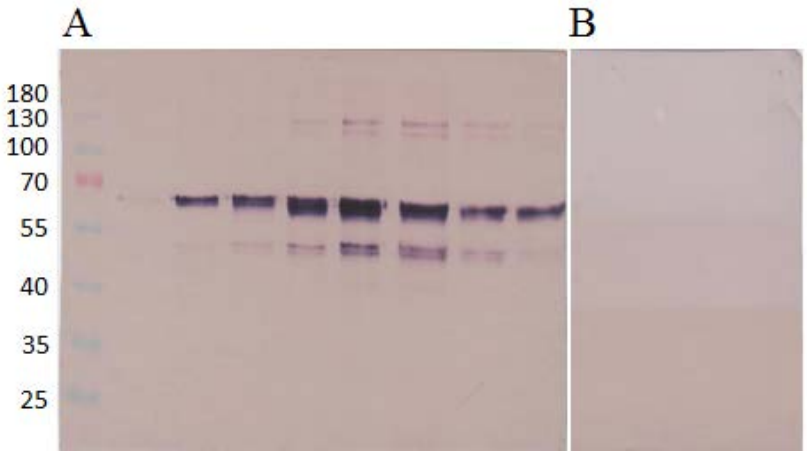

Figure 4.5. (A) Western blot result of cation-exclusion chromatography fractions separated by SDS-PAGE. M: PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, USA); lane 1-8; fractions 1-8, (B) lane 9-12; fractions 9-12.

Sucrose gradient centrifugation

As an alternative strategy, cleared yeast cell lysates were also loaded onto a sucrose density gradient (50-70% g/L) and ultracentrifuged. After centrifugation, the sucrose gradient separated in different phases (Fig 4.6A). Five hundred microliter fractions were collected through a piercing at the bottom of the tube, and analysed by SDS-PAGE and Western blotting (Fig 4.6B). The highest recovery of HPV16 SAF was from fractions representing 65%, 60% and 55% sucrose.

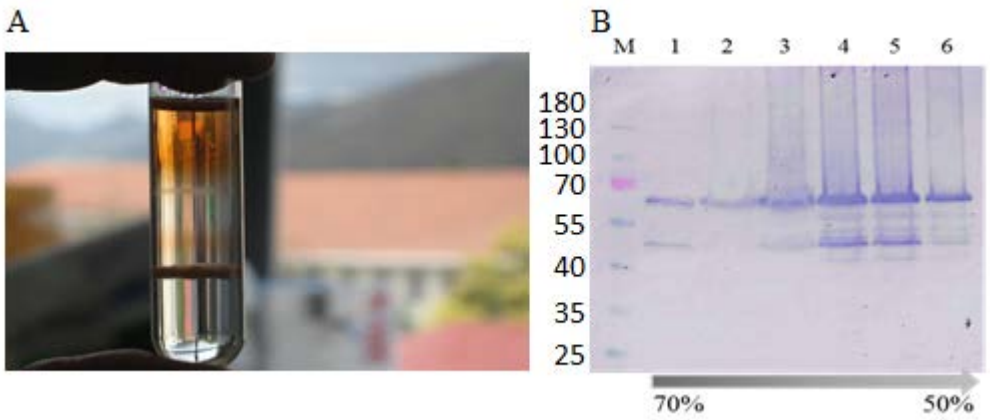

Figure 4.6. Isolation of HPV SAF chimeric monomeric proteins. (A) Yeast cell lysate loaded onto a 50-70% sucrose density gradient and ultracentrifuged. (B) Sucrose gradient fractions separated by SDS-PAGE followed by Western blotting. M: PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, USA); lane 1: pellet, lane 2: 70% sucrose, lane 3: 65% sucrose, lane 4: 60% sucrose, lane 5: 55% sucrose, lane 6: 50% sucrose.

In order to dilute the sucrose content in the sucrose gradient centrifuged samples, fractions 5 and 6 were dialyzed against distilled water for 4 hours. In addition, the cation-exchange chromatography fractions 1-8 were pooled and concentrated using dialysis tubing and PEG 35000. Both the concentrated cation-exchange chromatography sample and sucrose samples were used to prepare carbon grids for transmission electron microscopy.

Self-assembly of purified SAF monomer proteins

Semi-purified SAF monomer proteins self-assembled into recognizable secondary structures. It is evident from the frequency distribution chart that SAF proteins assembly mostly into capsomeres (~10 nm in diameter) with a lower frequency of other higher-ordered structures. The lowest frequency was T=1 VLPs (30 nm in diameter) (Fig. 4.7 A, B).

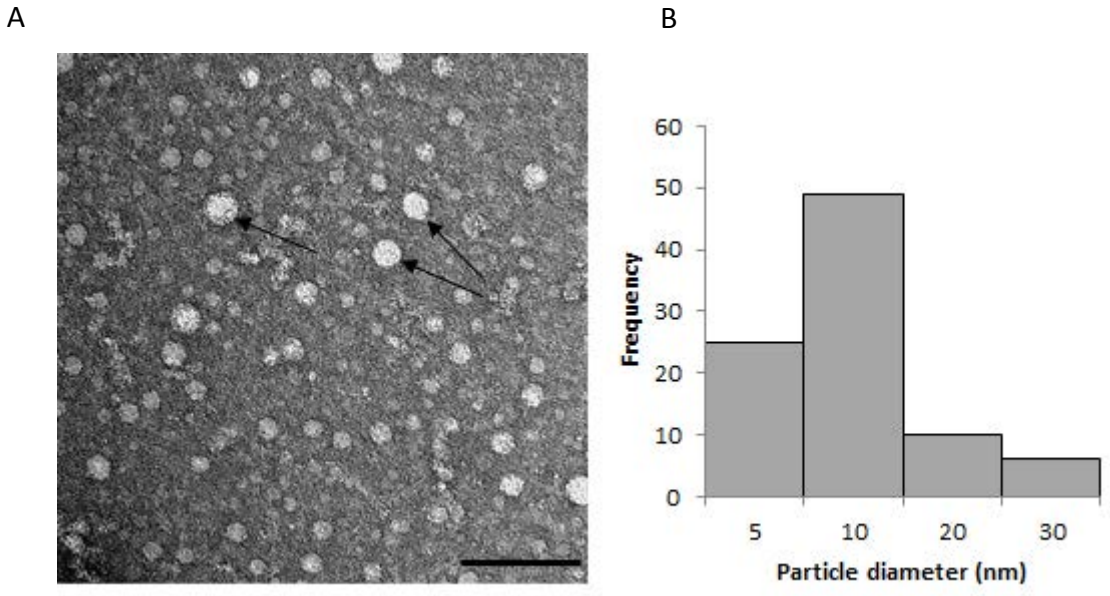

Figure 4.7. Analysis of the HPV16 L1/L2 (SAF) chimeric protein. A) TEM analysis. T=1 VLPs (25-30nm in diameter) are indicated by arrows against a background of smaller particles. A 100 nm bar for reference is located in the lower right corner of the picture. B) Diameter distribution of particles shown by frequency histogram.

Discussion

HPV L1 VLPs have been produced in various expression systems, such as bacteria, e.g. *Escherichia coli* (Chen *et al.*, 2001; Li *et al.*, 1997), *Lactococcus lactis* (Cortes-Perez *et al.*, 2009) and

Lactobacillus casei (Aires *et al.*, 2006), yeast such as *S. cerevisiae* (Woo *et al.*, 2008), *P. pastoris* (Liu *et al.*, 2007; Bazan *et al.*, 2009; Kotzé *et al.*, 2011; Zhao *et al.*, 2015) and *H. polymorpha* (Li *et al.*, 2009), baculovirus-infected insect cells (Kirnbauer *et al.*, 1992; Le Cann *et al.*, 1994), transgenic plants (Biemelt *et al.*, 2003), and mammalian cells (McLean *et al.*, 1990). Other studies have also reported on the production of VLPs consisting of both structural HPV proteins, L1 and L2, in human epithelial cells (Cianciarullo *et al.*, 2010) and insect cells (Kirnbauer *et al.*, 1993; Zhou *et al.*, 1991). In all of these expression systems, HPV type L1 assembled into relatively regular T=7 VLPs consisting of 72 pentamers. With the exception of expression in *E. coli*, smaller T=1 VLPs, consisting of 12 L1 pentamers (60 L1 monomers) and about 30-40 nm in diameter, have been observed but only when 10 or more amino acids have been removed from the amino-terminus of L1 (Chen *et al.*, 2001).

In this study, a chimeric protein was constructed by replacing the h4 helix of the HPV-16 L1 protein with a 13-amino acid peptide from the HPV-16 L2 protein, followed by the expression in methylotrophic yeasts *P. pastoris* and *H. polymorpha*. Electron microscopy (EM) showed that the chimeric L1/L2 protein (SAF) retained the ability to aggregate mainly into capsomeres (10 nm in diameter) and the occasional T=1 VLPs (25-30 nm in diameter) (Fig. 4.7 A, B). These secondary structures were present in samples from both the chromatography and sucrose gradient centrifugation. Bishop *et al.* (2007) reported the presence of the helix 4 near the C-terminus of HPV L1 as a prerequisite for the assembly of both T1 and T7 virus-like particles. Their helix 4 deletion mutants oligomerize into only capsomeres (Bishop, Dasgupta and Chen, 2007). Although other studies showed successful VLP formation following the expression of intact HPV-16 L1 proteins in *P. pastoris* (Liu *et al.*, 2007; Bazan *et al.*, 2009; Kotzé *et al.*, 2011; Zhao *et al.*, 2015), we did not include the expression of intact HPV-16 L1 in our study. Therefore, one cannot assume that the low frequency of T=1 VLPs and the absence of T=7 VLPs was attributed to the displacement of the helix 4. Although HPV-16 L1 had been expressed in *H. polymorpha*, no secondary structures were observed (Li *et al.*, 2009).

Analysis of EM also showed low frequencies of VLPs smaller than T=1 (20 nm in diameter) as well as particles smaller than capsomeres (5 nm in diameter). This could possibly be incomplete capsomeres or VLPs. Despite low yields of VLPs, our EM preparations did not inadequately assemble into amorphous aggregates.

Although our study did not investigate the immunogenicity of these secondary structures, Schädlic *et al.* (2009) showed that the humoral immunity elicited by T=1 VLPs was comparable to those elicited by T=7 VLPs (Schädlic *et al.*, 2009). Capsomeres were also highly immunogenic and capable of eliciting strong neutralizing antibody responses against both LI and L2 epitopes, but the responses were 20- to 40-fold lower than those by VLPs (Fligge *et al.*, 2001; Rose *et al.*, 1998; Thönes *et al.*, 2008). Schädlic *et al.* (2009) hypothesized that the lower antibody titers induced by capsomeres were the results of their smaller particle size compare to VLPs. Capsomeres have also provided protection against viral challenges in the canine oral papillomavirus model (Rose *et al.*, 1994; Yuan *et al.*, 2001). Despite their lower immunogenicity, capsomeres still offer great potential in future vaccine preparations. Chimeric SAF proteins displaying L2 epitopes offer simultaneously high titres of L1 specific neutralizing antibodies as well as cross-neutralizing antibodies against L2 (Varsani *et al.*, 2003; Rubio *et al.*, 2009; McGrath *et al.*, 2013). In addition to HPV L2 epitopes, L1 monomers bearing other heterologous epitopes within the helix 4 region can self-assemble into capsomeres that elicit antibody response specific against such non-HPV encoded epitopes. Thus, the HPV L1 helix 4 site can function as a novel antigen carrier within the L1 pentamer and thereby serve as a potential vaccine platform.

Although HPV type 16 L1 has been expressed in *H. polymorpha* before (Li *et al.*, 2009; (Liu, Yang and Yao, 2014), our study is the first report showing the formation of T=1 VLPs in this methylotrophic yeast. Previously, Liu *et al.* (2015) showed the formation of T=7 VLPs when HPV type 52 L1 was expressed in *H. polymorpha.*

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Chapter 5

Expression of rotavirus VP6 protein: A comparison amongst *Escherichia coli, Pichia pastoris* and *Hansenula polymorpha*

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Abstract

During this study, we successfully expressed a codon-optimized gene for rotavirus VP6 protein intracellularly in two methylotrophic yeasts, *Pichia pastoris* and *Hansenula polymorpha*, during methanol induction. Expressions were performed in shake flasks and subsequently scaled-up to 1.3 L bioreactors. The yields obtained in the yeasts were compared with that observed in *E. coli*. Despite producing the lowest biomass levels of all the expression systems in shake flasks, the highest VP6 concentration was obtained with *E. coli*. In shake flasks, *P. pastoris* yielded higher volumetric levels of VP6 than *H. polymorpha*, but specific production of VP6 was similar in both yeasts. In the controlled environment of bioreactors, yeast strains attained typical high cell densities, but also increased VP6 production compared to all shake flask cultures. Unlike in shake flask expressions, *H. polymorpha* outperformed both *P. pastoris* as well as *E. coli* during bioreactor cultivation. In all three VP6 systems, the VP6 production was growth-associated. In contrast to yeast expressions, bacterially expressed VP6 protein was found to be insoluble upon analysis. This is the first report of VP6 expressed in methylotrophic yeast and holds the promise for the inexpensive production of VP6 as a possible vaccine candidate or drug delivery mechanism.

Introduction

Gastroenteritis, resulting in severe diarrhea, is one of the leading causes of illness and deaths in children under the age of five years worldwide (Parashar, Bresee and Glass, 2003). Rotavirus (RV) infection accounts for one third of diarrhea-associated hospitalizations and more than 500 000 deaths annually in developing countries (Parashar *et al*., 1998; World Health Organization, 2007). More than half of those deaths occur in developing sub-Saharan Africa (World Health Organization, 2007). A rotavirus vaccine has been included in the South African Expanded Programme on Immunization (EPI) due to the positive outcome of vaccine trials in South Africa (Madhi *et al*., 2010) and elsewhere (Gray, 2011). Despite being highly effective, future vaccination in low income, high-burden countries may have a large financial impact. In addition, these countries also show lower vaccine efficacy than in high- and middle-income countries. This could be due to various factors, including the emergence of viral variants other than the vaccine strains. Booster doses may improve the rotavirus vaccine performance (Program for Appropriate Technology in Health: PATH, 2015). Therefore, there is a large demand for cheaper RV vaccine alternatives.

Rotavirus particles are icosahedral composed of three concentric protein layers: the inner layer, containing VP2 as the scaffold arranged as dimers, together with VP1 and VP3; the intermediate layer forming the inner capsid consists of 780 VP6 protein molecules arranged in 260 trimers, and the surface layer consisting of VP7 with VP4 spikes (Desselberger *et al.*, 2009; Mena *et al*., 2006). VP6 is the most abundant protein in the particle and contributes the greatest molecular mass (Parashar *et al.*, 1998). This protein is highly immunogenic (Svensson *et al*., 1987; Estes and Cohen, 1989) and contains conserved cross-reacting epitopes (Tang *et al*., 1997), making VP6 an attractive candidate for a potential RV vaccine (Estes and Cohen, 1989). Whether antibodies directed against VP6 are neutralizing the virus, remains controversial. While there are some studies showing that anti-VP6 antibodies do not induce passive protection in mice (Coste *et al*., 2001), others reported they were able to reduce viral shedding in mice challenged with RV (Burns *et al*., 1996; Corthésy *et al.*, 2006; Schwartz-cornil *et al*., 2002). Furthermore, a murine VP6 DNA vaccine (Herrmann *et al*., 2012; Chen *et al*., 1997) as well as the *Escherichia coli* (*E. coli*) expressed chimeric protein (Choi *et al*., 1999; Choi *et al*., 2000) induced protective immunity in mice, suggesting VP6 is a promising antigen in vaccine design.

Trimerization and assembly of VP6 into different structural conformations are intrinsic properties of this 45 kDa protein (Estes *et al*., 1987). Depending on the pH, ionic strength and

divalent cation concentration, VP6 alone can self-assemble into either spherical or tubular multimeric structures (Lepault *et al*., 2001). Although irregular, VP6 can form closed particles by itself (Mathieu *et al*., 2001). In addition to rods, it can also form icosahedral or virus-like particles (VLPs) when expressed in plants, resembling the morphology of dually expressed VP2 and VP6 VLPs, as observed in the insect cell-baculovirus expression system (O'Brien *et al.*, 2000).

In addition to its application as vaccine candidate, VP6 VLPs have also been applied as drug delivery tool (Redmond *et al*., 1991; Zhao *et al*., 2011). Frenchick *et al.* (1992) demonstrated that peptides coupled to VP6 spheres elicited a greater humoral response than the peptides alone, and that the spheres were not neutralized by anti-rotavirus antibodies (Redmond *et al*., 1991; Frenchick *et al*., 1992). RV VP6 multimeric structures have been expressed in a variety of different heterologous systems: mammalian cells (Gonzalez and Affranchino, 1995), insect cell/baculovirus systems (IC-BVS) (Crawford *et al*., 1994; Bertolotti-Ciarlet *et al*., 2003), the prokaryote *E. coli* (Choi *et al*., 2004; Zhao *et al*., 2011) the eukaryotic yeast *Saccharomyces cerevisiae* (Rodríguez-Limas *et al.*, 2011), and baculovirus-silkworm (Yao *et al*., 2012). More recently, the methylotrophic yeast *Pichia pastoris (P. pastoris)* has been investigated for the intracellular co-expression of VP2, VP4 VP6 and VP7 and subsequent VLP formation (Zhang and Lou, 2011).

Due to the inability of *E. coli* to effectively express complex eukaryotic proteins and the cost associated with mammalian and insect cell lines, yeast became the preferred alternative for VLP production because of the ease of manipulation, scalability and lower production cost (Federico, 2010). Methylotrophic yeasts, such as *P. pastoris* and *Hansenula polymorpha (H. polymorpha),* possess several advantages as the ideal expression system: (i) expression of recombinant proteins is under a tightly regulated and highly productive promoters (*AOX1/AOX2*) in *P. pastoris* (Cregg *et al*., 2000) and *MOX* in *H. polymorpha* (Gellissen, 2000); (ii) they can attain high-cell densities of 150 g L^{-1} dry cell weight in bioreactors (Cereghino and Cregg 1999); (iii) relatively low levels of endogenous protein are secreted into the medium (Cereghino and Cregg, 2000); and (iv) these yeasts tend not to hyperglycosylate recombinant proteins (Sudbery, 1996), rendering them less allergenic and more suitable as pharmaceuticals. *Hansenula polymorpha* is more thermotolerant than *P. pastoris* (37°C versus 30°C), which enables faster high-yield fermentation (Hollenberg and Gellissen, 1997).

Growing *P. pastoris* and *H. polymorpha* in bioreactors at high biomass yields can produce copious amounts of heterologous protein, due to the increased transcription efficiency from the

AOX1 and *MOX* promoters (Cereghino and Cregg, 2000). Bulk biomass was obtained with fed-batch feeding of both glycerol and methanol as carbon source in fermentor cultures. Methanol metabolism requires oxygen. The expression of foreign proteins is negatively affected by oxygen depletion. Bioreactors provide a controlled environment to monitor and adjust oxygen levels in the culture medium.

During this study, we set out to establish the capacity of *P. pastoris* and *H. polymorpha* to produce the VP6 protein and to compare the levels to that produced in *E. coli*. Expression of VP6 was performed in shake flasks as well as in 1.3 L bioreactors. High VP6 concentration is important for the production of multimeric structures as higher concentrations are prone to assemble more efficiently both *in vitro* and *in vivo* (Pattenden *et al*., 2005). The recombinant VP6 protein obtained following expression in *E. coli* BL21 (DE3), was used as a baseline reference. The VP6 amino acid sequence used during this work was derived from an emerging South African RV strain, G9P[6] (N. Page, personal communication), making this VP6 protein suitable to boost local vaccine efficacy. To our knowledge, this is the first report of RV VP6 expression levels in methylotrophic yeasts *P. pastoris* and *H. polymorpha*.

Materials and methods

Strains and maintenance media

E. coli DH5α strain was used as host to maintain and amplify synthesized plasmids as well as expression vectors for both yeasts. The *E. coli* DH5α parental strain and transformants were grown at 37°C on Luria Bertani (LB) agar plates (0.5% [$g.L^{-1}$] yeast extract, 1% [$g.L^{-1}$] tryptone, 1% [$g.L^{-1}$] NaCl and 1.5% [$g.L^{-1}$] agar) containing either 50 $\mu g.mL^{-1}$ ampicilin or 100 $\mu g.mL^{-1}$ kanamycin, depending on the plasmid used. Bacteria were grown in LB culture medium (0.5% [$g.L^{-1}$] yeast extract, 1% [$g.L^{-1}$] tryptone, 1% [$g.L^{-1}$] NaCl) and all plasmid preparations for cloning, transformation and sequencing were performed using cetyltrimethyl ammonium bromide (CTAB) (Del Sal *et al*., 1988). The *P. pastoris*_GS115 strains were obtained from Invitrogen (Life Technologies) and the *H. polymorpha* NCYC 495 strain from Jan Kiel, University of Groningen, The Netherlands. Both yeast species were cultured on YPD (1% [$g.L^{-1}$] yeast extract, 2% [$g.L^{-1}$] tryptone, 1% [$g.L^{-1}$] glucose) agar plates; *P. pastoris* was incubated at 30°C and *H. polymorpha* at 37°C. The *E. coli* BL21 (DE3) strain was used as bacterial expression strain and cultured on LB agar plates, selective for ampicilin resistance.

Construction of expression cassettes

The RV VP6 sequence used in this work was derived from a prevalent South African RV strain, G9P[6] (N. Page, personal communication). All DNA manipulations were performed as outlined by Sambrook *et al*. (1989). The rotavirus *VP6* gene was double-codon-optimized for both *P. pastoris* and *H. polymorpha,* and synthesized (GeneArt, Regensburg, Germany). The commercial codon-optimized *VP6* genes were closely examined for preferred codon usage in both yeasts to further reduce the usage of rare codons. This was done using an adaptation of the codon bias index for each of the two yeasts from Sharp *et al*. (1988). The pHIPX4-HNBESX plasmid (*leu2*, kan^R) (Gietl *et al*., 1994) was used as the backbone for the construction of all plasmids transformed into the *H. polymorpha* genome and the pBLHIS-IX plasmid (*his4*, amp^R) (Cereghino *et al.,* 2001) for the construction of all plasmids transformed into the *P. pastoris* genome. Appropriate restriction sites flanking the *VP6* gene were incorporated during synthesis to enable directional sub-cloning into the relevant shuttle vectors: *Bam*HI and *Hind*III were used for cloning into pHIP4X-HNBESX, and *Eco*RI and *Xho*I into pBLHIS-IX, resulting in pHIP4X-VP6 and pBLHIS-VP6, respectively. This allowed cloning of the *VP6* gene down-stream of the *P. pastoris* and *H. polymorpha* methanol-induced promoters, AOX and MOX, respectively. Shuttle vectors were sequenced using primers pHIPX4-F and pHIPX4-R (for pHIP4X-HNBESX) (Smith *et al.,* 2012) and 5'AOX and 3'AOX (for pBLHIS-IX) from Invitrogen (Life Technologies) to verify an open reading frame of the *VP6* gene.

For *E. coli* expression, the pProEx™ HTc vector was selected to clone *VP6*. The commercial *E. coli* codon-optimized *VP6* gene was further optimized using Sharp and co-workers' codon bias index replacing rare codons in the *VP6* gene with codons commonly used by *E. coli* (Sharp *et al.,* 1988). To sub-clone the optimized *VP6* gene into pProEx™ HTc, the pGA15-VP6 plasmid was linearized with *Nde*I and pProEx™ HTc was linearized with *Hin*dIII in parallel. Following digestion for 2 h at 37°C, Klenow enzyme was added to both digestions and incubated for another 1 h to generate blunt ends. The DNA from both digestions was recovered by precipitation and the linear pGA15-VP6 and pProEx™ HTc were again digested with *Xho*I and *Sal*I, respectively. The *VP6* gene was subsequently ligated into the pProEx™ HTc plasmid using T_4 DNA ligase resulting in the expression plasmid pProEx-VP6c. The pProEx-VP6c vector was sequenced using commercial primers to verify an open reading frame of the VP6 gene. All the enzymes used for cloning were provided by Fermentas (Thermo Scientific, Inqaba Biotec). Glycerol stocks of all desired clones were prepared for storage purposes.

Bacterial transformation

One tube of chemically prepared competent *E. coli* DH5α cells per transformation was thawed on ice. Once thawed, the ligation mix (10 µl) was added to BL21 (DE3) cells and the cell-DNA mixture was incubated on ice for 30 min followed by heat shock treatment at 42°C for 90 s. To allow the cells to recover, 1 ml of LB medium was added and incubated while shaking at 37°C for 1 h. The transformation was then plated in various dilutions onto selective LB agar plates and incubated overnight (o/n) at 37°C.

Yeast transformation

Transformation into *H. polymorpha* NCYC495 was carried out by electroporation according to Faber *et al*. (1994). Prior to electroporation, plasmids were linearized by digestion with *Stu*I, precipitated and complete digestion verified using agarose gel electrophoresis. Linear DNA (0.005-1.0 µg) was added to 60 µl of cell suspension and transferred to a pre-chilled 2 mm electroporation cuvette. For transformation, the cell suspension was pulsed approximately 10 ms with a field strength of 1.5 kV, 200 Ω and 25 µF using a Bio-Rad GenePulser®II. Following transformation, yeast cells were plated onto yeast nitrogen base (YNB) selective plates (0.17% [$g.L^{-1}$] YNB, 0.25% [$g.L^{-1}$] ammonium sulphate [$(NH_4)_2SO_4$], 0.5% [$g.L^{-1}$] glucose) followed by incubation at 37°C for 3 to 10 days. Transformation of plasmids into the *P. pastoris*_KM71 genome was carried out by electroporation according to Becker and Guarenthe (1991). Plasmids were linearized by digestion with *Sac*I, precipitated and complete digestion verified using agarose gel electrophoresis. The electro-competent cells (40-50 µl) were mixed with 0.005-1.0 µg of linear pBLHIS-VP6 plasmid DNA in a 2 mm electroporation cuvette. The cells were pulsed for 10 ms with a field strength of 1.5 kV, 200 Ω and 25 µF using a Bio-Rad GenePulser®II. Following electroporation, the cells were plated onto double strength YNB plates supplemented with 0.5% [$g.L^{-1}$] $(NH_4)_2SO_4$, 1% [$g.L^{-1}$] glucose and incubated at 30°C for 3-10 days.

PCR confirmation of positive recombinants

Recombinant colonies large enough for colony PCR as well as subsequent inoculation for storage purposes were selected. Each yeast colony was transferred to a 10 µl solution containing 2.5 $mg.mL^{-1}$ Zymolyase enzyme solution (ICN Biomedicals), 1.2 M Sorbitol and 0.1 M sodium phosphate buffer, pH 7.4, and the colony suspension incubated at 37°C for a minimum of 15 min. The PCRs were performed in a total volume of 20 µl, containing 0.5 µM of each primer, 0.05 U Taq DNA

Polymerase (NEB), 2 µl 10x ThermoPol buffer (NEB), 1.5 mM $MgCl_2$ and 1 µl colony mix as DNA template. Primers *5'AOX1* and 3' *AOX1* (Invitrogen) were used for screening *P. pastoris* transformants and pHIPX_F and pHIPX_R (Smith *et al.*, 2012) for *H. polymorpha*. The PCR conditions were: one cycle at 95 °C for 5 min, followed by 35 cycles at 95 °C, for 10 s, 50 °C (55 °C for *P. pastoris*) for 40 s, and 72 °C for 1 min 30 s, and a final elongation step at 72°C for 4 min.

Bacterial recombinants were screened using restriction enzyme analysis following inoculation from selective plates. Correct band patterns following agarose gel electrophoresis, verified the presence of *VP6* in plasmid pProEx™ HTc.

Gene expressions and growth conditions in shake flasks

The *H. polymorpha* colonies were cultured in 25 ml mineral medium (Van Dijken, Otto and Harder, 1976). Each recombinant selected was inoculated into three separate shake flasks with mineral media containing vitamin solution (Van Zutphen *et al*., 2008) and 5 mg ml^{-1} glucose, and incubated overnight at 37°C with shaking at 200 rpm. The following morning, the culture was diluted to an A_{600} of 0.1 and grown to the mid-exponential growth phase. This was repeated until the doubling time of wild-type is almost 1 h (Van Zutphen *et al*., 2008). As a negative control, wild type *H. polymorpha* NCYC495 containing only the pHIP4X-HNBESX plasmid was included. After pre-culturing, mid-exponential cells were diluted into minimal medium containing 0.5% [v/v] methanol to an A_{600} of 0.1 so as to induce expression. Samples for protein extraction were taken at 24 h, 48 h and 72 h.

Culturing and induction of protein expression in *P. pastoris*_GS115 cultures was according to the protocol described in the *Pichia* Expression kit (Invitrogen). Briefly, the recombinant strains were cultured, in triplicate, in 20 ml buffered glycerol-complex medium (BMGY: 1% [v/v] glycerol, 1% [$g.L^{-1}$] yeast extract, 2% [$g.L^{-1}$] peptone, 100 mM potassium phosphate, pH 6, 1.34% [$g.L^{-1}$] yeast nitrogen base and 4 x 10^{-5}% biotin) to generate biomass. When sufficient biomass had been obtained, 75 ml buffered methanol-complex medium (BMMY: 0.5% methanol, 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% yeast nitrogen base and 4 x 10^{-5}% biotin) in 500 ml baffled flasks covered with only a cotton wool plug to encourage O_2 diffusion was used for induction purposes. Cultures were incubated at 30°C while shaking at 200 rpm for both biomass generation and induction. Methanol (0.5% [v/v]) was added every 24 h to maintain induction. As a negative control, wild type *P. pastoris*_GS115 containing only the pBLHIS-IX plasmid was included. Samples for protein extraction were taken at 24 h, 48 h and 72 h.

Single colonies of recombinant BL21 (DE3) cells were cultured o/n at 37°C and agitation in 5 ml LB broth with 50 µg ml^{-1} ampicilin. The following day, cultures were diluted 1/100 in 200 ml LB and grown until an A_{600} of 0.5–1.0, followed by the addition of isopropyl β-D-thiogalactosidase (IPTG) to a final concentration of 0.6 mM to induce expression. Alternatively, recombinant BL21 (DE3) cells were also cultured in 120 ml phosphate/citric acid buffer, pH 6.3, used for *E. coli* high cell-density fermentation (Korz *et al.*, 1995). The medium consist of 0.1 M KH_2PO_4, 30 mM $(NH_4)_2HPO_4$ and 9 mM citric acid, supplemented with 3% glucose, 10 M $MgSO_4$, 1x trace metal solution, 15 µM Thiamine solution and 100 µg ml^{-1} ampicilin. Once the A_{600} reached 0.6, 1 mM IPTG is added to initiate protein production.

Yeast fermentation cultures

All fermentations were conducted in 1.3 L New Brunswick Bioreactors. Pre-cultures were prepared by inoculating a streak of colonies from YPD (1% [$g.L^{-1}$] yeast extract, 2% [$g.L^{-1}$] tryptone, 2% [$g.L^{-1}$] glucose) agar plates into 0.04 L of buffered glycerol complex medium (BMGY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4 x 10^{-5}% biotin, 1% glycerol) in baffled shake flasks. Pre-cultures were incubated overnight at either 30°C (for *P. pastoris)* or 37°C (for *H. polymorpha)* at 200 rpm. Cultures were then diluted to an A_{600} of 0.1 in an equal amount of fresh BMGY medium and subsequently cultured overnight at the same conditions to an A_{600}=2-6. This culture was used to inoculate 0.36 L of fermentation medium in the bioreactor to obtain a starting A_{600} = 0.2-0.6.

Both *P. pastoris* and *H. polymorpha* strains were grown in triplicate in fermentation basalt salt medium, supplemented with 4% [v/v] glycerol and 4.35 ml PTM_1 trace salts/L medium, according to the *Pichia* Fermentation protocol (Invitrogen Life Technologies, 2002). Culture conditions were maintained as follows: 30°C for *P. pastoris* and 37°C for *H. polymorpha*, pH 5.O with 28% ammonium hydroxide (NH_4OH) aeration rate of 1.0 volume of oxygen per volume of fermentation culture per minute (vvm) air and dissolved oxygen (DO) = 30%. DO is sensed by the DO electrode and controlled by a cascade effect between agitation rate (200-1000 rpm) and aeration rate (1 vvm), which is maintained by the proportional (P) and integral (I) controller. Once the glycerol is depleted from the glycerol batch phase (Day 1, 24 hrs post-inoculation), as indicated by a spike in the DO levels, a 50% [v/v] glycerol feed supplemented with 12 ml PTM_1 trace salts L^{-1}, is initiated (Day 2, 48 hrs post-inoculation). Glycerol feeding was carried out in a fed-batch mode based on the DO stat method, also referred to as ‘demand-feed’. This entails the controlled addition

of glycerol depending on the DO tension. As the yeast metabolized actively, glycerol became depleted and the DO tension began to rise. Once the DO > 30%, the glycerol feed was switched on. In the presence of glycerol, the metabolic pathway became activated and the DO began to decrease. When the DO dropped below 30%, the glycerol feed was switched off. The DO levels were restored to 30% by a mixture of agitation cascade and oxygen supplementation. As the DO increased beyond 30%, the glycerol feed switched on again. A cellular yield of 180-220 g L^{-1} wet cell weight (WCW) indicated the end of the glycerol fed-batch phase. The glycerol feed was terminated and replaced with 100% [v/v] methanol (Day 3, 72 hrs post inoculation; Day 1 induction), supplemented with 12 ml PTM_1 trace salts L^{-1}, to start the methanol fed-batch phase and proceeded for 5 consecutive days or until the WCW started to decrease. Methanol feeding in *P. pastoris* and *H. polymorpha* cultures was controlled similarly using the DO stat method.

Bacterial fermentation cultures

A single colony of *E. coli* was inoculated in triplicate in three 12 ml LB medium with 50 µg ml^{-1} ampicilin in a 50 ml tube and incubated overnight at 200 rpm and 37°C. Two of the cultures were spun for 10 min at 4000 rpm, their supernatant was discarded and the pellets were resuspended in the remaining culture. This culture was used to inoculate 630 ml 1x phosphate/citric buffer (0.1 M KH_2PO_4, 0.03 M $(NH_4)_2HPO_4$, 0.009 M citric acid), supplemented with 3% [v/v] glucose, 0.01 M $MgSO_4$, 1x trace metal solution, 0.015 mM Thiamine and 50 µg ml^{-1} ampicilin, in a 1.3-L bioreactor. Except for a pH of 6.8 and fermentation temperature of 37°C, the culturing conditions were identical to that of yeast.

The end of the batch phase was indicated by an increase in the DO levels (24 hours post-inoculation), followed by the start of the fed-batch phase. The 70% [v/v] glucose feed, enriched with 0.16 M $MgSO_4$, 1x trace metal solution, 0.14 mM Thiamine and 50 µg ml^{-1} ampicilin, lasted for 8 hours. Towards the end of the fed-batch phase (32 hours post-inoculation), the culturing temperature was lowered to 20°C and protein expression was induced by the addition of 3 ml 320 mg ml^{-1} yeast extract and a final IPTG concentration of 0.8 mM.

Crude protein extractions and SDS-PAGE

After 72 h of growth, triplicate yeast samples of 1 ml were taken to determine the wet cell weight (WCW) and dry cell weight (DCW) of each expression. DCW was determined by drying the WCW pellets at 60°C for 3 days or until no more weight loss was noted. In addition, 60 mg WCW from

each yeast culture was weighed for crude cell extracts. Cells were lysed and total protein was extracted and prepared for SDS PAGE gel electrophoresis as previously described by Smith *et al*. (2012). The first bacterial sample was taken within one hour after IPTG induction, and then hourly for 6 hours. Due to the low biomass obtained in bacterial expressions, only 6 mg and 10 mg from shake flask and bioreactor expressions, respectively, were used to prepare protein extracts. The bacterial WCW was resuspended in 2x SDS PAGE gel electrophoresis buffer (4% SDS, 10% [v/v] β-mercaptoethanol, 20% [v/v] glycerol, 0.002% [$g.L^{-1}$] bromophenol blue and 0.1 M Tris-HCl, pH 6.8) and boiled for 5 min. When the solubility of the VP6 protein was determined, 2 ml of bacterial culture was collected and centrifuged to isolate the bacterial cells. The cells were resuspended in suspension buffer (1 × PBS, pH 8.0, 100 mM NaCl, 0.2% [v/v] Triton X-100, 1 mM PMSF) followed by the addition of 1.5 mg [$g.L^{-1}$] lysozyme and incubated at room temperature for 20 min. Centrifugation at 10000 x g for 10 min at 4°C yielded two phases, of which the upper soluble phase was aspirated and saved for further use. The bottom phase was washed twice in 500 µl milli-Q water and the pellet resuspended in solubilization buffer (20 mM phosphate buffer, pH 8.0, 300 mM NaCl, 2% [v/v] SDS, 2 mM DTT, 1% [v/v] Triton X-100). After centrifugation for 10 min at maximum, the supernatant containing the insoluble protein fraction was aspirated and kept for further analysis (Drummond, 2011). Prior to SDS PAGE electrophoresis, equal volume of 2x SDS PAGE electrophoresis buffer was added to the soluble and insoluble fractions followed by boiling for 5 min.

SDS-polyacrylamide gel electrophoresis for western blot and densitometric analysis was performed as described previously (Smith *et al.*, 2012). Where bacterial proteins were analyzed to determine the solubility of the proteins, the acrylamide gels were stained with Coomassie Brilliant blue for 1 h followed by destaining with 50% [v/v] methanol and 10% [v/v] acetic acid until no more background was visible. For quantification purposes, VP6 from a crude lysate of recombinant SF9 cells at a predetermined concentration were used as a reference protein (provided by Albie van Dyk, North West University).

Western blot analysis

Proteins separated on acrylamide gels were transferred to nitrocellulose membranes (BioTrace™ NT, PALL) by electro-blotting using the Semi-Phor blotting apparatus (Hoefer Scientific Instruments) at 50 mA per minigel for 80 min (Khyse-Andersen, 1984). The membranes were blocked overnight at room temperature with 5% skim milk (Sigma) in 1x TBST (10 mM Tris-HCl,

pH 8.0, 150 mM NaCl and 0.05% [v/v] Tween-20) with gentle agitation. The following day, the membranes were probed with mouse anti-rotavirus VP6 antibodies (Cat no. 227144, US Biological, Massachusetts) diluted 1:5000 for 2 h, followed by another 2 h incubation with alkaline phosphatase-conjugated goat anti-mouse antibodies (Santa Cruz Biotechnology) diluted (1:5000). After each antibody incubation, membranes were washed three times for 5 min at room temperature in 1x TBST with gentle agitation. Prior to detection, membranes were equilibrated in 1 x detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.01 M $MgCl_2$, pH 9.5) for 5 min. Detection was carried out for 10 min using 1-step nitrobluetetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Fermentas).

Quantitative western blot

The same quantity of cells (60 mg of yeast cells, 6 mg of bacterial cells) were collected from every time point to ensure we do not introduce any bias with regards to VP6 protein present as a factor of the sampling. Equal volumes of cell lysate from every time point were loaded and immuno-detected using VP6 antibodies. Each blot was visualized for the gradual increase and decrease in VP6 band intensity over time. In case any irregular intensity was noted during the course of induction, the extraction was repeated. Once VP6 proteins were positively identified during Western Blot analysis, the protein yield from each time point in each culture was quantified in triplicate using densitometry. VP6 proteins expressed in SF9 cells were used as a reference for the determination of protein concentrations. Dilutions of the VP6 reference were analyzed in parallel with dilutions of yeast-expressed VP6 on the same SDS-acrylamide gel and nitrocellulose membrane. The amount of protein was normalized by comparing to known concentrations of SF9-expressed VP6. A Canon Canoscan LIDE 100 scanner was used to scan the nitrocellulose membranes at 300 dpi on grayscale. The area and intensity of protein species were quantified by densitometry analysis (Abramoff, Magalhães and Ram, 2004) as outlined in http://www.lukemiller.org/journal/2007/08/quantifying-western-blots-without.html. Shake flask expressions were performed in triplicate and each shake flask expression was quantified in triplicate. A simple T-test was performed to determine the significance between the protein yields in *P. pastoris* versus *H. polymorpha* and *p* values of less than 0.05 were considered significant.

Results and Discussion

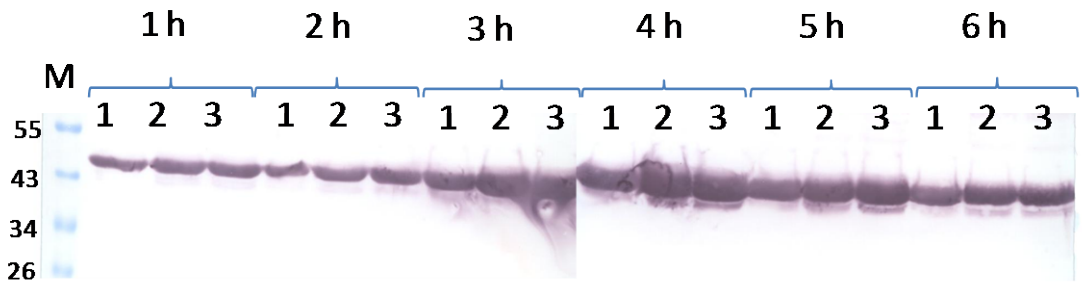
VP6 shake flask expressions

Recombinant *E. coli* BL21 (DE3) strains harboring the pProEx-VP6c plasmid were cultured and expression of the VP6 protein was monitored after induction with IPTG. A time course experiment was performed to monitor when VP6 production occurred, sampling hourly after induction for a period of 6 h. Production was noticed 1 hour after induction and peaked at 3-4 hours after induction, in both LB medium (not shown) and Fermentation medium (Figure 5.1A). After that, protein concentrations stayed relative constant with little decrease.

Once successful production of VP6 using *E. coli* BL21 (DE3) was observed, we determined the extent of VP6 protein solubility. *E. coli* BL21 (DE3) cells were lysed in an aqueous buffer to separate the soluble from the insoluble proteins. SDS PAGE analysis revealed that no induction occurred prior to the addition of IPTG (Figure 5.1B, lanes 2 and 3), but production of the VP6 protein followed after the addition of IPTG (Figure 5.1B, lane 7). However, all of the VP6 protein produced by *E. coli* BL21 (DE3) was insoluble (Figure 5.1B, lane 4).

Equal volumes of cell lysate, representing equal biomasses, from every time point were loaded to verify successful extraction of VP6 (Figure 5.2A). Various dilutions of the SF9 crude lysate containing the recombinant expressed VP6 reference protein were separated on SDS-PAGE gels and Western blots were performed to determine the optimal range for use as a standard curve (Figures 5.2B, C). The VP6 reference was subsequently loaded together with samples of crude lysate each time quantification was performed. Analysis of the Western blots involved plotting the concentrations of VP6 (ng) reference against the area under the profile plot for each band as assigned by ImageJ and the equation of the resulting standard curve was used to calculate the respective concentration of VP6 protein (ng μl^{-1}) visible on the blot. Protein samples were diluted to ensure that the area under the profile plot of each sample fell within the range determined by the standard curve.

A



B

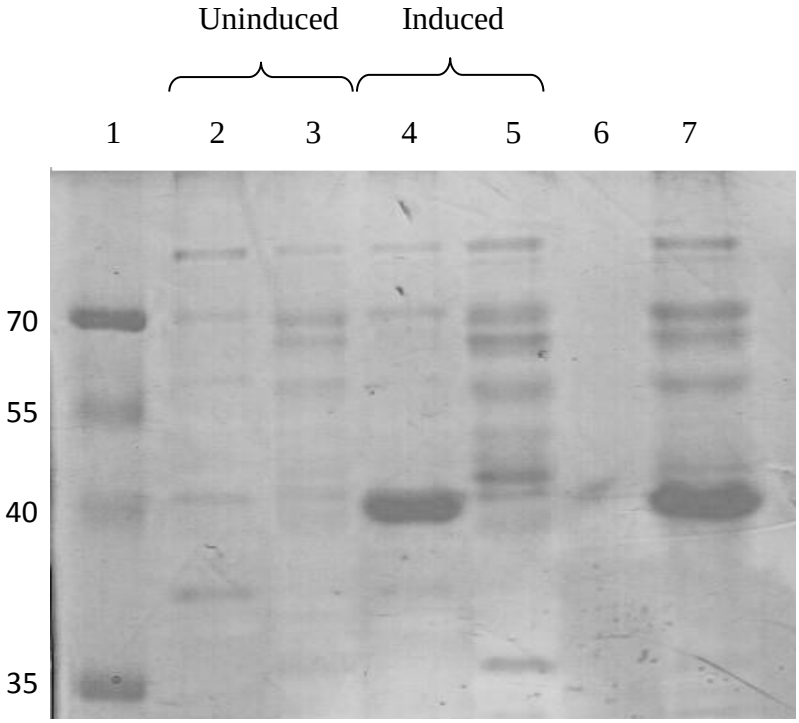


Fig. 5.1. VP6 expressed in *E. coli* BL21 (DE3). (A) Time course analysis of VP6 expressed in triplicate, cultured in high-cell density medium. Lane M: Pageruler™ (Thermo scientific); lane 1, 2 and 3: culture1, 2 and 3;1 h, 2 h, 3 h, 4 h, 5 h and 6 hours post-induction of VP6, respectively. (B) SDS PAGE analysis of the soluble and insoluble fractions of VP6. Lane 1: Pageruler™ (Thermo scientific); Lane 2: Uninduced insoluble fraction; Lane 3: Uninduced soluble fraction; Lane 4: Induced insoluble fraction; Lane 5: Induced soluble fraction; Lane 6: blank; Lane 7: Total protein fraction.

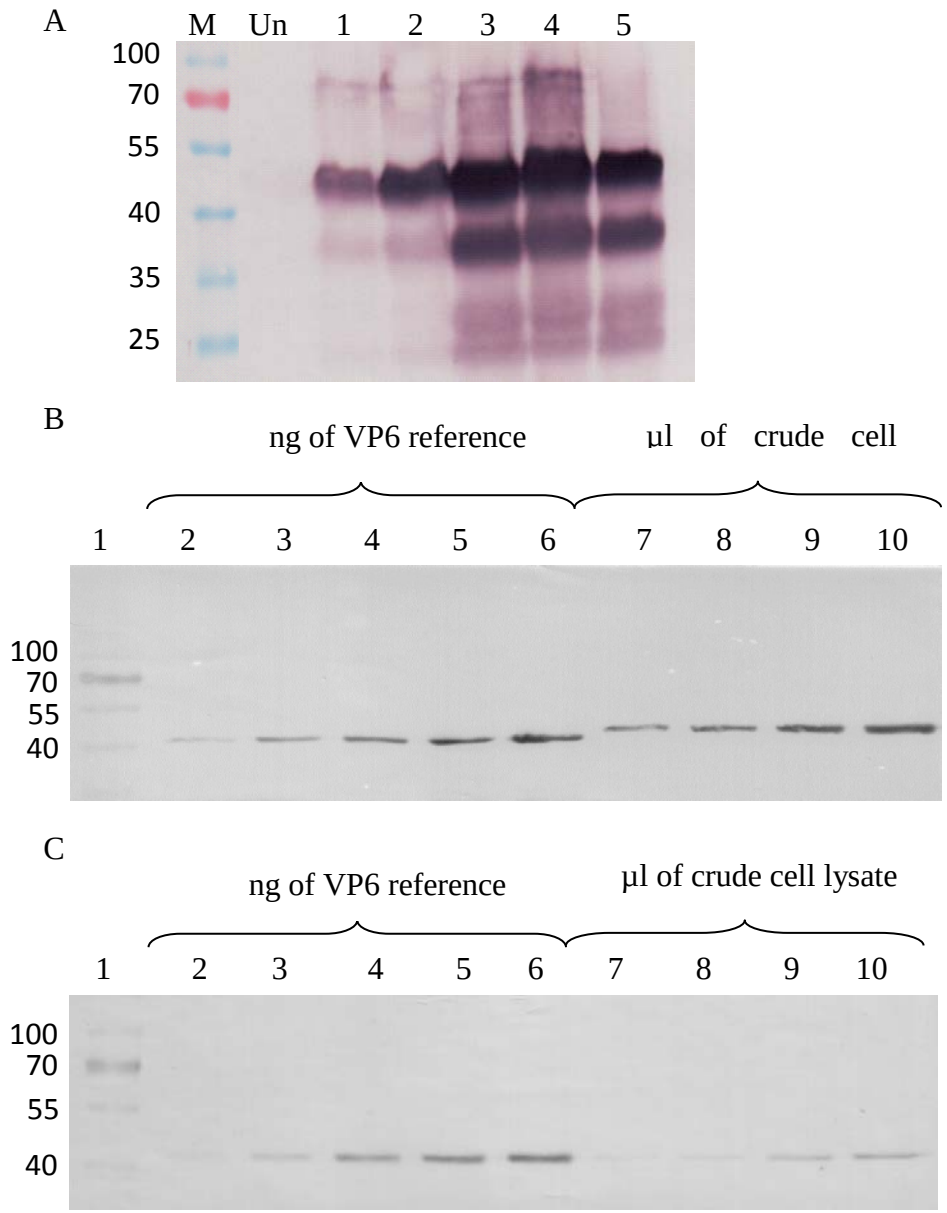


Fig. 5.2. Western blot of VP6 expressed in shake-flasks for quantification by densitometry. (A) Expressed by recombinant *P. pastoris_GS115*. Lane 1, Pageruler^{TM} (Thermo scientific); Lane 2, (uninduced), Equal volume yeast cell lysate before methanol induction; Lane 3-7, Equal volumes yeast cell lysates from day 1 to 5 post methanol induction. (B) Expressed by recombinant *E. coli* BL21 (DE3). Lane 1, Pageruler™ (Thermo scientific); lane 2, 80ng; lane 3, 160ng; lane 4, 320ng; lane 5, 480ng; Lane 6, 640 ng of VP6 reference; lane 7, 2.0 µl; lane 8, 3.0 µl; lane 9, 4.0µl; lane 10, 5.0µl; of *E. coli* VP6 crude cell lysate. (C) Expressed by recombinant *P. pastoris_GS115*. Lane 1, Pageruler™ (Thermo scientific); lane 2, 72ng; lane 3, 144ng; lane 4, 288ng; lane 5, 433ng; Lane 6, 578 ng of VP6 reference; lane 7, 0.5 µl; lane 8, 1.0 µl; lane 9, 2.0µl; lane 10, 3.0µl; of *P. pastoris* VP6 crude cell lysate.

Both volumetric and specific productivity of each of the production platforms evaluated in shake flasks are shown in Table 1. In both instances, *E. coli* significantly outperformed the two yeasts platforms in both growth media. *E. coli* showed the highest volumetric levels at an average of

97.60 mg VP6.L^{-1}.h^{-1} in LB broth and 36.58 mg.L^{-1}.h^{-1} in Fermentation medium. Analysis of the specific production revealed that *E. coli* produced an average 139.43 g VP6.kg^{-1}DCW.h^{-1} in LB media and 34.53 g.kg^{-1}.h^{-1} in Fermentation medium. In yeast, the average specific production was very much similar with *H. polymorpha* and *P. pastoris* yielding 0.174 g.kg^{-1}DCW.h^{-1} and 0.185 g.kg^{-1}.h^{-1}, respectively. The average volumetric VP6 productivity attained by *H. polymorpha* was the lowest at 0.37 mg.L^{-1}.h^{-1}, whereas *P. pastoris* produced almost five times more at an average of 1.688 mg.L^{-1}.h^{-1}. Comparing the VP6 concentration in yeasts, *P. pastoris* had an almost five times higher VP6 concentration than *H. polymorpha* (121.563 mg.L^{-1} versus 26.630 mg.L^{-1}). Since the different strains were cultured in different volumes, the amount of biomass produced in each expression system was normalized for the same volume (Table 5.1). The yeast strains produced the highest biomass, 2.13 g.L^{-1} and 9.14 g.L^{-1} for *H. polymorpha* and *P. pastoris*, respectively, compared to *E. coli*, 0.700 g.L^{-1} and 1.400 g.L^{-1}, with the lowest specific VP6 yield. Using both types of culture media, *E. coli* showed the highest VP6 yield ($Y_{p/x}$) per unit biomass (Table 5.1).

Scaled-up VP6 quantification in bioreactors

All three expression systems were evaluated in terms of their productivity in bioreactors. The latter provided a controlled environment for optimum expression of recombinant proteins. The bacterial strain, using only Fermentation medium, outperformed the two yeast systems with a volumetric productivity of 93.91 mg.L^{-1}.h^{-1} compared to 12.91 mg.L^{-1}.h^{-1} and 26.48 mg.L^{-1}.h^{-1} for *P. pastoris* and *H. polymorpha*, respectively (Table 5.1). Comparing specific productivity levels, *E. coli* had the highest with 3.36 g.kg^{-1}.h^{-1} compared to 0.09 g.kg^{-1}.h^{-1} (*P. pastoris*) and 0.293 g.kg^{-1}.h^{-1} (*H. polymorpha*). Volumetric productivity of all three expression systems and the specific productivity of only *H. polymorpha* were higher in bioreactors than in shake flasks. Fermentation in bioreactors resulted in higher concentrations of biomass for all three expressions compared to that obtained in shake flasks: 27.933 g.L^{-1} DCW (*E. coli*), 90.58 g.L^{-1} DCW (*H. polymorpha*) and 151.12 g.L^{-1} DCW (*P. pastoris*). *H. polymorpha's* biomass increased more than 45 times in bioreactors, whereas the biomass in *P. pastoris* and *E. coli* increased up to 20 times. Unlike in shake flasks, the lowest biomass concentration in bioreactors did not result in the highest VP6 concentrations. *E. coli* produced double the VP6 yield per unit biomass of *P. pastoris* (0.017 gVP6.g DCW^{-1} versus 0.009 g VP6.g DCW^{-1}), and *H. polymorpha* double the VP6 yield of *E. coli* (0.037 g VP6.g DCW^{-1} versus 0.017 g VP6.g DCW^{-1}) (Table 5.1).

Although rotavirus infection affects the developing world to a larger extent, it remains a high-cost global threat (Desselberger *et al*., 2009; Parashar, Bresee and Glass, 2003) that has been prioritized as a target for vaccine development (Ward *et al*., 2008). Unresolved safety concerns regarding living rotavirus vaccines have shifted the focus towards non-living vaccines as next generation candidates (Ward *et al*., 2008). Despite the fact that VP6 is not considered the primary antigen for protective immunity, studies have shown that this highly immunogenic protein does provide protection even though the mechanism is not fully understood (Corthésy *et al.,* 2006). Therefore, VP6 is an ideal candidate to be used as a booster to the current vaccine regime in high burden countries. Recently, the expression of VP2/4/6/7 VLP's was reported in *P. pastoris*, but not in *H. polymorpha*. Very few literature exists regarding VP6 production in *E. coli* reporting low yields (Choi *et al*., 2004; Zhao *et al*., 2011). This urged us to include *E. coli* thereby establishing the production potential of this well-exploited expression system and use the yield as a reference for comparison to that found in methylotrophic yeast.

Table 5.1. Different parameters of shake flask cultures as well as scaled-up fermentations in bioreactors for VP6 producing strains.

Parameter	Shake flask expressions				Up-scaled fermentation in 1.3L bioreactors		
	E coli LB media (0.2L)	*E coli* Fermentation media (0.12L)	*H polymorpha* Mineral media (0.025L)	*P pastoris* BMGY/BMMY (0.075L)	*E coli* Fermentation media (0.725L)	*H polymorpha* Mineral media (0.784L)	*P pastoris* BMGY/BMMY (0.883L)
OD_{600}	4.300	3.210	4.290	14.600	63.967	453.167	391.833
VP6 (mg/L)	292.799	186.931	26.630	121.563	469.572	3350.717	1432.830
Biomass (g/L)	0.700	1.400	2.130	9.140	27.933	90.577	151.123
#Yp/x (g VP6/g DCW)	0.418	0.105	0.013	0.013	0.017	0.037	0.009
Total volume MeOH (ml) added	-	-	0.0004	0.001	-	293.333	572.000
Yp/s (g VP6/g MeOH)	-	-	2.242	10.233	-	0.0114	0.0029
Volumetric productivity ($mg/L.h^{-1}$)	97.600	36.575	0.370	1.688	93.914	26.480	12.905
Specific productivity ($g/kg.h^{-1}$)	139.428	34.527	0.174	0.185	3.362	0.293	0.085

Yield = mass VP6 product produced per mass dry cell weight

*Yield = mass VP6 product produced per mass carbon source (methanol)

Despite less biomass, the VP6 concentrations produced by *E. coli* in shake flasks using both LB and Fermentation medium were higher than that obtained in yeast. This is also evident in the higher mass VP6 per mass dry cell weight ($Y_{p/x}$) obtained in *E. coli* compared to yeast. LB medium support cell density of *E. coli* up to an OD_{600} of 10. This was evident as high biomass and VP6 concentrations were obtained in LB shake flask cultures (Table 5.1) with an average A_{600} = 4.30 compare to 3.21 in more complex Fermentation medium (Table 5.1). Common growth medium such as LB cannot sustain high density cell growth in bioreactors with an OD_{600} exceeding 50; hence the reason why complex medium was used for high cell density fermentation. The latter is buffered by phosphates and organic salts, keeping the pH neutral for optimal protein expression (Expression Technologies Inc. 2003).

The huge difference in VP6 yield between shake flasks and controlled reactor *E. coli* cultures (Table 5.1), can be attributed to the different absorbance at which IPTG induction was initiated. High cell-density fermentation of *E. coli* entailed a batch phase during which the culture increased in biomass. The end of this phase was indicated by a sharp increase in DO as glucose levels started to drop. Biomass was further increased with the addition of glucose during an 8-hour fed-batch phase followed by IPTG induction at an average biomass of 23.456 g $DCW.L^{-1}$ (OD = 45.861). Bioreactor fermentations allowed constant growth conditions such as set temperature and DO levels as well as the fixed rate of glucose supplementation as carbon source. In contrast, the DO levels in shake-flask cultures was not maintained constant, glucose levels were not supplemented and induction was started only at an average biomass of 0.700 g $DCW.L^{-1}$ (OD = 0.748).

We monitored the stability of VP6 protein production following induction in *E. coli*. Ernst and Stroup (1988) expressed VP6 in *E. coli* using the JM10 strain and showed VP6 protein production peaked during the initial 3 h after induction with IPTG, followed by a sharp decline in VP6 protein until nothing was visible after 7 h. We noted a similar trend for the first 4 h, followed by a steady decline in VP6 yield (Figure 5.2A). The final VP6 concentrations produced in *E. coli* BL21 (DE3) culture were the highest ever reported for any culture volume, yielding an average concentration of 469.57 mg L^{-1} in bioreactors, volumetric production of 97.60 mg L^{-1} h^{-1} and specific production of 139.43 g kg^{-1} h^{-1} DCW in shake flasks (Table 5.1). Choi *et al*. (2004) produced 2 mg L^{-1} and 20 mg L^{-1}, using a codon-optimized VP6 in *E. coli* BL21 (DE3) and

Rosetta (DE3) placI, respectively. Similar levels of 20 mg L^{-1} were reported by Zhao *et al.* (2011) using *E. coli* BL21 (DE3) while we produced up to 292.799 mg VP6 per liter culture (Table 5.2). One possible explanation for this discrepancy in yield is the difference in biomass between our *E. coli* expressions and theirs. Although not very clear from the literature, it seems that induction with IPTG was started at a much lower OD (0.500 as opposed to 0.748 in our study) and maintained for an OD of 1.500 compared to 4.300 in this study.

Table 5.2. Rotavirus VP6 concentrations obtained in other studies compared to current study.

Authors	VP6 levels ($mg.L^{-1}$) obtained in *E. coli*	
	BL21 strain	Rosetta strain
Choi *et al.* 2004	2	20
Zhao *et al.* 2011	20	-
This study	293	-

Upon investigating the solubility of the VP6 produced by *E. coli*, we found that all the protein was insoluble (Figure 5.1B). This was in agreement with the findings documented by Aijaz and Rao (1996), where they described that the VP6 they produced in *E. coli* BL21 (DE3) formed inclusion bodies immediately following synthesis. The insoluble nature of VP6 in *E. coli* is likely due to the native trimetric conformation assumed by the protein (Mathieu *et al*., 2001), which might prove too difficult for *E. coli* to process. Despite the many advantages of heterologous expression in *E. coli*, the major disadvantage is the accumulation of partially folded or misfolded proteins during over-expression. Protein folding is a challenge in the highly reductive environment of the bacterial cytosol, where protein translation occur at a fast rate (Lorimer 1996). During over-expression of recombinant proteins in *E. coli*, folding modulators are required for large multi-domain proteins. The inability to interact with these modulators or to perform several eukaryotic post-translational modifications, result in the assembly of these insoluble proteins in inclusion bodies (Baneyx and Mujacic 2004).

Inclusion bodies are classified as either amorphous aggregates or may contain ordered structural segments of cross-β structures (Sawaya *et al.*, 2007). The latter resembles amyloid structures, which are associated with pathological disorders such as Alzheimer and Parkinson disease. Aggregation occurs when the intramolecular hydrophobic surfaces of the structural subunits of a partially folded protein interact with the intermolecular surface of neighboring subunits. This results in the exposure of hydrophobic areas, which are hidden in the native form

of the protein. These exposed hydrophobic areas have a very strong tendency for aggregation (Fink, 1998). The fact that the expressed VP6 protein was recovered from the insoluble part of the bacterial cell lysate, indicated that the protein was also trapped in inclusion bodies or amyloid structures. The rotavirus VP6 protein has a distinct β-barrel domain (Mathieu *et al.*, 2001) that predisposed for amyloid formation. Zhao *et. al.* (2011) reported the successful *in vitro* renaturation of VP6 followed by self-assembly into VLP's. They showed that VP6 can be prepared in large scale from *E. coli*, their inclusion bodies were solubilized and the VP6 was retrieved, refolded and self-assembled into VLP's (Zhao *et al.*, 2011).

However, recovering recombinant proteins from inclusion bodies entails a couple of cumbersome steps. Firstly, bacterial cells are lysed and inclusion bodies are separated from cell lysates by low-speed centrifugation. The latter step also sediments cell wall and outer membrane components, which are then subsequently removed by various detergent washes. Recombinant proteins are solubilized and folded into native and biological active proteins. Refolded proteins are separated by SDS-PAGE and the VP6 band is cut from the gel; VP6 is removed from the gel band by dialysis and concentrated. Alternatively, the unfolded protein can be further purified by gel filtration. Refolding recombinant proteins from inclusion bodies can be challenging and yields of correctly folded proteins can be low (Lilie, Schwarz and Rudolp, 1998). In contrast to soluble intracellular VP6 proteins, host cells are lysed and the supernatant is subjected to ammonium sulphate precipitation. After clarification, the preparation is loaded onto a size-exclusion chromatography column followed by anion ion-exchange chromatography (Kar and Roy, 2003).

The successful production of VP6 as part of a VLP in *S. cerevisiae* (Rodríguez-Limas *et al.*, 2011), prompted us to investigate the potential of *P. pastoris* and *H. polymorpha* to produce VP6 in shake flasks. The concentration of VP6, produced in shake flasks, in *P. pastoris* (121.56 mg L^{-1}) was much higher than that observed in *H. polymorpha* (26.63 mg L^{-1}) (Table 5.1). Both the yeast strains reached higher cell concentrations than *E. coli*, but produced VP6 at significantly lower concentrations. VP6 products were formed along with the growth of the microbial cells after induction with either IPTG or methanol, depending on the expression system (Figure 5.3). Product concentration is almost directly proportional to biomass in all three expression systems. There appears to be a notable trend in both our hands and that of other researchers when comparing the production potential between *H. polymorpha* and *P. pastoris* in shake-flask cultures using the same protein. In a previous study expressing human

papillomavirus (HPV) in shake flasks, we showed twice as much production of the target protein in *P. pastoris* (Smith *et al*., 2012). In another study, Mack *et al*. (2009) also compared the two platforms by expressing two mammalian proteins, showing that *P. pastoris* produced significantly more than *H. polymorpha* in both cases. Though only a hand-full of direct comparisons have been made between the two systems to date, it does appear as though *P. pastoris* outperforms *H. polymorpha* in shake-flask cultures. To obtain a true reflection in production potential and efficiency we determined the specific production of the two systems to dateand observed that there was no significant difference in the ability of *H. polymorpha* (0.17 g kg^{-1} DCW h^{-1}) and *P. pastoris* (0.19 g kg^{-1} DCW h^{-1}) to produce VP6 (Table 5.1). According to the methodology of Van Dijken, Otto and Harder (1976), culture volumes for *H. polymorpha* shake flask expressions (0.025 L) are three times less than the culture volumes for *P. pastoris* (0.075 L). Due to the nature of this expression, *H. polymorpha* yields more than 4-times lower cell concentration, measured in dry weight. Larger culturing volumes also explain the higher volumetric productivity observed for *P. pastoris* in shake flasks as expression last for the same duration in both yeast systems.

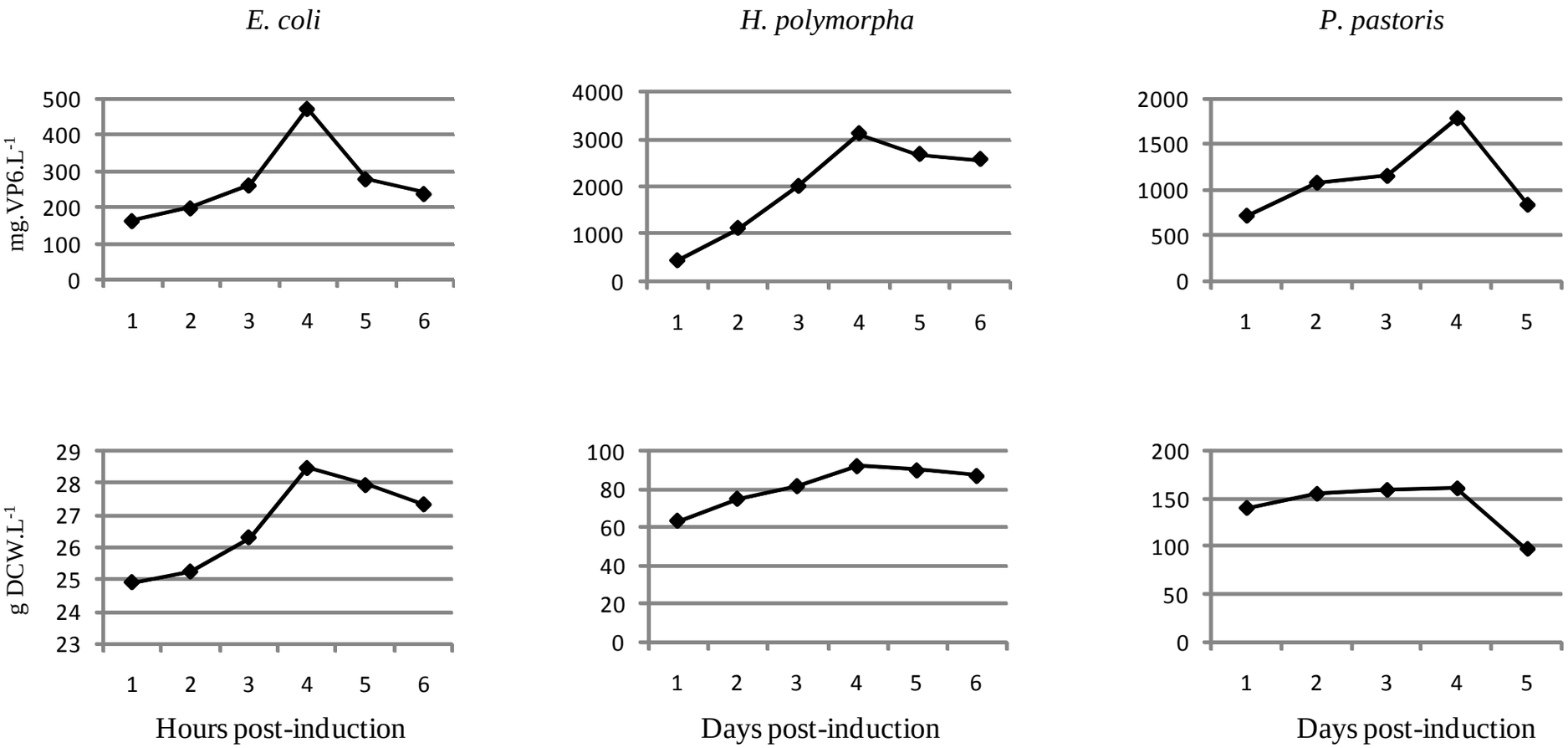


Fig. 5.3. Growth kinetics of *E. coli*, *H. polymorpha* and *P. Pastoris*. Bacterial cells were sampled every hour for 6 hours and yeast cells every 24 h for 5-6 days for biomass concentration (lower panel). Simultaneously, cell samples were lysed and rotavirus VP6 concentrations were determined (upper level) by Western blot analysis.

The true potential of the two yeast platforms was investigated with bioreactor studies. Recombinant protein expression in bioreactor cultures of methylotrophic yeasts is usually done using a three-stage process (Schenk *et al.*, 2007). The first is the batch phase with an unlimited repressing carbon source such as glycerol, followed by the fed-batch phase, when the carbon source is limited. During these first two phases, the biomass is increased. During the third phase, the yeasts cells are induced using methanol to express the protein. Due to the increased transcription efficiency from the *AOX1* promoter in fermentation cultures, the level of transcription can be up to 3-5 times greater in cells fed with methanol at growth-limiting rates compared to cells grown in excess methanol (Cereghino and Cregg, 2000). This phenomenon is evident in the increased biomass and VP6 concentrations of the bioreactor cultures compared to that of shake flasks (Table 5.1). The scaled up VP6 production in bioreactors was driven by the increase in cell growth of the host. Except for *H. polymorpha* which is marginally higher, the VP6 yield obtained per unit biomass in the rest of the scaled-up expressions is less than that obtained in shake flasks expression. Expressing rotavirus VP6 proteins in a controlled environment such as bioreactors, increased the volumetric productivity for all expression systems, but decreased specific productivity, except for *H. polymorpha*. In contrast to what has been observed thus far in shake flasks, *H. polymorpha* produced the highest VP6 concentration and the highest VP6 yield per unit biomass, of the three microbial systems compared. Between the two yeast systems, *H. polymorpha* also showed higher volumetric as well as specific productivity than *P. pastoris.* The production levels obtained with the yeasts under bioreactor conditions were significantly larger than obtained with *E. coli* in either shake flasks or bioreactors.

Since methanol is used to induce the production process, the amount of methanol used plays an important role in determining the expression platform of choice. The *P. pastoris* GS115 strain has a methanol utilization plus phenotype (Mut^+), which is known to utilize more methanol for protein induction than the other *Pichia* phenotypes (Veenhuis *et al.*, 1983). Our studies showed that *P. pastoris* used almost double the amount of methanol than *H. polymorpha* to induce expression, contributing to an $Y_{x/s}$ value = 0.0114 compared to an $Y_{x/s}$ value = 0.0029 in *P. pastoris* in bioreactors (Table 5.1). Therefore, *H. polymorpha* is more methanol-effective in producing large-scale rotavirus VP6 than *P. pastoris*.

Conclusion

Although *E. coli* is still one of the most widely used hosts for the production of heterologous proteins, protein products are often deposited in inclusion bodies that complicates downstream purification. Despite higher yields of VP6 in *E. coli* shake flask expressions, all the expressed protein was found to be insoluble and possibly linked to amyloid formation. Intermediate structures during amyloid formation might be cytotoxic when administered to eukaryotes and poses an additional threat in cases where insoluble inclusion bodies were used for any human purposes. Scaled-up fermentation of *H. polymorpha* proved to be most effective in expressing high concentrations rotavirus VP6 protein (Figure 3), outperforming both *E. coli* and *P. pastoris* bioreactor cultures. It is encouraging as it opens the possibility for inexpensive production of VP6 as a potential antigen and subsequent vaccine candidate. This could include VP6 alone, used as a carrier protein (Zhao *et al*., 2011) or in combination with the three remaining rotavirus structural proteins as Rodríguez-Limas and co-workers (2011) showed in *S. cerevisiae*.

Future work

Future work will need to incorporate the expression of VP2 as well, in *H. polymorpha*. Both genes will preferably be cloned into a single plasmid since multiple plasmids can pose a methabolic burden on the yeast and result in lower recombinant yields (Rodríguez-Limas, *et al.*, 2011).

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Chapter 6

Expression of a novel collagen-antimicrobial peptide fusion protein in yeast

In collaboration with Prof Marina Rautenbach, Department Biochemistry, Stellenbosch University

Introduction

Due to poor living conditions throughout Africa, large families are forced to live in confined spaces. Using candles, kerosene lamps and open fires as light and energy sources, the incidence of burn wounds are increasing. The rate of burn related child deaths is seven times higher in developing countries than in developed countries (World Health Organization, 2016). Patients with serious burn wounds need to be hospitalized immediately and treated with specialised sterilising wound dressings to minimize morbidity and mortality due to infection. Commercialized sterilising wound dressings are very costly and are therefore not readily available to people in developing countries. In addition, bacteria that infect burn wounds can become resistant against conventional antibiotics found in the wound dressings. Therefore, there is a great demand for alternative wound dressings that would be more affordable with a low probability to induce bacterial resistance.

Antimicrobial peptides (AMPs) are possible candidates to replace antibiotics in the treatment of burn wounds. AMPs are small peptides that form part of the natural immunity of most living organisms. They have antimicrobial activity against various bacteria, viruses, fungi and parasites with virtually no resistance (Peschel and Sahl, 2006). The group of antimicrobial peptides, namely cathelicidins, are synthesized as pre(signal)-pro-peptides in neutrophils. The carboxy-terminal domain represents the antimicrobial active peptide. Once the neutrophils are activated, the pro(signal)-peptide must be removed from the C-terminal peptide to unleash the microbicidal activity (Bals and Wilson, 2003) (see Fig. 6.1).

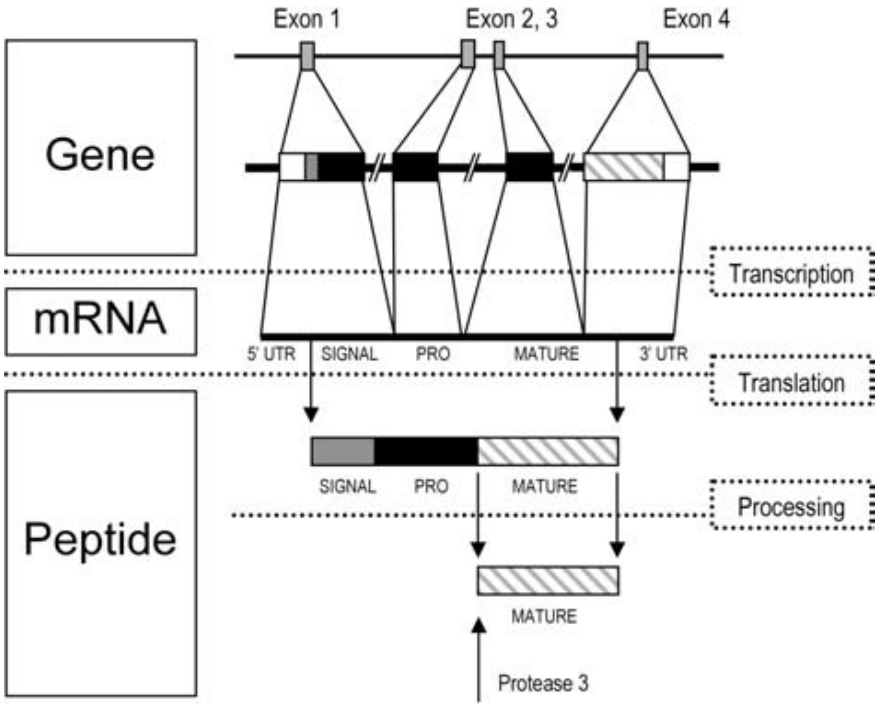


Figure 6. 1. Structure of the gene and peptide of LL-37/hCAP-18 as a prototypical example for the cathelicidin family. The C terminus represents the part with antimicrobial activity. The gene is represented schematically with the following individual components: White box, 5¢ untranslated region (5' UTR); grey box, signal sequence; black box, pro- sequence; shaded box, mature peptide; white box, 3' UTR (Bals and Wilson, 2003).

Apart from curbing the initial infection, the healing of the wound needs to be as rapid as possible to limit the exposure of open wounds to bacteria and fungi. Collagen enhances wound healing and therefore has been widely used in the management of severe burn wounds (Ruszczak and Schwartz, 1999). Previously, collagens were thought to function only as structural support, but it is now evident that its mode of action entails the following: 1) collagen signals and increases fibroblast production; 2) it has a hydrophilic property that encourages fibroblast infiltration to the wound and enhances the deposition of oriented, organized collagen fibers; 3) it assists in the uptake of fibronectin; 4) it helps to preserve cells such as leukocytes, macrophages, fibroblasts, and epithelial cells; and 5) it helps to maintain the chemical and thermostatic microenvironment of the wound (Doillon and Silver, 1986; Doillon *et al.*, 1988; Doillon *et al.*, 1984; Burton, Etherington and Peachey, 1978; Palmieri, 1992). Animal derived collagen, the most abundant protein in mammals, can be used for this application, but can cause allergies or an immune response in humans (Nokelainen *et al.*, 2001). Isolating sufficient quantities from animal tissue is also not feasible.

An efficient large-scale system for the production of both human α1 (type 1) collagen (collagen found in most connective tissue) and selected AMPs would have a valuable medical application in burn wound treatment. Yeasts can grow rapidly on inexpensive media to high cell densities, and can be modulated to secrete modified recombinant proteins of interest. The authors hypothesize that a cost-effective method would be to express AMPs linked to collagen via an amino acid sequence containing an enzymatic cleavage site, as fusion proteins in yeast such as *Pichia pastoris* and *Hansenula polymorpha.* Once it is expressed, the recombinant protein can be purified and applied in wound dressings. The collagen part of the fusion protein will associate with the collagen in the wound in such a way that it exposes the cleavage site. Enzymes in the wound, as part of the natural immune response, will cut the linker sequence and release the AMPs. The latter targets bacteria in the wound and also helps with wound healing.

Material and Methods

Design of a fusion collagen-AMP cassette

The human collagen alpha-1(I) chain sequence (Accession number XP_005257115) was used as template to select a suitable candidate for the collagen part of the recombinant fusion protein. The collagen part was then linked to one of the cathelicidin peptides, protegrin, via a linker sequence containing a cleavage site(s) for the neutrophil elastases present in the burn wound.

Cloning and expression of the collagen-protegrin cassette

The codon-optimised collagen-protegrin gene was sub-cloned from the commercial bacterial cloning vector into pBLHIS–IX (*his4, amp*^{R}) (Cereghino *et al.*, 2001) and pHIPX4–HNBESX (*leu2, kan*^{R}) (Gietl *et al.*, 1994), integration-expression plasmids of *P. pastoris* and *H. polymorpha*, respectively. Both cloning vectors without the collagen-protegrin insert were transformed into the respective yeast hosts to verify the expression of the fusion protein in positive cultures. In addition, yeast cells were also transformed with only ligase buffer (without any vector DNA) to verify the absence of any endogenous proteins with similar molecular weight secreted in the yeast cultures. All these cultures served as negative controls. To mediate extracellular secretion of the collagen-protegrin protein, the collagen-protegrin cassette was cloned downstream of the α-factor secretion signal of *Saccharomyces cerevisiae*.

Cloning and transformation of *H. polymorpha* NCYC 495 (Jan Kiel, University of Groningen, The Netherlands) and *P. pastoris*_KM71 (Invitrogen, Carlsbad, CA, United States) genomes were carried out as previously described by Smith *et al.* (2012). After growth on selected agar plates, several colonies were screened according the PCR protocol of the *Pichia* Expression Kit (Invitrogen, Carlsbad, CA, United States) for *P. pastoris* transformants. Alternatively, the pHIPX4-F and pHIPX4-R primers were used for *H. polymorpha* transformants (Smith *et al.*, 2012). Positive yeast transformants were cultured in shake-flasks and expression was initiated by the methanol inducible promoters *AOX1* and *MOX* in *P. pastoris* and *H. polymorpha*, respectively. Transformants expressing well in shake flasks were up-scaled to express in bioreactors as per fermentation protocols previously described (Bredell *et al.*, 2016; Bredell et al., 2017, under review). Expression was monitored at regular intervals to determine optimal time post-induction to harvest the expressed protein.

Analytical methods

Supernatant extracts

Supernatant fluid (SNF) samples of positive yeast cultures were collected prior to induction and daily during induction. Bigger unwanted proteins present in the supernatant fluids were precipitated using one of the following techniques: i) SNF was heated to 80ºC, centrifuged at 15000 × *g* for 10 min, and SNF freeze dried; or ii) 100% acetonitrile (ACN) was diluted to 50% using SNF, centrifuged at 15000 × *g* for 10 min at 4ºC, and SNF freeze dried.

Detection of collagen-protegrin fusion proteins using tricine-sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Silver staining

Freeze dried samples were analysed for the presence of the collagen-protegrin fusion protein using tricine-SDS-PAGE under denaturing conditions (Schägger, 2006). Gels were stained using Silver stain (O'Connell and Stults, 1997) and the correct-sized polypeptide construct was verified using an appropriate protein marker.

Detection of collagen-protegrin fusion protein using Liquid Chromatography-Mass Spectrometry (LC-MS)

Freeze dried SNF samples were reconstituted in 50% ACN and subjected to LC-MS (Stellenbosch University, Central Analytical Facility) to provide highly accurate molecular weight information on the fusion molecules.

Detection of antimicrobial activity against <u>Micrococcus luteus</u>

To verify the antimicrobial activity of the released protegrin, Gram-positive bacteria *Micrococcus luteus* were plated onto LB agar plates. Supernatant extracts, either untreated or treated with commercial elastase, were spotted onto the agar plates and incubated at the appropriate temperature of the bacterial strain. Once the elastases have cut the protegrin from the rest of the fusion protein, it will become activated and acted upon the *M. luteus*. Antimicrobial activity was detected as zones of growth inhibition on the carpet of yellow bacterial growth.

Nano-liquid chromatography-mass spectrometry (nano-LC-MS) of tricine-SDS PAGE gel bands

Treated SNF samples were loaded onto a tricine-SDS PAGE gel. After electrophoresis, the correct-sized band (~8 kDa) was cut out from the acrylamide gel using a clean scalpel. The acrylamide gel piece were treated with trypsin following the Trypsin Digest Protocol for MS Sample Preparation (http://www.sun.ac.za/caf/Trypsin Digest Protocol for MS Sample Preparation). Dried peptides were dissolved in 5% ACN in 0.1% formic acid and 10 µl injections were made for nano-LC-MS using a Thermo Scientific EASY-nLC II connected to a LTQ Orbitrap Velos mass spectrometer (Stellenbosch University, Central Analytical Facilities, Proteomics lab). Thermo Proteome Discoverer 1.3 (Thermo Scientific, Bremen, Germany) was used to identify proteins via an automated database searching all tandem spectra against the sequence of the fusion protein provided. Proteins were positively identified when at least 2 tryptic peptides match the provided sequence, a Mascot/Sequest score of more than $p>0.05$, as determined by Proteome Discoverer 1.3 (Stellenbosch University, Central Analytical Facilities, Proteomics lab).

Results

Screening for positive transformants

Following transformation, several colonies were screened for the correct size insert (625 base pairs). Only *H. polymorpha* had positive transformants (Fig. 6.2).

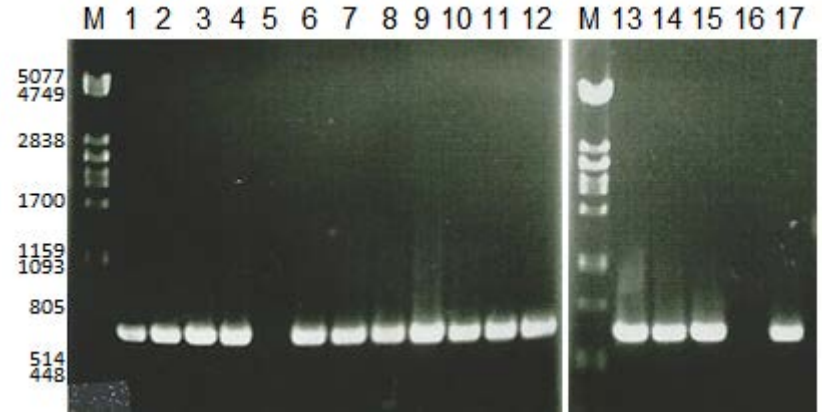


Fig. 6.2. Agarose gel showing PCR products of *H. polymorpha* transformed colonies. M: *lambda* DNA, digested using Pst1, Lane 1-3, 5-15: 625 bp PCR product of positive transformed colonies; Lane 4: No PCR product, negative transformed colony; Lane 16: Negative control; Lane 17: Positive control.

Designing and construction of the collagen-protegrin fusion gene

One of the main obstacles in expressing collagen was solubility as the protein is naturally very hydrophobic (Shoulders and Raines, 2009). The collagen part needed to be long enough for efficient attachment to the existing collagen matrix in the wound. Excessively long fragments will aggregate into inclusion bodies of the yeast and complicate downstream purification of the recombinant proteins. In addition to neutrophil elastases, contaminating microbial pathogens in the wound also secrete various proteases, which are capable of cleaving the linker region and releasing the protegrin.

In order to find a suitable collagen candidate, it has to have the following criteria: 1) long enough to attach to existing collagen; 2) contain polar amino acids to ensure solubility; 3) high frequency of the repetitive amino acid sequence of glycine-proline-X or glycine-X-hydroxyproline where “X” represents any amino acid to ensure a right handed triple helix formation; and low frequency of elastase cleavage sites (*A/G/V). The human collagen alpha-1(I) chain sequence (Accession number XP_005257115) (National Center for Biotechnology

Information, 2017a) was used as template to find suitable candidate collagen sequences. Each of the 12 possible candidates (Fig. 6.2, highlight in yellow) was assessed for the different criteria mentioned above. Candidate sequence 2 was chosen since it was significantly long (40 aa), has minimum elastase cleavage sites (2), has the most abundant helix turns (13) and contained polar amino acids.

MFSFVDLRLL LLLAATALLT HGQEEGQVEG QDEDIPPITC VQNGLRYHDR DVWKPEPCRI
CVCDNGKVLC DDVICDETKN CPGAEVPEGE CCPVCPDGSE SPTDQETTGV EGPKGDTGPR
GPRGPAGPPG RDGIPGQPGL PGPPGPPGPP GPPGLGGNFA PQLSYGYDEK STGGISVPGP 1.
MGPSGPRGLP GPPGAPGPQG FQGPPGEPGE PGASGPMGPR GPPGPPGKNG DDGEAGKPGR 2.
PGERGPPGPQ GARGLPGTAG LPGMKGHRGF SGLDGAKGDA GPAGPKGEPG SPGENGAPGQ
MGPRGLPGER GRPGAPGPAG ARGNDGATGA AGPPGPTGPA GPPGFPGAVG AKGEAGPQGP 3.
RGSEGPQGVR GEPGPPGPAG AAGPAGNPGA DGQPGAKGAN GAPGIAGAPG FPGARGPSGP 4.
QGPGGPPGPK GNSGEPGAPG SKGDTGAKGE PGPVGVQGPP GPAGEEGKRG ARGEPGPTGL
PGPPGERGGP GSRGFPGADG VAGPKGPAGE RGSPGPAGPK GSPGEAGRPG EAGLPGAKGL
TGSPGSPGPD GKTGPPGPAG QDGRPGPPGP PGARGQAGVM GFPGPKGAAG EPGKAGERGV
PGPPGAVGPA GKDGEAGAQG PPGPAGPAGE RGEQGPAGSP GFQGLPGPAG PPGEAGKPGE 5.
QGVPGDLGAP GPSGARGERG FPGERGVQGP PGPAGPRGAN GAPGNDGAKG DAGAPGAPGS
QGAPGLQGMP GERGAAGLPG PKGDRGDAGP KGADGSPGKD GVRGLTGPIG PPGPAGAPGD
KGESGPSGPA GPTGARGAPG DRGEPGPPGP AGFAGPPGAD GQPGAKGEPG DAGAKGDAGP 6.
PGPAGPAGPP GPIGNVGAPG AKGARGSAGP PGATGFPGAA GRVGPPGPSG NAGPPGPPGP 7.
AGKEGGKGPR GETGPAGRPG EVGPPGPPGP AGEKGSPGAD GPAGAPGTPG PQGIAGQRGV
VGLPGQRGER GFPGLPGPSG EPGKQGPSGA SGERGPPGPM GPPGLAGPPG ESGREGAPGA 8.
EGSPGRDGSP GAKGDRGETG PAGPPGAPGA PGAPGPVGPA GKSGDRGETG PAGPAGPVGP 9. 10.
VGARGPAGPQ GPRGDKGETG EQGDRGIKGH RGFSGLQGPP GPPGSPGEQG PSGASGPAGP 11.
RGPPGSAGAP GKDGLNGLPG PIGPPGPRGR TGDAGPVGPP GPPGPPGPPG PPSAGFDFSF 12.
LPQPPQEKAH DGGRYYRADD ANVVRDRDLE VDTTLKSLSQ QIENIRSPEG SRKNPARTCR
DLKMCHSDWK SGEYWIDPNQ GCNLDAIKVF CNMETGETCV YPTQPSVAQK NWYISKNPKD
KRHVWFGESM TDGFQFEYGG QGSDPADVAI QLTFLRLMST EASQNITYHC KNSVAYMDQQ
TGNLKKALLL QGSNEIEIRA EGNSRFTYSV TVDGCTSHTG AWGKTVIEYK TTKTSRLPII
DVAPLDVGAP DQEFGFDVGP VCFL

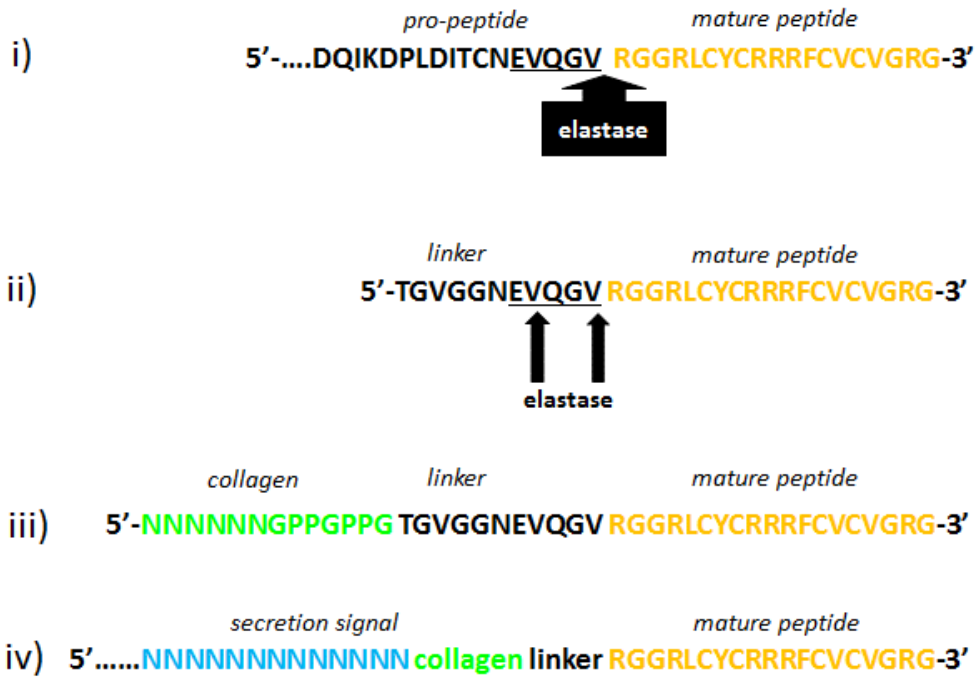
Figure 6.3. The human collagen alpha-1(I) chain sequence (Accession number XP_005257115) (National Center for Biotechnology Information, 2017) used as template to identify 12 possible collagen candidate sequences, marked 1-12 (yellow highlight), in the collagen-antimicrobial peptide fusion protein.

The amino acid sequence of mature protegrin (PG-1) is RGGRLCYCRRRFCVCVGRG (Accession no. AAB27599.1) (National Center for Biotechnology Information, 2017b) (Fig 6.3). Using the pro-PG-1 as a template (Fig. 6.3), a linker sequence of 11 amino acids containing the tetrapeptide EVQSV at the end of the pro-PG-1, was added to the N-terminus of the mature protegrin. This 11-aa sequence served as a

linker between the collagen and protegrin with the tetrapeptide EVQSV as one of the preferred cleavage site for elastases (Fig. 6.4A). Also, the valine in the elastase cleavage site should preferably be preceded by an amino acid with a β-turn, and followed by a small amino acid to ensure the exposure of the valine for elastase cleavage. The complete collagen-protegrin construct (Fig.6.4B) was commercially codon-optimized and synthesized to ensure optimal expression by both *H. polymorpha* and *P. pastoris.* The commercial codon-optimized gene was further optimized using Sharp and co-workers' codon bias index replacing rare codons in the gene with codons commonly used by both yeasts (Sharp *et al.* 1988).

Figure 6.4. Comparison of different cathelicidin antimicrobial peptides and their pre- and pro-sequences. The tetrapeptide EVQSV of protegrin (PG-1), indicated in a red box, is the preferred cleavage site for elastases (Bals and Wilson, 2003).

A



B

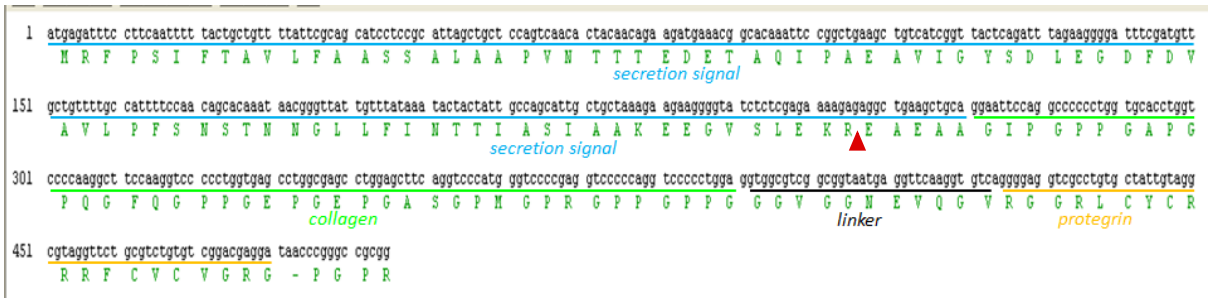


Figure 6.5. Collagen-protegrin peptide design (A) Schematic presentation of the novel approach. i) Amino acid sequence of the pro-sequence and mature peptide, protegrin. Original cleavage site of neutrophil elastases to release the active mature peptide is indicated. ii) Amino acid sequence of the mature protegrin (19 aa) with 11-aa linker sequence attached to the N terminal-end. Possible cleavage sites for elastases are indicated. iii) Attachment of the 40 amino acid-collagen sequence to the N terminal-end of the linker. iv) Attatchment of the α-factor secretion signal sequence from *Saccharomyces cerevisiae* to the N terminal-end of the collagen to allow for extracellular secretion of the collagen-protegrin peptide. (B) Nucleotide - and amino acid sequence of the complete collagen-protegrin construct. Kex2 signal cleavage site indicated as red arrow (▲).

Detection of putative collagen-protegrin fusion protein using tricine-SDS-PAGE and LC-MS

Suprnatant fluid from PCR-positive cultureSupernatant samples were first treated with either 80ºC heat or ACN to precipitate any unwanted large proteins present in the culture fluid. After centrifugation, SNF samples were freeze dried and resuspended in 2 x loading dye (Bredell *et al.,* 2016) before loading onto a tricine-SDS-PAGE gel. The theoretical molecular weight (MW) of the putative fusion protein is 7,23 kDa. There was only one protein specie visible on the SDS-PAGE of shake-flask SNF which correspond to a MW of <10 kDa (Fig. 6.6 A, Lane 1-3). The protein specie was absent in cultures transformed with vectors without the fusion insert (Fig. 6.6 A, Lane 4-6). Similarly, the protein specie was also absent in cultures transformed without any vector DNA (Fig. 6.6 A, Lane 7). Supernatant fluid from bioreactor cultures also showed the presence of protein species larger than 10 kDa on silver-stained SDS-PAGE.

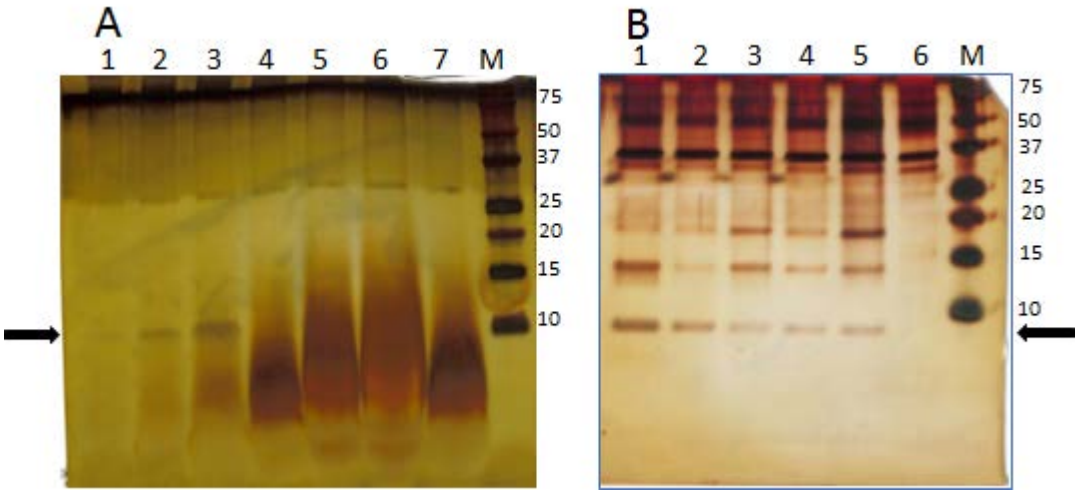


Figure 6.6. Silver stained tricine-SDS-PAGE gels showing the presence of protein species in the SNF of *H. polymorpha* cultures. (A) Supernatant fluid of positive shake-flask cultures expressing the putative fusion protein (indicated with arrow) (Lane 1-3: 1 µl, 5 µl, 10 µl). Supernatant fluid of shake-flask cultures transformed with vectors without the fusion insert (Lane 4-6: 1 µl, 5 µl, 10 µl). Supernatant fluid of shake-flask cultures transformed without any vector DNA (Lane 7). (B) Supernatant fluid of 1.3 L bioreactor cultures expressing the putative fusion protein (indicated with arrow) as well as other protein species >10 kDa (Lane 1-5: day 1-5 post-inoculation). No proteins were expressed during the glycerol fed-batch stage (Lane 6). Kaleidoscope™ Prestained Protein standard (BIO-RAD, CA, USA) was used as marker.

The reconstituted SNF in 50% ACN was subjected to LC-MS for protein identification and characterization. Theoretically, the average molecular mass of the fusion protein is 7 234.13 Da and the collagen and protegrin part of the fusion protein is 5 034.46 and 2 217.68 Da, respectively. The LC-MS spectrum revealed the presence of a dominant species of 7 608.23 Da (Fig. 6.7).

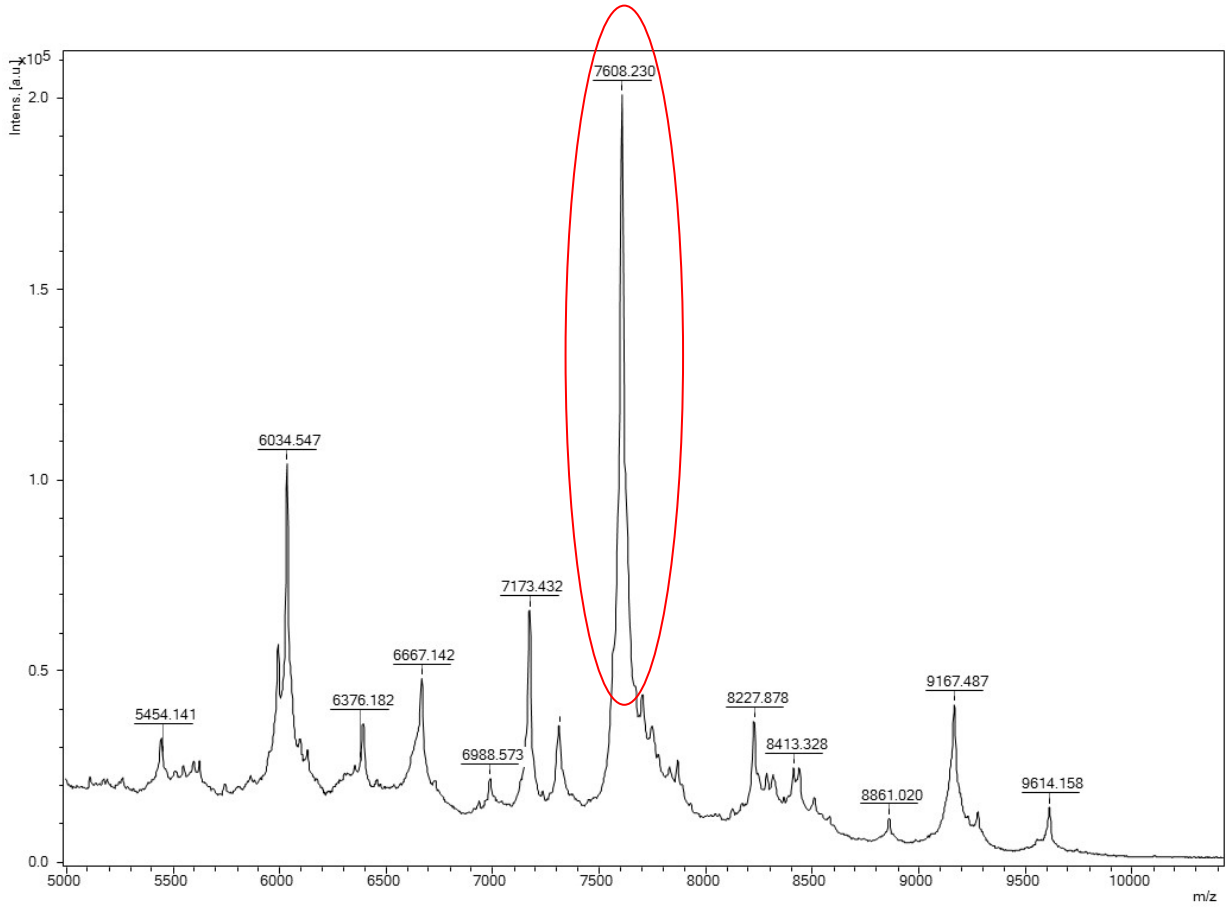


Figure 6.7. Chromatogram of the LC-MS showing the molecular weight of peptides present in the culture of positive transformed *Hansenula polymorpha* cultures. According to their masses, neither of the peptides present resembled the putative fusion protein, nor the collagen or protegrin part.

Detection of antimicrobial activity against Micrococcus luteus

Supernatant fluids from both positive transformed *H. polymorpha* cultures as well as negative control cultures were spotted onto agar plates covered with *M. luteus* bacteria. Antimicrobial activity, seen as growth inhibition zones, was detected for both positive and negative cultures.

Extracts treated with 80ºC heat showed bigger inhibition zones than those treated with 50% ACN (Fig. 6.8).

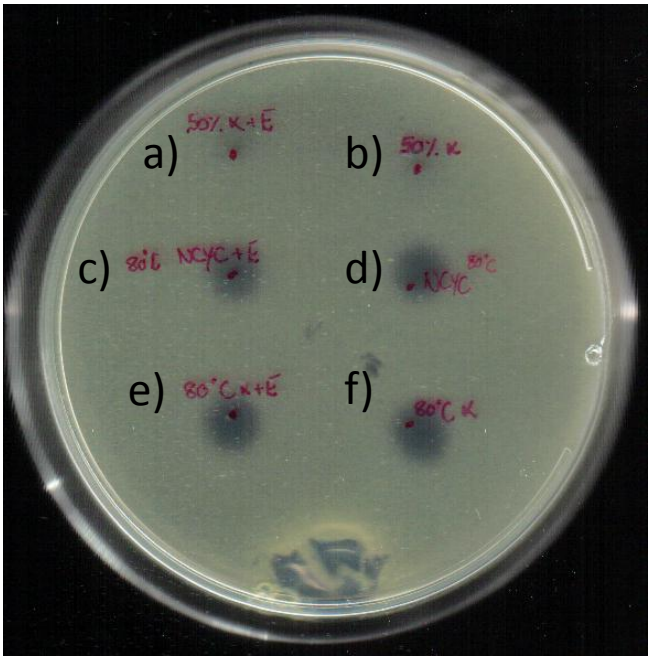


Figure 6.8. An agar plate covered with *M. luteus* showing growth inhibition zones. a) SNF from positive transformed yeast culture treated with 50% ACN and elastase, b) SNF from positive transformed yeast culture treated with 50% ACN only, c) SNF from untransformed yeast culture treated with 80ºC and elastase, d) SNF from untransformed yeast culture treated with 80ºC only, e) SNF from positive transformed yeast culture treated with 80ºC and elastase, and f) SNF from positive transformed yeast culture treated with 80ºC only.

Positive identification of fusion protein using proteomics

According to the Thermo Proteome Discoverer 1.3, the proteins recovered from the acrylamide gel band corresponded to the provided sequence of the fusion protein with a Mascot/Sequest score of p = 1353.74. Also, proteins were considered positive when they were identified with at least two tryptic peptides per protein. Amino acid sequences indicated in red of the collagen-linker-protegrin fusion protein were positively identified:

GIPGPPGAPG PQGFQGPPGE PGEPGASGPM GPRGPPGPPG **TGVGGNEVQG V**

collagen linker

RGGRLCYCR RRFCVCVGRG

protegrin

Only the unique linker sequence and half of the protegrin sequence were identified from the sample.

Discussion

Combining a collagen α-chain with an antimicrobial peptide (AMP) in a wound dressing can have a positive impact on wound treatment. The collagen part can aid in wound healing and the AMP in fighting infectious agents present in the wound. Expressing these two peptides as a fusion protein extracellularly in *Hansenula polymorpha,* would ease downstream purification from the supernatant culture fluid (SNF).

The design of the fusion protein is novel and it has not been expressed in any expression system. Therefore, the authors had no idea how this fusion protein will perform once it is expressed in the culture SNF. Theoretically, the MW of the fusion protein is 7.23 kDa. Tricine-SDS PAGE gels revealed the expression of a single protein specie < 10 kDa in positive transformed *H. polymorpha* cultures (Fig. 6.6 A). Since this protein specie was absent in yeast cultures transformed with vectors without the fusion insert, one can assume that the single protein specie is the putative fusion protein. Absence of any other protein species in cultures transformed without vector DNA, verified the presence of endogenous proteins in the yeast cultures. The slower migration pattern of the fusion protein at a distance equals <10 kDa could be attributed to the enrichment of proline amino acids in the collagen. It is known that excessive proline residues in a protein can cause structural rigidity to the primary sequence causing a decrease in the electrophoretic mobility of the protein (Dr Vishal Singh Negi, University of Hawai‘i at Mānoa, Peronal communication). Yet, positive identification of the fusion protein still needed to be confirmed. Absence of expression in *Pichia pastoris* cultures indicated that this yeast either failed to express the fusion protein or the putative protein could have been encapsulated in inclusion bodies that were not isolated

LC-MS identified a dominant species of 7 608.23 Da that could represent the fusion protein if the protein was inefficiently cleaved (Fig. 6.7). Yeast endo-protease Kex2 removes the α-factor secretion signal sequence from the fusion protein, and thereby releases the mature form of the secreted protein (Julius *et al.,* 1984). Cleavage occurs at the * of the amino acid sequence E-K-R-*-E-A-E-A. The E-A repeats are then further trimmed by the STE13 protease. Often the cleavage by Kex2 and/or STE13 is not efficient, leaving unwanted

overhangs at the amino terminus of the expressed proteins (Brake *et al.*, 1984; Ghosalk ar, Sahai and Srivastava, 2008). This could have resulted in the larger protein species. Initially, large unwanted proteins present in the SNF were removed through precipitation using AcN and heat, but smaller peptides could still be present. LC-MS showed a variety of smaller peptides present suggesting additional purification steps were needed before subjecting the SNF to LC-MS. One option is to purify the peptide of interest using high-performance liquid chromatography (HPLC) by separating the fusion protein peak from the rest of the other impurities.

In order to purify the putative fusion polypeptide, large quantities of this construct were needed and were therefore expressed in 1.3 L bioreactors. Due to the long methanol induction phase during the fermentation process (5 days), the culture fluid showed an increase of contaminating proteins present while the putative polypeptide band decreased (Fig. 6.6 B). This could be attributed to the secretion of endogenous proteins in the culture fluid, including proteases, during the extended growth curve. The latter could be detrimental to proteins present with the greatest impact on smaller proteins and peptides such as the putative fusion protein. Maximum yield of the putative protein was expressed after the first day of methanol induction, after which the yield started to decline.

Growth inhibition zones on *M. luteus* bacterial plates would usually indicate the presence of activated protegrin. However, clear inhibition zones were also detected when untransformed *H. polymorpha* cultures (NCYC) were spotted onto bacterial growth. Since no fusion protein was secreted in untransformed cultures, these inhibition zones suggest the possible secretion of endogenous antimicrobial agents in yeast. However, no natural host defense peptides have been found in any yeast. Yet, there are reports of proteases present in yeast cultures that could acted on the *M. luteus* bacteria and leave inhibition zones (Wang, Li and Wang, 2016). This data reiterated the need to include additional purification steps before analysing to remove any possible proteases.

Digesting the acrylamide gel band that correspond to the mass of the putative fusion protein, followed by proteomic analysis, revealed the possible identification of the fusion protein. Although only half of the protegrin sequence and the unique linker sequence were identified from the sample, the Mascot/Sequest score of 1353.74 was significant. Also, the fusion protein was identified with two tryptic peptides. Together with the SDS-PAGE results,

this is the first evidence of the possible successful expression of a recombinant collagen-protegrin fusion protein in yeast.

Future work

In order to obtain the fusion protein in bulk, it has to be cultured in bioreactors and soon after methanol induction, to harvest the SNF. Purification of the fusion proteins from SNF needs to be further optimized. Size-exclusion chromatography followed by ion-exchange chromatography will be considered in the process to separate the fusion protein from the rest of the contaminating proteins present in the culture SNF.

Once the fusion protein is purified, it will be confirmed using liquid chromatography–mass spectrometry (LC-MC) and antimicrobial activity needs to be re-accesed.

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Chapter 7

Conclusions

Self-assembly of biological systems is a ubiquitous phenomenon in nature. It describes the spontaneous association of numerous individual entities through non-covalent interactions, including hydrogen and ionic bonds, and hydrophobic and van der Waals interactions, into well-defined structures without instructions from an external source. Although these interactions are weak, in large numbers they dominate in large numbers the structural and conformational behavior of the assembly (Zhang, 2002). The spectrum of such self-assembly structural proteins include viral proteins which self-assemble into VLPs (López-Sagaseta *et al*,. 2016) and α-chains that form procollagen (Köster *et al.*, 2008).

Many viruses encode proteins which self-assemble into nanoparticles and thereby encapsulate the viral genome as a prerequisite for infection. If these proteins assemble in the absence of the viral genome, non-infectious non-replicating VLPs are obtained that closely resemble intact virions (López-Sagaseta *et al.*, 2016). Due to their close resemblance with infectious virions, some VLPs have been developed into successful vaccine candidates, such as the current RV and HPV vaccines (Plotkin, 2014). Thus, the first two aims of this thesis were to express structural proteins involved in VLP formation of both HPV and RV in two methylotrophic yeasts, *P. pastoris*_KM71 and *H. polymorpha*_NCYC495. Expressions were performed in shake flasks and subsequently scaled-up to bioreactors.

The first aim of this PhD study was to express a codon-optimized human papillomavirus-16 L1/L2 chimeric (SAF) protein intracellularly in two methylotrophic yeasts, *P. pastoris* and *H. polymorpha*. Firstly, two different feeding strategies in fed-batch cultures of *P. pastoris* Mut^S expressing SAF, were evaluated: a predetermined feed rate versus feeding based on the oxygen consumption by maintaining constant dissolved oxygen levels. We have adopted a closed-loop proportional-integral (PI) controller to maintain a constant DO of 30%. Our results were consistent with Kotzé *et al.* (2011) that the DO stat control was the preferred feeding strategy compared to the predetermined feeding option in *P. pastoris* Mut^+ (X-33) cultures. They showed that the DO controlled feeding produced both higher volumetric production as well as biomass of HPV type 16 L1 protein. Our Mut^S cultures also showed a significant increase in biomass when methanol was fed using the DO stat control. In other studies the predetermined feeding strategy was superior over other methods of methanol

feeding only because the culture growth rate was kept constant (Trinh, Phue and Shiloach, 2003; Minning *et al.,* 2001). In our study the growth rate was neither monitored nor maintained but rather the DO value was indicative of how much methanol to add to the culture. However, our data confirmed that of Chung (2000) that the DO stat method was found to be effective in cultures growing under a single carbon source (Chung, 2000). In our study methanol was the sole carbon source during the induction of the recombinant HPV L1/L2 chimeric proteins.

Secondly, we used the DO stat feeding strategy to compare the biomass yield, methanol consumption and SAF production in two *P. pastoris* strains, namely KM71 (Mut^S) and GS115 (Mut^+). Methanol served both as inducer of recombinant protein expression as well as carbon source. In general, Mut^S strains have a slower growth rate, lower productivity and hence, a slower methanol consumption rate than Mut^+ strains (Chiruvolu, Gregg and Meagher, 1997; Cregg and Madden, 1988; Kim *et al.,* 2009; Romanos, Scorer and Clare, 1992; van den Burg, de Wit and Vervoort, 2001). Maintaining optimum methanol concentrations (preferably at 4 $g.L^{-1}$) is critical during the successful product formation in *P. pastoris* fermentation. Accumulation of raw methanol is toxic to yeast cells (Cregg *et al.,* 1989) moreover, excess of methanol oxidation by-products can lead to cellular stress and ultimately cell death (Couderc and Baratti, 1980; Cregg and Madden, 1988; Kern *et al.,* 2007; van der Klei, Bystrykh and Harder, 1990; Zhang *et al.,* 2000). When methanol levels are too low, it may not be enough to initiate transcription (Cereghino and Cregg, 2000) as well as reduce the productivity by triggering the proteolytic degradation of the recombinant proteins (Kupcsulik *et al.,* 2001). Smaller culture volumes require slower flow rates of methanol addition and therefore, accurate controling of methanol levels is more challenging. Overfeeding can easily occur and thereby cell growth can be inhibited. The latter cause the DO to increase further which results in more overfeeding.

Higher biomass concentration and methanol feeding rate in *P. pastoris* Mut^+ cultures confirmed that *P. pastoris* Mut^+ strains had faster growth rates on methanol than *P. pastoris* Mut^S strains. Higher methanol concumption has proved to be easier to control. However, SAF protein concentration and protein yield on methanol substrate were almost double of what was obtained for the Mut^+ strain making Mut^S an attractive host for the production of recombinant proteins. Several other studies support Mut^S strains to be superior in the production of some recombinant proteins (Krainer *et al.,* 2012; Orman, Calik and Ozdamar,

2009; Pla *et al.*, 2006). Scaling up the expression of SAF to 10 L bioreactors resulted in a 2-fold higher SAF concentrations than in the 1.3 L-cultures.

Thirdly, we evaluated the expression of HPV type 16 L1/L2 chimeric proteins in *P. pastoris*_KM71 (Mut^S) versus *H. polymorhpa* strain NCYC495 in bioreactors. *H. polymorpha* exceeded the expression levels of SAF per biomass in *P. pastoris*_KM71 by more than 5-fold. However, when *H. polymorpha* was compared with *P. pastoris* wild type X33 (Mut^+) for the production of mammalian proteins, *P. pastoris* yielded more than 3-fold more protein than *H. polymorpha* (Mack *et al.*, 2009). Our results showed the maximum concentration SAF protein expressed in both yeasts ever reported before, 48.96 $mg.L^{-1}$ and 132.10 $mg.L^{-1}$ in *P. pastoris*_KM71 and *H. polymorpha*, respectively. Previously the highest concentration HPV-16 L1 protein reported in those two platforms was 14.2 $mg.L^{-1}$ (Bazan *et al.*, 2009) and 78.6 $mg.L^{-1}$ (Li *et al.*, 2009), respectively.

Western blot analysis showed more degradation of expressed proteins in *H. polymorpha* cultures than observed in *P. pastoris* cultures (Mack *et al.*, 2009). Increased methanol utilization and higher culturing temperature during *H. polymorpha* fermentation could have stressed the yeast cells and triggered excess protease production which resulted in more degradation. Despite the observed degradation, *H. polymorpha* still produced higher yields of SAF per methanol substrate than *P. pastoris*.

The SAF protein was semi-purified from the yeast cell lysate using either chromatography or sucrose density gradient, and then subjected to electronmicroscopy. Upon transmission electron microscopy (TEM), mostly secondary structures ~10nm in diameter were observed with the occasional formation of T=1 VLPs (25-30nm in diameter). Although HPV16 L1 has been expressed in *H. polymorpha* before, our study is the first report showing the formation of T=1 VLPs in this methylotrophic yeast (Liu, Yang and Yao, 2014; Li *et al.*, 2009). Chimeric SAF proteins displaying L2 epitopes offer simultaneously high titres of L1

specific neutralizing antibodies as well as cross-neutralizing antibodies against L2. Although capsomeres offer lower immunogenicity than VLPs, it still offer great potential in future vaccine preparations (Fligge *et al.*, 2001; Rose *et al.*, 1998; Thönes *et al.*, 2008).

The second aim of this PhD study was to express a codon-optimized RV VP6 protein, of which the sequence derived from an emerging South African RV strain, intracellularly in

E. coli and yields were then compared with that observed in the two methylotrophic yeasts, *P. pastoris*_KM71 and *H. polymorpha*. *E. coli* is the most widely used prokaryotic system for the expression of heterologous proteins. In our RV VP6 expressions, *E. coli* showed higher volumetric and specific productivities, in both shake-flasks and bioreactors, compare to the methylotrophic yeasts *P. pastoris* and *H. polymorpha*. Shorter culture times contributed to these higher productivities in *E. coli*. Both yeasts reached maximum VP6 production after 4 days post-induction in bioreactors whereas bacteria reached it after only 4 hours. Although *E. coli* had a lower biomass than yeasts in shake-flasks, it produced the highest VP6 yield per biomass.

Expression of a recombinant protein may impart a metabolic burden on the microorganism causing a decrease in growth rate (Bentley *et al.*, 1990). Therefore, high cell-density culture methods were designed to boost *E. coli* growth (Korz *et al.*, 1995). LB media can support cell densities of OD_{600} up to 7.0 in shake-flask cultures (Sezonov, Joseleau-Petit and D'Ari, 2007). However, scaled-up cultures in bioreactors with cell densities of OD_{600} > 60, require high cell-density media that was buffered to keep the pH constant for maximum protein production. Growing the cultures to higher densities in bioreactors increased both the biomass and the volumetric yield of the VP6 protein across all three expression platforms. VP6 products was almost directly proportional to biomass in all three expression systems (Fig. 5.3). Bioreactors provided a controlled environment not only with adapted media but also good aeration and oxygen supplementation. Unlike in shake flask expressions, ideal culture conditions in bioreactors favored *H. polymorpha* for high VP6 yield per culture volume (3350.717 $mg.L^{-1}$) as well as yield per unit biomass (0.037 $g.g^{-1}$) that outperformed both *E. coli* as well as *P. pastoris*_KM71. The latter Mut^S phenotype has a slower growth rate which metabolizes methanol poorly, making the addition of methanol in smaller cultures more challenging.

Despite *E. coli*'s poor performance in comparison with methylotrophic yeasts, the *E. coli* VP6 levels produced in our study were the highest ever reported. Despite good yields of RV VP6 in *E. coli*, all the protein expressed by *E. coli* BL21 (DE3) in our study was deposited into insoluble aggregates known as inclusion bodies. This usually happens as a result of misfolded proteins which is biologically inactivate (Villaverde and Carrio, 2003). The insoluble nature of VP6 in *E. coli* is likely due to the native trimetric conformation

assumed by the protein (Mathieu *et al*., 2001), which might prove too difficult for *E. coli* to process.

Reducing the rate of protein production in *E. coli* grants newly transcribed proteins the opportunity to fold properly. The most common way of slowing down the production rate is to lower the culture temperature from 37ºC to 20ºC during the induction phase (Schein and Noteborn 1988). In our study, the low culture temperature still resulted in the formation of inclusion bodies. Misfolded VP6 proteins, with their distinct β-barrel domain (Mathieu *et al.*, 2001), found in inclusion bodies resembles amyloid structures which are associated with pathological disorders such as Alzheimer and Parkinson disease. Misfolded proteins in insoluble inclusion bodies might be cytotoxic for any human use. According to literature, inclusion bodies could be solubilized and the VP6 retrieved, refolded and self-assembled into VLP's (Zhao *et al.*, 2011). However, isolation from inclusion bodies and refolding the recombinant proteins can be challenging resulting in low yields of correctly folded proteins (Lilie, Schwarz and Rudolp, 1998). In contrast to bacterial expressions, VP6 proteins expressed in yeast were found to be soluble upon analysis making methylotrophic yeast very desirable for expression recombinant proteins.

Since methanol was used to induce the production process, the amount of methanol used played an important role in determining the expression platform of choice. Our bioreactor studies showed that *P. pastoris* used almost double the amount of methanol than *H. polymorpha* to induce expression, contributing to almost 4 times less VP6 yield per substrate ($Y_{p/s}$) than *H. polymorpha* (Chapter 5, Table 5.1). Therefore, *H. polymorpha* was more methanol-effective in producing large-scale rotavirus VP6 than *P. pastoris*. This is the first report of VP6 expressed in methylotrophic yeast and holds the promise for the inexpensive production of VP6 either as a potential antigen, or possible vaccine candidate or drug delivery mechanism (Zhao *et al*., 2011).

In addition, the same recombinant technology was applied to express a novel antimicrobial peptide (AMP)-collagen fusion peptide possibly used in the treatment of burn wounds. Once this fusion protein is expressed, it can be purified and applied in wound dressings. We hypothesize that the collagen part of the fusion protein will associate with the collagen in the wound encouraging wound healing. As part of the natural immune response, elastases present in the wound will release the AMP from the collagen targeting invading

bacteria. Although SDS-PAGE results showed the presence of a single correct-sized protein band and LC-MS presented a dominant species relating to the putative collagen-AMP fusion protein, these results were not conclusive. Preliminary proteomic data is the first evidence of the possible successful expression of a recombinant collagen-protegrin fusion protein in yeast.

Current vaccines against both HPV and RV are still relevant in terms of circulating genotypes but as new genotypes emerge vaccines will need to be updated. This was obtained through the inclusion of the cross-neutralizing L2 epitope in HPV L1 VLPs, and a VP6 protein isolated from a local gastroenteritis patient in RV secondary structures.

In conclusion, this PhD study confirmed that methyloptrophic yeasts remains a very promising producer of frecombinant proteins, and even more so self-assembling proteins. Despite *E. coli*'s good yields, these proteins are often deposited into insoluble aggregates which require downstream purification. Methylotrophic yeast, in particular *H. polymorpha*, showed increased production of structural viral proteins, RV VP6 and HPV type 16 SAF. *H. polymorpha* not only exceeded the expression of viral proteins in *E. coli* and *P. pastoris* strains, KM and GS115, but was also more methanol-effective in producing large-scale viral proteins. Purifying the HPV-16 SAF protein from H. *polymorpha* cultures resulted in the formation of capsomeres (10nm in diameter) and the occasional putative T=1 VLPs (25-30nm in diameter). Large-scale expression in *H. polymorpha* holds promise for the manufacturing of cheaper vaccines in developing countries as it resulted in increased level of expressed viral proteins. Methanol is highly flammable and hazardous and, therefore, undesirable for large-scale fermentations. Effective alternatives for induction are needed to replace methanol for industrial scale fermentations (Stadlmayr *et al.*, 2010). Alternative inducible promoters, such as the constitutively *GAP* promoter, can be induced without methanol and provides continuous transcription of the gene of interest.

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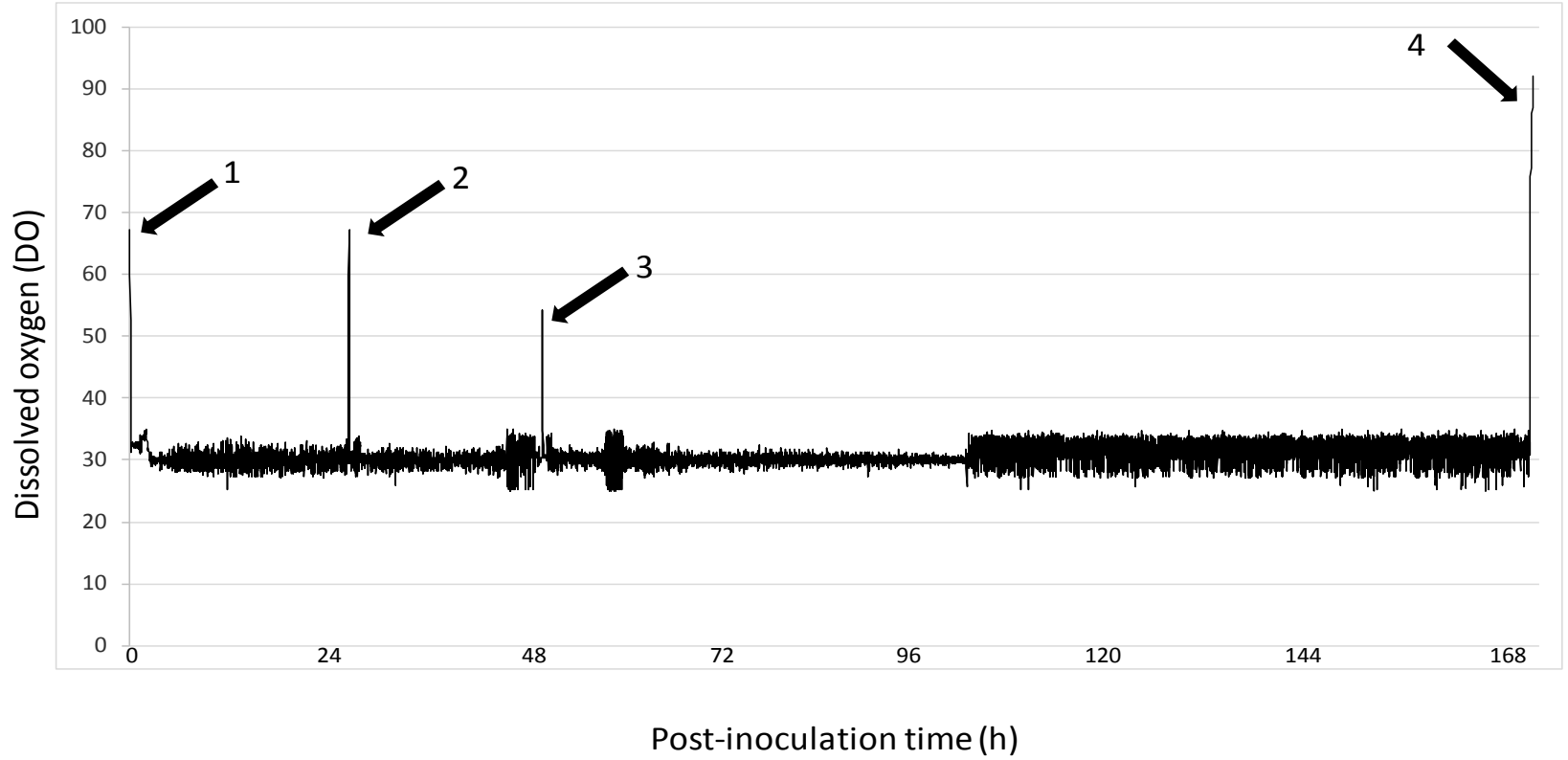

DO graph representing a typical yeast fermentation. Initially, the bioreactor's DO sensor is calibrated using nitrogen until the DO is 0% and pure oxygen (O_2) to saturate it to 100%. Therefore, the DO will be near 100% at the beginning of the fermentation process and starts to decline after inoculation when the yeast metabolizes the access glycerol in the media and consumes O_2. 1) DO is high at the beginning of the fermentation process. 2) After 24 hours post-inoculation, the glycerol in the media is depleted and the DO starts to increase. This spike indicates the end of the batch phase, beginning of the glycerol fed-batch phase. 3) When the glycerol feed is interrupted and the DO shows an increase, it indicates glycerol is limited and the methanol fed-batch phase can immence (48 h post-inoculation). 4) The end of the fermentation process is indicated by an increase in DO.

Appendix B

Comparing cytosolic expression to peroxisomal targeting of the chimeric L1 / L2 (ChiΔH-L2) gene from human papillomavirus type 16 in the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha**

Smith JJ, Burke A, Bredell H, van Zyl WH and Görgens JF.

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