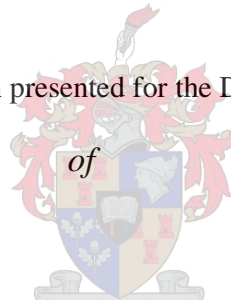


Recombinant Hepatitis B
surface antigen production in
Aspergillus niger

by

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

By targeting Hepatitis B surface antigen (HBsAg) production through the secretory pathway in *Aspergillus niger*, completely assembled and properly folded HBsAg, was obtained. This was achieved by implementing a gene fusion strategy using the highly expressed catalytic domain of the glucoamylase gene ($GlaA_{G2}$) fused to the HBsAg *S* gene. The inducible glucoamylase promoter ($GlaA_p$) was used to control transcription in *A. niger*. The gene fusion strategy resulted in the cleavage of the fused product by the KEX2-like protease, and intracellular accumulation of HBsAg was observed, while glucoamylase was secreted. Full assembly and maturation of HBsAg occurred after cleavage of the fused product in the Golgi complex. Several intracellular glucoamylase degradation products indicated bottlenecks/ limitations in the secretory pathway caused by the over-expression of the fused $GlaA_{G2}::S$ gene. These findings were demonstrated by increases in HBsAg production through over-expression of protein disulfide isomerase (PDI) and calnexin (CLX) as well as in cultivations at reduced growth rates (μ).

An improved methodology using the gene fusion technology to identify and select high HBsAg producing transformants was developed through the use of a double mutant *A. niger* host strain, MGG029- $\Delta aamA$, lacking two major amylolytic enzymes, glucoamylase and acid amylase. The screen based selection on high extracellular glucoamylase production on solid state cultures using starch as a selection pressure. The high efficiency obtained through selecting for high extracellular glucoamylase transformants and the associated correlation between intracellular HBsAg production and secreted glucoamylase in batch/ fed-batch fermentation reflected the ability of the screen to indirectly quantify high HBsAg producing transformants through extracellular glucoamylase determination.

Through the application of the glucoamylase screen two high HBsAg producing transformants varying in μ_{\max} were identified; (1) the ‘fast-growing high-biomass producing’ transformant ($\mu_{\max} = 0.11 \text{ h}^{-1}$), and (2) the ‘slow-growing high-glucoamylase producing’ transformant ($\mu_{\max} = 0.08 \text{ h}^{-1}$). These two transformants were evaluated in carbon-limited exponential fed-batch fermentations as well as used as hosts together with the *A. niger* D15 strain in chaperone/ foldase over-production studies. In carbon-limited exponential fed-batch fermentations HBsAg production was most efficient at $0.07 < \mu < 0.08 \text{ h}^{-1}$, and provided a method for increasing HBsAg production levels up to 81 %.

The over-production of CLX and PDI resulted in the highest percentage increase (88 % and 107 %, respectively) and therefore reflected their critical involvement with regards to efficient HBsAg assembly using gene fusion. The combinations of BiP and associated combinations with CLX and PDI failed to improve HBsAg production levels. Reductions of up to 40 % were observed upon BiP over-production and therefore indicated unfavourable responses towards HBsAg assembly and production, which were triggered upon BiP over-expression using gene fusion. HBsAg production levels were further increased through the cultivation of the CLX and PDI over-producing transformants in carbon-limited exponential fed-batch fermentation. Highest HBsAg production levels of $352 \pm 21.3 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ ($6237.2 \pm 437.2 \mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$) and $330.2 \pm 24.4 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ ($5760.4 \pm 531.9 \mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$) in CLX and PDI over-expressing transformants were observed. These findings, together with the potential application of the solid-state glucoamylase screen for the identification of high HBsAg producers over-producing chaperones/ foldases, demonstrates the usefulness and application of gene fusion in *A. niger* MGG029- $\Delta aamA$ transformants as a technology for other recombinant virus like particles (VLPs).

OPSOMMING

Die produksie van Hepatis B oppervlak antigeen (HBsAg) deur middel van die uitscheidingsmeganisme in *Aspergillus niger*, het verseker dat die geproduseerde HBsAg struktureel korrek en volledig gevou was. Hierdie resultaat is verkry deur geenfusie waartydens die katalitiese domain van die glukoamilase geen (*GlaA_{G2}*), wat in hoë konsentrasies uitgedruk word, verbind is aan die HBsAg S geen. Transkripsie in *A. niger* is gekontroleer deur die induseerbare glukoamilase promoter (*GlaA_p*). Hierdie geenfusie strategie het veroorsaak dat die saamgesmelte produk deur 'n KEX2 gelykvormige protease gesny is en intrasellulêre akkumulاسie van HBsAg waargeneem is, terwyl glukoamilase uitgeskei is. Struktureel volledige montering van HBsAg het voorgekom nadat die saamgevoegde produk in die Golgi apparaat gesny is. Die vorming van verskeie intrasellulêre glukoamilase afbraak-produkte het gedui op beperkinge in die uitscheidingsmeganisme, wat veroorsaak is deur die oormatige uitdrukking van die saamgevoegde *GlaA_{G2}::S* geen. Hierdie bevindinge is gestaaf deur verhoogde HBsAg produksie as gevolg van oormatige uitdrukking van proteïen disulfied isomerase (PDI) en calnexin (CLX) asook kultivering teen verlaagde groei tempo's (μ).

Die gebruik van die geenfusie strategie deur gebruik te maak van 'n dubbel mutante *A. niger* gasheer stam, MGG029- $\Delta aamA$, waarvan twee hoof amolitiese ensieme – glukoamilase en suur amilase nie uitgedruk word nie - het aanleiding gegee tot die ontwikkeling van 'n verbeterde metode om hoë produserende HBsAg transformante te identifiseer en uit te kies. Selektoring vir hoë ekstrasellulêre glukoamilase produksie was gedoen deur die beskikbaarheid van stysel aan te wend as seleksie meganisme op soliede kulture. Die hoë effektiwiteit verkry deur seleksie van transformante met hoë ekstrasellulêre glukoamilase aktiwiteit en die geassosieerde korrelasie tussen

intracellulêre HBsAg produksie en glukoamilase uitskeiding in geenvoer of voerfermentasie, het die vermoë van die siftingsmetode aangedui om indirek hoë HBsAg-produkerende transformante te kwantifiseer deur ekstrasellulêre glukoamilase te bepaal. Deur die aanwending van bogenoemde glukoamilase siftingsmetode, is twee hoë HBsAg-produkerende transformante met verskille in μ_{maks} geïdentifiseer: (1) die vinnig groeiende transformant wat hoë biomassa produseer ($\mu_{\text{maks}} = 0.11 \text{ h}^{-1}$) en (2) die stadig groeiende transformant met hoë glukoamilase produksie ($\mu_{\text{maks}} = 0.08 \text{ h}^{-1}$). Laasgenoemde twee transformante is geëvalueer in koolstofbeperkte eksponensiële voerfermentasies en ook as gebruik tesame met die *A. niger* D15 stam in “chaperone/foldase” verhoogde produksie studies. Tydens koolstofbeperkte voerfermentasies was HBsAg produksie die hoogste teen $0.07 < \mu < 0.08 \text{ h}^{-1}$. Sodoende is ‘n metode ontwikkel om HBsAg produksie met tot soveel as 81 % te verhoog.

Oormatige produksie van CLX en PDI het gelei tot die hoogste persentasiegewyse verhoging (88 % en 107 %, respektiewelik) en sodoende hul betrokkenheid by korrekte HBsAg formasie deur middel van geenfusie bevestig. Kombinasies van BiP en geassosieerde kombinasies met CLX en PDI kon nie HBsAg produksie verhoog nie. Oormatige produksie deur middel van geenfusie van BiP het tot vermindering van tot 40 % in HBsAg produksie gelei en sodoende ongunstige toestande ten opsigte van effektiewe HBsAg formasie en produksie aangedui. HBsAg produksie is verder verhoog deur die kweek van die CLX en PDI oormatig produserende transformante in koolstofbeperkte eksponensiële voerfermentasies. Die hoogste HBsAg produksie van $352 \pm 21.3 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ ($6237.2 \pm 437.2 \mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$) en $330.2 \pm 24.4 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ ($5760.4 \pm 531.9 \mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$) is waargeneem in CLX en PDI oormatig produserende transformante. Hierdie bevindinge, tesame met die potensiële aanwending van die glukoamilase siftingsmetode vir die identifikasie van hoë HBsAg

produseerders wat “chaperone/foldase” oormatig produseer, illustreer die bruikbaarheid en aanwending van geenfusie in *A. niger* MGG029- Δ *aamA* transformante, as ‘n metode vir die produksie van ander rekombinante virusagtige deeltjies.

PREFACE

This dissertation is presented as a compilation of manuscripts. Each manuscript is introduced separately and is written accordingly to reflect a ‘stand alone’ representation for journal submission.

DEDICATION

My Wife, Anelet and
Daughter, Sarah-Anne

and

All my Teachers

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ABBREVIATIONS

<i>A.</i>	<i>Aspergillus</i>
<i>alcA</i>	Alcohol dehydrogenase gene
<i>amds</i>	Acetamidase enzyme encoding gene
<i>amyB</i>	Taka-amylase A gene
BiP	Heavy chain binding protein
<i>bipA</i>	Heavy chain binding protein encoding gene
<i>bla</i>	Ampicillin resistance gene
bp	base pair
BV	Bacular Virus
CHO	Chinese Hamster Ovary
CLX	Calnexin
<i>clxA</i>	Calnexin encoding gene
DCU	Digital Control Unit
DNA	Deoxyribonucleic acid
dO ₂	dissolved Oxygen
DW	Dry Weight
<i>E.</i>	<i>Escherichia</i>
ELISA	Enzyme-linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum-associated Protein Degradation
<i>exlA</i>	1,4-beta-endoxylanase A
FAB	fragment, antigen binding
FDA	Food and Drug Administration
GFP	Green Fluorescent Protein
GLA	Glucoamylase
<i>glaA</i>	Glucoamylase encoding gene
<i>GlaA_{G2}</i>	Gene encoding the Glucoamylase G2 form (AA 1-514)
<i>gpdA</i>	glyceraldehyde-3- phosphate dehydrogenase gene
GRAS	Generally Recognized As Safe
<i>H.</i>	<i>Hansenula</i>
HBsAg	Hepatitis B surface Antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HepB	Hepatitis B
HEWL	Hen Egg-White Lysozyme
hLF	human Lactoferrin
<i>HmB</i>	Hygromycin B
<i>hph</i>	hygromycin B phosphotransferase
HPV	Human Papilloma Virus
HSP70	Heat Shock 70 Protein family
MM	Minimal Medium
P	Promoter region
<i>P.</i>	<i>Pichia</i>

PDA	Potato Dextrose Agar
PDI	Protein Disulphide Isomerase
<i>pdiA</i>	Protein Disulphide Isomerase encoding gene
PNPG	4-Nitrophenyl alpha-D-Glucopyranoside
<i>pyrG</i>	Orotidine 5-monophosphate decarboxylase encoding gene
rBV	recombinant Baculovirus
RNA	Ribonucleic acid
S	Hepatitis B S protein
<i>S.</i>	<i>Saccharomyces</i>
t-PA	Tissue plasminogen activator
trpC	trifunctional gene in Cryptophan biosynthesis
UPR	Unfolded Protein Response
VLP	Virus-like Particle
VP	Viral Protein
WHO	World Health Organisation

NOMENCLATURE

C_s	Glucose concentration in reactor, ($\text{g}_{\text{glucose}} \cdot \text{L}^{-1}$)
C_{sf}	Glucose feed concentration, ($\text{g}_{\text{glucose}} \cdot \text{L}^{-1}$)
C_x	Biomass concentration, ($\text{g}_{\text{DW}} \cdot \text{L}_{\text{broth}}^{-1}$)
D	Dilution rate, (h^{-1})
m_s	Non-growth-associated maintenance coefficient, ($\text{g}_{\text{Glucose}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$)
M_{sf}	Mass of glucose feed, ($\text{g}_{\text{glucose}}$)
M_x	Biomass, (g_{DW})
P_{sf}	Feed pump set point (%)
q_s	Specific glucose consumption rate, ($\text{g}_{\text{Glucose}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$)
r_s	Glucose consumption rate, ($\text{g}_{\text{Glucose}} \cdot \text{h}^{-1}$)
t	Time, (h or s)
V	Reactor volume, (L_{broth})
V_{sf}	Volume of glucose feed, ($\text{g}_{\text{glucose}}$)
Y_{xs}	Biomass yield coefficient, ($\text{g}_{\text{DW}} \cdot \text{g}_{\text{glucose}}^{-1}$)
μ	Specific growth rate during fed-batch phase, (h^{-1})
μ_{set}	Set specific growth rate during fed-batch phase, (h^{-1})

INTRODUCTION

The development of non-replicating vaccines is an emerging option for safe, effective vaccines, several of which contain virus-like particles (VLPs). Considerable attention is being paid to the development of such vaccines as a means of curbing the spread of infectious disease (Hansson *et al.*, 2000). VLPs, including the Hepatitis B surface antigen (HBsAg) have been efficiently produced in microbial systems such as *Pichia pastoris*, *Hansenula polymorpha* and *Saccharomyces cerevisiae*.

The filamentous fungus *Aspergillus niger* has gained much interest as an alternative host to the commonly employed yeasts for the production of mammalian and higher eukaryotic gene products achieving varying degrees of success (Gwyne *et al.*, 1987; Upshall *et al.*, 1987; Ward *et al.*, 1995; Gouka *et al.*, 1997a; Svetina *et al.*, 2000; James *et al.*, 2007), and has previously been identified as a candidate VLP expression system through the evaluation of HBsAg (Plüddemann and van Zyl, 2003). Plüddemann and van Zyl, (2003), demonstrated the ability of *A. niger* to synthesize and assemble complex intracellular HBsAg into correctly-folded VLPs. However, despite high expression levels of the HBsAg *S* gene, production levels were low amounting to 0.4 mg.L_{culture}⁻¹ (Plüddemann and van Zyl, 2003). These results suggest that limitations with the production of recombinant HBsAg do not appear to be at the level of transcription but rather on a (post)translational level as observed in other studies expressing higher recombinant eukaryotic gene products (Jeenes *et al.*, 1994; Nyssönen and Keränen, 1995; Gouka *et al.*, 1997a; Plüddemann and van Zyl, 2003).

In direct contrast to the process mechanisms of the Hepatitis B virus in infected cells where efficient secretion is observed (Simon *et al.*, 1988; Ganem and Prince, 2004),

intracellular accumulation of HBsAg is observed in *A. niger* (Plüddemann and van Zyl, 2003). Similar findings have been observed in *S. cerevisiae* (Hamsa and Chattoo, 1994), *P. pastoris* (Cregg *et al.*, 1987), *H. polymorpha* (de Roubin *et al.*, 1991) and plant cells (Smith *et al.*, 2002), and therefore is indicative of the limitations realized in recombinant microbial expression systems.

With the continuous development and improvement of gene technology, several strategies have been developed, serving as tools for enhancing heterologous protein production levels. These include, vector design and construction, codon optimization (Gouka *et al.*, 1996), choice of promoter (Van den Hondel *et al.*, 1991), the generation of fusion constructs (Jeenes *et al.*, 1993; Gouka *et al.*, 1997b; Spencer *et al.*, 1998), over expression of chaperone proteins (Ngiam *et al.*, 2000; Punt *et al.*, 1998), the isolation of protease deficient mutants (Archer and Peberdy, 1997; van den Hombergh *et al.*, 1997) high-throughput screening for hyper-producing transformants/ mutants (Weenink *et al.*, 2006), and the optimisation of media and cultivation conditions (Schrickx *et al.*, 1993; MacKenzie *et al.*, 1994; Xu *et al.*, 2000; Conesa *et al.*, 2001; James *et al.*, 2007).

The dissertation includes a **Literature Review**, which provides an introduction to VLP vaccine systems with specific reference to HBsAg as a model VLP. The significance of VLP technology is also discussed and the potential expression systems evaluated. This also includes the possible challenges and solutions involved in recombinant production of higher eukaryotic gene products and VLPs in filamentous fungi with specific focus on *Aspergillus*. In this dissertation *A. niger* is used as the production host. HBsAg is used as a model VLP and production is targeted through the secretory pathway as

performed by Hepatitis B infected cells with the main aim of further developing the VLP technology in *A. niger*. In **Manuscript 1** a gene fusion approach is evaluated as a means of targeting HBsAg production through the secretory pathway. In **Manuscript 2** an improved methodology to identify and select high HBsAg producing transformants is evaluated. The effects of growth rate (μ) in glucose-limited exponential fed-batch fermentations on HBsAg production is evaluated in **Manuscript 3** and in **Manuscript 4** the effects of modifying chaperone/ foldase levels on HBsAg production levels is evaluated. Finally, in **Concluding remarks and Prospects**, the results of this dissertation are discussed in more general terms with a summary of the major conclusions as well as recommended future prospects.

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LITERATURE REVIEW

VIRUS-LIKE PARTICLE (VLP) PRODUCTION IN THE FILAMENTOUS

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VLPs as vaccine production systems

Vaccination is considered as an efficient means of preventing infectious diseases and through vaccination programmes, the incidence of several fatal diseases has drastically decreased (Liljeqvist and Stahl, 1999). Vaccines have often been based on attenuated or chemically inactivated live viruses. However, the potential for incomplete inactivation of the virus or reversion of an attenuated vaccine strain to a more virulent state has lead to some concern (Smithburn, 1949; Caplen *et al.*, 1985; Noad and Roy, 2003). The potential development of disease in vaccinated individuals, with the use of attenuated or chemically inactivated live viruses, is particularly relevant for viruses with a high degree of genetic exchange (Noad and Roy, 2003). Given these circumstances, the development of non-replicating vaccines is an emerging option for safe and effective vaccines, several of which contain virus-like particles (VLPs).

VLPs are a highly effective type of subunit vaccine, which are produced by recombinant expression of viral capsid proteins or envelope proteins (Miyanochara *et al.*, 1986; Delchambre *et al.*, 1989; Gheysen *et al.*, 1989; French *et al.*, 1990). VLPs are highly ordered, repetitive structures mimicking the native structural assembly and

antigenic properties of the parental virus. The VLPs are devoid of viral genetic material but have the authentic conformation of viral capsid proteins seen with attenuated virus vaccines, without any of the risks associated with viral reversion, recombination or reassortment (Noad and Roy, 2003). Additional benefits of VLP vaccines include high efficiency in eliciting a protective response, where VLPs not only stimulate B-cell-mediated immune responses, but have also shown to be highly effective at stimulating CD4 proliferative responses and cytotoxic T lymphocyte (CTL) responses (Schirmbeck *et al.*, 1996; Boisgerault *et al.*, 2002; Paliard *et al.*, 2000; Murata *et al.*, 2003). Furthermore, a high immunogenic response would result in lower vaccine doses and is likely to be a major contribution towards VLP effectiveness (Noad and Roy, 2003).

VLP based vaccines for many diseases affecting humans and animals have been licensed or are in pipeline development (Noad and Roy, 2003; Grgacic and Anderson, 2006) (Table 1). These VLP based vaccines can be categorized as lipid-envelope VLPs and non-envelope VLPs (Noad and Roy, 2003) (Table 2). The hepatitis B vaccine, among others (Table 2), is an example of a lipid-envelope VLP vaccine, which was first licensed for use in humans in 1984 by Merck (Recombivax HB®) (Valenzuela *et al.*, 1982; McAleer *et al.*, 1984; Hilleman, 2001; Hilleman, 2003) (Table 1). These enveloped VLPs are assembled from envelope proteins, which bud from intracellular compartments and thus contain the cellular lipids that make up the viral lipoprotein envelope (Grgacic and Anderson, 2006). The expression and assembly of the major capsid proteins of papillomaviruses, parvoviruses, caliciviruses, circoviruses and polyomaviruses are examples of non-enveloped VLPs (Table 2). These non-enveloped VLPs are relatively robust and simple in structure and comprise of one or two major capsid proteins of viruses which do not have a lipid envelope. Recently, VLP vaccines

against the human papillomavirus (HPV), Gardasil® and Cerevix® by Merck and GlaxoSmithKline (GSK), respectively were licensed (Andrawiss, 2005; Schiller and Lowly, 2006) (Table 1).

The successful development of VLPs as candidate vaccines has initiated interest in chimeric VLPs as novel and effective carrier systems or fusion platforms for the delivery of protein epitopes targeted at other diseases. Chimeric VLPs provide a means for the incorporation of heterologous antigens or proteins into VLPs. The formation of chimeric VLPs is possible through the genetic engineering of VLP gene sequences and co-expression of fused antigens or protein epitopes directly to VLP-forming proteins. This strategy offers enormous potential through the implementation of a chimeric VLP that carries multiple protein or antigen epitopes eliciting an immune response to multiple diseases simultaneously (Bisht *et al.*, 2001; Netter *et al.*, 2001; Noad and Roy, 2003; Grgacic and Anderson, 2006; Greco *et al.*, 2007; Michel *et al.*, 2007). The full account of the range of chimeric vaccines, vaccine status in the developmental pipeline, the advantages and disadvantages and technical considerations have previously been described (Boisgerault *et al.*, 2002; Grgacic and Anderson, 2006). Alternative applications of VLPs involve the *in vitro* assembly and disassembly of VLPs through chemical manipulation. This quality could be exploited for conjugation purposes of foreign genes or chemical agents to reassembled VLPs (Goldmann *et al.*, 1999; Goldmann *et al.*, 2000).

Table 1. Examples of VLPs used for vaccines and vaccine development (Noad and Roy, 2003).

Virus	Particle composition	Type/expression system	Size	Vaccine Status	References
HBV	Small envelope protein (HBsAg)	rec VLP (yeast) (Recombivax-HB;	22 nm	Licensed	McAleer <i>et al.</i> , 1984; Andre and Safary, 1987
	Small envelope protein (HBsAg)	rec VLP (potato)	17 nm	Preclinical	Kong <i>et al.</i> , 2001
	PreS1+2 and HBsAg	rec VLP (CHO cells) (sci-B Vac; Bic	22 nm	Licensed Licensed (developing world)	Yap <i>et al.</i> , 1992; Shouval <i>et al.</i> , 1994; Madalinski <i>et al.</i> , 2001; Yap <i>et al.</i> , 1996 Krugman <i>et al.</i> , 1971
HPV	HBsAg	Native SVP (plasma) rec VLP (mammalian cells;	22 nm	Licenced	Zhou <i>et al.</i> , 1991; Kimbauer <i>et al.</i> , 1992; Koutsky <i>et al.</i> , 2002; Villa <i>et al.</i> , 2005
	L1, major capsid protein	baculovirus; yeast) gardasil, Cervarix	40 - 50 nm		
HEV	Truncated major capsid protein (ORF2)	rec VLP (baculovirus)	23.7 nm		Li <i>et al.</i> , 1997; Li <i>et al.</i> , 2005; Purcell <i>et al.</i> , 2003; Emerson and Purcell, 2001
Influenza	HA, NA, matrix	rec VLP (baculovirus)	80 - 120 nm	Preclinical	Pushko <i>et al.</i> , 2005; Galarza <i>et al.</i> , 2005; Latham and Galarza, 2001
HCV	Core, E1, E2	rec VLP (baculovirus)	40 - 60 nm	Preclinical	Baumert <i>et al.</i> , 1998; Jeong <i>et al.</i> , 2004; Lechmann <i>et al.</i> , 2001; Murata <i>et al.</i> , 2003
Poliovirus	Capsid (VP0,1,3)	rec VLP (baculovirus)	27 nm	None	Brautigam <i>et al.</i> , 1993
HIV	Pr55gag, envelope	rec VLP (baculovirus; mammalian cells; yeast)	100 - 120 nm	Preclinical	Sakuragi <i>et al.</i> , 2002; Gheysen <i>et al.</i> , 1989; shioda and Shibuta, 1990; Deml <i>et al.</i> , 2005; Doan <i>et al.</i> , 2005
Ebola virus; Marburg virus	Glycoprotein (GP) and matrix (VP40)	rec VLP (mammalian cells)	Filovirus-like particle	Preclinical	Swenson <i>et al.</i> , 2005; Warfield <i>et al.</i> , 2003; Warfield <i>et al.</i> , 2005
Norwalk virus	capsid	rec VLP (baculovirus; transgenic potatoes)	38 nm	Phase I	Ball <i>et al.</i> , 1999; Mason <i>et al.</i> , 1996; Tacket <i>et al.</i> , 2003
Rotavirus	VP2, VP6, VP7	rec VLP (baculovirus)	70 - 75 nm	Preclinical	Vieira <i>et al.</i> , 2005; Bertolotti-Ciarlet <i>et al.</i> , 2003; Crawford <i>et al.</i> , 1994
SARS coronavirus	S, E and M	rec VLP (baculovirus)	100 nm	Preclinical	Mortola and Roy, 2004

Abbreviations: HBV, hepatitis B virus; HPV, human papilloma virus; HEV, hepatitis E virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; SARS, severe acute respiratory syndrome; rec, recombinant.

Table 2. VLP production for structurally diverse virus families (Grgacic and Anderson, 2006).

Family	Virus	Genome	Capsid structure			References
			Envelope	Major Proteins	Minor Proteins	
<i>Caliciviridae</i>	Norwalk, Hawaii, Grimsby, Burwash Landing, White Rivier, Florida, rabbit haemorrhagic disease, Hepatitis E	SsRNA	No	1	1-2	Jiang <i>et al.</i> , 1992; Laurent <i>et al.</i> , 1994; Pletneva <i>et al.</i> , 1998; Hale <i>et al.</i> , 1999; Belliot <i>et al.</i> , 2001; Li <i>et al.</i> , 1997
<i>Picornaviridae</i>	Polio	SsRNA	No	4	0	Brautigam <i>et al.</i> , 1993
<i>Flaviviridae</i>	Hepatitis C	SsRNA	Yes, 2 proteins	1		Baumert <i>et al.</i> , 1998
<i>Retroviridae</i>	HIV, SIV, FIV, BIV, visna, FeLV, BLV, rous sarcoma	SsRNA	Yes, 2 proteins	1	0	Delchambre <i>et al.</i> , 1989; Gheysen <i>et al.</i> , 1989; Yamshchikov <i>et al.</i> , 1995; Overton <i>et al.</i> , 1989; Rasmussen <i>et al.</i> , 1990; Morikawa <i>et al.</i> , 1991; Thomsen <i>et al.</i> , 1992; Rafnar <i>et al.</i> , 1998; Kakker <i>et al.</i> , 1999; Johnson <i>et al.</i> , 2001
<i>Paramyxoviridae</i>	Newcastle disease	SsRNA	Yes, 2 proteins	4	0	Nagy <i>et al.</i> , 1991
<i>Bunyaviridae</i>	Hantaan	Segmented ssRNA	Yes, 2 proteins	1	0	Betenbaugh <i>et al.</i> , 1995
<i>Orthomyxoviridae</i>	Influenza A	Segmented ssRNA	Yes, 3 proteins	2	3	Latham and Galarza, 2001
<i>Birnaviridae</i>	Infectious bursal disease	Segmented ssRNA	No	3	0	Kibenge <i>et al.</i> , 1999; Fernandez-Arias <i>et al.</i> , 1998
<i>Reoviridae</i>	Bluetongue, rotavirus	Segmented ssRNA	No	4	2-4	French <i>et al.</i> , 1990; French and Roy, 1990; Crawford <i>et al.</i> , 1994
<i>Parvoviridae</i>	Porcine parvovirus, mink enteritis parvovirus, canine parvovirus, B19, adeno-associated	SsDNA	No	1	2	Lopez de Turiso <i>et al.</i> , 1992; Martinez <i>et al.</i> , 1992; Brown <i>et al.</i> , 1991; Christensen <i>et al.</i> , 1994; Hoque <i>et al.</i> , 1999
<i>Circoviridae</i>	Chicken anaemia, porcine circovirus	SsDNA	No	1	0	Noteborn <i>et al.</i> , 1998; Nawagitgul <i>et al.</i> , 2000
<i>Papillomaviridae</i>	Papillomavirus	SsDNA	No	1	1	Zhou <i>et al.</i> , 1991; Kirnbauer <i>et al.</i> , 1992; Kirnbauer <i>et al.</i> , 1993
<i>Polyomaviridae</i>	SV40, JC	SsDNA	No	1	2	Kosukegawa <i>et al.</i> , 1996; Chang <i>et al.</i> , 1997
<i>Hepadnaviridae</i>	Hepatitis B	Discontinuous dsDNA	Yes, 3 proteins	1	0	Miyano-hara <i>et al.</i> , 1986; McAleer <i>et al.</i> , 1984; Kunke <i>et al.</i> , 1993

Abbreviations: BIV, bovine immunodeficiency virus; BLV, bovine leukemia virus; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; SV40, simian virus-40.

The Hepatitis B virus and Hepatitis B Surface Antigen

Hepatitis B, caused by the infectious Hepatitis B virus (HBV), is a serious liver infection causing liver failure, cirrhosis or cancer of the liver (Hepatitis B Foundation, <http://www.hepb.org>), and is often characterized by jaundice, abdominal pain, liver enlargement and fever. HBV interferes with the function of the liver in replicating hepatocytes causing the immune system to be activated leading to liver inflammation. An estimated 400 million people are chronically infected with HBV worldwide, of which 1 million carriers are estimated to die annually due to HBV and its consequences (WHO, 2004). This disease is transmitted by blood and body fluids including urine, saliva/nasopharyngeal fluids, semen, and menstrual fluids (Alter et al., 1977; Davison et al., 1987). Treatment is generally ineffective (Hepatitis B Foundation, <http://www.hepb.org/>) and thus prevention and vaccination remain the best alternative to curb the spread of disease. The World Health Organisation (WHO) has recommended that a Hepatitis B vaccine be included in routine immunization schedules for all children in all countries (Vryheid et al., 2001).

Hepatitis B virus

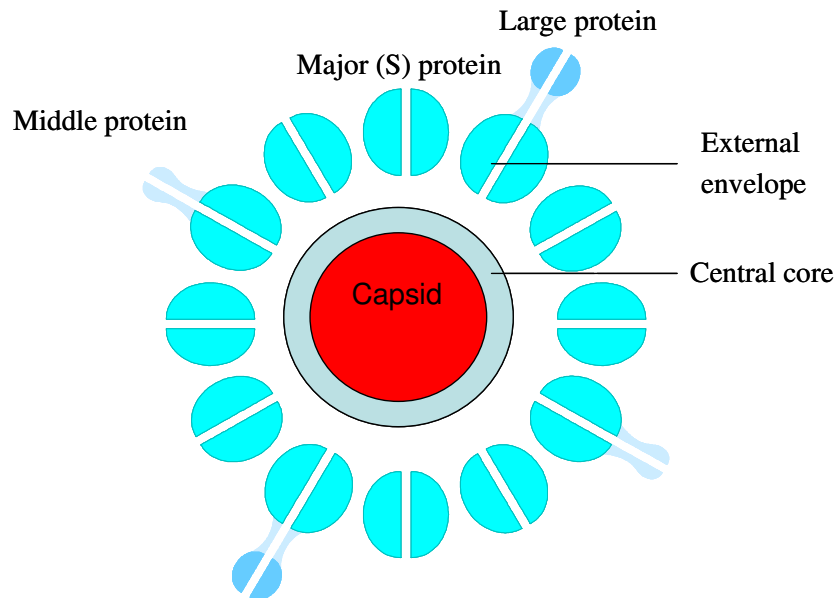
HBV, classified in the family *Hepadnaviridae* (hepatropic DNA viruses), genus *Orthohepadnavirus*, is a small envelope virus containing a partially double stranded, circular DNA genome (Huovila et al., 1992; Purcell, 1994; Shepard et al., 2006). The Hepatitis B virion, known as the Dane particle, is a spherical particle (diameter of 42 – 47 nm), comprising of a central core and external envelope. The central core or capsid comprises of ~ 180 Hepatitis B core proteins arranged in an icosahedral arrangement (Nassal and Schaller, 1993; Crowther et al., 1994), which surrounds at least one Hepatitis B polymerase protein as well as the infectious HBV genome (Gerlich and

Robinson, 1980). The external envelope consists of three proteins, designated major (S), middle (M) and large (L) protein, respectively. One Dane particle contains 300 to 400 major protein molecules and 40 to 80 middle and large protein molecules (Tiollais *et al.*, 1985), which together with the central core form the infectious viral particle (virion) (Ganem and Varmus, 1987). All three envelope proteins are glycosylated, type II transmembrane proteins that form multimers, and with further self assembly form non-infectious spherical or tubular particles that stimulate the production of antibodies in the infected host. These multimers are stabilised by disulphide bridges, which are formed by cysteine residues (Seeger and Mason, 2000). A schematic of the cross section of the hepatitis B virus is as shown in **Figure 1A** while **Figure 1B** shows the 3D structure of the virus resolved from X-ray crystallography studies.

The majority of these non-infectious self-assembling particles are in the spherical form, and termed hepatitis B surface antigens (HBsAg) (Peterson, 1987). These 22 nm subviral particles consist almost entirely of S proteins with approximately 100 monomers forming the HBsAg particle (Ganem and Varmus, 1987). HBsAg characterisation studies have revealed that the HBsAg S protein consists of 226 amino acids presented in an unglycosylated form (24 kDa) and a *N*-glycosylated form (27 kDa) (Huovila *et al.*, 1992). The primary structure includes three significant hydrophobic regions involved in HBsAg S protein characterization, intra- and inter-HBsAg assembly, and intracellular transport and secretion (Berting *et al.*, 1995; Prange *et al.*, 1995a). The HBsAg S protein is initially synthesised as a transmembrane polypeptide, where both translocation and export of the protein can proceed in the absence of any other viral gene products (Eble *et al.*, 1987). With a composition of ~ 75 % protein and ~ 25 % lipid by mass (Gavilanes *et al.*, 1982), the lipid-protein

interactions are responsible for HBsAg structural assembly and antigenicity (Gavilanes *et al.*, 1990; Gomez-Gutierrez *et al.*, 1994; Prange *et al.*, 1995b).

A



B

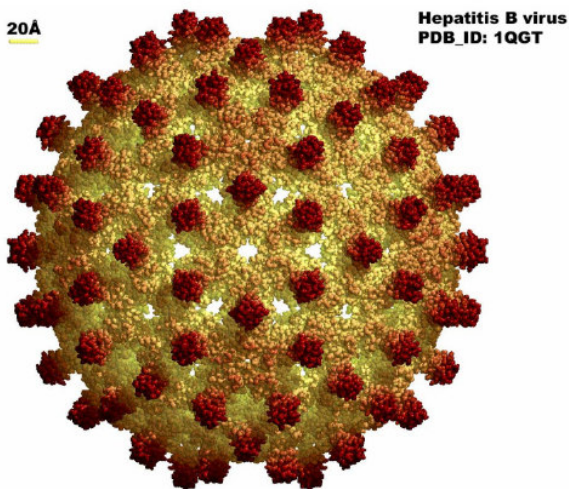


Figure 1. (A) a schematic of the cross section of a hepatitis B virus. (B) the structure of the hepatitis B virus as solved by x-ray crystallography, (taken from <http://www.virology.wisc.edu/-virusworld/covers.php>).

In the HBV life cycle, the synthesis of HBsAg S proteins is tightly regulated at the transcriptional and translational level (Tiollais and Buendia, 1991). Newly synthesised HBsAg S proteins are translocated into the lumen of the ER where disulphide-linked dimers are rapidly formed (Huovila *et al.*, 1992). Dimers are then transported to a post-ER/pre-Golgi compartment, where higher oligomers are formed resulting in the assembly of the HBsAg particle. The HBsAg particle matures by budding into this post-ER/pre-Golgi compartment (Huovila *et al.*, 1992; Prange and Streeck, 1995). The particle is then rapidly exported from the cell via the constitutive pathway of vesicular transport and the Golgi where further modification of the oligosaccharides occurs (Simon *et al.*, 1988). HBsAg particles are produced in excess of 10^3 - to 10^6 -fold in comparison to the infectious virion (Ganem and Prince, 2004), forming an effective shielding strategy contributing towards the survival of the virus against the antibodies produced by the infected host.

Brief history of treatment for the Hepatitis B disease

The first Hepatitis B vaccine (HEPTAVAX®) was licensed in 1981 and was a subunit vaccine isolated from the blood of hepatitis B infected donors (Prince, 1982). In hepatitis B patients, non-infectious 22 nm HBsAg particles are expressed in significant amounts, eliciting a strong immune response (Dekleva, 1999). These blood-derived particles were purified to levels exceeding 99 % and used as a vaccine. However the supply of suitable human plasma was inconsistent and unreliable. Together with extensive purification methods and product screening requirements, manufacturing lead times of more than 1 year for the production of 1 batch were of the norm (Dekleva, 1999). During the 1980s there was also growing concerns about HIV infection through blood-derived products (Francis *et al.*, 1986) which led to pressures to develop

recombinant vaccines for human use. The first commercially manufactured recombinant hepatitis B VLP (RECOMBIVAX HB®, Merck), which was licensed in 1984 (Valenzuela *et al.*, 1982; McAleer *et al.*, 1984; Hilleman, 2001; Hilleman, 2003), was cloned and expressed in *Saccharomyces cerevisiae* (Miyanojara *et al.*, 1983; McAleer *et al.*, 1984). GSK has produced a recombinant hepatitis B vaccine, ENGERIX B®, which is also currently in the market, among others. In addition, there is now a combined vaccine for both hepatitis A and B, known as TWINRIX®, which is bivalent in nature, containing antigenic components used in producing HAVRIX® (Hepatitis A inactivated vaccine) and ENGERIX-B® (Hepatitis B, recombinant vaccine), by GSK.

Potential expression systems for VLP vaccines

Various expression systems have demonstrated the ability to produce VLPs and these include: (1) *Escherichia coli* and other bacteria (Liew *et al.*, 2010); (2) various species of yeast including *S. cerevisiae* (Miyanojara *et al.*, 1983; McAleer *et al.*, 1984), *Pichia pastoris* (Cregg *et al.*, 1987), and *Hansenula polymorpha* (de Roubin *et al.*, 1992); (3) the baculovirus/insect cell system (Crawford *et al.*, 1994; Bertolotti-Ciarlet *et al.*, 2003; Mortola and Roy, 2004; Vieira *et al.*, 2005); (3) transgenic plant systems (Mason *et al.*, 1996; Smith *et al.*, 2002); and (4) various mammalian cell lines (Warfield *et al.*, 2003; Swenson *et al.*, 2005; Warfield *et al.*, 2005). Each expression system offers its own advantage, however there are also limitations which have to be considered when choosing the expression system (Awram *et al.*, 2002) (Table 3).

Table 3. Comparison of various systems for expression of foreign proteins (Awram *et al.*, 2002).

	Growth media/expense	Equipment costs	Level of protein production	Purification difficulty	Sensitivity to growth conditions*	Post translational Processing#
Bacteria	Moderate	Moderate	High	Easy to moderate	Moderate	Poor
Insect cells	Moderate to high	Moderate	Moderate to high	Easy to moderate	Moderate	Moderate
Animal cells	High	High	Low	Moderate to difficult	High	Excellent
Yeast cells	Moderate	Moderate	High	Easy to moderate	Moderate	Moderate to excellent
Plants	Low	Low	Low	Moderate to difficult	Low	Moderate

*Shear forces, pH, temperature, oxygen; #expression of mammalian proteins

The advantage of using *E. coli* for heterologous protein production is its ability to grow rapidly to high densities on inexpensive substrates. Genetic tools are well characterised (Baneyx, 1999) and large scale cultivation technologies are readily available. However, limitations regarding glycosylation and accumulation of misfolded proteins exist (Baneyx, 1999) (Table 3).

Ease of expression and cultivation, ability to scale-up and cost of production on inexpensive media have made yeast systems a popular choice (Sudbery, 1996) (Table 3). These systems include *S. cerevisiae*, *P. pastoris* and *H. polymorpha*, which generally provide an appropriate environment for eukaryotic post-translational processing and secretion, resulting in a product that is often similar to the native protein (Sudbery, 1996). Production of VLPs in yeast systems include the HIV-1 Gag protein (Jacobs *et al.*, 1989; Sakuragi *et al.*, 2002), anti-malarial antigens (Brady *et al.*, 2001),

poliovirus subviral proteins (Rombaut and Jore, 1997), HBsAg (Valenzuela *et al.*, 1982), and HPV-16 vaccine (Koutsky *et al.*, 2002). With respect to HBsAg production, in batch cultivations, *S. cerevisiae* has obtained yields of $\sim 2 \text{ mg.L}^{-1}_{\text{culture}}$ ($280 \text{ }\mu\text{g.g}_{\text{DW}}^{-1}$) (Gu *et al.*, 1991), which has been significantly improved by using a fed-batch process, resulting in HBsAg levels of 10 to 20 $\text{mg.L}^{-1}_{\text{culture}}$ ($670 \text{ }\mu\text{g.g}_{\text{DW}}^{-1}$) (Hsieh *et al.*, 1988; Gu *et al.*, 1991). Similar results were obtained with *H. polymorpha*: in batch cultures, HBsAg production reached levels of $1.6 \text{ mg.L}^{-1}_{\text{culture}}$ ($46 \text{ }\mu\text{g.g}_{\text{DW}}^{-1}$), while the implementation of fed-batch cultivation substantially increased HBsAg production levels to 8 to 9 $\text{mg.L}^{-1}_{\text{culture}}$ ($225 \text{ }\mu\text{g.g}_{\text{DW}}^{-1}$) (de Roubin *et al.*, 1992). In shake flask cultivation, recombinant *P. pastoris* obtained HBsAg production levels of $270 \text{ }\mu\text{g.g}_{\text{DW}}^{-1}$ while large scale fed-batch cultivation resulted in a significant increase in HBsAg production to $380 \text{ mg.L}^{-1}_{\text{culture}}$ ($6.4 \text{ mg.g}_{\text{DW}}^{-1}$) (Cregg *et al.*, 1987).

Recombinant baculovirus (rBV) expression vectors are extensively used for the expression of a variety of recombinant proteins in insect cells. These include cytosolic, nuclear, mitochondrial, membrane-bound and secreted proteins (Kost and Condreay, 1999). Recombinant BVs have been extensively used to express a variety of viral proteins such as VP1, VP2 and VP3 of the polyomavirus (Chang *et al.*, 1997; An *et al.*, 1999), Norwalk virus capsid protein (Bertolotti-Ciarlet *et al.*, 2002), HIV-1 Gag protein (Nermut *et al.*, 1994; Zhao *et al.*, 1994) and HPV capsid proteins (Kirnbauer *et al.*, 1993; Christensen *et al.*, 1994; Touze *et al.*, 1998; Baek *et al.*, 2010). Despite the high level of expression of recombinant proteins, the baculovirus system in terms of growth medium is relatively expensive (Table 3). Similar to yeast systems, baculoviruses are limited to high mannose glycoprotein modification, and this is sometimes inconsistent in comparison to mammalian cell lines. Nonetheless, baculovirus expression systems

provide a great tool for the investigation of functional and antigenic properties of rBV expressed proteins (Possee, 1999) and are currently leading the field, along with yeast in VLP production (Harro *et al.*, 2001, Koutsky *et al.*, 2002; Grgacic and Anderson, 2006).

Mammalian cells have also been used in the production of VLPs (MacNab *et al.*, 1976; Michel *et al.*, 1984) and have been successful due to appropriate modifications and authentic assembly of higher eukaryotic gene products. One commonly used cell line that has successfully produced VLPs is the Chinese Hamster Ovary (CHO) cell line (Michel *et al.*, 1984). HBsAg vaccine production in these cells as a vaccine proved to be highly immunogenic in humans and provided rapid protection against HBV (Tron *et al.*, 1989). Other mammalian cell lines include African green monkey kidney CV-1 cells transformed with an origin-defective SV-40 mutant virus and murine-fibroblasts (Shibahara *et al.*, 1985; Kaufman, 1990). However, the production of vaccines using this expression platform requires both complex and costly equipment, methodology and culture media (Marino, 1991). These cells are sensitive to shear forces, culture pH and temperature and therefore scale-up to large-scale production levels is complex and costly (Table 3). Mammalian cells grow significantly slower than microbial cells (average doubling time of 18 to 48 hours compared to 20 to 90 minutes for microbial cells) with production levels that are relatively low compared to other expression platforms. There are also fears relating to the safety of vaccines derived from mammalian cell lines. Retroviruses in particular also tend to include unwanted host cell membrane proteins in their envelope during assembly (Grgacic and Anderson, 2006). Due to these complications mammalian cell cultures are not always preferred for VLP production.

The rationale of producing VLPs in plants is significant and thus has gained much interest recently. The local production of recombinant plants producing VLPs would reduce logistics concerning distribution, cold-chain storage and transport, and costs significantly (Mett *et al.*, 2008; Sharma *et al.*, 2010). The scale of production is flexible and easily changed to market requirements (Herbers and Sonnewald, 1999). Giddings *et al.*, (2000) estimated that the cost of producing recombinant proteins in plants could be 10 to 50 - fold lower than in *E. coli*. In addition, plants do not harbour human infectious pathogens therefore sterility is not a big concern. VLP production in plants has been evaluated for HBsAg (Richter *et al.*, 2000), Norwalk virus (Tacket *et al.*, 2000), HIV (Zhang *et al.*, 2002), influenza (Beachy, 1999; D' Aoust *et al.*, 2008), and Rotavirus (O' Brien *et al.*, 2000). Mason *et al.*, (1992) demonstrated the assembly of HBsAg particles in transgenic tobacco, and even though production levels were lower compared to other expression systems, oral administration of raw potatoes induced an antibody response that was greater than those required for protection (Kong *et al.*, 2001). This technology is promising, taking into account the long culturing times and low production levels, but there are issues regarding regulatory affairs, which include quality control and validation of the manufacturing process.

In comparison to the aforementioned expression systems, filamentous fungi are hosts that have gained much interest for expressing mammalian and higher eukaryotic gene products, due to several potentially advantageous characteristics. The genus *Aspergillus* is a prodigious exporter of homologous protein and is able to produce a broad range of different enzymes in large quantities (Jeenes *et al.* 1991; Van den Hondel and Punt 1991). Through advancements in fungal genetics and the knowledge gained of the biochemical pathways, it has been demonstrated that *Aspergillus* have the ability to

express mammalian and higher eukaryotic gene products in a stable, correctly folded and functional form, through efficient post-translational modifications. Hypermethylation and disulfidation appears to be less of a problem in comparison to that in yeast and bacterial expression systems. Most filamentous fungi are transformed by plasmids that integrate into the fungal genome, suggesting potentially superior long-term stability of the fungal transformants. Examples of mammalian and higher eukaryotic gene products produced in *Aspergillus* include: insulin, human interferon α -2, interleukin-6, human lactoferrin, hen egg white lysozyme (HEWL), β 2-microglobulin, mucus proteinase inhibitor, porcine pancreatic phospholipase A₂, thaumatin, pancreatic phospholipase, tissue plasminogen activator, and bovine chymosin (Table 4). In addition, *Aspergillus* is generally robust during cultivation and can grow on various substrates and organic compounds (Jeenes *et al.* 1991; Van den Hondel and Punt 1991), resulting in cultivation methods that are substantially cheaper than various other expression systems. Detailed safety evaluations and toxicity tests of various homologous and heterologous protein preparations from *Aspergillus niger* and *Aspergillus oryzae* have indicated that there are no reasons for safety concerns in the human consumption of these products (Barbesgaard *et al.*, 1992; Greenough *et al.*, 1996; Lane *et al.*, 1997; Coenen *et al.*, 1998). *A. niger* has therefore been granted GRAS (Generally Regarded As Safe) status since 1973 by the United States Food and Drug Administration (FDA). For these reasons, filamentous fungi have been identified as acceptable candidate hosts, for the production of recombinant proteins of pharmaceutical interest.

Table 4. Mammalian and higher eukaryotic gene products expressed in *Aspergillus*

Product	Source	Expression host	Production levels	Reference	
Insulin	Human	<i>A. niger</i>	776 mU.L ⁻¹	Mestric <i>et al.</i> , 1996	
Interferon alpha-2	Human	<i>Aspergillus nidulans</i>	0.2 mg.L ⁻¹	MacRae <i>et al.</i> , 1993	
			1 mg.L ⁻¹	Gwynne <i>et al.</i> , 1989	
Interleukin-6	Human	<i>A. nidulans</i>	4.8 mg.L ⁻¹	Contreras <i>et al.</i> , 1991	
Lactoferrin	Human	<i>Aspergillus oryzae</i>	2 g.L ⁻¹	Ward <i>et al.</i> , 1995	
Lysozyme	HEWL	<i>A. niger</i>	1 mg.L ⁻¹	Archer <i>et al.</i> , 1990	
microglobulin	Human	<i>A. nidulans</i>	117 □g.L ⁻¹	O'Herrin <i>et al.</i> , 1996	
Mucus proteinase inhibitor	Human	<i>A. niger</i>	3 mg.L ⁻¹	Mikosch <i>et al.</i> , 1996	
Porcine pancreatic phospholipase A ₂	Human	<i>A. niger</i>	10 mg.L ⁻¹	Roberts <i>et al.</i> , 1992	
			Plant		
			<i>(Thaumatococcus danielli)</i>	<i>A. oryzae</i>	50 ng.L ⁻¹
Thaumatococcus danielli		<i>Aspergillus awamori</i>	5 - 7 mg.L ⁻¹	Faus <i>et al.</i> , 1998	
			100 mg.L ⁻¹	Moralejo <i>et al.</i> , 1999	
Pancreatic phospholipase	Pig	<i>A. niger</i>	10 mg.L ⁻¹	Roberts <i>et al.</i> , 1992	
Chymosin	Calf	<i>A. oryzae</i>	0.16 mg.L ⁻¹	Dunn-Colemann <i>et al.</i> , 1991	
		<i>A. awamori</i>	1.3 g.L ⁻¹	Tsuchiya <i>et al.</i> , 1993	
Tissue plasminogen activator	Human	<i>A. nidulans</i>	1 mg.L ⁻¹	Upshall <i>et al.</i> , 1987	
		<i>A. niger</i>	12 - 25 mg.L ⁻¹	Wiebe <i>et al.</i> , 2001	

Consequently, filamentous fungi have been identified as potential platforms for recombinant VLP production. Plüddemann and van Zyl, (2003) demonstrated *A. niger*'s ability to synthesize and assemble complex immunogenic viral proteins into correctly-folded VLPs. HBsAg, which is well documented and characterised, was used as the model VLP in the study. *A. niger* was transformed with the HBV *S* gene encoding the major viral envelope protein under control of the constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter (Plüddemann and van Zyl, 2003). Production of the 24 kDa HBsAg S protein, as well as a 48 kDa dimer was observed. HBsAg was localized in the intracellular membrane-associated protein fraction. Similar findings have been observed in *S. cerevisiae* (Hamsa and Chattoo, 1994), *P. pastoris* (Cregg *et al.*, 1987), *H. polymorpha* (de Roubin *et al.*, 1991) and plant cells (Smith *et al.*, 2002). The yield of HBsAg, with a diameter of 22 nm, from mycelium of the recombinant *Aspergillus* strain was estimated to be 0.4 mg.L^{-1} culture in shake flask cultivations. HBsAg production in batch culture was enhanced ~ 9-fold by optimizing the bioprocessing parameters, agitation intensity and dissolved oxygen (dO_2) concentration and were low compared to the production of homologous proteins (James *et al.*, 2007). Despite these low levels of intracellular HBsAg, high mRNA levels were recorded (Plüddemann and van Zyl, 2003). These results suggest that limitations with the production of recombinant HBsAg do not appear to be at the level of transcription but rather on a (post)translational level as observed in other studies expressing higher recombinant eukaryotic gene products (Jeenes *et al.*, 1994; Nyyssönen and Keränen, 1995; Gouka *et al.*, 1997a; Plüddemann and van Zyl, 2003). Various reasons have been suggested for these low heterologous protein yields including incorrect folding or processing of the protein during posttranslational modification, up regulation of the unfolded protein response (UPR) and Endoplasmic

Reticulum associated degradation (ERAD), and proteolytic degradation (Archer and Peberdy, 1997; Gouka *et al.*, 1997a; Plüddemann and van Zyl, 2003).

The aforementioned findings indicate the potential of the filamentous fungi, *A. niger*, as a host for VLP production. Furthermore, alternate VLPs for emerging diseases may also be developed to meet urgent needs of the society by taking advantage of the versatility of this platform technology. However, aforementioned findings also reflect new challenges and opportunities using biomolecular and bioprocessing methods to further develop the VLP technology in the filamentous fungi *A. niger*.

Challenges and opportunities involved in new research and development

The improvement of fungal production strains through developing genetic and bioprocessing technologies remains an important objective in present and future research. These approaches towards fungal strains producing recombinant products will be powerful tools for improving production levels, producing novel tailored compounds or directing the synthesis of desired products, especially in the context of a VLP production platform using the filamentous fungus, *A. niger*. However, this will only become feasible with the development of efficient methods to introduce and control gene expression in filamentous fungi (Meyer, 2008).

Classical strain improvement has been used since the early 1950's to increase yield, titre, productivity or general ease of use of filamentous fungi and has been responsible for virtually all the published hyper-producers to date. However, before classical strain improvement strategies are implemented, introducing the desired gene product of interest to the fungus host strain and the selection of hyper-producing transformants

often represents a challenge. The manipulation of genomic DNA can be achieved through several methods, one of which is of interest in this context, random integration. Random integration focuses on introducing DNA into the genome resulting in randomly integrated copies of the heterologous gene into the chromosome of the transformed strain. A common feature of this process is the duplication of the integrated DNA, producing multiple copies of the target gene (Verdoes *et al.*, 1993). This strategy is often favoured due to the ease of implementation, as well as the ability to attain high copy number insertions. Significant variance in recombinant gene products is generally expected amongst transformants through the implementation of random integration techniques. This is attributed to vector copy number and/ or site of integration (Verdoes *et al.*, 1994).

It has been shown that vector copies generally integrate in only one chromosome (Verdoes *et al.*, 1993), and that plasmid vectors can be integrated into regions of the chromosome which are transcribed with different efficiencies as suggested in the studies of Thompson and Gasson (2001) and Plüddemann and van Zyl (2003). A specific risk of random insertion is the possibility of a pleiotropic effect, such as disruption of a chromosomal gene that has a central function and thereby the transformation negatively influences growth or production. It is therefore essential to screen transformants with the objective of selecting hyper-producing transformants. To increase the probability of identifying hyper-producers, these screens require a large number of transformants. The cultivation and analysis of a large number of transformants is often laborious and time consuming. Furthermore, high value recombinant products are often costly to assay. Through the implementation of a screening strategy that allows for the cost effective, rapid, robust and high-throughput

evaluation of transformants, recombinant production levels can be significantly increased. Rational selection techniques, not involving the product of interest, but associated biochemical characteristics is a possible solution and has been successfully implemented in mutagenesis studies. Such techniques involve, for example, direct colony selection after a bioassay overlay for better penicillin producers (Vournakis and Elander, 1983), laccase production (Weenink *et al.*, 2006), and citrate specific indicator (R-di-methylaminobenzaldehyde) assays for citrate hyper-producers (Mattey, 1992).

Considering the high production potential of the fungal cell and the many similarities among the protein processing machinery of the filamentous fungi and other expression platforms such as yeasts and higher eukaryotes, the often low production levels obtained from heterologous proteins when the *A. niger* production system is employed, poses an intriguing challenge (reviewed by Jeenes *et al.* 1991; Van den Hondel *et al.* 1991; Gwynne and Devchand 1992). This bears some concern considering scale-up options. The host cell may not only be stressed by environmental conditions but also by the over-expression of the heterologous protein (Mattanovich *et al.* 2004), where limitation can be experienced at levels of transcription, translation, secretion, and degradation by proteases. Since usually highly inducible fungal transcription-control regions are used, the mRNA levels of heterologous genes, especially in multicopy strains, are not considered limiting (Gouka *et al.*, 1997a). However, high transcription rates of heterologous genes often overburden the protein production capacity so that high intracellular concentrations of misfolded heterologous proteins accumulate, leading to stress reactions as well as direct loss of product. Consequently, limitations involved with the production of heterologous protein are suggested to be associated at the post-translational level, which includes inefficient translation initiation and

elongation, translocation, folding, transport, processing or secretion of the protein (Ward *et al.* 1990; Turnbull *et al.* 1989; Tsuchiya *et al.* 1992; Broekhuijsen *et al.* 1993). Moreover, when heterologous proteins are finally produced efficiently, the threat of degradation by proteases is a major concern. *Aspergilli* can secrete a diversity of proteases (Mattern *et al.* 1992; Van den Hombergh *et al.* 1995) and it has been shown that proteases are responsible for the degradation of many heterologous proteins including human interleukin-6 (Broekhuijsen *et al.* 1993), bovine chymosin (Van Hartingsveldt *et al.* 1990) and porcine pancreatic phospholipase A (Roberts *et al.* 1992).

Appropriate strain improvement strategies for the further development of A. niger as an expression host for VLP production.

On the basis of limitations observed for the production of heterologous proteins, strain improvement strategies have been developed to alleviate the limiting factor(s) and putative blockages, with the aim of increasing the production yields of heterologous proteins in *A. niger* (Archer *et al.* 1994). Below several strain improvement strategies considered for this study are discussed.

Promoters

For effective heterologous expression, the common strategy involves the introduction of multiple copies of the gene product of interest, expressed under the transcriptional control of an efficient promoter into a high-protein-producing strain. Various promoters from filamentous fungi have been isolated which include constitutive and inducible promoters (Van den Hondel *et al.*, 1991). Each has their advantages and disadvantages: Constitutive promoters are functional during growth and therefore

unsuitable for over-expression of foreign proteins that might be toxic to the host cells, while inducible promoters are repressed by various simple, ready-to-use carbon sources. Needless to say, the success of applying either a constitutive or inducible promoter often depends on either the protein to be expressed, the fungal host strain and/or cultivation conditions.

A commonly used constitutive promoter is the *gpdA* promoter from *A. nidulans* (Punt *et al.*, 1988). The *gpdA* promoter is based on the glyceraldehyde-3-phosphate-dehydrogenase (GPD), which is a key enzyme in glycolysis and gluconeogenesis and in *A. nidulans* up to 5 % of the soluble cellular proteins consists of GPD (Punt *et al.*, 1990). The expression signals of the *gpdA* gene are therefore very strong, making this a popular promoter for heterologous expression. Examples of transcriptional control using the *gpdA* gene promoter include the production of thaumatin (Hahm and Batt, 1990), human lysozyme and HEWL (Archer *et al.*, 1990), Tissue plasminogen activator (t-PA) (Wiebe *et al.*, 2001), and HBsAg particles in *A. niger* (Plüddemann *et al.*, 2003).

In view of inducible promoters, various options are available: the alcohol dehydrogenase I (*alcA*) promoter, the glucoamylase (*glaA*) promoter of *A. niger*, *A. oryzae* and *A. awamori*; the Taka-amylase A gene promoter (*amyB*) of *A. oryzae* and the *A. awamori* 1,4- β -endoxylanaseA (*exlA*) promoter (Nunberg *et al.*, 1984; Fowler *et al.*, 1990; Hata *et al.*, 1992; Gouka *et al.*, 1996; Kanemori *et al.*, 1999). The alcohol dehydrogenase I (*alcA*) promoter is one of the strongest inducible promoters available. It shows low expression on glucose, but is strongly induced by ethanol or threonine under glucose depleted conditions (Devchand *et al.*, 1989; Gwynne *et al.*, 1989; Hintz and Lagosky, 1993). This expression system was used to produce human lactoferrin in

A. nidulans (Ward *et al.*, 1992). Both the *glaA* and *amyB* promoters are inducible by starch or maltose and repressed by xylose or glycerol, while the *exIA* promoter is strongly inducible by D-xylose. The *glaA* promoter has been studied using green fluorescent protein (GFP) as a reporter, as well as with the aid of deletion analyses (Fowler *et al.*, 1990; Santerre Henriksen *et al.*, 1999). Studies have revealed that the promoter is induced to high levels when the fungus is grown on starch or maltose, to intermediate levels when grown on glucose, but that it is repressed in the presence of xylose (Santerre Henriksen *et al.*, 1999). The promoter region is 1966 bp and two regions essential for transcription have been identified. Region I (between position –318 and –562) is required for high-level expression and region II (which lies within the first 214 bp upstream of the translation initiation site) directs low-level expression and is the minimum region that can act as a promoter (Fowler *et al.*, 1990). Putative binding sites for the protein that mediates carbon catabolite repression (CREA) have also been identified (Archer and Peberdy, 1997; Kelly, 1994). Examples of transcriptional control using the *glaA* gene promoter include the production of chymosin (Cullen *et al.*, 1987; Ward *et al.*, 1990; Tsuchiya *et al.*, 1994), human lysozyme and HEWL (Archer *et al.*, 1990; Jeenes *et al.*, 1994; MacKenzie *et al.*, 1994), cytokine human interleukin-6 (hIL6) (Broekhuijsen *et al.*, 1993; Gouka *et al.*, 1997a), human interferon α -2 (Gwynne *et al.*, 1987), Tissue plasminogen activator (t-PA) (Wiebe *et al.*, 2001), Human lactoferrin (hLF) (Ward *et al.*, 1995), Porcine pancreatic phospholipase A₂ (Roberts *et al.*, 1992), and antibody fragments (Frenken *et al.*, 1998).

Proteases deficient hosts

Filamentous fungi produce proteases which is one of the major factors contributing towards the low yield of heterologous proteins (Archer and Peberdy, 1997; Van den

Hombergh *et al.*, 1997). The evaluation of the *Aspergillus* proteolytic spectrum has revealed the presence of a number of aspartic proteases, alkaline and semi-alkaline serine proteases and serine carboxypeptidases (Jarai, 1997; Van den Hombergh *et al.*, 1997). Protease production is either localized intracellularly or in the extracellular medium (Jarai, 1997). Intracellular accumulation of proteases can be associated with the cell wall or in lytic compartments such as the vacuole (Jarai, 1997). These proteases are constitutively expressed and are probably involved in non-specific protein degradation and turnover, as well as in the activation of the precursors of several vacuolar enzymes (Jarai *et al.*, 1994). Extracellular protease expression increases significantly upon derepression with respect to carbon and/or nitrogen source in *A. niger* (Kudla *et al.*, 1990; Kelly, 1994; Tilburn *et al.*, 1995).

To reduce heterologous protein degradation by proteases, the construction and use of protease-deficient host strains has become a favored strategy (Van den Hombergh *et al.*, 1997). The construction of protease-deficient host strains has been achieved by either disrupting the interfering proteases genes or by classical mutagenesis. Disruption of the protease *pepA* gene in *A. awamori* reduced extracellular proteolytic activity to 20 % of that of the wild-type (Berka *et al.*, 1990; Mattern *et al.*, 1992) and the expression of chymosin resulted in improved production levels (Ward *et al.*, 1993). Similar studies regarding the deletion of the protease *pepE* gene resulted in the reduction of the total intracellular proteolytic activity to 32 % (Van den Hombergh *et al.*, 1997). Several protease-deficient UV mutants of *A. niger* have also been isolated and characterized through mutagenesis studies (Mattern *et al.*, 1992; Van den Hombergh *et al.*, 1995; Van den Hombergh *et al.*, 1997). These mutants have varying levels of proteolytic activity ranging from 80 % to as little as 1-2 % of that of the parental wild-

type strain and when expression of human interleukin-6 and porcine phospholipase A2 was evaluated active recombinant protein was secreted in both cases (Roberts *et al.*, 1992; Broekhuijsen *et al.*, 1993). Mutagenesis has also been employed to yield strains that acidify the medium less, thereby preventing induction of acid protease expression and degradation of heterologous protein products (Gordon *et al.*, 2000). This kind of protease deficient, non-acidifying strain is commonly used in the expression of heterologous proteins, particularly non-fungal proteins, and was also utilized for the expression of hepatitis B viral proteins (Pluddeman and van Zyl, 2003; Gordon *et al.*, 2000; Wiebe *et al.*, 2001). In general, a mutagenesis-based selection program with classical genetic strain improvement coupled to the cloning and targeted disruption of certain protease genes can provide a selection of host strains with diverse protease spectra and low proteolytic activity which can be employed in heterologous protein expression (Jarai, 1997). As already mentioned, yield reduction of target heterologous proteins can be attributed not only to co-secreted proteases, but due to intracellular degradation (Gouka *et al.*, 1996) and the release of intracellular proteases from hyphal disruption (Archer *et al.*, 1992). A reduction in specific protease activity can be dependant on culture morphology and associated level of autolysis in cultivations (Papagianni and Young, 2002; Ahamed *et al.*, 2005). Fungal proteases are also pH regulated where acid proteases are only expressed at an acidic pH (Denison, 2000). Thus, development of an optimal production medium and improved bioreactor operation strategies may benefit the heterologous protein production significantly (Wang *et al.*, 2003). Protease activity was most influenced by initial glucose concentration and dissolved oxygen (Wang *et al.*, 2003). Proteolytic activity can be reduced by growing cultures of recombinant *Aspergillus* strains in buffered media containing glucose as the carbon source at high concentrations to prevent depletion of

preferred carbon source and acidification or alkalisation of the medium with concomitant protease induction (Bartling *et al.*, 1996). Use of rich media, typically containing peptide nitrogen, induces protease production by *A. niger* (Archer *et al.*, 1990; Ahamed *et al.*, 2005); productivity of secreted egg lysozyme by a recombinant strain of *A. niger* was reduced in such rich media (Ward *et al.*, 2005). Cultivation pH control has also been a successful strategy. The study of O'Donnell *et al.* (2001), demonstrated a 6-fold reduction in protease activity and a 10-fold increase in recombinant protein yield in cultivations controlled at pH 6 compared to cultivations without pH control. In the case of HEWL production by *A. niger* HEWL under the control of the *A. niger glaA* promoter, control of pH during filamentous growth at pH 4.0 reduced protease activity 5-fold (Ward *et al.*, 2005). Protease inhibitors in growth medium could potentially reduce protease activity, however for large scale production processes this is unlikely to be a cost-effective option (Archer and Peberdy, 1997).

Gene fusion strategies

Gene fusion is one strategy that has been widely employed to enhance heterologous protein production in *A. niger* as was demonstrated for bovine prochymosin (Ward *et al.*, 1990), porcine pancreatic phospholipase A2 (Roberts *et al.* 1992), human interleukin-6 (Contreras *et al.* 1991; Broekhuijsen *et al.* 1993), HEWL (Jeenes *et al.*, 1993), human lactoferrin (Ward *et al.*, 1992; Ward *et al.*, 1995), human tissue plasminogen activator (Wiebe *et al.*, 2001), phytases from *A. awamori* (Martin *et al.*, 2003), and humanized immunoglobulin G1-kappa antibodies (Ward *et al.*, 2004) (Table 5). The observed increases in heterologous protein production varies from 5 to 1000 -fold, depending on the protein and strain, resulting in heterologous protein levels varying from 1 to 2000 mg.L⁻¹. Levels up to 1–2 g.L⁻¹ were obtained for chymosin (Dunn-Coleman *et al.*,

1991) and lactoferrin (Ward *et al.*, 1995) when high-level-production strains were subjected to several rounds of mutagenesis. Ward *et al.*, (1995) expressed a gene fusion comprising the genes encoding *A. niger glaA* and bovine chymosin to significantly increase chymosin yields.

Table 5. Production of heterologous proteins by filamentous fungi using a gene-fusion approach (Gouka, 1996).

Host	Promoter	Pre(Pro) seq.	'Carrier'-gene	KEX2	Protein level	Improvement factor	References
BOVINE PROCHYMOSIN							
<i>A. nidulans</i>	<i>A. niger glaA</i>	<i>glaA</i>	-		3 mg.l ⁻¹		Cullen <i>et al.</i> , 1987
	<i>A. niger glaA</i>	chymosin	-		6.2 mg.l ⁻¹		
<i>A. niger</i>	<i>A. niger glaA</i>	<i>glaA</i>	-		11.3 mg.l ⁻¹		Van Hartingsveldt <i>et al.</i> , 1990
	<i>A. niger glaA</i>	<i>glaA</i> (prepro)	-		4.1 mg.l ⁻¹		
	<i>A. niger glaA</i>	<i>glaA</i> (prepro)	<i>A. niger glaA</i> ₁₋₇₁	-	10.2 mg.l ⁻¹		
	<i>A. niger glaA</i>	<i>glaA</i>	-		8 mg.l ⁻¹		
	<i>A. niger glaA</i>	<i>glaA</i>	-		8 mg.l ⁻¹		
<i>A. awamori</i>	<i>A. awamori glaA</i>	<i>glaA</i> (prepro)	<i>A. awamori glaA</i> ₁₋₆₁₄	-	140 mg.l ⁻¹	10 - 20 x	Ward <i>et al.</i> , 1990
	<i>A. awamori glaA</i>	<i>glaA</i> (prepro)	<i>A. awamori glaA</i> ₁₋₆₁₄	-	1 g.l ^{-1(a,b)}	100 x	Dunn-Coleman <i>et al.</i> , 1991
	<i>A. oryzae glaA</i>	chymosin	-		70 μg.l ⁻¹		
<i>A. oryzae</i>	<i>A. oryzae glaA</i>	<i>glaA</i> (prepro)	<i>A. oryzae glaA</i> ₁₋₆₀₃	-	30 - 80 μg.l ⁻¹		Tsuchiya <i>et al.</i> , 1994
	<i>A. oryzae glaA</i>	<i>glaA</i> (prepro)	<i>A. oryzae glaA</i> ₁₋₅₁₁	-	0.1 - 0.3 mg.l ⁻¹	5 x	
	<i>A. oryzae glaA</i>	<i>glaA</i> (prepro)	<i>A. oryzae glaA</i> ₁₋₅₁₁	-	150 mg/kg ^(c)	500 x	
<i>A. awamori</i>	<i>A. awamori amyA/B</i>	<i>amyA/B</i>	<i>A. awamori amyA/B</i>	-	50 mg.l ⁻¹		Korman <i>et al.</i> , 1990
Fab ANTIBODY FRAGMENTS							
	<i>T. reesei cbh1</i>	<i>cbh1</i>	-		0.3 - 1 mg.l ⁻¹		
<i>T. reesei</i>	<i>T. reesei cbh1</i>	<i>cbh1</i>	<i>T. reesei cbh1</i> ₁₋₄₆₆	-	5 - 40 mg.l ⁻¹	5 - 100 x	Nyysönen <i>et al.</i> , 1993
	<i>T. reesei cbh1</i>	<i>cbh1</i>	<i>T. reesei cbh1</i> ₁₋₄₆₆	-	50 - 150 mg.l ^{-1(d)}	> 150 x	Nyysönen <i>et al.</i> , 1995
HEN EGG WHITE LYSOZYME							
	<i>A. niger glaA</i>	HEWL	-		12 mg.l ⁻¹		
<i>A. niger</i>	<i>A. nidulans gpdA</i>	HEWL	-		1 mg.l ⁻¹		Archer <i>et al.</i> , 1990b
	<i>A. niger glaA</i>	HEWL	-		50 mg.l ⁻¹		
	<i>A. niger glaA</i>	<i>glaA</i> (prepro)	<i>A. niger glaA</i> ₁₋₄₉₈	+	1 g.l ⁻¹	> 20 x	Jeenes <i>et al.</i> , 1993

Table 5 (continued). Production of heterologous proteins by filamentous fungi using a gene-fusion approach (Gouka, 1996).

Host	Promoter	Pre(Pro) seq.	'Carrier"-gene	KEX2	Protein level	Improvement factor	References
HUMAN INTERLEUKIN-6							
	<i>A. niger glaA</i>	<i>glaA</i>	-		25 $\mu\text{g.l}^{-1}$		
	<i>A. niger glaA</i>	hIL6	-		< 1 $\mu\text{g.l}^{-1}$		Carrez <i>et al.</i> , 1990
<i>A. nidulans</i>	<i>A. niger glaA</i>	<i>glaA</i> (prepro)	-		2 $\mu\text{g.l}^{-1}$		
	<i>A. niger glaA</i> / <i>A. nidulans gpdA</i>	<i>glaA</i>	<i>A. niger glaA</i> ₁₋₆₁₄	+	5 mg.l^{-1}	200 x	Contreras <i>et al.</i> , 1991
	<i>A. niger glaA</i>	<i>glaA</i>	-		ND		
<i>A. niger</i>	<i>A. niger glaA</i>	hIL6	-		ND		Carrez <i>et al.</i> , 1990
	<i>A. niger glaA</i>	<i>glaA</i> (prepro)	-		ND		
	<i>A. nidulans gpdA</i>	hIL6 (prepro)	-		ND ^(a)		
<i>A. niger</i>	<i>A. nidulans gpdA</i>	<i>glaA</i> (prepro)	-		ND ^(a)		
	<i>A. nidulans gpdA</i>	<i>glaA</i> (prepro)	<i>A. niger glaA</i> ₁₋₅₁₄	+	15 $\text{mg.l}^{-1(a)}$	> 1000 x	Broekhuijsen <i>et al.</i> , 1993
	<i>A. nidulans gpdA</i>	<i>glaA</i> (prepro)	<i>A. niger glaA</i> ₁₋₅₁₄	-	> 40 $\text{mg.l}^{-1(a)}$	> 1000 x	
	<i>A. nidulans alcA</i>	unknown	-		< 1 - 5 mg.l^{-1}		Hintz <i>et al.</i> , 1995
<i>A. nidulans</i>	<i>A. nidulans alcA</i>	unknown	<i>A. niger glaA</i>	+	> 100 mg.l^{-1}	20 - 100 x	
HUMAN LACTOFERRIN							
<i>A. nidulans</i>	<i>A. nidulans alcA</i>	lactoferrin	-		5 mg.l^{-1}		Ward <i>et al.</i> , 1992a
<i>A. oryzae</i>	<i>A. oryzae amy</i>	<i>amy</i>	-		25 mg.l^{-1}		Ward <i>et al.</i> , 1992b
<i>A. awamori</i>	<i>A. awamori glaA</i>	<i>glaA</i> (prepro)	<i>A. awamori glaA</i> ₁₋₄₉₈	+	> 250 mg.l^{-1}	> 10 - 50 x	Ward <i>et al.</i> , 1995
<i>A. awamori</i>	<i>A. awamori glaA</i>	<i>glaA</i> (prepro)	<i>A. awamori glaA</i> ₁₋₄₉₈	+	> 2 $\text{g.l}^{-1(b)}$	> 80 - 400 x	
HUMAN LYSOZYME (HLz)							
	<i>T. reesei</i> TR1	HLz			0.5 - 2 mg.l^{-1}		
<i>T. geodes</i>	<i>T. reesei</i> TR1	Synthetic prepro			0.25 - 2 mg.l^{-1}		Baron <i>et al.</i> , 1992
	<i>T. reesei</i>	Synthetic prepro	<i>S. hindustanus ble</i>	-	10 - 150 mg.l^{-1}	10 - 150 x	
PORCINE PANCREATIC PROPHOSHOLIPASE A2							
	<i>A. niger glaA</i>	PLA2	-		ND ^(a)		
<i>A. niger</i>	<i>A. niger glaA</i>	<i>glaA</i> (prepro)	<i>A. niger glaA</i> ₁₋₆₁₄	-	10 $\text{mg.l}^{-1(a)}$		Roberts <i>et al.</i> , 1992

ND – Not decetable, ^(a) Protease mutant strain, ^(b) high levels obtained by mutagenesis, ^(c) solid state fermentation, ^(d) submerged fermentation

The gene fusion strategy fuses the genomic sequences encompassing the complete or partial coding region of a homologous, highly expressed and well secreted protein often termed ‘the carrier protein’ to the N-terminal end of the heterologous protein of interest (Ward *et al.*, 1990). The highly expressed and well secreted glucoamylase gene (*glaA*) of *A. niger* and *A. awamori* are commonly used as the ‘carrier protein’ for gene fusions. The glucoamylases can be divided into three domains: an N-terminal catalytic domain, a C-terminal starch-binding domain and a flexible *O*-glycosylated linker region that separates the two domains (Archer, 1994). The starch-binding domain shuffling in *A. niger* glucoamylase significantly affects the insoluble starch binding and hydrolysis (Cornett *et al.*, 2003), while the C-terminal starch-binding domain can be efficiently replaced by the heterologous protein (Broekhuijsen *et al.*, 1993; Jeenes *et al.*, 1993; Ward *et al.*, 1995) and the linker region permits the catalytic domain and the rest of the fusion protein to fold independently. A positive effect of the fusion is caused through the identification of the genomic sequences encompassing the coding region of the highly expressed carrier protein by the host cells protein processing machinery, and therefore encouraging high expression of the fused cassette (Le Loir *et al.*, 2005). The N-terminal carrier protein is then believed to facilitate passage of the heterologous protein through the processing machinery of the host, aiding heterologous protein maturation and stabilization by facilitating translocation and subsequent folding in the Endoplasmic Reticulum (ER) (Gouka *et al.*, 1997b; Bermudez-Humaran *et al.*, 2003). Also, by creating a passage through the fungal protein processing pathway for the heterologous protein under the mask of the carrier protein, intracellular proteolytic degradation is reduced (Gouka *et al.*, 1997b), and protein localization such as secretion (Le Loir *et al.*, 2005) or intracellular accumulation (Gordon *et al.*, 2000; Sojikul *et al.*, 2002) can be realized. The fusion protein is then cleaved further along the protein

processing pathway, resulting in the production of the separate proteins. Cleavage occurs either by autocatalytic processing of the heterologous protein by an unknown fungal protease (Ward *et al.*, 1990; Baron and Tiraby, 1992; Roberts *et al.*, 1992; Nyssönen *et al.*, 1993; Tsuchiya *et al.*, 1994; Nyssönen and Keränen 1995; Punt *et al.*, 2002) or by a KEX2-like protease, for which a dibasic amino acid recognition site had been introduced specifically into the fusion protein (Calmels *et al.*, 1991; Contreras *et al.*, 1991; Broekhuijsen *et al.*, 1993; Ward *et al.* 1995; Mikosch *et al.*, 1996; Gouka *et al.*, 1997b). The latter has proven an effective method of obtaining correctly processed mature heterologous proteins because an effective kexin-like maturase has been identified in *A. niger* (Jalving *et al.*, 2000). The separation of the heterologous protein from the homologous carrier protein is usually achieved by inserting a recognition sequence for KEX2-like endopeptidases, which cleaves proteins at two adjacently located basic amino acids, preferentially lys-arg (Radzio and Kueck, 1997).

Modifying chaperone levels

Newly synthesized membrane-bound and/or secreted proteins must fold and assemble correctly in order to travel along the secretory pathway and eventually reach their appropriate cellular destinations. Unfolded and/or misfolded proteins are prevented from leaving the ER, which may lead to the saturation or overloading of the secretory pathway (Lodish *et al.* 1983; Shuster 1991; Parekh *et al.* 1995; Shusta *et al.* 1998; Smith *et al.* 2004). This in turn induces the synthesis of folding enzymes (and associated UPR) and/or an ER-associated protein degradation response (ERAD), which degrades those proteins that fail to reach the correct conformation (Conesa *et al.*, 2001). This action of folding and/or maturation of proteins into their native form are assisted *in vivo* by helper proteins named chaperones and foldases. Foldases catalyze

slow, often rate limiting, covalent changes, such as disulphide bond formation and proline isomerisation, which are essential for obtaining a functional conformation. Molecular chaperones are not regarded as catalysts but as assisting proteins that transiently and non-covalently bind to hydrophobic amino acid stretches, stabilizing the immature protein, thus preventing it from aggregating with other unfolded/misfolded proteins. In normal cells, the controlled concentrations of foldases and chaperones are likely to be sufficient for proper folding and assembly of proteins destined for secretion. However, in heterologous expression systems where there is a greater flux of proteins being translocated into the ER, the folding, assembly, and secretion machinery may become saturated, leading to improperly folded structures or protein aggregates that are not secreted (Ngiam *et al.*, 2000).

The overproduction of certain helper proteins has become a plausible strategy in reducing UPR and ERAD associated cellular stress and thus enhancing heterologous protein production efficiency. The effect of chaperone levels on heterologous protein production has been assessed for a number of systems (Dorner *et al.*, 1989; Dorner *et al.*, 1992; Hsu and Betenbaugh, 1997). The genes for several ER chaperones and foldases have been isolated from filamentous fungi; namely *bipA* (from a family of binding proteins, BiP) (van Gemeren *et al.*, 1997); *pdiA* (from a family of protein disulfide isomerase) (Jeenes *et al.*, 1997; Ngiam *et al.*, 2000); and a family of calnexins (Conesa *et al.*, 2001), which have been over-expressed as a strategy to improve the heterologous protein production (Table 6).

Table 6. Effect of the modification of the levels of ER resident enzymes on heterologous protein production (Conesa *et al.*, 2002).

Chaperone	Protein	Organism	Effect	References
BiP	Three human proteins	CHO cells	BiP levels inversely correlated with protein production levels	Dorner <i>et al.</i> , 1992 Dorner <i>et al.</i> , 1988
BiP	Antibodies	Baculovirus	BiP overexpression increased intracellular but not secreted Ig protein levels	Hsu <i>et al.</i> , 1994
BiP	GLA:IL6	<i>A. niger</i>	BiP overexpression increased intracellular but not extracellular levels of fusion proteins	Punt <i>et al.</i> , 1998
BiP	GLA::scFV4715	<i>A. niger</i>	BiP overexpression had no effect	van Gemeren <i>et al.</i> , 1998
BiP	Cutinase	<i>A. awamori</i>	BiP overexpression had no effect	van Gemeren <i>et al.</i> , 1998
BiP	Three heterologous proteins	Yeast	Lower BiP levels resulted in lower secretion levels. Higher BiP levels did not increase secretion levels	Robinson <i>et al.</i> , 1996
BiP	Chymosin TMT	Yeast	BiP overexpression increased level of secreted chymosin. TMT unaffected	Harmsen <i>et al.</i> , 1996
PDI	PDGF	Yeast	PDI overexpression increased PDGF secretion and lowered intracellular accumulation	Robinson <i>et al.</i> , 1994
PDI	Lysozyme	Yeast	PDI overexpression increased intracellular and secreted protein levels	Hayano <i>et al.</i> , 1995
PDI	Antistasin	Yeast	PDI overexpression increased protein production	Schultz <i>et al.</i> , 1994
PDI	Lysozyme, Glucoamylase	<i>A. niger</i>	<i>PDIA</i> overexpression did not influence the secretion levels of both proteins	Ngiam <i>et al.</i> , 2000
BiP Calnexin	MnP	<i>A. niger</i>	Calnexin overexpression increases MnP production. BiP overexpression decreases MnP production	Conesa <i>et al.</i> , 2002
BiP Calnexin Calreticulin	HCV envelope proteins	Mammalian	The three chaperones interact, but when overexpressed, no effect on secretion levels	Choukhi <i>et al.</i> , 1998
BiP Calnexin Calreticulin Erp57	Myc-SERT	Baculovirus	Increased production with Calnexin, less with BiP and Calreticulin. Erp57 no effect	Tate <i>et al.</i> , 1999

The molecular chaperone, BiP (also known as the glucose regulated protein GRP78) is a member of the heat shock 70 protein family (HSP70), which is localized in the lumen of the ER (Bole *et al.*, 1986; Munro and Pelham, 1986) and is involved in several functions in the ER: (1) assists in protein folding, (2) is involved in translocation, and (3) assists in the ERAD pathway (Brodsky *et al.*, 1999; Gething, 1999; Matlack *et al.*, 1999). In filamentous fungi, as in other organisms, the BiP encoding gene has a basal expression level under normal growth conditions and is over-expressed in situations of cellular stress such as glucose starvation, heat shock and conditions typical of the UPR (Mori *et al.*, 1992; van Gemeren *et al.*, 1997; Techel *et al.*, 1998; Ngiam *et al.*, 2000). The correlation between BiP induction and secretion efficiency remains unclear. Enhanced *bipA* mRNA levels have been observed in various *Aspergillus* strains expressing recombinant extracellular proteins (Punt *et al.*, 1998); inefficient secretion of single chain antibodies in *A. awamori* coincided with an increased BiP production (Frenken *et al.*, 1998) and similarly, a two-fold induction in *bipA* mRNA levels was measured in two *A. niger* strains producing the HEWL (Ngiam *et al.*, 2000). However, contradicting results were obtained when the BiP levels remained unchanged when interleukin-6 was produced in *A. niger* (Punt *et al.*, 1998). In terms of over-expressing the Bip chaperone, the production of several homologous and heterologous proteins fused to *glaA* were investigated, where the over-expression of *bipA* resulted in increased levels of unprocessed fusion proteins in the cell lysates in *A. niger* (Punt *et al.*, 1998). However, no improvement of the secretion of the fusion proteins was observed. Similar results were obtained in *A. awamori*, where *bipA* over-expression had no effect on the production of cutinase variants (van Gemeren *et al.*, 1998) (Table 6). Although no clear relationship between BiP induction and a particular characteristic of the overproduced protein can be deduced from this data, they do suggest that protein overproduction may lead to increased levels of unfolded proteins, and thus result in *bipA* over-expression.

The foldase, disulphide isomerase (PDI), from the thioredoxin superfamily, is known to be an essential protein that catalyzes the refolding of denatured and reduced RNase, through the oxidation, reduction and isomerisation of protein disulphides (Laboissiere *et al.*, 1995; Noiva, 1999; Ngiam *et al.*, 2000; Wilkinson and Gilbert 2004). PDI behaves as a chaperone as it inhibits the aggregation of misfolded proteins (Gilbert, 1997; Wang, 1998; Ferrari and Söling, 1999). These functions are crucial during protein maturation in the ER (Gilbert, 1997; Wang, 1998; Ferrari and Söling, 1999) as demonstrated by the study of Moralejo *et al.* (2001) who showed that modifying the level of PDI was critical for production of thaumatin in *A. awamori*. Maximum levels of thaumatin (5-fold increase) was obtained in a strain with two to four fold more PDI than in the parental strain, with no significant change in production of the native proteins acid phosphatase and α -amylase. However, characterization of the *pdiA*-like gene *prpA* of *A. niger* var. *awamori* has also shown that although the *prpA* gene was induced in cells secreting bovine prochymosin, the overproduction of *prpA* had no effect on chymosin production (Wang and Ward, 2003). Similarly, an increased level in *pdiA* transcripts was also observed in two *A. niger* strains overproducing the heterologous protein, HEWL. However, over-expression of *pdiA* did not increase secreted yields of HEWL in *A. niger*, suggesting that *pdiA* itself is not limiting for secretion of this protein (Ngiam *et al.*, 2000) (Table 6).

Calnexin (CLX) is a lectin-like, glycan-dependent molecular chaperone and is a major component of the ER quality control system. Similar to BiP, CLX also has several intracellular functions; (1) facilitates the folding of glycosylated proteins (Helenius *et al.*, 1997; Conesa *et al.*, 2002) by slowing folding events and allowing them to occur in a controlled manner, (2) retains immature or unassembled structures in the ER, (3) prevents aggregation, (4) facilitates the catalysis of disulfide bond formation, and (5) potentially aids in the sorting of malformed substrates for degradation (Pearse and Hebert, 2009). Over-production of CLX has led to improved heterologous protein yields; CLX over-production

enhanced the levels of functional serotonin transporter produced using the baculovirus expression system (Tate *et al.*, 1999). Similarly *clx* over-expression increased manganese peroxidase levels in *A. niger* 5-fold (Conesa *et al.*, 2002). In contrast, a similar approach in mammalian cells had no effect on production yields of two hepatitis C (HCV) envelope proteins (Choukhi *et al.*, 1998) (Table 6).

It is clear that results concerning the over-expression of chaperones are far from conclusive and frequently seem contradictory. The process of protein folding is a complex network of interactions which is dependent on numerous factors which include the characteristics of the folding proteins, the environment in the ER and the availability of specific co-factors. Chaperones may co-bind or act sequentially in protein folding as reported by Molinari *et al.* (2002), who observed a sequential involvement of the CLX and BiP/PDI chaperone systems in mammalian cells. CLX bound to newly synthesized proteins and thus facilitated folding as well as prevented aggregation and premature degradation. BiP and PDI were also involved in this phase, but were also essential in directing terminally misfolded proteins, after their release from CLX, for degradation in the ERAD pathway (Molinari *et al.*, 2002). Similarly, Jannaitipour *et al.*, (1998) reported the co-interaction of CLX and BiP in the folding of acid phosphatase in *Schizosaccharomyces pombe* and Gillece *et al.*, (1999) and Mayer *et al.*, (2000) report on the synergistic effect and cooperative action of BiP and PDI in protein folding.

Appropriate bioprocessing strategies for the further development of A. niger as a production host for VLP production.

In order to develop a successful, cost effective cultivation process for *A. niger*, cultivation parameters as well as the mode of operation are of paramount importance to ensure reproducible cultivations and maximizing product yield. This is not a simple task due to the

inherent complications in filamentous fungi cultivations. Filamentous fungi are morphologically complex organisms, differing in structure during various cultivation periods in their life cycle, differing in form between surface and submerged growth, differing also with the nature of the cultivation parameters.

The optimisation and control of cultivation parameters, such as inoculum, temperature, pH, agitation, dissolved oxygen (dO_2), aeration, and medium composition are critical, as their influence on growth, morphology and product formation can be used to provide reproducible cultivations and maximize product yield (James, 2006 MSc thesis). However, as illustrated in Figure 2, complex interactions exist between the cultivation conditions, the productivity and the morphology in fermentations of filamentous microorganisms. Thus extensive knowledge of the host strain and product of interest is a prerequisite for the successful development of a cultivation process.

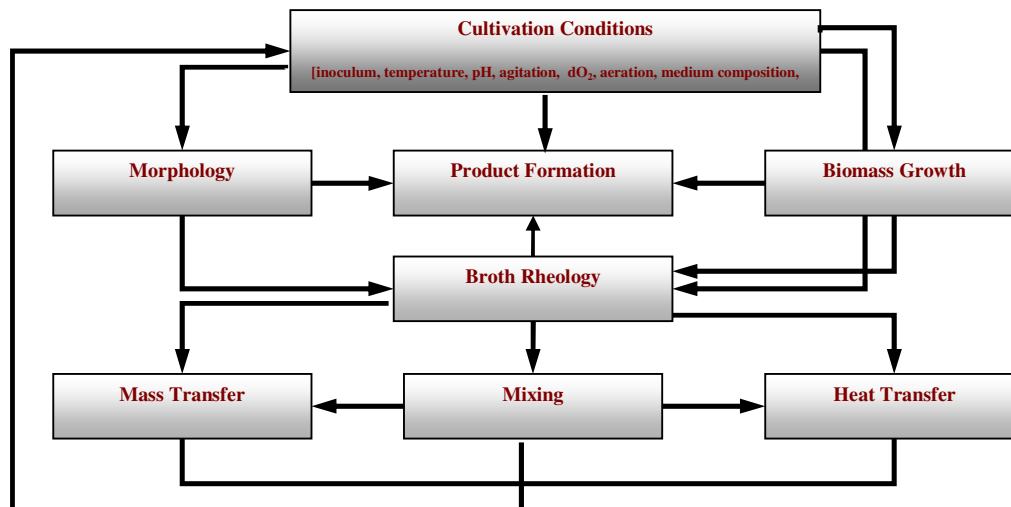


Figure 2. Complex interactions between process conditions, productivity, and morphology in submerged fermentations of filamentous microorganisms (adapted from Metz, 1976).

Batch, fed-batch, and continuous cultivations are generally the three modes of operation in submerged cultivation. Each have their advantages. Batch systems can be used to produce different products at different periods of the cultivation and are fairly easy and cost effective to execute. Due to the reduced culturing period, batch systems have a lower risk of contamination. Batch systems have an increased genetic stability due to a freshly produced inoculum used for each batch.

Continuous cultures are favourable because a steady state is obtainable through stable physical, chemical and biological variables. With the advent of the genomic era has come the knowledge of microbial genomes, and the technology to study global protein, mRNA and metabolite profiles has been developed. These so called 'omic' technologies provide the possibility to characterize cell physiology at a molecular level, providing temporal, spatial and even real-time information. The continuous chemostat is an ideal system for such studies as it is a continuous culture system that provides a constant environmental background (Hoskisson and Hobbs, 2005). Bull (1974) and Pirt (1975) listed some of the most evident advantages of the continuous chemostat cultivation: (1) Environmental conditions can be maintained constant; (2) The specific growth rate can be varied by changing the growth-limiting substrate made available to the microorganism for consumption, (3) The specific growth rate can be maintained for long periods of time; (4) Steady state biomass concentration can be set and maintained constant by the use of different concentrations of the growth-limiting substrate in the influent medium; and (5) Substrate-limited growth rate can be studied in a constant environment, whereas substrate-limited growth in batch culture is transient and is accompanied by changing growth rate.

Fed-batch fermentations are used extensively in industrial applications and are often described as a production technique between batch and continuous fermentation (Longobardi, 1994).

Through an accurate feed rate, and appropriate feed composition (Jong *et al.*, 1995), fed-batch offers many advantages over batch and continuous cultures. However, this requires knowledge of the microorganism and its requirements as well as batch fermentation data that will contribute to the understanding of its physiology with product formation. Through the implementation of fed-batch fermentations the cultivation time is often extended in comparison to batch, contributing to high biomass concentrations. For growth associated products this is often favorable causing volumetric productivity improvements (Agrawal *et al.*, 1989). Through the implementation of growth-limiting substrate feed, allowing sufficient nutrients solely for the production of the product of interest, catabolic repression and the production of by-products that are generally related to the presence of high concentrations of substrate can also be avoided. When high concentrations of substrate are present, the oxidative capacity of the cells is exceeded, and due to the Crabtree effect, products other than the one of interest are produced, reducing the efficacy of the carbon flux. Moreover, these by-products prove to even "contaminate" the product of interest, such as ethanol production in baker's yeast production, and to impair the cell growth reducing the fermentation time and its related productivity (<http://userpages.umbc.edu/~gferre1/fedbatch.html>). Various feeding strategies are available to fed batch cultivations. Some examples include continuous and pulsed feeding (Bhargava *et al.*, 2003), pH regulation (Tulin *et al.*, 1992), shot feeding (Larsson *et al.*, 1993), single or multi-substrate (Tulin *et al.*, 1992), and a linear increase, or exponential feed (Gu *et al.*, 1991; Ramirez *et al.*, 1995). For a growth-associated product, a direct correlation between biomass formation and the product of interest generally exists. This correlation can also relate to the specific growth rate of the host. It is therefore beneficial to apply an exponential feeding strategy that allows growth at an optimum and constant specific growth rate as demonstrated for the production of HBsAg in *S. cerevisiae* (Agrawal *et al.*, 1989; Alfafara *et al.*, 1992). A

10-fold increase in HBsAg was reported using this strategy in comparison to a linear feed to which the same volume and total substrate was added.

This could also be achieved using a continuous cultivation, however there are concerns regarding the accumulation of undesired mutations in prolonged cultivation (Withers *et al.*, 1994; Swift *et al.*, 2000; van de Vondervoort *et al.*, 2004). Also, in all filamentous fungi cultivations there are complications achieving homogenous cultivations. These complications are accentuated in prolonged fungal cultivations as in continuous cultures and are attributed to the morphology and surface properties of hyphae and spores. Poulsen (2005) discusses the challenges in obtaining and maintaining homogenous submerged cultures of filamentous fungi in bioreactors. Mycelia tend to adhere to bioreactor surfaces, such as probes, baffles and on walls at and above the liquid-gas interface of the bioreactor. This is termed as wall growth and is unfavorable as it contributes to culture heterogeneity. Most problems that lead to culture heterogeneity can be alleviated with special cultivation strategies as discussed by Larsen *et al.* (2004) and Poulsen (2005). Variable agitation and cooling of head space are some strategies that can be used to reduce wall growth (Larsen *et al.*, 2004), however these strategies do not promise 100% success as additional cultivation issues may arise from species- or strain-specific properties (Zangirolami *et al.*, 2000).

Concluding remarks

Evidence suggests that the factors influencing heterologous protein yield are complex and include biochemical and biophysical properties of the protein itself; its influence on, and modification of, the host's own cellular functions and the choice and deployment of those sequences that are necessary for efficient processing and production. Therefore development strategies must be individually designed and assessed for each heterologous protein

production system. Apart from implementing the above-mentioned strategies as tools for enhancing heterologous protein production levels, other strategies are available. These include mutagenesis (MacKenzie *et al.*, 2000; Yaver *et al.*, 2000), signal sequences (Verdoes *et al.*, 1994; Jarai *et al.*, 1997; Conesa *et al.*, 2002), the introduction of a large number of gene copies (Christensen *et al.*, 1988; Finkelstein *et al.*, 1989; Fowler *et al.*, 1990; Punt *et al.*, 1991b; Verdoes *et al.*, 1994), improving the capacity of the glycosylation machinery (Conesa *et al.*, 2002). Another field of research with considerable promise is that of the ‘-omics’ technologies. These technologies deal with an overall analysis of gene expression (transcriptomics), protein (proteomics) and metabolite (metabolomics) production at the level of the complete organism. Together with advanced microscopy technologies, such as fluorescence resonance energy transfer and fluorescent lifetime imaging, these technologies will add value in visualizing metabolic pathways and protein–protein interactions in living systems and hence direct research towards improved heterologous protein production levels.

This project aims to address the challenges discussed above in an attempt to identify factors involved in improving HBsAg production in the expression system *A. niger*. Specific aims of the dissertation are discussed next.

Project Aims

Manuscript 1

The intracellular accumulation of recombinant HBsAg in *A. niger* and similarly in *S. cerevisiae* (Hamsa and Chattoo, 1994), *P. pastoris* (Cregg *et al.*, 1987), *H. polymorpha* (de Roubin *et al.*, 1991) and plant cells (Smith *et al.*, 2002), suggests that the production process in the abovementioned expression systems differs to the efficiently secreted Hepatitis B virus in infected cells. This may be indicative of the processing limitations in recombinant

microbial systems. In this study we investigate the option of targeting HBsAg production through the secretory pathway of *A. niger*. The gene fusion strategy is implemented using the highly expressed glucoamylase gene (*GlaA*) fused to the HBsAg *S* gene. The inducible glucoamylase promoter (*GlaA_p*) is used to control transcription in the *A. niger* D15 host. The study focuses on the mechanism of the fusion protein and associated HBsAg processing. Further objectives include determining (1) the impact of the glucoamylase promoter on HBsAg production, and (2) the stability of the HBsAg product in intracellular and extracellular extracts. Batch fermentation data is presented to quantify results.

Manuscript 2

It is well recognized that the successful enhancement of heterologous protein levels is largely based on the development of effective strains, transformants and/ or mutants. In Manuscript 2 we aim to develop an improved methodology to identify and select high HBsAg producing transformants. Three screening methods for the identification of high HBsAg producing transformants are evaluated in conjunction with conventional selection (*amdS* selection marker) based on acetamide selection; (1) transformant selection based on acrylamide utilization through expression of the *amdS* gene, (2) transformant selection based on high growth on starch as reported by Weenink *et al.* (2006), and (3) transformant selection based on high extracellular glucoamylase activity using starch as a selection pressure. Batch fermentation data is included to quantify growth, glucoamylase and HBsAg production levels of selected high-HBsAg producing transformants.

Manuscript 3

In this study, we evaluate the effects of growth rate (μ) in glucose-limited exponential fed-batch fermentations on HBsAg production of two HBsAg producing transformants, previously identified (Manuscript 2 of this dissertation); (1) the 'fast-growing high-biomass producing' transformant, DAHB21#20, and (2) the 'slow-growing high-glucoamylase producing' transformant, DAHB21#40. Through glucose-limited exponential fed batch fermentation the effects of μ over a range of 0.03 to 0.1 h⁻¹ on specific and volumetric intracellular HBsAg production as well as biomass concentration and extracellular glucoamylase activity are evaluated. Additional analysis on the effect of μ in fed batch fermentation on substrate consumption (q_s) and the maintenance coefficient (m_s) is also performed and provides insight towards determining the requirements for efficient HBsAg production.

Manuscript 4

In this manuscript we test the hypothesis that limitations/ bottlenecks, at a (post)translational level in the secretory pathway using gene fusion, are negatively affecting HBsAg production, and can be demonstrated by increases in HBsAg production through the over-production of chaperones/ foldases. The effects of over-producing the chaperones BiP and CLX, the foldase PDI, and the combinations thereof on the production of recombinant HBsAg are evaluated in *A. niger* expressing the HBsAg fusion cassette. Transformants expressing modified chaperone and foldase levels created are evaluated for intracellular HBsAg, extracellular glucoamylase activity, and biomass production. To compare the strategies of growth regulated cultivations and modifying chaperone levels as a means of enhancing HBsAg production levels in *A. niger*, highest HBsAg producing transformants expressing modified chaperone and foldase levels are evaluated in batch and glucose-limited exponential fed batch fermentations. As an additional objective of this study the potential application of the solid-state glucoamylase

screen using starch as a selection pressure (previously described in Manuscript 2 of this dissertation) for the identification of high HBsAg producers over-producing chaperones/foldases is evaluated.

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RECOMBINANT HEPATITIS B SURFACE ANTIGEN PRODUCTION IN *ASPERGILLUS NIGER*: EVALUATING THE STRATEGY OF GENE FUSION

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Aspergillus niger, gene fusion, inducible *GlaA* promoter, HBsAg, Glucoamylase, KEX2-like processing, fermentation.

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ABSTRACT

This study reports that by targeting HBsAg production through the secretory pathway in *Aspergillus niger*, completely assembled and properly folded HBsAg, was obtained. This was achieved by implementing a gene fusion strategy using the highly expressed catalytic domain of the glucoamylase gene (*GlaA_{G2}*) fused to the HBsAg *S* gene. The inducible glucoamylase promoter (*GlaA_p*) was used to control transcription in the *A. niger* D15 host. The gene fusion strategy resulted in the cleavage of the fused product by the KEX2-like protease, and intracellular accumulation of HBsAg was observed, while glucoamylase was secreted. Greater than 80 % of intracellular HBsAg accumulation was associated with the membrane fraction with the remainder resided in the cytoplasmic fraction. Immunodetection using a monoclonal HBsAg antibody could not detect the fused *GlaA_{G2}::S* product in intracellular and extracellular fractions, and therefore suggested that full assembly and maturation of HBsAg occurred after cleavage of the fused product in the Golgi complex. Several breakdown products showing an immunoreactive response to the glucoamylase polyclonal antibody, indicated a level of intracellular degradation. Intracellular HBsAg extracts were reduced significantly in extracellular extracts (91 % degradation), while 77 % of the HBsAg remained stable in fresh medium. These findings supported the hypothesis that the glucoamylase degradation products were probably not as a result of proteolytic degradation but possibly accrued by a protein saturated secretory pathway. The choice of carbon source proved to be significant, where HBsAg production levels were affected by the induction levels of the glucoamylase promoter. Highest HBsAg production was observed in inducing substrates of starch and its degradation products (maltodextrin and maltose). However residual glucose accumulation in the mid exponential phase in starch based cultivations reduced HBsAg production. In order to further improve HBsAg production in starch based cultures, the rate between glucose consumption by the host and hydrolysis of starch by glucoamylase requires

optimization. This study demonstrates the potential of *A. niger* as a candidate expression technology for VLP production using gene fusion.

INTRODUCTION

The development of non-replicating vaccines is an emerging option for safe and effective vaccines, several of which contain virus-like particles (VLPs). VLPs are a highly effective type of subunit vaccine, which are produced by recombinant expression of viral capsid proteins or envelope proteins (Miyanojara *et al.*, 1986; Delchambre *et al.*, 1989; Gheysen *et al.*, 1989; French *et al.*, 1990). Various expression systems have demonstrated the ability to produce VLPs and these include: (1) *Escherichia coli* and other bacteria (Liew *et al.*, 2010); (2) various species of yeast including *Saccharomyces cerevisiae* (McAleer *et al.*, 1984; Miyanojara *et al.*, 1986), *Pichia pastoris* (Cregg *et al.*, 1987), and *Hansenula polymorpha* (de Roubin *et al.*, 1991); (3) the baculovirus/insect cell system (Crawford *et al.*, 1994; Bertolotti-Ciarlet *et al.*, 2003; Mortola and Roy, 2004; Vieira *et al.*, 2005); (3) transgenic plant systems (Mason *et al.*, 1996; Smith *et al.*, 2002); and (4) various mammalian cell lines (Warfield *et al.*, 2003; Swenson *et al.*, 2005; Warfield *et al.*, 2005). VLPs are highly ordered, repetitive structures mimicking the native structural assembly and antigenic properties of the parental virus. The Hepatitis B vaccine, among others, is an example of a VLP vaccine, which was first licensed for use in humans in 1984 by Merck (Recombivax HB®) (Valenzuela *et al.*, 1982; McAleer *et al.*, 1984; Hilleman, 2001; Hilleman, 2003).

In the present study the filamentous fungi, *Aspergillus niger*, was considered as an expression system for VLP production through expression of the Hepatitis B surface antigen (HBsAg). *A. niger* has become one of several fungal species developed for the production of both homologous and heterologous enzymes. As with other fungal species, *A. niger* is capable of

producing high concentrations of proteins (e.g., $>20 \text{ g.L}^{-1}$ glucoamylase (GLA); Withers *et al.*, 1998). *A. niger* has also demonstrated the processing of eukaryotic and higher gene products in a correctly folded and functional form, efficient disulfide bridge formation and does not over-glycosylate heterologous proteins as observed in yeast (Upshall *et al.*, 1987; Harvey and McNeil, 1994; Kinghorn and Unkles, 1994). The transformants obtained are usually stable, as the incoming DNA readily integrates into the genome upon transformation (van den Hondel and Punt, 1991). These advantageous characteristics, combined with the available cultivation technologies (Jeenes *et al.*, 1991), have resulted in large scale production methods that are substantially cheaper than various other expression systems (Bodie *et al.*, 1994; Davies, 1994), and are indicative of the potential of *A. niger* as a host for VLP production.

Plüddemann and van Zyl, (2003), demonstrated the ability of *A. niger* to synthesize and assemble complex intracellular Hepatitis B surface antigens (HBsAg) into correctly-folded VLPs, under control of the constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA_p*) promoter. Maximum production levels obtained in controlled batch fermentations were a few milligrams per litre culture and were low compared to the production of homologous proteins (James *et al.*, 2007). Despite these low levels of intracellular HBsAg, high mRNA levels were recorded (Plüddemann and van Zyl, 2003). These results suggest that limitations with the production of recombinant HBsAg do not appear to be at the level of transcription but rather on a (post)translational level as observed in other studies expressing higher recombinant eukaryotic gene products (Jeenes *et al.*, 1994; Nyssönen and Keränen, 1995; Gouka *et al.*, 1997a; Plüddemann and van Zyl, 2003). Various reasons have been suggested for these low heterologous protein yields including incorrect folding or processing of the protein during posttranslational modification, up regulation of the

unfolded protein response (UPR) and Endoplasmic Reticulum associated degradation (ERAD), and proteolytic degradation (Archer and Peberdy, 1997; Gouka *et al.*, 1997a; Plüddemann and van Zyl, 2003).

To elucidate the issues revolving around these low HBsAg yields we refer to the Hepatitis B virus life cycle. In the Hepatitis B virus life cycle, newly synthesised S proteins are carried through the secretory pathway of the infected host, resulting in the assembly and maturation of the HBsAg in the ER/ Golgi complex (Huovila *et al.*, 1992). This is followed by the rapid exportation from the cell (Simon *et al.*, 1988), where HBsAg particles are produced in excess of 10^3 - to 10^6 -fold in comparison to the infectious virion (Ganem and Prince, 2004). The intracellular accumulation of recombinant HBsAg in *A. niger* and similarly in *S. cerevisiae* (Hamsa and Chattoo, 1994), *P. pastoris* (Cregg *et al.*, 1987), *H. polymorpha* (de Roubin *et al.*, 1991) and plant cells (Smith *et al.*, 2002), suggests that the production process in the abovementioned expression systems differs to that of the Hepatitis B virus life cycle.

Gene fusion is one strategy that can be used to direct production through the secretory pathway as is performed in the Hepatitis B virus life cycle. This is achieved by fusing a homologous and well secreted protein often termed 'the carrier protein' to the N-terminal end of the heterologous protein of interest. Gene fusion has been used successfully for a number of expression systems including *A. niger* to enhance production of higher recombinant eukaryotic gene products as was demonstrated for bovine prochymosin (Ward *et al.*, 1990), porcine pancreatic phospholipase A2 (Roberts *et al.* 1992), human interleukin-6 (Contreras *et al.* 1991; Broekhuijsen *et al.* 1993), hen egg-white lysozyme (Jeenes *et al.*, 1993), human lactoferrin (Ward *et al.*, 1992, Ward *et al.*, 1995), human tissue plasminogen activator (Wiebe *et al.*, 2001), phytases from *Aspergillus awamori* (Martin *et al.*, 2003), and humanized

immunoglobulin G1-kappa antibodies (Ward *et al.*, 2004). The observed increases in heterologous protein production varied from 5 to 1000 -fold, depending on the protein and strain, resulting in heterologous protein levels varying from 1 to 2000 mg.l⁻¹ (Ward *et al.*, 1990; Contreras *et al.*, 1991; Broekhuijsen *et al.*, 1993; Archer, 1994; Gouka *et al.*, 1997b). Besides facilitating target protein localization e.g. secretion (Le Loir *et al.*, 2005) or intracellular accumulation (Gordon *et al.*, 2000; Sojikul *et al.*, 2003), additional advantages of this method include; (1) identification of the genomic sequences encompassing the coding region of the carrier protein by the host cells protein processing machinery, therefore encouraging high expression of the fused cassette (Le Loir *et al.*, 2005), (2) aiding heterologous protein maturation and stabilization by facilitating translocation and subsequent folding in the ER (Gouka *et al.*, 1997b; Bermudez-Humaran *et al.*, 2003), and (3) preventing intracellular proteolytic degradation by creating a passage through the fungal protein processing pathway for the heterologous protein under the mask of a homologous gene product (Gouka *et al.*, 1997b).

In this study we evaluate gene fusion as a strategy to produce correctly folded and assembled HBsAg. HBsAg production was directed through the secretory pathway in *A. niger* as is performed in the Hepatitis B virus life cycle. The gene fusion strategy was implemented using the highly expressed glucoamylase gene (*GlaA*) fused to the HBsAg *S* gene. The inducible glucoamylase promoter (*GlaA_p*) was used to control transcription in the *A. niger* D15 host. The study focuses on the mechanism of the fusion protein and associated HBsAg processing. Further objectives include determining (1) the impact of the glucoamylase promoter on HBsAg production, and (2) the stability of the HBsAg product in intracellular and extracellular extracts. Batch fermentation data is presented to quantify results.

MATERIALS AND METHODS

Strains

A. niger D15 (uridine auxotrophic (*pyrG*), protease-deficient (*prtT*), nonacidifying (*phmA*) mutant) was used as the recipient strain for gene fusion transformations (Gordon *et al.*, 2000).

A. niger D15 (*pBluescript-pyrGamdS*) was used as the reference strain (Plüddemann, 2003).

Plasmid and transformation procedures

The plasmid used for transformation, *pAHB19*, (*bla pyrG amdS GlaA_p-GlaA_{SS}-GlaA_{G2}-S-trpC_T*), constructed by Plüddemann, (2003), contains the *A. niger* glucoamylase promoter (*GlaA_p*), secretion signal fused to a sequence encoding the truncated *A. niger* glucoamylase catalytic domain (*GlaA_{G2}*) and the *Aspergillus nidulans trpC* terminator. The *S* gene is fused to the truncated *A. niger GlaA_{G2}* form (514 aa) lacking the starch-binding domain (Broekhuijsen *et al.*, 1993), and the dibasic proteolytic cleavage site “Lys-Arg” separates the *GlaA_{G2}* and Hepatitis B sequences resulting in the cleavage of the fusion protein into separate proteins by a KEX2-like protease (Broekhuijsen *et al.*, 1993). The *A. nidulans* acetamidase (*amdS*) and *A. niger* orotidine-5'-decarboxylase (*pyrG*) genes are used as selection markers (provided by Prof P Punt TNO, Zeist, The Netherlands, Figure 1).

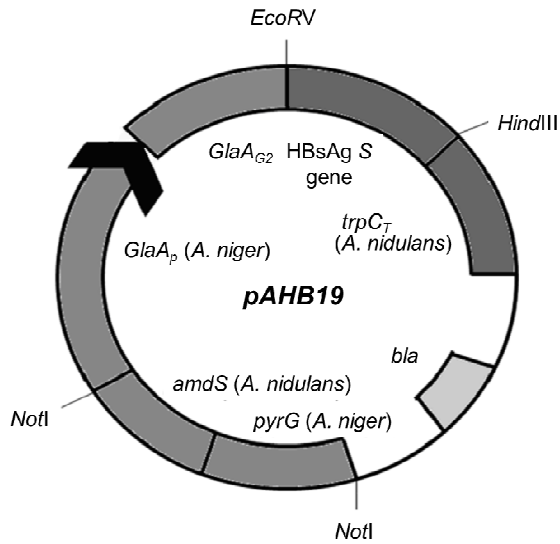


Figure 1. Schematic representation of the *pAHB19* expression vector containing the fusion gene that consists of the sequence coding for the *A. niger* *GlaA_{G2}* (aa 1-514) and the HBsAg *S* gene, regulated by the *A. niger* *GlaA_p*. The *E. coli* β -lactamase (*bla*), *A. nidulans* acetamidase (*amdS*) and *A. niger* orotidine-5'-decarboxylase (*pyrG*) genes were used as selectable markers. The *trpC_T* (a trifunctional gene in tryptophan biosynthesis) was used as the gene terminator.

A. niger D15 was transformed following the protoplasting method described by Punt and van den Hondel (1992), using Novozym 234 (Sigma-Aldrich). Resulting DAHB19 transformants were selected for on minimal medium (MM) (Bennett and Lasure, 1991) without uridine containing 1.2 M sorbitol as osmotic stabiliser, 1.5 % agar (Oxoid) and 10 mM acetamide as sole nitrogen source (Kelly and Hynes, 1985). Transformants were selected for multicopy integration of the expression cassettes through the use of acrylamide plates (Verdoes *et al.*, 1993).

Inoculum preparation

Fungal spores were obtained from a densely conidiating culture grown on potato dextrose agar (PDA) at 30 °C for 120 h and harvested with physiological saline solution (NaCl, 0.9 % w.v⁻¹). Shake flask cultures were inoculated with a spore concentration of 1×10⁶ spores.ml⁻¹ culture. Batch fermentations were inoculated with a shake flask pre-culture amounting to 10 % of the fermentors's total working volume.

Media and cultivations

All cultivations were grown on minimal medium without uridine (Plüddemann and van Zyl, 2003) containing 3 % (w.w⁻¹) soluble starch or glucose and supplemented with 0.1 % (w.w⁻¹) casamino acids (Difco), 0.8 % (w.w⁻¹) tryptone and 0.8 % (w.w⁻¹) yeast extract. For induction studies 3 % soluble starch was substituted with 3 % xylose, sucrose, fructose, glucose, maltose, maltodextrin, and insoluble starch, respectively. Shake flask cultivations were performed in 1 L Erlenmeyer flasks (un-baffled) containing 250 ml medium at 30 °C on a rotary shaker (Innova 2300, New Brunswick Scientific, Edison, USA) at 180 rpm. Batch fermentations were performed in 2 L bench-top bioreactors (INFORS AG, Switzerland) with a working volume of 1.5 L. The fermentation temperature was maintained at 30 °C (± 0.1 °C), aeration at 0.8 vvm, dissolved oxygen was maintained above 25 % saturation by varying impeller speed (starting rate of 150 rpm), through a cascaded controller. The impeller speed was ramped by 200 rpm for 1 min every hour to reduce wall growth. pH was controlled above a value of 4 using NH₄OH (25 % w.v⁻¹).

Sample preparation

Shake flasks were sampled in 24 hr increments while batch fermentations were sampled at intervals of 3 to 8 h. Samples containing ± 50 g of cultivation medium were withdrawn and

biomass concentration was measured by vacuum filtering 25 g sample on dry and pre-weighed filters (Whatman no.1), followed by washing with 40 ml 0.9 % (v.w⁻¹) NaCl solution and drying to a constant weight at 105 °C for 24 to 48 h. The remaining 25 g sample was ground with liquid nitrogen in a mortar and pestle to a fine powder, and an aliquot of 0.3 g mycelia powder was stored at -80 °C in a 2 ml microcentrifuge tube. Ground samples were suspended in protein extraction buffer with or without 0.1 % Triton X-100 (Plüddemann and van Zyl, 2003) and homogenized for 1 min at 4 °C. Cytoplasmic proteins were isolated using extraction buffer without the non-ionic detergent Triton X-100, and total intracellular proteins including membrane-associated proteins were isolated by the addition of Triton X-100 to the extraction buffer. For protein concentration, both intracellular and extracellular fractions were precipitated with 30-60 % ammonium sulfate fractionation, followed by size exclusion using a 10 000 MW spin concentrators (Vivaspin, Sartorius, Göttingen, Germany).

SDS Page and Western Blot

Proteins were separated in a 12 % SDS polyacrylamide gelelectrophoresis (PAGE) gel (Bio-Rad, Hercules, CA) and visualised by means of silver staining (ProteoSilver™ Plus Silver Stain Kit, Sigma-Aldrich). For Western blotting, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Pharmacia Biotech) in transfer buffer [25 mM Tris-HCl (pH 7.5), 192 mM glycine, 15 % (v.v⁻¹) methanol] using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). The HBsAg primary antibodies were monoclonal mouse anti-Hepatitis B surface antigen (1:5000 dilution; DakoCytomation, USA) and secondary antibodies were anti-mouse immunoglobulins conjugated to horseradish peroxidase (1:10 000 dilution). *S. cerevisiae*-derived Engerix B Hepatitis B virus vaccine (20 µg.ml⁻¹; GlaxoSmithKline Biologicals, Rixensart, Belgium) was used as the reference protein. For the immunodetection of glucoamylase, primary polyclonal antibodies raised against glucoamylase

(TNO, Zeist, The Netherlands) was used at a dilution of 1:20 000 and secondary antibodies were anti-mouse immunoglobulins conjugated to horseradish peroxidase (1:10 000 dilution). Amyloglucosidase (Fluka 10113; activity 120 U.mg⁻¹) served as the reference protein. Detection was performed with 1-Step™ TMB-Blotting (Pierce Biotechnology, Rockford, USA).

HBsAg concentration determination

A “sandwich” type enzyme immunoassay based on monoclonal anti-HBsAg (HBsAg 3.0 EIA, Bio-Rad, Hercules, CA) was used to quantify completely assembled and properly folded HBsAg as per manufacturers specification.

Glucoamylase Activity

The activity of glucoamylase was measured by the method of Withers *et al.* (1998) using freshly prepared 0.1 % (v.v⁻¹) 4-nitrophenyl- α -D-glucopyranoside (PNPG) (Sigma-Aldrich N-1377) as substrate in 0.1 M Sodium Acetate buffer (pH 4.3). To commence the reaction, 100 μ l PNPG was added to 50 μ l sample (or GlaA standard) in a flat bottom microtitre plate and incubated for 20 min at 25 °C. The reaction was terminated by the addition of 150 μ l Borax (0.1 M Na₂B₄O₇.10 H₂O) solution, and absorbance at 400 nm was measured using a BioTek Power wave^{HT} microtitre plate reader (BioTek Instruments Inc., USA) referenced with distilled water. Background absorbance was normalized with the addition of 150 μ l Borax solution to 50 μ l sample prior to the addition of 100 μ l PNPG, in a duplicate assay. The measured background absorbance was subtracted from the absorbance readings obtained from the incubated samples and converted into glucoamylase activity units using a standard curve (Amyloglucosidase Fluka 10113).

Glucose Concentration

Glucose concentration was measured using an HPIC (CarboPac™ PA1 column, Dionex, MA, USA) and ACCUTREND glucose strips (Roche Diagnostics Ltd, East Sussex, UK).

Protein analysis

The concentration of total protein was determined by the Bradford method (Bradford, 1976) according to Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) as per manufacturers specification using bovine serum albumin as standard.

RESULTS

Transformation of *GlaA_{G2}::S* gene fusion construct into *A. niger*

The HBsAg fused gene construct, *GlaA_{G2}::S*, in expression vector *pAHB19* was transformed into $\pm 7.3 \times 10^9$ *A. niger* D15 protoplasts. A total of 53 uridine prototrophic and acetamide utilising transformants were obtained after purification on MM (Bennett and Lasure, 1991) containing 10 mM acetamide as sole nitrogen source (Kelly and Hynes, 1985). Subsequently 18 positive transformants showing efficient growth and sporulation on MM agar plates containing 10 mM acrylamide were selected (Verdoes *et al.*, 1993).

No extracellular HBsAg above a detection limit of 0.32 pg.ml^{-1} culture broth was observed for the 18 transformants screened in triplicate shake flask cultivations (data not shown). Intracellular accumulation of HBsAg was observed in 15 transformants. Highest total HBsAg production levels ($34 \text{ }\mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$; combined in the membrane associated and non-membrane fractions) were observed by transformant DAHB19#7 (Figure 2). A high percentage of HBsAg was observed in the intracellular membrane fraction (83 %), while the

remainder was detected in the intracellular cytoplasmic fraction ($5.9 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$) from transformant DAHB19#7 (data not shown).

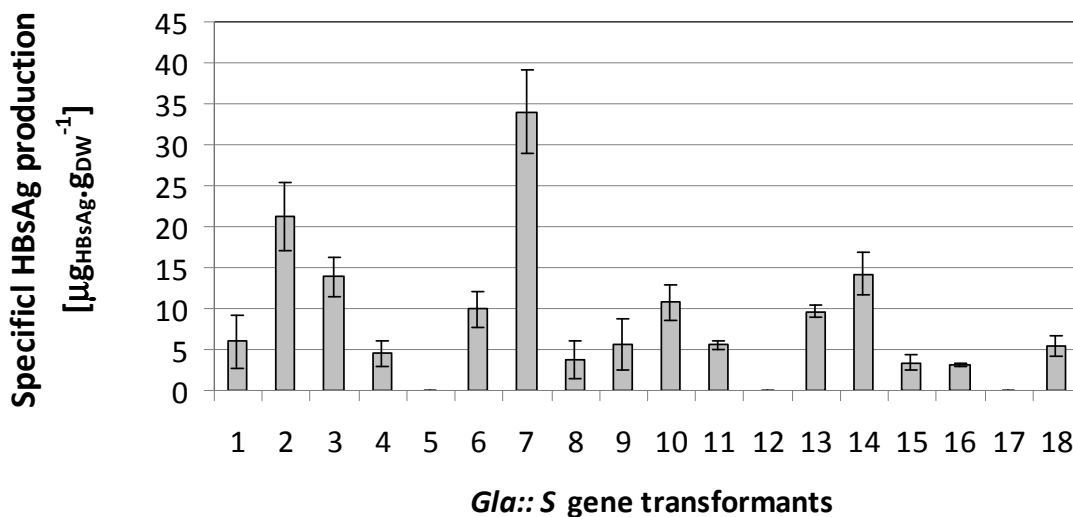


Figure 2. Screening of $Gla_{G2}::S$ gene transformants in shake flask cultivations for HBsAg production. Analysis is performed after 96 h of cultivation. Error bars represent the standard deviation of the mean of triplicate cultivations.

Production of HBsAg, Glucoamylase and Glucoamylase-HBsAg fused protein by transformant DAHB19#7

Western blot analysis for HBsAg, glucoamylase and glucoamylase-HBsAg fused product detection was performed on culture supernatant and intracellular fractions of the highest intracellular HBsAg producing $Gla_{G2}::S$ gene transformant, DAHB19#7. Western blot analysis using a monoclonal HBsAg antibody, raised against human serum-derived HBsAg, only detected completely assembled and properly folded HBsAg, while unfolded polypeptides were not recognized (Sunil Kumar *et al.* 2005). Results showed the intracellular accumulation of a 24 kDa protein species that did not appear in the analysis of the corresponding reference

strain sample (Figure 3A). This protein also appeared in the Engerix vaccine HBsAg protein, confirming that the 24 kDa protein corresponds to the HBsAg S protein (Figure 3A). The size of the glucoamylase-HBsAg fused product is estimated to be ~ 97 kDa and could not be detected with monoclonal HBsAg antibodies in both the intracellular cytoplasmic protein fractions (data not shown) and in fractions including membrane associated proteins (Figure 3A).

Western blot analysis for glucoamylase detection, using polyclonal glucoamylase antibodies, revealed two protein products in the culture supernatant with molecular masses of approximately 90 kDa and 70 kDa, in both the reference strain and *GlaA_{G2}::S* gene transformant DAHB19#7. Quantification through “sandwich” type ELISA using polyclonal glucoamylase antibodies detected an ~ 1.47 fold increase in extracellular glucoamylase activity of transformant DAHB19#7 in comparison to the parent strain (data not shown). In the intracellular fraction, similar protein bands as well as several smaller products that did not appear in the reference strain, were observed in *GlaA_{G2}::S* gene transformant DAHB19#7 (Figure 3B).

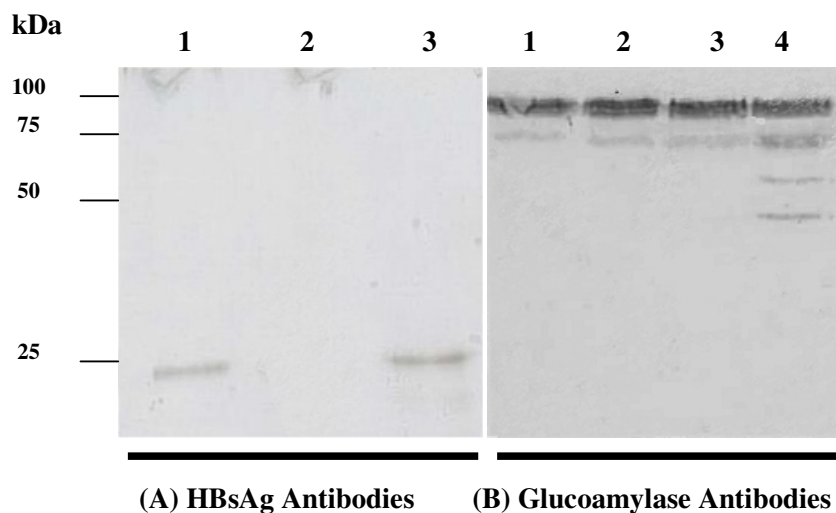


Figure 3. Western blot analysis from shake flask cultivations after 96 h. (A) Immunodetection using monoclonal HBsAg antibodies: lane 1, Hepatitis B virus vaccine, Engerix B; lane 2, total intracellular fraction from prototrophic reference strain (D15-pyrGamdS); lane 3, total intracellular fraction from *GlaA_{G2}::S* gene transformant DAHB19#7. (B) Immunodetection using polyclonal glucoamylase antibodies; lane 1, extracellular fraction from prototrophic reference strain (D15-pyrGamdS); lane 2, total intracellular fraction from prototrophic reference strain (D15-pyrGamdS); lane 3, extracellular fraction from *GlaA_{G2}::S* gene transformant DAHB19#7; lane 4, total intracellular fraction from *GlaA_{G2}::S* gene transformant DAHB19#7.

Intracellular and extracellular stability of HBsAg in *A. niger*

The stability of HBsAg in potential intracellular and extracellular *A. niger* proteases was evaluated by monitoring the concentration of intracellular HBsAg extracts in (1) culture filtrate (96 h spent *A. niger* D15 culture medium), and (2) fresh culturing medium. Total intracellular extracts of *GlaA_{G2}::S* gene transformant DAHB19#7 after 96 h of growth in shake flask cultivations was prepared (200 ml solution). 100 ml of the solution was used as control in an equal volume of fresh culturing medium, while the remaining 100 ml was re-introduced into an equal volume of 96 h spent *A. niger* D15 culture medium. The two solutions were incubated at 30 °C for 32 h. Samples taken at 0, 2, 4, 8, 16, 24, and 32 h were quantified by ELISA. HBsAg concentrations in spent culture filtrate were reduced by 3 %

after two h, 28 % after eight h and 91 % after 32 h with a degradation rate of $1.16 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$ (Figure 4). In comparison, intracellular HBsAg concentration in fresh medium remained relatively stable with $\pm 23 \%$ degradation after 32 h at a degradation rate of $0.22 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$ (Figure 4).

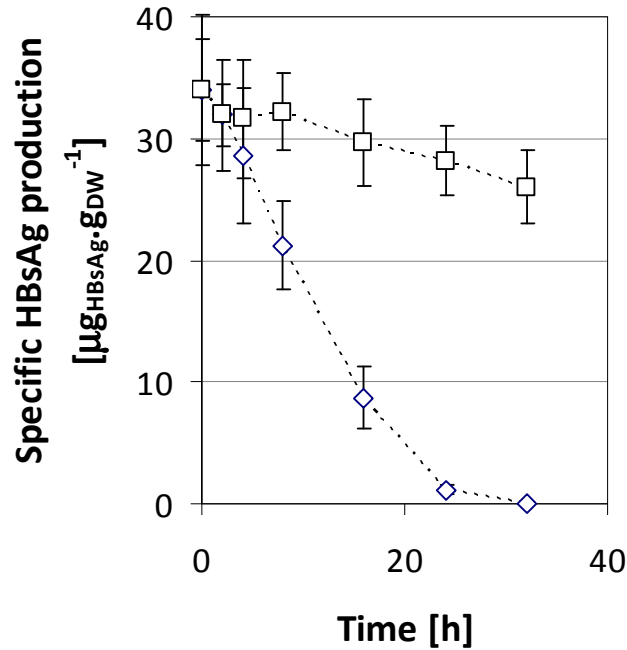


Figure 4. Protease susceptibility assay. Concentration of extracted HBsAg concentration ($\mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$) (□) incubated in intracellular extraction buffer at $30 \text{ }^\circ\text{C}$ and (◇) re-introduced into culture filtrate with intracellular extraction buffer and incubated at $30 \text{ }^\circ\text{C}$. Error bars represent standard deviation of the mean of triplicate samples.

Impact of glucoamylase promoter on biomass and HBsAg production on various carbon sources

Biomass and HBsAg production by transformant DAHB19#7 on various carbon sources at $30 \text{ }^\circ\text{C}$ after 96 h of incubation were determined in triplicate shake flask cultivations (Figure 5). Use of insoluble starch, soluble starch and starch derived polysaccharides (maltose and maltodextrin) resulted in high specific HBsAg production with the maximum levels of $38 \pm$

1.34 and $36 \pm 3.05 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ observed with maltose and maltodextrin, respectively. Specific HBsAg levels on insoluble and soluble starch were similar and equal to $88 \pm 2\%$ of that obtained in maltose cultivations (34.16 ± 3.26 and $33.77 \pm 2.19 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$, respectively). Biomass levels were highest in maltodextrin cultures and lowest in insoluble starch cultures ($10.0 \text{ g}_{\text{DW}} \cdot \text{kg}_{\text{Broth}}^{-1}$ and $7.6 \text{ g}_{\text{DW}} \cdot \text{kg}_{\text{Broth}}^{-1}$, respectively). Glucose cultures obtained highest biomass concentrations ($10.2 \text{ g}_{\text{DW}} \cdot \text{kg}_{\text{Broth}}^{-1}$) and HBsAg production was recorded at 42 % of the levels reached in maltose cultures. Lower biomass levels and trace amounts of HBsAg were measured in fructose, sucrose and xylose cultures ($< 9.1 \text{ g}_{\text{DW}} \cdot \text{kg}_{\text{Broth}}^{-1}$ and $< 6.5 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$, respectively Figure 5).

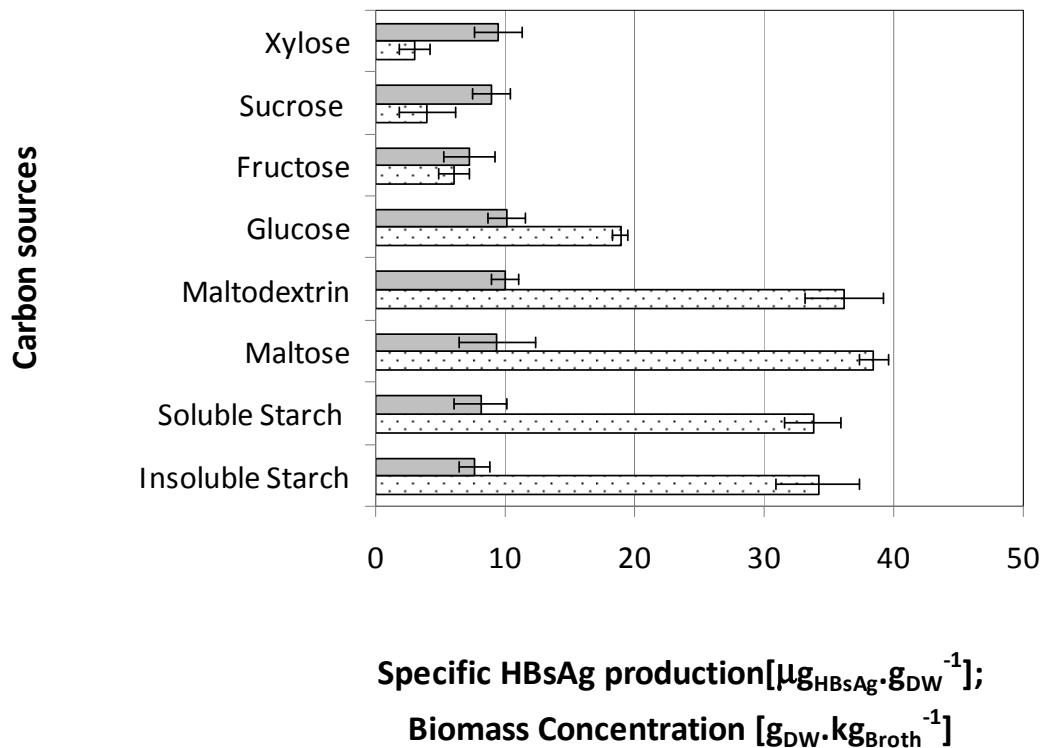


Figure 5. Analysis of HBsAg and biomass production on various carbon sources after 96 h of shake flask cultivation at 30 °C. Shown are data for: grey columns, biomass concentration ($\text{g}_{\text{DW}} \cdot \text{kg}_{\text{Broth}}^{-1}$); white columns, specific HBsAg production ($\mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$). Error bars represent the standard deviation of the mean of triplicate cultivations.

Evaluating HBsAg production in controlled batch fermentations of transformant DAHB19#7

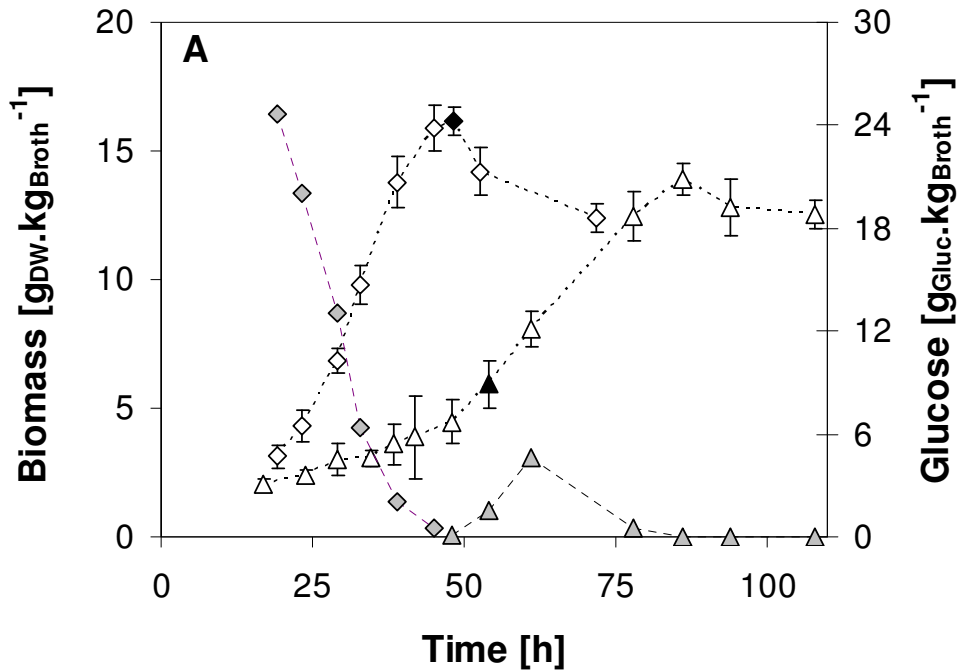
Biomass, intracellular HBsAg, extracellular glucoamylase and total cellular protein (intracellular and extracellular) production during batch fermentation of transformant DAHB19#7 was determined on starch and glucose substrates. The highest maximum specific growth rate (μ_{max}) of $0.08 \pm 0.003 \text{ h}^{-1}$ was obtained in batch culture with glucose as carbon source, resulting in a maximum biomass concentration of $16.2 \pm 0.8 \text{ g}_{\text{DW}} \cdot \text{kg}_{\text{Broth}}^{-1}$ (Figure 6A). Glucose depletion was observed after 48 h. In batch fermentation with soluble starch as carbon source, a lower μ_{max} was obtained ($0.03 \pm 0.004 \text{ h}^{-1}$) with a maximum biomass

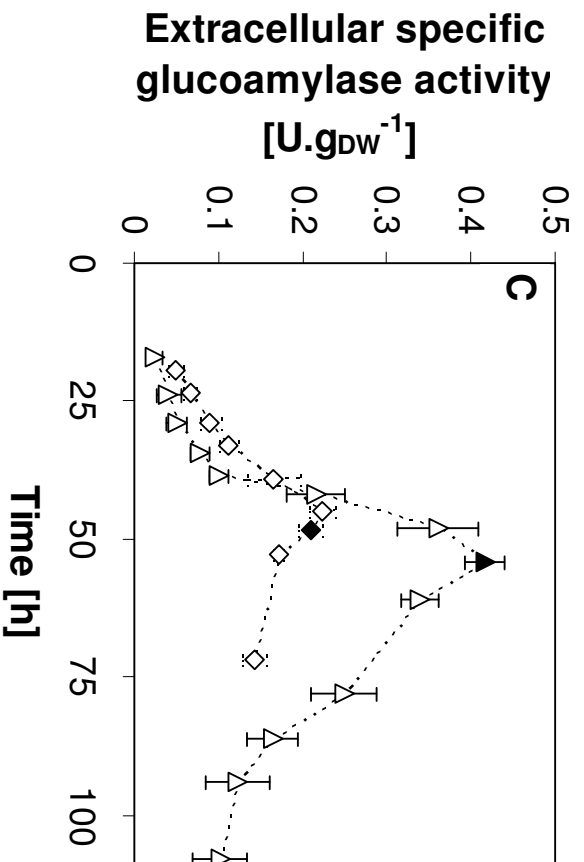
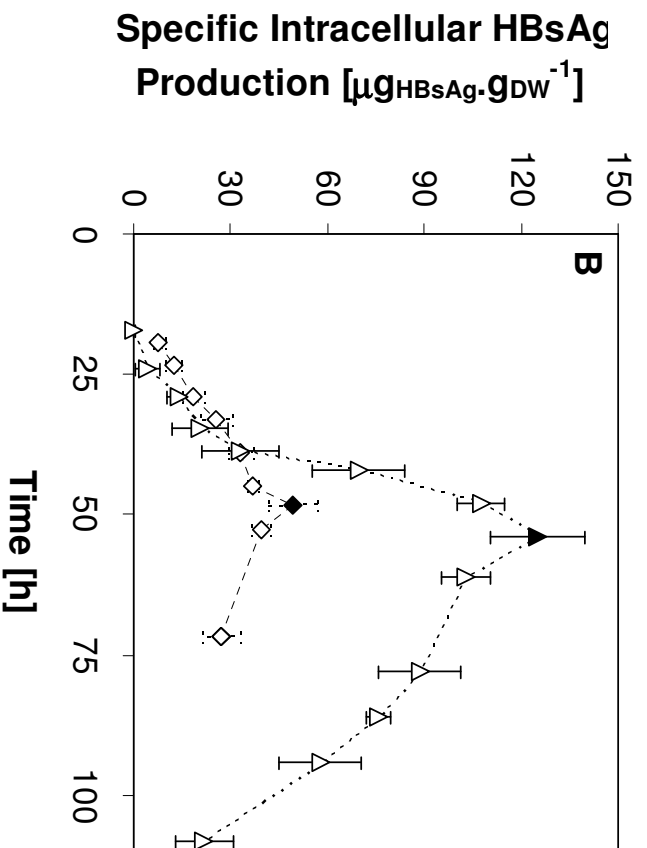
concentration of $13.9 \pm 0.6 \text{ g}_{\text{DW}} \cdot \text{kg}_{\text{Broth}}^{-1}$ after 86 h. Residual glucose accumulation was observed in the starch-based cultures between 54 and 86 h of cultivation, starting during the mid exponential growth phase. Residual glucose accumulation increased to a maximum of $4.65 \pm 1.3 \text{ g}_{\text{Gluc}} \cdot \text{kg}_{\text{Broth}}^{-1}$ (61 h), followed by a steady decrease with total depletion corresponding to the maximum biomass concentration (86 h; Figure 6A).

Intracellular HBsAg production in glucose batch fermentations was low with a maximum production rate of $1.432 \text{ } \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$ and maximum levels of $49 \pm 7.6 \text{ } \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ parallel to glucose depletion (48 h). The maximum HBsAg production levels in batch culture with starch at $124.8 \pm 14.5 \text{ } \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ were approximately 2.5-fold higher than batch fermentations with glucose. Maximum intracellular HBsAg production rates in starch-based batch culture were observed between 39 and 54 h of cultivation ($5.8 \text{ } \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$). Maximum HBsAg concentration in starch-based batch culture coincided with the onset of glucose accumulation, and subsequently decreased during the 54 to 86 hour time frame of the culture.

In glucose batch fermentations low extracellular glucoamylase activity was measured with a maximum of $0.22 \pm 0.02 \text{ U} \cdot \text{g}_{\text{DW}}^{-1}$ and a production rate of $0.007 \text{ U} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$ in the exponential growth phase. In starch cultivations, extracellular glucoamylase activity was highest at $0.41 \pm 0.03 \text{ U} \cdot \text{g}_{\text{DW}}^{-1}$ and a maximum production rate of $0.02 \text{ U} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$ between 39 and 54 h of cultivation. Similar to the HBsAg production profiles, glucoamylase activity in cultures grown on glucose were reduced after glucose depletion, whereas under starch conditions glucoamylase activities diminished with the accumulation of residual glucose in the culture broth (Figure 6C).

Total protein analysis (intracellular and extracellular) in glucose cultivations showed maximum protein concentrations of $111 \pm 8.7 \text{ mg}_{\text{protein}} \cdot \text{g}_{\text{DW}}^{-1}$ after 45 h of cultivation and a maximum production rate of $2.2 \text{ mg}_{\text{protein}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$. A lower production rate of $1.23 \text{ mg}_{\text{protein}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$ was observed in starch cultivations with a maximum of $113 \pm 16.1 \text{ mg}_{\text{protein}} \cdot \text{g}_{\text{DW}}^{-1}$ after 86 h of cultivation (Figure 6D).





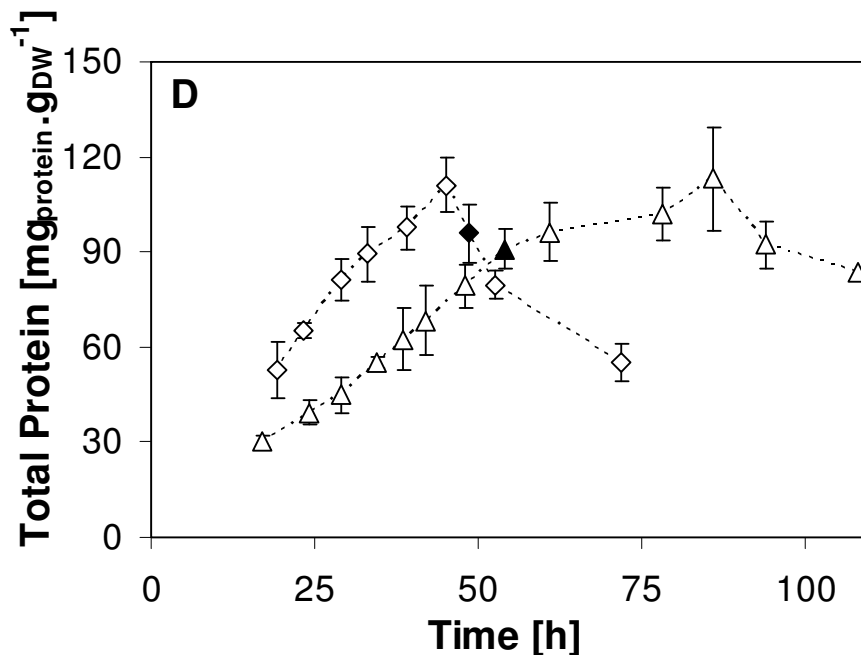


Figure 6. Batch fermentations of *A. niger* $GlaA_{G2}::S$ gene transformant DAHB19#7: Shown is data regarding cultivations grown on; ◇ glucose, and △ starch. Time course data of: (A) Dry biomass concentration (open symbols) and glucose concentration (grey symbols); (B) specific intracellular HBsAg production (open symbols); (C) specific extracellular glucoamylase activity (open symbols); and (D) total cellular protein (intracellular and extracellular) production are shown (open symbols). Closed symbols for △ and ◇ represent glucose depletion in glucose cultivations, while closed symbols for △ represent residual glucose in starch cultures. Error bars represent the standard deviation of the mean of triplicate cultivations. Points without error bars have an associated deviation that is < 10 % of the value of the point.

DISCUSSION

The aim of this study was to evaluate the strategy of gene fusion as a means of producing recombinant HBsAg in *A. niger* by targeting production through the secretory pathway as is performed by the Hepatitis B virus in infected cells. The gene fusion cassette was constructed by fusing the highly expressed $GlaA_{G2}$ gene (Le Gal-Coeffet *et al.*, 1995) to the HBsAg S gene. The glucoamylase-HBsAg fused cassette was under transcriptional control of the inducible $GlaA_p$ and the ubiquitous KEX2 protease cleavage site was introduced between the S gene and $GlaA_{G2}$ to facilitate cleavage (Contreras *et al.*, 1991).

Targeting HBsAg through the secretory pathway of *A. niger* using the gene fusion approach resulted in completely assembled and properly folded HBsAg, as recognized by monoclonal antibodies raised against human serum-derived HBsAg. Unfolded polypeptides were not recognized (Sunil Kumar *et al.* 2005). HBsAg was localized intracellularly in membrane associated extracts, while glucoamylase was secreted. Intracellular accumulation of HBsAg is common in *S. cerevisiae* (Hamsa and Chattoo, 1994), *P. pastoris* (Cregg *et al.*, 1987), *H. polymorpha* (de Roubin *et al.*, 1991), *A. niger* (Plüddemann, 2003) and plant cells (Smith *et al.*, 2002) and emphasizes alternative processing of HBsAg VLPs in microbial systems in comparison to infected cells. The HBsAg is inherently hydrophobic in nature (Vassileva *et al.*, 2001) and has been reported to progress no further than ER/Golgi in recombinant microbial systems (Smith *et al.*, 2002). This results in the dilation of the ER membrane network upon HBsAg expression of various recombinant expression systems (Smith *et al.*, 2002), while in infected cells the HBsAg buds through the host cell membrane and into the bloodstream. This is indicative of the limitations of HBsAg processing in microbial systems in comparison to infected cells.

Several breakdown products showing an immunoreactive response to the glucoamylase polyclonal antibody, were detected in intracellular extracts. These glucoamylase products did not correspond to the starch binding domain (G1) and catalytic domain (G2) glucoamylases and did not appear in the corresponding extracellular fraction of transformants or in the intracellular fraction of the parent strain. Similar results were documented in the study of Plüddemann, (2003) and Conesa *et al.*, (2000) and indicates a level of intracellular degradation. Possible explanations to these findings can be attributed to intracellular

proteolytic degradation (Jarai *et al.*, 1994; Gouka *et al.* , 1996) or the bottlenecks caused by unfolded and/or misfolded proteins that accrue within the secretory pathway (Wang, 2005).

The *A. niger* parent strain to HBsAg producing transformants, *A. niger* D15 is a non-acidifying protease deficient strain, which would suggest that degradation due to proteolytic activity would be limited. Protease activities in *A. niger* D15, were reduced by mutagenesis and not by specific protease gene deletions, resulting in the reduction but not complete deletion of protease activity (Mattern *et al.*, 1992). In addition, the use of complex media typically containing peptide nitrogen as used in this study, induces protease production by *A. niger* (Archer *et al.*, 1990; Ahamed *et al.*, 2005; Ward *et al.*, 2006). Productivity of secreted egg lysozyme by a recombinant strain of *A. niger* was reduced in such complex media (Archer *et al.*, 1990; Ward *et al.*, 2006). The lack of stability (91 % degradation over 32 hr) of intracellular HBsAg extracts re-introduced into 96 hr spent culture filtrate in this study is evidence that HBsAg is vulnerable to extracellular protease activity. In contrast, intracellular HBsAg production remained relatively stable at 30 °C for 32 hours, where 77 % of the HBsAg produced remained correctly folded and assembled. This would suggest that the breakdown products showing an immunoreactive response to glucoamylase were not a consequence of fused product degradation by intracellular proteases.

Bottlenecks have been reported to accrue in recombinant microbial systems expressing higher eukaryotic gene products (Gouka *et al.* , 1996). These are a result of a greater flux/ overload of proteins being translocated into the Endoplasmic Reticulum (ER), where the folding, assembly, and processing machinery may become saturated, leading to improperly folded structures (Ngiam *et al.*, 2000). Unfolded and/or misfolded proteins are prevented from leaving the ER (Lodish *et al.* 1983; Shuster 1991; Parekh *et al.* 1995; Shusta *et al.* 1998;

Smith *et al.* 2004) and may explain the breakdown products showing an immunoreactive response to the glucoamylase. The accumulation of unfolded and/or misfolded proteins induces the up-regulation of the unfolded protein response (UPR) and/or an ER-associated protein degradation response (ERAD). The UPR controls the expression of genes of several ER-resident chaperones and foldases and numerous other genes involved in other secretory functions (Valkonen *et al.*, 2003), while the ERAD degrades those proteins that fail to reach the correct conformation (Conesa *et al.*, 2001).

Immunodetection using a monoclonal HBsAg antibody could not detect the fused *Gla*_{G2}::*S* product in intracellular and extracellular fractions. These observations indicate that full assembly and maturation of HBsAg occurred after cleavage of the fused product in the Golgi complex, as it has been reported in *A. niger* that dibasic cleavage by the KEX2-like processing site occurs in the Golgi complex (Brigance *et al.*, 2000). This process differs in infected cells: hepatitis B *S* protein is inserted into the ER membrane and subsequently translocated to the ER lumen where disulphide-linked dimers are rapidly formed (Huovila *et al.*, 1992). These dimers are transported to a post-ER/pre-Golgi compartment where the formation of oligomer crosslinks occurs, resulting in the formation of pseudoviral particles, which are then exported from the cell via vesicular transport (Huovila *et al.*, 1992; Prange and Streeck, 1995). Despite the apparent difference in HBsAg processing through the secretory pathway using the gene fusion approach in *A. niger* in comparison to infected cells, results indicate completely assembled and properly folded HBsAg.

The inducible glucoamylase promoter had a significant impact on HBsAg production in *A. niger*. HBsAg production was induced highest on starch or its degradation products maltodextrin and maltose, concurring with the studies of Carrez *et al.* (1990), Fowler *et al.*

(1990), van den Hondel *et al.* (1992), Schrickx *et al.* (1993), Withers *et al.*, (1998), Santerre Henriksen *et al.* (1999), and Siedenberg *et al.* (1999). HBsAg production levels of 40 to 50 % lower were found in cultivations based on glucose as carbon source.

In controlled batch fermentation using glucose as the carbon source, HBsAg production was measured to have a growth associated correlation with biomass production, where biomass and HBsAg production ceased after glucose depletion. Similar findings were observed in *S. cerevisiae* (Kim *et al.*, 2009) but not for *P. pastoris* where a slower growth rate is essential for efficient particle assembly (Cregg *et al.*, 1987; Vassileva *et al.*, 2001). The galactose-inducible *GALI* promoter was used for HBsAg production in *S. cerevisiae*, while the methanol induced *AOX1* promoter was used for *P. pastoris*. This reflects the contrasting ability of control promoters have on HBsAg assembly and production in various expression systems. Nonetheless, the fundamentals remain the same; under conditions where the inducing substrate was depleted, synthesis of HBsAg was apparently down regulated to lower constitutive levels (Kim *et al.*, 2009).

Despite highest HBsAg production levels in starch based fermentation, the accumulation of residual glucose remains a challenge as this coincided with the reduction in extracellular glucoamylase activity and HBsAg production mid-way through the exponential phase. The phenomenon of residual glucose accumulation indicates that the rate of starch hydrolysis is greater than glucose consumption of the host organism, as discussed in the study of Ganzlin and Rinas, (2008). In the presence of the starch substrate, the production of glucoamylase is used to catalyze the cleavage of starch into glucose units (starch hydrolysis), which in turn is consumed by the host organism. In the event of high glucoamylase production, the rate of starch hydrolysis is increased, resulting in an abundant supply of glucose. The high rate of

glucoamylase synthesis produced for starch hydrolysis is no longer justified, and glucoamylase production is down regulated by the inducible glucoamylase promoter and even repressed by catabolite repression if glucose levels reach a certain threshold (Ruijter and Visser, 1997). Ganzlin and Rinas, (2008) reported that in *A. niger* fed-batch cultivations, under control of the inducible glucoamylase promoter, when implementing a high maltose feed rate, residual extracellular glucose was detected resulting in higher biomass formation and lower levels of the reporter protein compared to cultures fed at a lower feed rate with no glucose accumulation. Evidence therefore suggests that through the control of the inducible glucoamylase promoter there appears to be a fine balance between the up regulation and down regulation of glucoamylase-HBsAg fused gene expression. In order to further enhance HBsAg production levels of this system in starch based cultivations, we suggest continuous or fed batch fermentation be implemented, where the rate of starch hydrolysis can be equilibrated with glucose consumption.

This study serves as a proof-of-concept that by targeting HBsAg production through the secretory pathway in *A. niger*, completely assembled and properly folded HBsAg, is obtained. This also demonstrates the potential of *A. niger* using gene fusion as a candidate expression technology for VLP production. The mechanism of HBsAg processing in *A. niger* using the gene fusion approach, as in other microbial systems, appears to differ from infected cells. As a result, bottlenecks within the secretory pathway were evident and remain to be quantified in future studies. The induction levels of the glucoamylase promoter affected HBsAg production, where the choice of carbon source proved to be significant. Highest HBsAg production was observed in starch based cultivations. However, in order to further improve HBsAg production, the rate between glucose consumption by the host and hydrolysis of starch by glucoamylase requires optimization.

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MANUSCRIPT 2

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IMPROVED HEPATITIS B SURFACE ANTIGEN PRODUCTION IN ASPERGILLUS NIGER THROUGH THE EVALUATION OF GENETIC SELECTION USING GENE FUSION AND A STARCHED BASED SELECTION PRESSURE

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Running Title: Improved Hepatitis B surface antigen production in *Aspergillus niger* through the evaluation of genetic selection using gene fusion and a starched based selection pressure

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ABSTRACT

The aim of this study was to develop an improved methodology to identify and select high Hepatitis B surface antigen (HBsAg) producing transformants. A double mutant *Aspergillus niger* strain, MGG029- $\Delta aamA$, lacking two major amylolytic enzymes, glucoamylase and acid amylase, was used as the expression host. The expression vector introduced into the host included the catalytic domain of *A. niger* glucoamylase fused to the HBsAg *S* gene and the *Aspergillus nidulans* Acetamidase gene (*amdS*) selection marker. This facilitated HBsAg *S* gene expression and HBsAg assembly through the secretory pathway as well as served as a medium for selection. In conjunction with conventional selection based on acetamide utilization through *amdS* gene expression, three selection methods for the identification of high HBsAg producing transformants were evaluated: (1) selection based on improved growth on acrylamide through the expression of the *amdS* gene resulted in 20 % of selected transformants obtaining high HBsAg production levels, (2) selection based on improved growth on starch resulted in 40 % of transformants obtaining high HBsAg production levels, and (3) selection based on improved extracellular glucoamylase activity using starch as a selection pressure resulted in 86 % of transformants obtaining high HBsAg production levels. In glucose batch fermentation, two high HBsAg producing transformants were evaluated; high biomass producing transformant DAHB 21#20 and a high extracellular glucoamylase producing transformant, DAHB 21#40. The growth rate of these two transformants differed with highest extracellular glucoamylase and HBsAg production levels observed in the “slower-growing high-glucoamylase producing” transformant DAHB 21#40. A strong correlation between intracellular HBsAg and secreted glucoamylase was evident for both transformants. The high efficiency obtained through selecting for high extracellular glucoamylase transformants and the associated correlation between intracellular HBsAg production and secreted glucoamylase in batch fermentation reflects the ability of the

glucoamylase screen to indirectly quantify high HBsAg producing transformants through extracellular glucoamylase determination.

INTRODUCTION

Virus like particles (VLPs), including the Hepatitis B surface antigen (HBsAg) have been efficiently produced in microbial systems such as *Pichia pastoris*, *Hansenula polymorpha* and *Saccaromyces cerevisiae*. The filamentous fungus *Aspergillus niger* has gained much interest as an alternative host to the commonly employed yeasts for the production of mammalian and higher eukaryotic gene products achieving varying degrees of success (Gwyne *et al.*, 1987; Upshall *et al.*, 1987; Ward *et al.*, 1995; Gouka *et al.*, 1997a; Svetina *et al.*, 2000; James *et al.*, 2007). These accomplishments are largely based on effective strain and cultivation improvement strategies that have been developed: vector design and construction, codon optimization (Gouka *et al.*, 1996), choice of promoter (Van den Hondel *et al.*, 1991), the generation of fusion constructs (Jeenes *et al.*, 1993; Gouka *et al.*, 1997b; Spencer *et al.*, 1998), over expression of chaperone proteins (Ngiam *et al.*, 2000; Punt *et al.*, 1998), the isolation of protease deficient mutants (Archer and Peberdy, 1997; van den Hombergh *et al.*, 1997), and the optimisation of media and cultivation conditions (Schrickx *et al.*, 1993; MacKenzie *et al.*, 1994; Xu *et al.*, 2000; Conesa *et al.*, 2001; James *et al.*, 2007).

Although the abovementioned strategies have been successful in increasing the production levels of heterologous proteins in several *A. niger* expression systems, often these levels are still lower than those obtained for homologous proteins (James *et al.*, 2007). Introducing the desired gene product of interest to the fungus host strain and the selection of hyper-producing transformants often represents a challenge (Meyer, 2007). The manipulation of genomic DNA can be achieved through several methods, one of which is of interest in this context, random

integration. This strategy is often favoured due to the ease of implementation, as well as the ability to attain high copy number insertions of the gene product of interest (Verdoes *et al.*, 1993). Significant variance in recombinant gene products is generally expected amongst transformants through the implementation of random integration techniques. This is attributed to vector copy number and/ or site of integration (Verdoes *et al.*, 1994). It has been shown that vector copies generally integrate in only one chromosome (Verdoes *et al.*, 1993), and that plasmid vectors can be integrated into regions of the chromosome which are transcribed with different efficiencies as suggested in the studies of Thompson and Gasson (2001) and Plüddemann and van Zyl (2003). A specific risk of random insertion is the possibility of a pleiotropic effect, such as disruption of a chromosomal gene that has a central function and thereby the transformation negatively influences growth or production. It is therefore essential to screen transformants with the objective of selecting hyper-producing transformants. To increase the probability of identifying hyper-producers, these screens require a large number of transformants. The cultivation and analysis of a large number of transformants is often laborious and time consuming. Furthermore, high value recombinant products are often costly to assay. Through the implementation of a screening strategy that allows for the cost effective, rapid, robust and high-throughput evaluation of transformants, recombinant production levels can be significantly increased. Rational selection techniques, not involving the product of interest, but associated biochemical characteristics have been successfully implemented in mutagenesis studies. Such techniques involve, for example, direct colony selection after a bioassay overlay for better penicillin producers (Vournakis and Elander, 1983), laccase production in *A. niger* using a gene fusion approach (Weenink *et al.*, 2006), and citrate specific indicator (R-di-methylaminobenzaldehyde) assays for citrate hyper-producers (Mattey, 1992).

In this study we aim to develop an improved methodology to identify and select high HBsAg producing transformants. The double mutant *A. niger* strain, lacking two major amyolytic enzymes, glucoamylase and acid amylase, grows poorly on starch. However through the gene transfer of a glucoamylase gene, growth is restored (Weenink *et al.*, 2006). The expression vector introduced into the host organism includes the highly expressed and well secreted catalytic domain of *A. niger* glucoamylase (*GlaA_G*) fused to the HBsAg *S* gene as well as a selection marker, the *Aspergillus nidulans* Acetamidase gene (*amdS*). Three screening methods for HBsAg producing transformants are evaluated in conjunction with the conventional *amdS* selection based on acetamide selection; (1) transformant selection based on acrylamide utilization through expression of the *amdS* gene, (2) transformant selection based on high growth on starch as reported by Weenink *et al.* (2006), and (3) transformant selection based on high extracellular glucoamylase activity using starch as a selection pressure. Batch fermentation data is included to quantify growth, glucoamylase and HBsAg production levels of selected high-HBsAg producing transformants.

MATERIALS AND METHODS

Strains, plasmid and transformation procedures

A. niger MGG029- Δ *amA* (provided by Prof. P. Punt, TNO, Zeist, The Netherlands) was used as the recipient strain for transformation (Gordon *et al.*, 2000). The plasmid used for transformation, pAHB19, (*bla amdS GlaA_P-GlaA_{SS}-GlaA_{G2}-S-trpC_T*), constructed by Plüddemann, (2003), contains the *A. niger* glucoamylase promoter (*GlaA_P*), secretion signal fused to a sequence encoding truncated *A. niger* *GlaA_{G2}* and the *A. nidulans* *trpC_T* terminator. The HBsAg *S* gene is fused to the truncated *A. niger* *GlaA_{G2}* form (514 aa), lacking the starch-binding domain (Broekhuijsen *et al.*, 1993), and the dibasic proteolytic cleavage site “Lys-Arg” separates the *GlaA_{G2}* and Hepatitis B *S* gene sequences resulting in the cleavage of the

fusion protein into separate proteins by a Kex2-like protease (Broekhuijsen *et al.*, 1993). The *A. nidulans amdS* gene is used as a selection marker (provided by Prof P Punt TNO, Zeist, The Netherlands).

A. niger MGG029- Δ *aamA* was transformed following the protoplasting method of Punt and van den Hondel (1992), using Novozym 234 (Sigma-Aldrich). Resulting transformants were selected for on minimal medium (MM) (Bennett and Lasure, 1991) containing 1.2 M sorbitol as osmotic stabiliser, 1.5 % agar (Oxoid) and 10 mM Acetamide as sole nitrogen source (Kelly and Hynes, 1985).

Inoculum preparation, media and cultivations

Fungal spores were obtained from a densely conidiating culture grown on potato dextrose agar (PDA) at 30 °C for 120 h and harvested with physiological saline solution (NaCl, 0.9 % w/v). Shake flask cultures were inoculated with a spore concentration of 1×10^6 spores.ml⁻¹ culture. Batch fermentations were inoculated with a shake flask pre-culture amounting to 10 % of the fermentor's total working volume. All cultivations were grown on MM (Plüddemann and van Zyl, 2003) containing 3 % (w.w⁻¹) glucose or soluble starch and supplemented with 0.1 % (w.w⁻¹) casamino acids (Difco), 0.8 % (w.w⁻¹) tryptone and 0.8 % (w.w⁻¹) yeast extract. Shake flask cultivations were performed in 1 L Erlenmeyer flasks (un-baffled) containing 250 ml medium at 30 °C on a rotary shaker (Innova 2300, New Brunswick Scientific, Edison, USA) at 180 rpm. Fermentations were performed in 2 L bench-top bioreactors (INFORS AG, Switzerland) with a working volume of 1.5 L. The fermentation temperature was maintained at 30 °C (\pm 0.1°C), aeration at 0.8 vvm, dissolved oxygen was maintained above 25 % saturation by varying impeller speed (starting rate of 150 rpm),

through a cascaded controller. The impellor speed was ramped by 200 rpm for 1 min every hr to reduce wall growth. pH was controlled above a value of 4 using NH_4OH (25 % v.v⁻¹).

Sample preparation

Shake flasks were sampled in 24 h increments while bioreactor cultivations were sampled at intervals of 3 to 8 h. Samples containing ± 50 g of cultivation medium were withdrawn and biomass concentration was measured by vacuum filtering 25 g sample on dry and pre-weighed filters (Whatman no.1), followed by washing with 40 ml 0.9 % (w.v⁻¹) NaCl solution and drying to a constant weight at 105 °C for 24 to 48 h. The remaining 25 g sample was ground to a fine powder with liquid nitrogen in a mortar and pestle, and an aliquot of 0.3 g mycelia powder was stored at -80 °C in a 2 ml microcentrifuge tube. Ground samples were suspended in protein extraction buffer with 0.1 % Triton X-100 (Plüddemann and van Zyl, 2003) and homogenized for 1 min at 4 °C. Total intracellellur proteins including membrane-associated proteins were isolated by the addition of Triton X-100 to the extraction buffer.

HBsAg concentration determination

A “sandwich” type enzyme immunoassay based on monoclonal anti-HBsAg (HBsAg 3.0 EIA, Bio-Rad, Hercules, CA) was used to quantify completely assembled and properly folded HBsAg as per manufacturers specification.

Glucoamylase Activity

The activity of glucoamylase was measured by the method of Withers *et al.* (1998) using freshly prepared 0.1 % (v.v⁻¹) 4-nitrophenyl- α -D-glucoopyranoside (PNPG) (Sigma-Aldrich N-1377) as substrate in 0.1 M Sodium Acetate buffer (pH 4.3). To commence the reaction, 100 μl PNPG was added to 50 μl sample (or GlaA standard) in a flat bottom microtitre plate and

incubated for 20 min at 25 °C. The reaction was terminated by the addition of 150 µl Borax (0.1 M Na₂B₄O₇·10 H₂O) solution, and absorbance at 400 nm was measured using a BioTek Power wave^{HT} microtitre plate reader (BioTek Instruments Inc., USA) referenced with distilled water. Background absorbance was normalized with the addition of 150 µl Borax solution to 50 µl sample prior to the addition of 100 µl PNPG, in a duplicate assay. The measured background absorbance was subtracted from the absorbance readings obtained from the incubated samples and converted into glucoamylase activity units using a standard curve (Amyloglucosidase Fluka 10113).

Glucose Concentration

Glucose concentration was measured using an HPIC (CarboPacTM PA1 column, Dionex, MA, USA) and ACCUTREND glucose strips (Roche Diagnostics Ltd, East Sussex, UK).

RESULTS

Starch non-utilizing *A. niger* strain

The starch non-utilizing *A. niger* strain MGG029- $\Delta aamA$ lacks the two major amyolytic enzymes, glucoamylase and acid amylase (*AamA*) (Weenink *et al.*, 2006). The double-mutant strain grew poorly on starch with a severely reduced growth rate in comparison to *A. niger* strain MGG029, in which only the glucoamylase is deleted (data not shown). Residual growth of MGG029- $\Delta aamA$ is apparent (specific growth rate (μ_{max}) = 0.007 h⁻¹) and is possibly sustained by the constitutively low expression of undeleted glucoamylases (0.008 U.g_{DW}⁻¹) and other starch degrading enzymes e.g. alpha Glucosidase (Figure 1).

Construction of starch utilizing *A. niger* strain expressing an unfused truncated *A. niger* *GlaA_{G2}* gene construct

For use as a positive control, expression vector pAN56-8 under control of the inducible *A. niger* *GlaA_p* promoter and secretion signal fused to a sequence encoding the truncated *A. niger* *GlaA_{G2}* gene was transformed into *A. niger* MGG029- Δ *aamA*. This *A. niger* transformant, carrying vector pAN56-8 (*bla amdS GlaA_p-GlaA_{SS}-GlaA_{G2}-trpC_T*), showed improved growth on starch, an increased growth rate (0.024 h⁻¹) and high glucoamylase activity (0.073 U.g_{DW}⁻¹) in shake flask cultivations (Figure 1).

Construction of starch utilizing *A. niger* strains expressing a fused *GlaA_{G2}::S* gene construct

To produce HBsAg in *A. niger*, the HBsAg *S* gene was fused to the *GlaA_{G2}* gene. The fusion gene *GlaA_{G2}::S* was placed under control of the inducible *GlaA_p* of *A. niger* resulting in the expression vector pAHB19 (*bla amdS GlaA_p-GlaA_{SS}-GlaA_{G2}-S-trpC_T*). The *A. nidulans amdS* gene was introduced into the fused gene expression vector, pAHB19, and used as a selection marker. Expression vector pAHB19 was transformed into starch non-utilizing *A. niger* MGG029- Δ *aamA* protoplasts resulting in *GlaA_{G2}::S* fused gene transformants DAHB 21. Following transformation, a primary screen on selective medium, containing acetamide was implemented (Punt and van den Hondel, 1992). Selection was based on the expression of the *amdS* gene and the associated ability of transformants to utilize acetamide as sole carbon and/or nitrogen source (Hynes, 1972; Hynes, 1973). This resulted in the identification of 154 transformants with improved growth on acetamide and therefore represented potential HBsAg producing transformants. These 154 transformants were used to evaluate the three selection methods, as a means to identify high HBsAg producers.

Application of genetic selection system and identification of high producing HBsAg transformants

To identify high producing HBsAg transformants, three screening methods were evaluated in conjunction with conventional selection based on *amdS* expression and acetamide selection; (1) transformant selection based on acrylamide utilization through expression of the *amdS* gene, (2) transformant selection based on high growth on starch, and (3) transformant selection based on high glucoamylase activity using starch as a selection pressure.

(i) Conventional *amdS* selection based on acrylamide utilization

The 154 acetamide utilizing transformants identified in the primary screen were subjected to a secondary screen using acrylamide as the selection pressure. Based on colony size on agar plates, ten *GlaA_{G2}::S* fused gene transformants were selected, representing multicopy number gene insertion (Van den Hondel *et al.*, 1992), and preferred site of integration for acetamidase production.

These ten *GlaA_{G2}::S* fused gene transformants (DAHB 21# 2, 9, 20, 28, 32, 45, 58, 89, 114, and 139) were cultivated in submerged shake flask cultivations on inducing (starch) MM for 196 h, where sampling was performed every 24 h (Figure 1). Higher growth rates were obtained in the transformants, ranging between 0.015 h⁻¹ to 0.024 h⁻¹, in comparison to the starch non-utilizing parent strain MGG029- $\Delta aamA$ (0.007 h⁻¹). None of the selected ‘acrylamide utilizing’ transformants proved to have a growth rate higher than the starch utilizing *A. niger* strain expressing the unfused *GlaA_{G2}* gene product (Figure 1). Significant intracellular HBsAg levels (>25 $\mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$) were obtained in two *GlaA_{G2}::S* fused gene transformants (DAHB 21# 20, and 45). Levels were highest in DAHB 21# 20 at approximately $52 \pm 5.1 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$, corresponding to a high growth rate and extracellular

glucoamylase activity (0.017 h^{-1} and $0.049 \text{ U} \cdot \text{g}_{\text{DW}}^{-1}$, respectively) (Figure 1). No extracellular HBsAg was detected in transformants (data not shown).

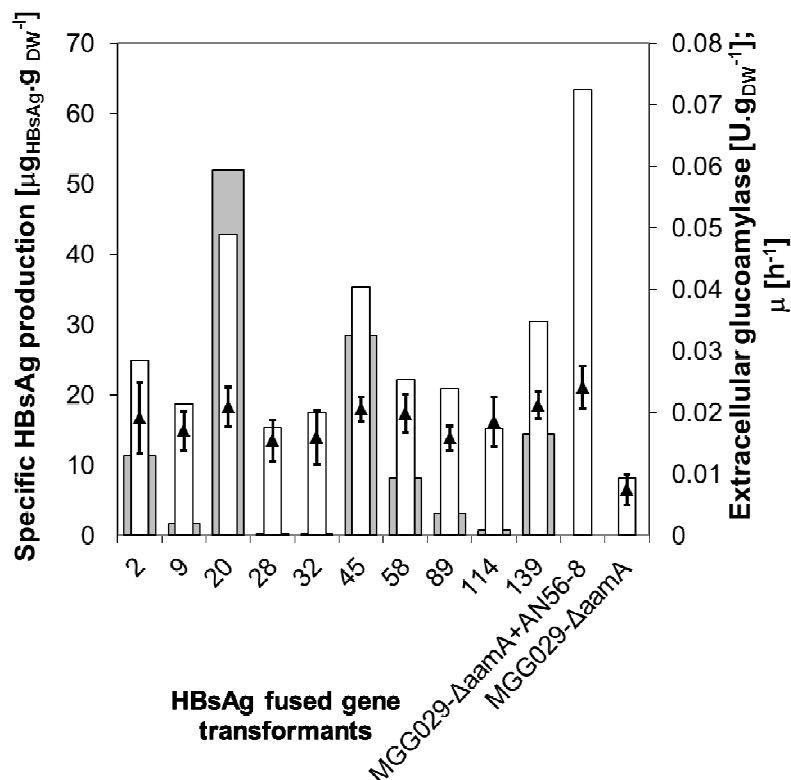


Figure 1. Screening of selected ‘acrylamide utilizing’ $Gla_{G2}::S$ fused gene transformants in shake flask cultivations for HBsAg production. Analysis is performed after 96 h of cultivation. Shown are data for: grey columns, specific intracellular HBsAg production ($\mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$); white columns, specific extracellular glucoamylase activity ($\text{U}_{\text{GlaA}} \cdot \text{g}_{\text{DW}}^{-1}$); \blacktriangle , maximum specific growth rate (μ_{max} , h^{-1}). Error bars represent the standard deviation of the mean of triplicate cultivations. Points without error bars have an associated deviation that is $< 10\%$ of the value of the point.

(ii) Transformant selection based on high growth on starch

The second selection criterion was adopted from Weenink *et al.* (2006) where the selection was based on $Gla_{G2}::S$ fused gene transformants showing improved growth on starch. A 10

μl drop of a spore solution amounting to 1×10^5 spores. ml^{-1} of each transformant was placed on solid MM containing 1 % starch as carbon source, incubated for 172 h at 30 °C and then stored for 48 h at 4 °C. Photographs followed by area measurements of biomass colonies permitted *GlaA_{G2}::S* fused gene transformant selections (Figure 2).

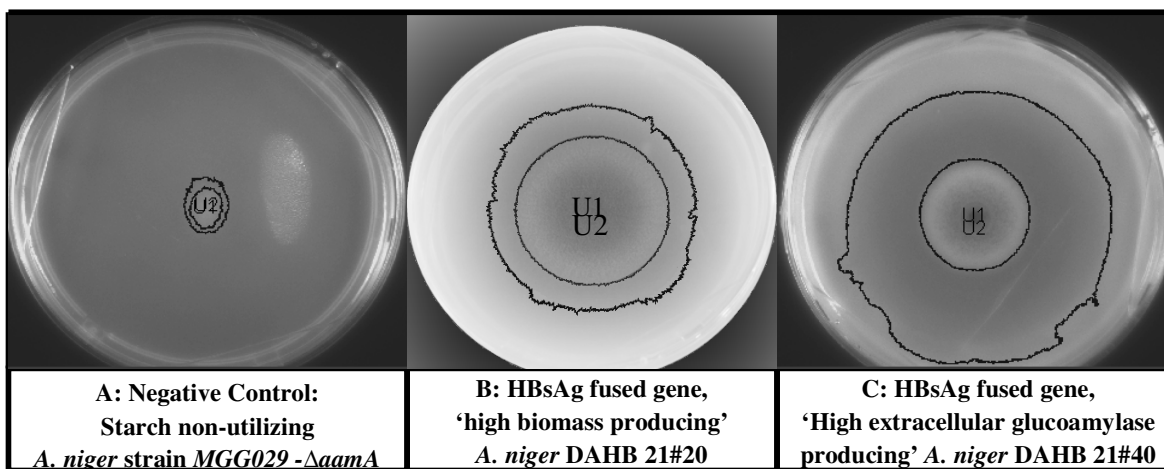


Figure 2. Screening of *GlaA_{G2}::S* fused gene transformants on solid medium containing 1 % starch as carbon source for HBsAg production. Each Petri dish (\varnothing 9 cm) was inoculated with 10 μl of a spore solution amounting to 1×10^5 spores. ml^{-1} of each transformant, incubated for 172 h at 30°C and then stored for 48 h at 4°C. Photographs followed by area measurements of biomass colonies and extracellular glucoamylase activity zones permitted *GLA_{G2}::S* transformant selections (ChemiDoc XRS+ System, Bio-Rad, Johannesburg).

Ten *GlaA_{G2}::S* fused gene transformants were identified as transformants with growth improvement on starch (DAHB 21# 2, 8, 13, 20, 21, 45, 59, 97, 126, and 151). These transformants were cultivated (as described above) in submerged shake flask cultivations on inducing (starch) MM for 196 h (Figure 3). High biomass levels (data not shown) and growth rates ranging between 0.019 h^{-1} to 0.023 h^{-1} were recorded. Extracellular glucoamylase activity amongst *GlaA_{G2}::S* transformants varied between 0.027 and 0.049 $\text{U} \cdot \text{g}_{\text{DW}}^{-1}$, with DAHB 21#20 obtaining the highest levels. High intracellular HBsAg levels ($>25 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$) were associated with high extracellular glucoamylase activity and were only observed in

four *GlaA_{G2}::S* fused gene transformants (DAHB 21# 20, 21, 45 and 126) (Figure 3). HBsAg levels were highest in transformant DAHB 21# 20. No extracellular HBsAg was detected in transformants (data not shown).

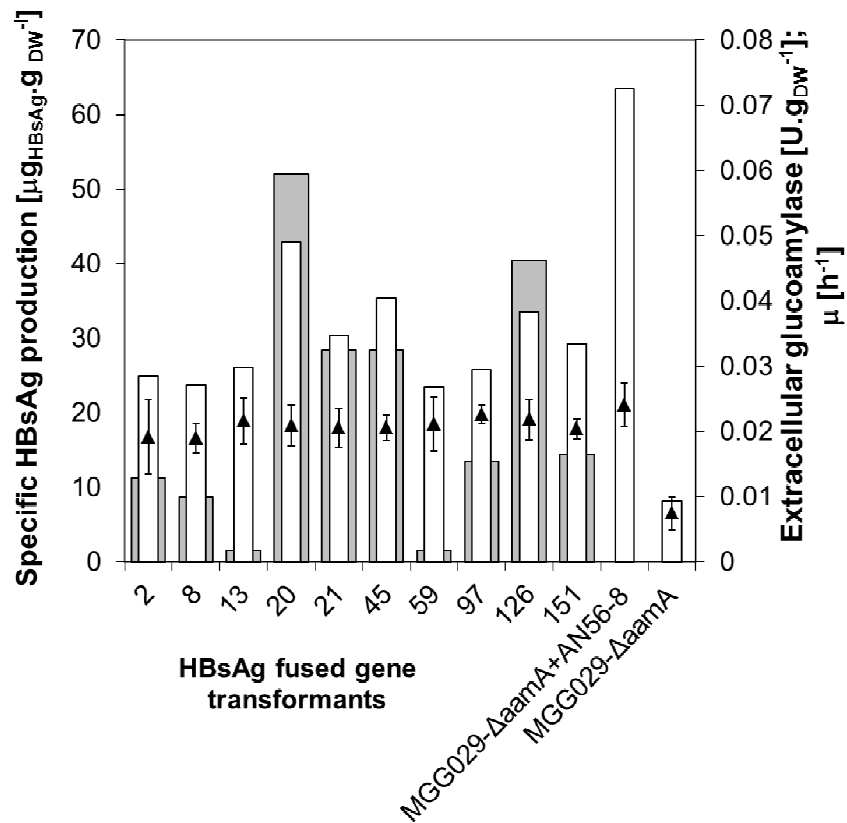


Figure 3. Screening of ‘high biomass producing’ *GlaA_{G2}::S* fused gene transformants in shake flask cultivations for HBsAg production. Analysis is performed after 96 h of cultivation. Shown are data for: grey columns, specific intracellular HBsAg production ($\mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$); white columns, specific extracellular glucoamylase activity ($\text{U}_{\text{GlaA}} \cdot \text{g}_{\text{DW}}^{-1}$); \blacktriangle , maximum specific growth rate (μ_{max} , h^{-1}). Error bars represent the standard deviation of the mean of triplicate cultivations. Points without error bars have an associated deviation that is < 10 % of the value of the point.

(iii) Transformant selection based on high extracellular glucoamylase activity using starch as a selection pressure

The third transformant selection criterion was based on the hypothesis that a selection based on high extracellular glucoamylase activity through a starch selection pressure would correlate with high HBsAg production levels. Transformants were cultivated on MM as previously described. Area measurements of biomass colonies and extracellular glucoamylase activity zones permitted *GlaA_{G2}::S* transformant selections based on specific extracellular glucoamylase activity ($\text{mm}^2_{\text{Glucoamylase}} \cdot \text{mm}^2_{\text{Biomass Growth}}^{-1}$) (Figure 2). Seven *GlaA_{G2}::S* fused gene transformants were selected with high extracellular glucoamylase activity (DAHB 21#20, 40, 62, 100, 103, 128, and 145). In liquid shake flask cultivations, all transformants produced high levels of extracellular glucoamylase activity, greater than $0.037 \text{ U} \cdot \text{g}_{\text{DW}}^{-1}$ (Figure 4), showing an acceptable correlation between specific extracellular glucoamylase activity ($\text{mm}^2_{\text{Glucoamylase}} \cdot \text{mm}^2_{\text{Biomass Growth}}^{-1}$) on agar plates and extracellular glucoamylase activity in submerged shake flask data (data not shown). Transformant DAHB 21#40 obtained the highest glucoamylase activity, $0.046 \text{ U} \cdot \text{g}_{\text{DW}}^{-1}$. Six transformants selected (DAHB 21#20, 40, 100, 103, 128, and 145) achieved high HBsAg production levels greater than $25 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$, with the highest levels obtained from DAHB 21#40 ($77 \pm 8.0 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$) (Figure 4). No extracellular HBsAg was detected in transformants (data not shown). Biomass levels of ‘high glucoamylase producing’ transformants varied (data not shown). Similarly, the growth rates were in the range of 0.014 and 0.021 h^{-1} , with DAHB 21#40 attaining a growth rate of 0.015 h^{-1} (Figure 4).

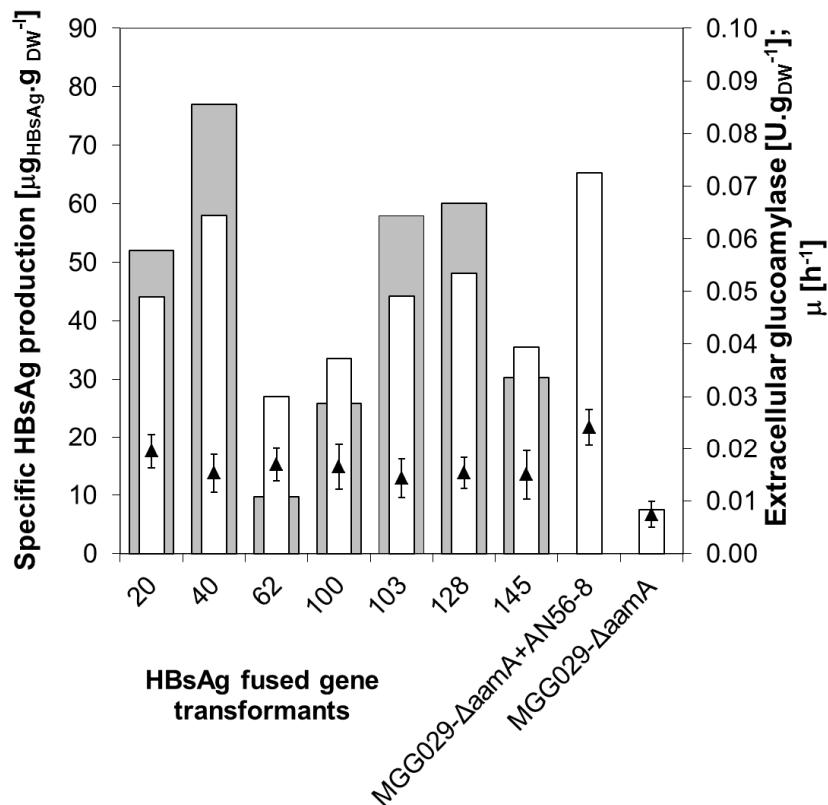


Figure 4. Screening of high extracellular glucoamylase producing *GlaA_{G2}::S* fused gene transformants in shake flask cultivations for HBsAg production. Analysis is performed after 96 h of cultivation. Shown are data for: grey columns, specific intracellular HBsAg production ($\mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$); white columns, specific extracellular glucoamylase activity ($\text{U}_{\text{GlaA}} \cdot \text{g}_{\text{DW}}^{-1}$); \blacktriangle , maximum specific growth rate (μ_{max} , h^{-1}). Error bars represent the standard deviation of the mean of triplicate cultivations. Points without error bars have an associated deviation that is $< 10\%$ of the value of the point.

Efficiency of screening methods for identification and selection of high HBsAg producing transformants

All three screening methods were able to identify HBsAg producing transformants. However, there were varying degrees of success in terms of HBsAg production levels and the percentage transformants identified as high HBsAg producers from each method. Out of the

10 transformants selected through the ‘acrylamide utilizing’ screen through *amdS* gene expression, 20 % were identified as high HBsAg producing transformants ($> 25 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$; Table 1). The screening methodology based on selecting for improved growth on starch yielding ‘high biomass producing’ transformants identified 40 %, while through the selection of high extracellular glucoamylase activity on starch, 86 % of the transformants selected were identified as high HBsAg producing transformants ($> 25 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$; Table 1).

Table1: Summary of screening methods evaluated

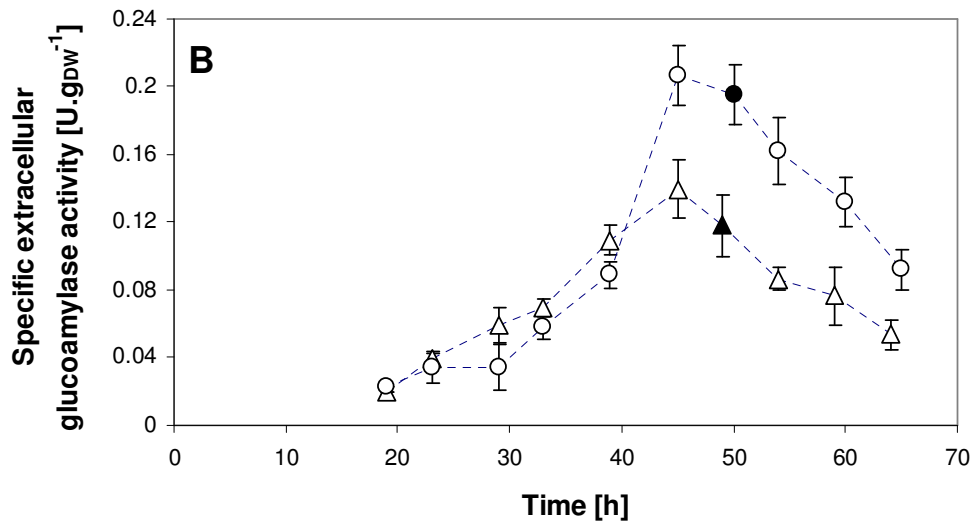
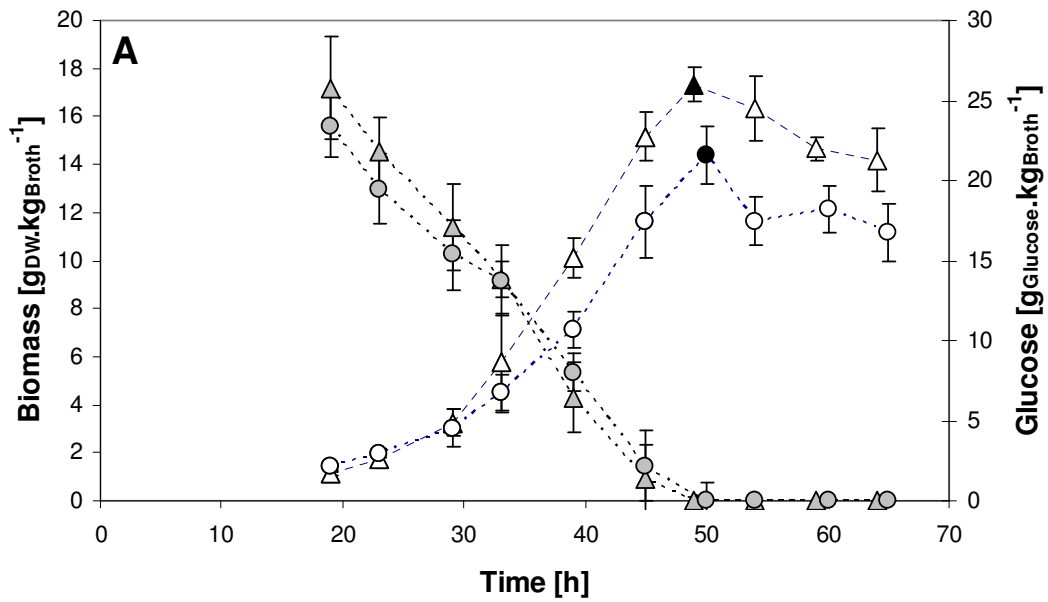
Screening strategy	No. of transformants selected from 154	No. of transformants $> 25 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$	Percentage of high HBsAg producing transformants ($>25 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$) selected (total transformants, 154)
1. ‘Acrylamide utilization’ through <i>amdS</i> expression	10	2	20
2. ‘high biomass producing’	10	4	40
3. ‘high extracellular glucoamylase activity’	7	6	86

Batch fermentation of two highest HBsAg producing transformants

From the three screening methodologies evaluated in this study, two high HBsAg producing transformants were identified: DAHB 21#20 that was identified using all three selection methods and DAHB 21#40 that was recognized while screening for ‘high extracellular glucoamylase producing’ transformants. To evaluate and compare the performance of the two high HBsAg producing transformants, batch fermentations were conducted. A carbon substrate of 3 % glucose in a rich media was used for cultivation to ensure rapid offline analysis of carbon consumption. Growth profiling of transformants followed accelerated

trends compared to shake flask cultivations (Figure 5A). Maximum biomass concentrations and growth rates differed significantly between transformants, with the ‘high biomass producing’ DAHB 21#20 obtaining the highest at $17.32 \pm 0.73 \text{ g}_{\text{DW}} \cdot \text{kg}_{\text{Broth}}^{-1}$ and $0.11 \pm 0.009 \text{ h}^{-1}$, and ‘high extracellular glucoamylase producing’ DAHB 21#40 obtaining $14.35 \pm 1.2 \text{ g}_{\text{DW}} \cdot \text{kg}_{\text{Broth}}^{-1}$ and $0.08 \pm 0.007 \text{ h}^{-1}$, respectively (Figure 5A). Similar glucose consumption rates were observed for both transformants, with glucose depletion correlating to maximum biomass concentrations ($\sim 49 \text{ h}$ for the ‘high biomass producing’ transformant and $\sim 50 \text{ h}$ for the ‘high extracellular glucoamylase producing’ transformant). Biomass yield coefficients, however, differed ($Y_{\text{XS}}^{\text{‘high biomass producing’}} = 0.56 \pm 0.015 \text{ g}_{\text{DW}} \cdot \text{g}_{\text{Glucose}}^{-1}$ and $Y_{\text{XS}}^{\text{‘high glucoamylase producing’}} = 0.51 \pm 0.02 \text{ g}_{\text{DW}} \cdot \text{g}_{\text{Glucose}}^{-1}$; Figure 5A). Extracellular glucoamylase activity profiles followed a growth associated trend, with the highest levels obtained in the ‘high extracellular glucoamylase producing’ transformant ($0.21 \pm 0.021 \text{ U} \cdot \text{g}_{\text{DW}}^{-1}$) (Figure 5B). Glucoamylase activity in the ‘high biomass producing’ transformant reached 67 % of the levels obtained in the ‘high extracellular GlaA producing’ transformant ($0.14 \pm 0.018 \text{ U} \cdot \text{g}_{\text{DW}}^{-1}$). Similar to the extracellular glucoamylase activity profiles, intracellular HBsAg production levels also followed a growth associated trend with the highest levels occurring after $\sim 50 \text{ h}$ (Figure 5C). The ‘high extracellular glucoamylase producing’ transformant achieved highest levels of $219 \pm 8.5 \text{ } \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$, while the ‘high biomass producing’ transformant obtained 68 % of the levels obtained in the ‘high glucoamylase producing’ transformant with $143 \pm 12.5 \text{ } \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$. The decrease in both extracellular glucoamylase activity and HBsAg production levels in transformants correlated to glucose depletion suggesting that glucose as carbon source was the rate limiting nutrient. Furthermore, linear correlations between intracellular HBsAg and extracellular glucoamylase activity were observed in the two *GlaA_{G2}::S* transformants during the exponential growth phase in glucose batch fermentation (Figure 6). The significance of the two correlations were confirmed through linear regression

analysis, with $p_{\text{values}} < 0.05$ of the x variables and intercepts at a 95 % confidence level and R^2 values ≥ 0.95 explaining more than 95 % of the total variation (Figure 6). However, no significant difference was observed between the two correlations ($p > 0.05$).



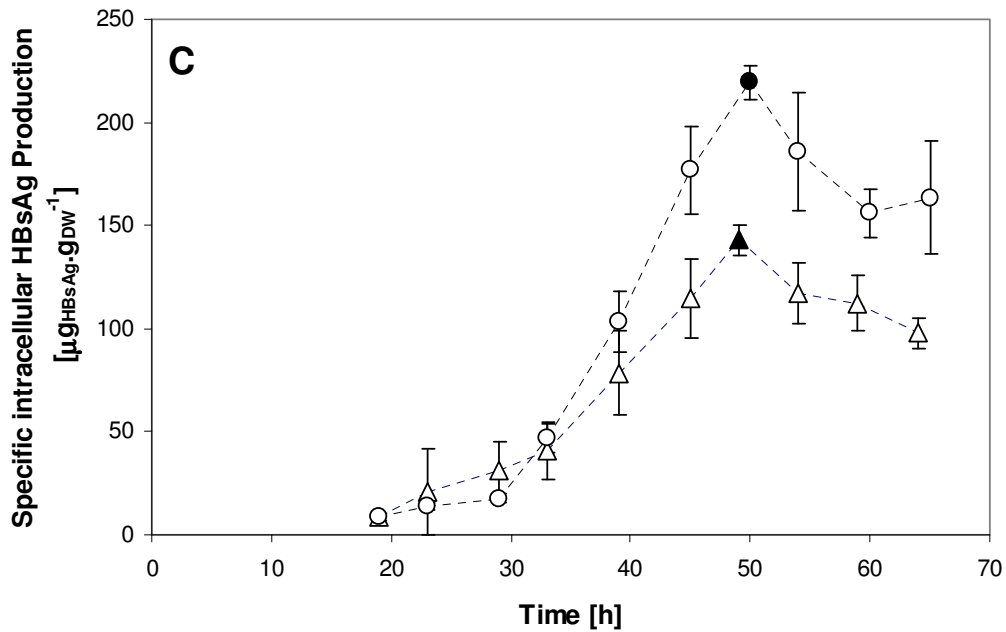


Figure 5. Glucose batch fermentation of *A. niger*: Shown is data regarding transformants (carrying the *GlaA_{G2}::S* fusion cassette and controlled by the inducible *GlaA_p* promoter); Δ, ‘fast-growing high-biomass producing’ transformant DAHB 21#20; and ○, ‘slow-growing high-glucoamylase producing’ transformant DAHB 21#40. Time course data of: (A) Dry biomass concentration (open symbols) and glucose concentration (grey symbols); (B) specific extracellular glucoamylase activity (open symbols); (C) specific intracellular HBsAg production (open symbols). Closed symbols for Δ and ○ represent glucose depletion in glucose cultivations. Error bars represent the standard deviation of the mean of triplicate cultivations.

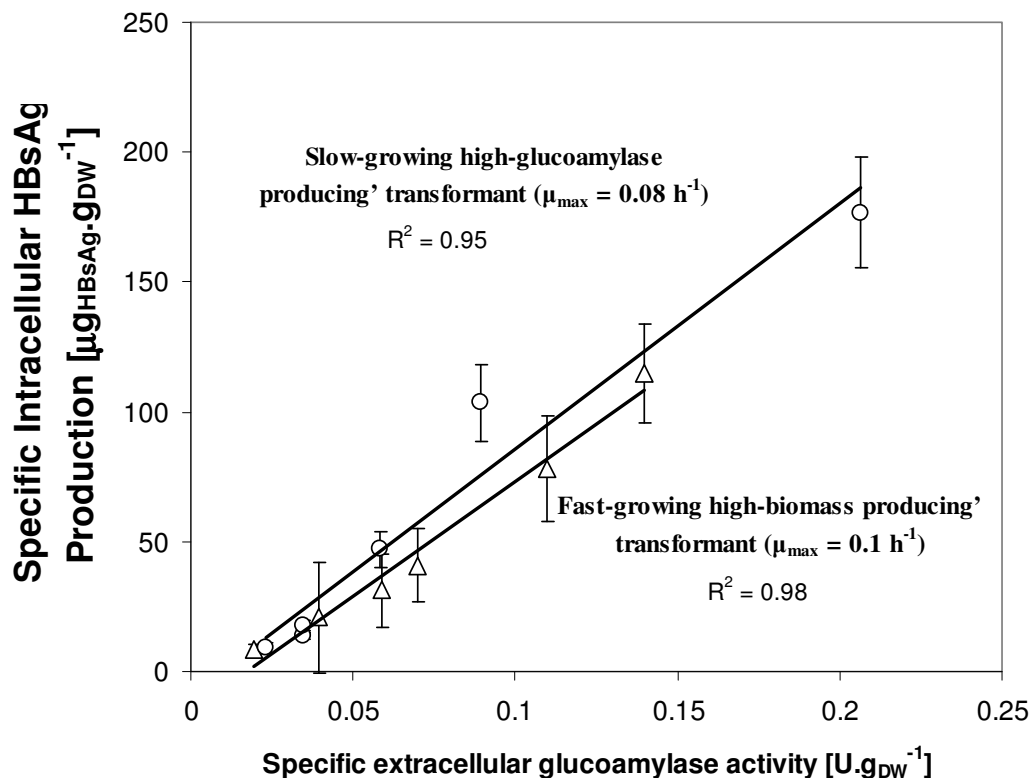


Figure 6. Correlation between specific intracellular HBsAg production and specific extracellular glucoamylase activity. Shown is data regarding glucose batch fermentation of *A. niger* transformants: Δ , 'fast-growing high-biomass producing' transformant DAHB 21#20; and \circ , 'slow-growing high-glucoamylase producing' transformant DAHB 21#40 during the exponential growth phase. Error bars represent the standard deviation of the mean of triplicate cultivations.

DISCUSSION

The aim of this study was to develop an improved methodology to identify and select high HBsAg producing transformants. Gene fusion, using the expression vector carrying the glucoamylase carrier gene fused to the HBsAg *S* gene as well as the inclusion of the *amdS* selection marker, was used not only to facilitate HBsAg *S* gene expression and HBsAg assembly through the secretory pathway but also to serve as a medium for selection. In conjunction with conventional selection based on *amdS* expression and associated acetamide utilization, transformants were screened using varied selection methods; (1) transformant

selection based on acrylamide utilization through expression of the *amdS* gene, (2) transformant selection based on high growth on starch as reported by Weenink *et al.*, (2006), and (3) transformant selection based on high extracellular glucoamylase activity on starch.

Conventional selection systems, in the form of selectable markers, are often incorporated within the plasmid vector carrying the gene product of interest. The majority of these systems are based on either complementation of auxotrophic mutant strains with the corresponding wild type gene and/ or dominant selection marker genes, which confer resistance to a fungicide (reviewed by Van den Hondel and Punt 1991; Turner 1994). The *amdS* gene, inserted into the fused *GlaA_{G2}::S* gene expression vector, is a commonly used dominant marker that encodes the enzyme acetamidase (E.C.3.5.1.4) which hydrolyses acetamide and acrylamide. Transformant selection using this approach is directed towards the transformant's ability to produce acetamidase and therefore utilize acetamide or acrylamide as sole carbon and/or nitrogen source (Hynes, 1972; Hynes, 1973). In *A. nidulans* one copy of the *amdS* gene is sufficient for growth on acetamide, however growth on acrylamide is only possible if several copies of the gene are present (Van den Hondel *et al.*, 1992). The identification of high HBsAg producing transformants in *amdS* selection based on acrylamide utilization therefore represents transformants with improved acetamidase production.

The low efficiency of 20 % in identifying high HBsAg producing transformants using this approach suggests that there is no strict correlation between acetamidase production and HBsAg production. In the study of Plüddemann (2003), *amdS* selection based on acrylamide utilization resulted in the identification of transformants with high transcription levels of the fused *GlaA_{G2}::S* gene, but no extracellular or intracellular HBsAg could be detected. These results suggest that limitations with the production of this higher recombinant eukaryotic gene product do not appear to be at the level of transcription but rather on a (post)translational level

(Plüddemann, 2003). Similar conclusions have been reported (Jeenes *et al.*, 1994; Nyysönen and Keränen, 1995; Gouka *et al.*, 1997a; Plüddemann and van Zyl, 2003). Therefore, any correlations that may have existed at a transcriptional level between *amdS* and fused *GlaA_{G2}::S* gene expression using selection based on acrylamide utilization could differ significantly at a posttranslational level, where bottlenecks can accrue through the secretory pathway and thus affect the fused glucoamylase-HBsAg products.

The screening criterion proposed by Weenink *et al.* (2006), regarding ‘high biomass producing’ transformants, bases selection on the transformants ability to grow on starch using an expression host that lacks two major amylolytic enzymes, glucoamylase and acid amylase. Through the expression of the fused *GlaA_{G2}::S* gene, growth of the transformant on starch is obtained through the production of glucoamylase which is required for starch to glucose hydrolysis. Thus, improved growth should reflect improved HBsAg production levels using starch as a selection pressure. This hypothesis relies on the following assumptions: (1) high biomass production reflects high glucoamylase production, and (2) glucoamylase activity is proportional to HBsAg production. With a 40 % efficiency in identifying high HBsAg producing transformants and a 2-fold improvement in comparison the ‘acrylamide utilizing’ screen, suggests that there is merit in the abovementioned hypothesis: high biomass production is indicative of higher glucoamylase production levels (Figure 3), and for HBsAg production in *A. niger*, using *GlaA_{G2}::S* gene fusion, glucoamylase is proportional to HBsAg production (Figure 6). But the 40 % success rate in identifying high HBsAg producers also indicates that the indirect quantification of HBsAg production in potential HBsAg producing transformants through improved growth on starch reflects an inefficiency using this method.

The third screening criterion selects specifically for high extracellular glucoamylase producing transformants, with the hypothesis that extracellular glucoamylase activity is directly proportional to correctly folded and assembled HBsAg. Higher extracellular glucoamylase producing transformants were identified in comparison to the 'high biomass producing' selection criterion and resulted in 86 % of all transformants selected obtaining high HBsAg levels, with the highest at $77 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ (DAHB 21#40). Transformants with a greater area of glucoamylase activity in relation to the area of biomass produced on starch agar selection plates could represent transformants with optimum copy number and preferred site of integration for the high expression and production of the fused *GlaAG2::S* gene product. More importantly this could also represent transformants with increased glucoamylase secretion efficiency. If this is the case, selecting for high extracellular glucoamylase producing transformants could represent transformants with the most efficient processing of the fused product. These findings also suggest that by selecting for 'high biomass' producing transformants, identification of high HBsAg producing transformants may be masked. Whether this is indicative of the extra sensitivity incurred by directing selection specifically towards the area of glucoamylase activity in relation to the area of biomass produced on starch agar selection plates is plausible. However, due to a direct proportional relationship between HBsAg production and glucoamylase activity, this has allowed for the indirect quantification of HBsAg production through extracellular glucoamylase determination. Any bottlenecks that accrue during expression or translation of the fused product in the secretory pathway could possibly be reflected in the quantification of extracellular glucoamylase.

From the three selection methods evaluated in this study, two high HBsAg-producing transformants were identified; DAHB 21#20 that was identified using all three selection methods and DAHB 21#40 that was only recognized while screening for high extracellular

glucoamylase. During the cultivation of these two transformants in glucose batch fermentations it became more apparent that two distinctive growth phenotypes had been identified. Transformant DAHB 21#20, showing evidence of high acetamidase, and biomass, production levels, had a higher growth rate and biomass yield coefficient in comparison to the high extracellular glucoamylase producing transformant DAHB 21#40, but the HBsAg production levels and extracellular glucoamylase levels were lower. The morphologies of the two transformants were pelleted and indistinguishable (data not shown), indicating that differences in growth rate were not caused by a morphological alteration. Possible explanations for this phenomenon could include variance in plasmid copy number (Verdoes *et al.*, 1994), site of integration (Plüddemann, 2003) and/ or posttranscriptional alteration of the fused *GlaA_{G2}::S* gene (Weenink *et al.*, 2006).

HBsAg production and assembly was highest at a lower growth rate in the 'high glucoamylase producing' transformant. The specific product formation rate of a protein can increase consistently with increasing growth rate, as shown for laccase in *A. niger* (Weenink *et al.*, 2006), Fab fragment production in *Pichia pastoris* (Zhang *et al.*, 2005) and β -galactosidase production in *Escherichia coli* (Sanden *et al.*, 2003). Whereas for other proteins, such as α -galactosidase of *P. pastoris*, cellobiohydrolase (Pakula *et al.*, 2005), laccase (Rautio *et al.* 2007) in *Trichoderma reesei* and HBsAg in *A. niger* in this study, the specific product formation rate is highest at lower growth rates. Efficient processing of the heterologous protein of interest may be compromised by the saturation or overloading of the secretory machinery caused by unfolded and/or misfolded proteins, which are prevented from leaving the Endoplasmic reticulum (ER) (Lodish *et al.* 1983; Shuster 1991; Parekh *et al.* 1995; Shusta *et al.* 1998; Smith *et al.* 2004; Damasceno *et al.*, 2007). Further investigation is necessary to determine the relation between growth rate and HBsAg assembly as a reduced growth rate

may enhance the efficiency of *A. niger* to synthesize and produce HBsAg by alleviating the speculated pressures of over expression or unfolded protein overload in HBsAg-producing *A. niger* transformants.

In this study we were able to select and identify high HBsAg producing transformants, using a modified/enhanced selection system, based on high extracellular glucoamylase activity using starch as a selection pressure. The genetic selection system, making use of the glucoamylase-HBsAg *S* gene fusion strategy and a starch non-utilizing *A. niger* host, increased intracellular HBsAg production levels 4-fold compared to levels previously reported (Manuscript 1 of this dissertation). Through the production of correctly folded antigenic HBsAg we demonstrate that this technology is rapid, robust, and versatile for the production of mammalian and higher eukaryotic gene products in *A. niger* using the fused gene approach and glucoamylase carrier protein. Through the application of molecular tools (e.g. modification of chaperone levels), as well as the development of cultivation parameters (e.g. exponential fed batch), the reported HBsAg production levels are expected to increase significantly. The further development of this technology is suggested as it is an exciting candidate for recombinant subunit vaccine production in *A. niger*.

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MANUSCRIPT 3

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THE EFFECT OF GROWTH RATE IN GLUCOSE-LIMITED EXPONENTIAL FED-BATCH FERMENTATION ON HEPATITIS B SURFACE ANTIGEN PRODUCTION
IN *ASPERGILLUS NIGER*

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ABSTRACT

The effects of growth rate (μ) in glucose-limited exponential fed-batch fermentations on HBsAg production in *Aspergillus niger* was evaluated, using two HBsAg producing transformants, (1) the ‘fast-growing high-biomass producing’ transformant ($\mu_{\max} = 0.11 \text{ h}^{-1}$), and (2) the ‘slow-growing high-glucoamylase producing’ transformant ($\mu_{\max} = 0.08 \text{ h}^{-1}$). Both transformants made use of the gene fusion approach, using the expression vector containing the *GlaA_{G2}* carrier gene fused to the HBsAg *S* gene, under control of the *GlaA_p* promoter. In comparison to batch fermentation, glucose-limited exponential fed-batch fermentations at $\mu \approx \mu_{\max}$, intracellular HBsAg production levels were enhanced by $\sim 20 \%$ for both transformants. The culturing of the ‘fast-growing high-biomass producing’ transformant, at a reduced $\mu = 0.08 \text{ h}^{-1}$, obtained highest specific HBsAg production levels of $\sim 240 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$. Highest intracellular HBsAg production levels in ‘slow-growing high-glucoamylase producing’ transformant cultivations of $\sim 250 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ were detected at μ ranging between $0.07 < \mu < 0.08 \text{ h}^{-1}$. At low μ , estimated m_s formed ~ 25 and 30% , respectively, of the q_s . The high non-growth associated substrate consumption for maintenance under these conditions potentially limited production of both biomass and proteins. These limitations were reflected in the significantly lower biomass levels, extracellular glucoamylase activities and intracellular HBsAg production levels for both transformants at $\mu = 0.03 \text{ h}^{-1}$. HBsAg production in relation to extracellular glucoamylase activity was most efficient for both transformants at $0.07 < \mu < 0.08 \text{ h}^{-1}$. Due to significantly higher biomass levels, volumetric HBsAg production levels were highest ($4483 \pm 340 \mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$) in ‘fast-growing high-biomass producing’ transformant fermentations. HBsAg production levels using this approach were increased 1.8 fold in comparison to batch fermentations. Thus glucose-limited exponential fed-batch fermentation at an optimum μ ($0.07 < \mu < 0.08 \text{ h}^{-1}$) for the current *A.*

niger HBsAg production system may provide a method for efficient HBsAg processing, resulting in increased HBsAg production levels.

INTRODUCTION

In the early 1980s, the first licensed vaccines against the Hepatitis B virus (HBV) became available and were produced from the serum of chronic carriers. These serum-derived vaccines were effective and safe, but were also expensive and in relatively short supply because of the shortage of human carrier plasma that met the requirements for vaccine production (Prince and Vnek, 1982). From 1986, advancements in recombinant technology have led to the success of current vaccination programs against Hepatitis B through the development of effective, recombinant hepatitis B surface antigens (HBsAg) that assemble into virus-like particles (VLPs). Initially, the production of such vaccines was restricted to baker's yeast, *Saccharomyces cerevisiae*, but with improvements in gene technology and biotechnological methods, alternative expression systems have been identified. This includes the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha* (Gleeson *et al.*, 1986; Roggenkamp *et al.*, 1986; Hollenberg and Gellissen, 1997; Gellissen, 2002) which are currently used in the production of several vaccines against different subtypes of the HBV (Gellissen and Melber, 1996; Brocke *et al.*, 2005; Melmer *et al.*, 2008).

The filamentous fungi, *Aspergillus niger* has also emerged as a potential alternative expression system for VLP vaccine production due to several advantageous characteristics: vector design and construction, codon optimization and choice of promoter (Van den Hondel and Punt, 1991; Gouka *et al.*, 1996; Plüddemann and van Zyl, 2003), the generation of fusion constructs (Gouka *et al.*, 1997b; Spencer *et al.*, 1998; Plüddemann and van Zyl, 2003; Manuscript 1 & 2 of this dissertation), over expression of chaperone proteins (Punt *et al.*,

1998; Ngiam *et al.*, 2000), the isolation of protease deficient mutants (Archer and Peberdy, 1997; van den Hombergh *et al.*, 1997), and the optimisation of media and cultivation conditions (MacKensie *et al.*, 1994; Xu *et al.*, 2000; Conesa *et al.*, 2001; James *et al.*, 2007).

It has been previously reported that by targeting HBsAg production through the secretory pathway in *A. niger*, completely assembled and properly folded HBsAg, was obtained (manuscript 1 and 2 of this dissertation). This was achieved by implementing a gene fusion strategy using the highly expressed catalytic domain of the glucoamylase gene (*GlaA_{G2}*) fused to the HBsAg *S* gene. The inducible glucoamylase promoter (*GlaA_p*) was used to control transcription. The gene fusion strategy resulted in the cleavage of the fused product by the KEX2-like protease, and intracellular accumulation of HBsAg was observed, while glucoamylase was secreted (manuscript 1 of this dissertation). However, several breakdown products showing an immunoreactive response to the glucoamylase polyclonal antibody were detected. Similar results were documented in the study of Plüddemann, (2003) and Conesa *et al.*, (2000) and indicated a level of intracellular degradation. Possible explanations to these findings were attributed to the bottlenecks that accrue within the secretory pathway (Wang, 2005). In heterologous expression systems, where there is a greater flux of proteins being translocated into the Endoplasmic Reticulum (ER), the folding, assembly, and processing machinery may become saturated, leading to improperly folded structures (Ngiam *et al.*, 2000).

In Manuscript 2 of this dissertation, using a starch substrate as a selection pressure for the expression of the *GlaA_{G2}* fused to the HBsAg *S* gene in an *A. niger* strain lacking native glucoamylase and acid amylase, two high HBsAg producing transformants were identified: (1) the 'fast-growing high-biomass producing' transformant ($\mu_{\max} = 0.11 \text{ h}^{-1}$), and (2) the

‘slow-growing high-glucoamylase producing’ transformant ($\mu_{\max} = 0.08 \text{ h}^{-1}$). Highest specific and volumetric intracellular HBsAg production correlated to high extracellular glucoamylase and was observed in a HBsAg-producing *A. niger* transformant, with an inherently reduced μ (manuscript 2 of this dissertation). This has led to the hypothesis that a reduced μ may enhance the efficiency of *A. niger* to synthesize and produce HBsAg.

In this study, we evaluated the effects of μ in glucose-limited exponential fed-batch fermentations on HBsAg production of two HBsAg producing transformants, previous identified (Manuscript 2 of this dissertation); (1) the ‘fast-growing high-biomass producing’ transformant, DAHB21#20, and (2) the ‘slow-growing high-glucoamylase producing’ transformant, DAHB21#40. Through glucose-limited exponential fed batch fermentation the effects of μ over a range of 0.03 to 0.1 h^{-1} on specific and volumetric intracellular HBsAg production as well as biomass concentration and extracellular glucoamylase activity were evaluated. Additional analysis on the effect of μ in fed batch fermentation on substrate consumption (q_s) and the maintenance coefficient (m_s) is also performed and provides insight towards determining the requirements for efficient HBsAg production.

MATERIALS AND METHODS

Strains

Two HBsAg-producing *A. niger* transformants, which were identified in Manuscript 2 of this dissertation, are evaluated in this study; (1) the ‘fast-growing high-biomass producing’ transformant, DAHB21#20, and (2) the ‘slow-growing high-glucoamylase producing’ transformant, DAHB21#40. These transformants were developed as previously described in Manuscript 2 of this dissertation.

Inoculum preparation, media and cultivations

Fungal spores were obtained from a densely conidiating culture grown on potato dextrose agar (PDA) at 30 °C for 120 h and harvested with physiological saline solution (NaCl, 0.9 % w.v⁻¹). Shake flask cultures were inoculated with a spore concentration of 1×10⁶ spores.ml⁻¹. Batch fermentations were inoculated with a shake flask pre-culture amounting to 10 % of the fermentor's total working volume. All cultivations were grown on minimal medium (MM) (Plüddemann and van Zyl, 2003) containing 3 % (w.w⁻¹) glucose and supplemented with 0.1 % (w.w⁻¹) casamino acids (Difco), 0.8 % (w.w⁻¹) tryptone and 0.8 % (w.w⁻¹) yeast extract. Shake flask cultivations were performed in 1 L Erlenmeyer flasks (un-baffled) containing 250 ml medium at 30 °C on a rotary shaker (Innova 2300, New Brunswick Scientific, Edison, USA) at 180 rpm. Fermentations were performed in 2 L bench-top bioreactors (INFORS AG, Switzerland) with a working volume of 1.5 L. The fermentation temperature was maintained at 30 °C (± 0.1°C), aeration at 0.8 vvm, dissolved oxygen was maintained above 25 % saturation by varying impeller speed (starting rate of 150 rpm), through a cascaded controller. The impellor speed was ramped by 200 rpm for 1 min every hr to reduce wall growth. pH was controlled above a value of 4 using NH₄OH (25 % w.v⁻¹).

Exponential feed calculations

Exponential fed-batch cultivations were used to evaluate a range of four different set growth rates (μ_{set}) (0.03, 0.05, 0.07, 0.08, and 0.1 h⁻¹). During the carbon limited exponential feeding phase, the mass of residual glucose in the fermentor (M_s) was maintained close to zero and μ_{set} was controlled by directly controlling the mass of concentrated glucose substrate added in the fed-batch cultivation. This approach of controlling the mass of concentrated glucose substrate added in the fed-batch cultivation (equation 3) while $M_s \approx 0$, was used to regulate the production of biomass in the fermentor (equation 1) through the control of glucose substrate

consumption by the filamentous fungi. This results in the control of the μ_{set} at a constant value under both transient and steady state conditions for biomass concentration (Martinez *et al.*, 1998; Kim *et al.*, 2000; Cheng *et al.*, 2003; Sun *et al.*, 2007; Chen *et al.*, 2008).

Biomass calculations

The biomass produced at time 1 ($M_{x(t1)}$) was used to calculate a predicted biomass profile for a particular μ_{set} using the principals of equation 1 (refer to derivations in Appendix A), to predict the biomass production at time 2 ($M_{x(t2)}$).

$$M_{x(t2)} = M_{x(t1)} e^{\mu_{\text{set}}(t_2 - t_1)} \quad \text{Equation 1.}$$

Assuming the growth yield, Y_{XS} is constant throughout the exponential feeding phase, and applying the principals of equation 2 to obtain equation 3 (refer to derivations in Appendix A), the amount of glucose at a specific time point required for the production of biomass at μ_{set} , was calculated;

$$Y_{xs} = \frac{\text{mass of new biomass formed}}{\text{mass of glucose substrate consumed to produce new biomass}} \quad \text{Equation 2.}$$

$$M_{sf(t2)} = \frac{(M_{x(t2)} - M_{x(t1)})}{Y_{XS}} + M_{sf(t1)} \quad \text{Equation 3.}$$

where $M_{sf(t2)} - M_{sf(t1)}$ is the predicted mass of glucose required at time 2 to produce new biomass, $M_{x(t2)} - M_{x(t1)}$ at μ_{set} . The mass of glucose in the fermentor (M_s) was maintained at approximately zero for the duration of the feeding phase, where it was assumed that M_s was consumed by the organism as fast as it entered the fermentor to control μ_{set} . The amount of

glucose fed was controlled and logged by an algorithm on the fermentor digital control unit (DCU) which exponentially increased the pump dosage every 10 seconds according to the glucose profile calculated (Equation 1 & 3). This data was cross referenced gravimetrically. Calculations regarding the exponential feeding methodology are presented in Appendix B.

Rate calculations

The specific rate of substrate consumption (q_s) was calculated using the following rate equation, Equation 3:

$$q_s = \frac{r_s}{M_x} \quad \text{Equation 3.}$$

Where q_s ($\text{g}_{\text{Glucose}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$) is the specific rate of substrate consumption, r_s ($\text{g}_{\text{Glucose}} \cdot \text{h}^{-1}$) is the rate at which the substrate is consumed, and M_x (g_{DW}) is the mass of biomass cultivated. The non-growth-associated maintenance coefficient, m_s , ($\text{g}_{\text{Glucose}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$) and $Y_{\text{XS}}^{\text{max}}$ were calculated using the linear equation of substrate utilization, introduced by Pirt (1965) and shown in Equation 4.

$$-q_s = \frac{\mu}{Y_{\text{XS}}^{\text{max}}} + m_s \quad \text{Equation 4.}$$

q_s , $Y_{\text{XS}}^{\text{max}}$, and m_s were estimated by linear regression, assuming that the energy requirement for m_s and the $Y_{\text{XS}}^{\text{max}}$ are constant over the different μ and that no metabolites other than carbon dioxide and water are produced.

Sample preparation

Shake flasks were sampled in 24 hr increments while bioreactor cultivations were sampled at intervals of 3 to 8 h. Samples containing ± 50 g of cultivation medium were withdrawn and

biomass concentration was measured by vacuum filtering 25 g sample on dry and pre-weighed filters (Whatman no.1), followed by washing with 40 ml 0.9 % (w.v⁻¹) NaCl solution and drying to a constant weight at 105 °C for 24 to 48 h. The remaining 25 g sample was ground to a fine powder with liquid nitrogen in a mortar and pestle, and an aliquot of 0.3 g mycelia powder was stored at -80 °C in a 2 ml micro-centrifuge tube. Ground samples were suspended in protein extraction buffer with 0.1 % Triton X-100 (Plüddemann and van Zyl, 2003) and homogenized for 1 min at 4 °C. This was followed by clarification at 13 000 rpm and 4 °C. Extraction of supernatant resulted in samples containing total intracellular proteins including membrane-associated proteins.

HBsAg concentration determination

A “sandwich” type enzyme immunoassay based on monoclonal anti-HBsAg (HBsAg 3.0 EIA, Bio-Rad, Hercules, CA) was used to quantify completely assembled and properly folded HBsAg as per manufacturers specification.

Glucoamylase Activity

The activity of glucoamylase was measured by the method of Withers *et al.* (1998) using freshly prepared 0.1 % (v.v⁻¹) 4-nitrophenyl- α -D-glucoopyranoside (PNPG) (Sigma-Aldrich N-1377) as substrate in 0.1 M Sodium Acetate buffer (pH 4.3). To commence the reaction, 100 μ l PNPG was added to 50 μ l sample (or glucoamylase standard) in a flat bottom micro-titre plate and incubated for 20 min at 25 °C. The reaction was terminated by the addition of 150 μ l Borax (0.1 M Na₂B₄O₇.10 H₂O) solution, and absorbance at 400 nm was measured using a BioTek Power wave^{HT} micro-titre plate reader (BioTek Instruments Inc., USA) referenced with distilled water. Background absorbance was normalized with the addition of 150 μ l Borax solution to 50 μ l sample prior to the addition of 100 μ l PNPG, in a duplicate assay. The

measured background absorbance was subtracted from the absorbance readings obtained from the incubated samples and converted into glucoamylase activity using a standard curve (Amyloglucosidase Fluka 10113).

Glucose Concentration

Glucose concentration was measured using a high performance ion chromatography (HPIC) (CarboPac™ PA1 column, Dionex, MA, USA) and ACCUTREND Glucose strips (Roche Diagnostics Ltd, East Sussex, UK).

RESULTS

Growth regulated, glucose-limited exponential fed-batch fermentation

The ‘fast-growing high-biomass producing’ transformant, DAHB21#20, and the ‘slow-growing high-glucoamylase producing’ transformant, DAHB21#40 were cultured under glucose-limited exponential fed-batch conditions at various μ ($0.03 \pm 0.005 - 0.1 \pm 0.003 \text{ h}^{-1}$). Triplicate fed-batch cultivations were performed for all exponential feeding rates in the range of 0.07 and 0.1 h^{-1} , duplicate fermentations were performed for exponential feeding rates of 0.03 and 0.05 h^{-1} . In glucose batch fermentation the ‘fast-growing high-biomass producing’ transformant, DAHB21#20, and the ‘slow-growing high-glucoamylase producing’ transformant, DAHB21#40 obtained maximum specific growth rate (μ_{\max}) of 0.08 and 0.11 h^{-1} , respectively, and Y_{XS}^{\max} of 0.56 and $0.51 \text{ g}_{\text{DW}} \cdot \text{g}_{\text{Glucose}}^{-1}$, respectively (Manuscript 2 of this dissertation, Table 1). The exponential feed was determined on the amount of glucose required at specific time points for the increase in biomass at the desired μ . The exponential glucose feed was started upon depletion (between 19 and 21 h) of an initial glucose concentration of $10 \text{ g}_{\text{Glucose}} \cdot \text{kg}_{\text{broth}}^{-1}$ and was stopped after biomass levels ceased to increase and residual glucose accumulation was observed in the fermentor. Thereafter, fermentations

were monitored and sampled for an additional 10 h before the run was stopped. The residual glucose concentration was below the detection limit ($5 \text{ mmol.l}_{\text{broth}}^{-1}$) for all cultivations during the exponential feeding phase.

Table1: Performance of HBsAg-producing *A. niger* strains in glucose based batch fermentations (manuscript 2 of this dissertation)

<i>A. niger</i> strains	Maximum growth rate [h ⁻¹]	Maximum Biomass [g _{DW} ·kg _{Broth} ⁻¹]	Y _{XS} [g _{DW} ·g _{Glucose} ⁻¹]	Maximum Glucoamylase [U·g _{DW} ⁻¹]	Maximum HBsAg [μg _{HBsAg} ·g _{DW} ⁻¹]
‘fast-growing high-biomass producing’ transformant, DAHB21#20	0.11 ± 0.009	17.32 ± 0.73	0.56	0.14 ± 0.018	143 ± 12.5
‘slow-growing high-glucoamylase producing’ transformant, DAHB21#40	0.08 ± 0.007	14.35 ± 1.2	0.51	0.21 ± 0.021	219 ± 8.5

Biomass levels were highest in glucose-limited exponential feed cultivations of the ‘fast-growing high-biomass producing’ transformant at μ above 0.07 h^{-1} (Figure 1A). Highest levels of $18.60 \pm 1.1 \text{ g}_{\text{DW}}\cdot\text{kg}_{\text{Broth}}^{-1}$ were recorded at a μ of 0.08 h^{-1} . In comparison, biomass levels were lower in ‘slow-growing high-glucoamylase producing’ transformant fermentations, with highest levels recorded at 0.07 h^{-1} ($15.91 \pm 0.45 \text{ g}_{\text{DW}}\cdot\text{kg}_{\text{Broth}}^{-1}$). A notable decrease in biomass levels was observed between μ of 0.03 and 0.05 h^{-1} in both transformants (Figure 1A).

Similar extracellular glucoamylase activities were recorded in ‘fast-growing high-biomass producing’ and ‘slow-growing high-glucoamylase producing’ transformant cultivations between the regulated μ of 0.07 and 0.08 h^{-1} (Figure 1B). Highest extracellular glucoamylase

activities in the exponential fed-batch cultivations of the ‘fast-growing high-biomass producing’ transformant were observed at a μ of 0.08 h^{-1} ($0.23 \pm 0.02 \text{ U.g}_{\text{DW}}^{-1}$). In ‘slow-growing high-glucoamylase producing’ transformant fed-batch fermentations highest glucoamylase activities ($0.23 \pm 0.03 \text{ U.g}_{\text{DW}}^{-1}$) were at a μ of 0.07 h^{-1} . A notable decrease in extracellular glucoamylase activity was observed in both transformants at a μ below 0.05 h^{-1} ($\sim 0.1 \text{ U.g}_{\text{DW}}^{-1}$ at 0.03 h^{-1}), and in ‘fast-growing high-biomass producing’ transformant cultivations when the μ approached μ_{max} (0.1 h^{-1} ; $0.18 \pm 0.02 \text{ U.g}_{\text{DW}}^{-1}$; Figure 1B).

Intracellular (specific and volumetric) HBsAg production followed similar trends to extracellular glucoamylase activities for both transformants. Highest specific and volumetric HBsAg production levels were observed in the ‘fast-growing high-biomass producing’ transformant exponential fed-batch cultivations at a μ of 0.08 h^{-1} ($241 \pm 19 \mu\text{g}_{\text{HBsAg.g}_{\text{DW}}^{-1}}$ and $4483 \pm 308 \mu\text{g}_{\text{HBsAg.kg}_{\text{Broth}}^{-1}}$, respectively) (Figure 1C and 1D). Highest specific and volumetric HBsAg production levels in ‘slow-growing high-glucoamylase producing’ transformant cultivations were obtained at a μ of 0.07 h^{-1} ($251 \pm 35 \mu\text{g}_{\text{HBsAg.g}_{\text{DW}}^{-1}}$; $3989 \pm 274 \mu\text{g}_{\text{HBsAg.kg}_{\text{Broth}}^{-1}}$, respectively) (Figure 1C and 1D). In comparison, glucose-limited fed-batch fermentations of the ‘slow-growing high-glucoamylase producing’ at a μ of 0.07 h^{-1} obtained highest specific HBsAg production levels (Figure 1C), while cultivations of the ‘fast-growing high-biomass producing’ transformant at 0.08 h^{-1} obtained highest volumetric HBsAg levels (Figure 1D).

Figure 1E depicts the ratio of intracellular HBsAg production and extracellular glucoamylase activity using the maximum values measured in the various glucose-limited exponential fed-batch fermentations. This data, reflecting the ratio between maximum intracellular HBsAg production and maximum extracellular glucoamylase activity, indicates that between the μ of

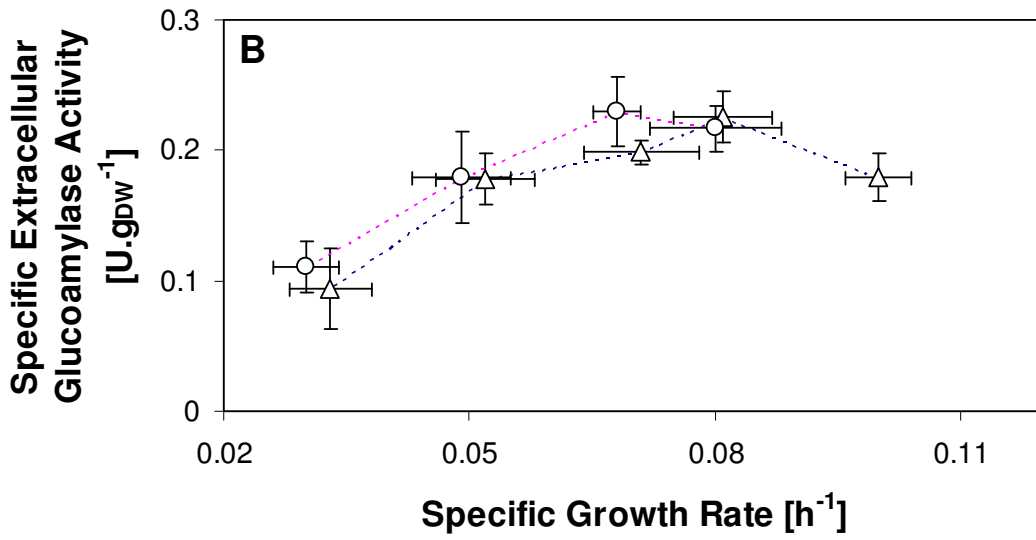
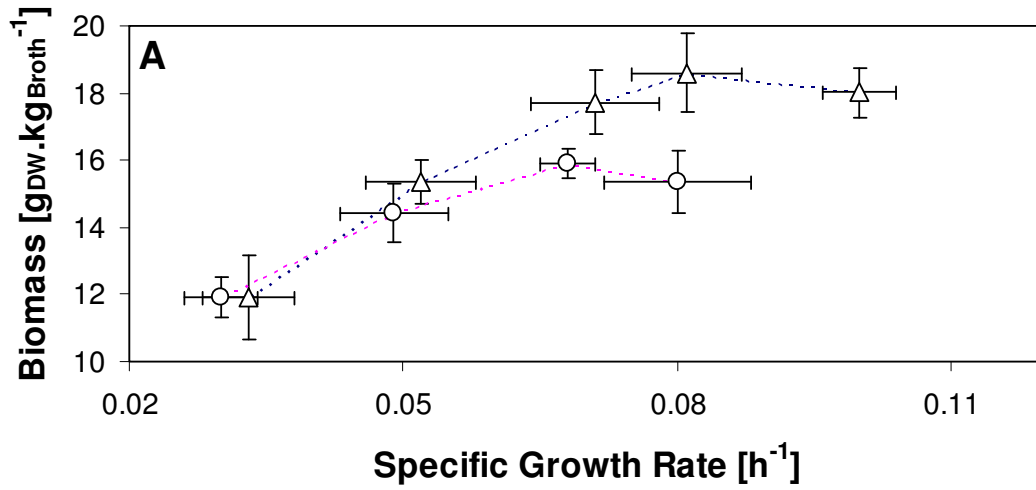
0.07 and 0.1 h⁻¹ the glucose-limited exponential fed-batch fermentation of the ‘fast-growing high-biomass producing’ transformant achieved a ratio of $\sim 1050 \pm 92 \mu\text{g}_{\text{HBsAg}} \cdot \text{U}^{-1}$. A decrease in μ resulted in a significant decrease in this ratio to $610 \pm 101 \mu\text{g}_{\text{HBsAg}} \cdot \text{U}^{-1}$ at a μ of 0.03 h⁻¹. Similarly in ‘slow-growing high-glucoamylase producing’ transformant cultivations, between the μ of 0.07 and 0.08 h⁻¹, a ratio between maximum intracellular HBsAg production and maximum extracellular glucoamylase activity was equal to that obtained in ‘fast-growing high-biomass producing’ transformant cultivations ($1045 \pm 79 \mu\text{g}_{\text{HBsAg}} \cdot \text{U}^{-1}$). A decrease in μ resulted in a significant decrease to a low level of $587 \pm 82 \mu\text{g}_{\text{HBsAg}} \cdot \text{U}^{-1}$ at a μ of 0.03 h⁻¹.

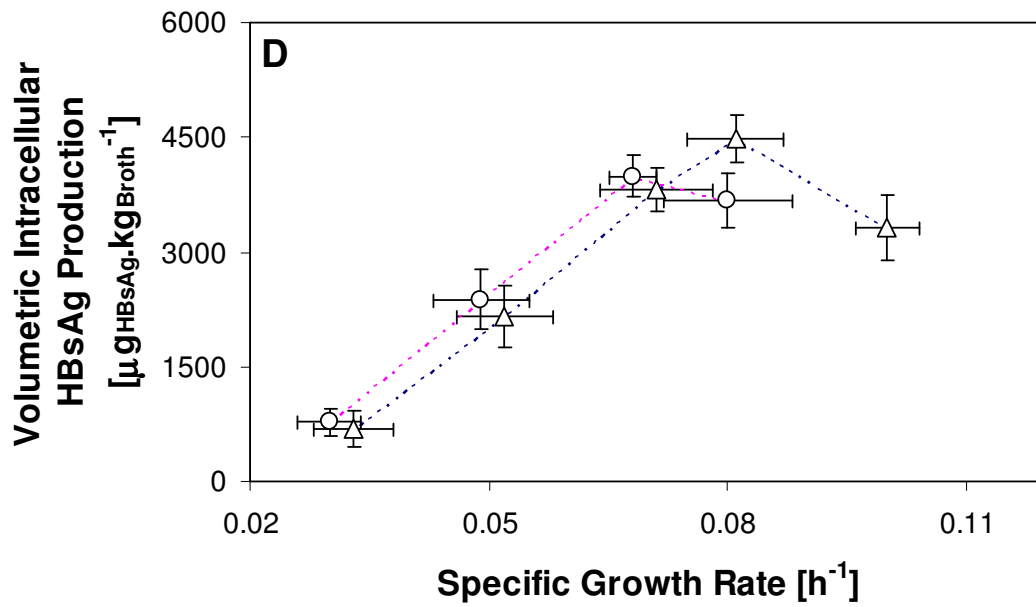
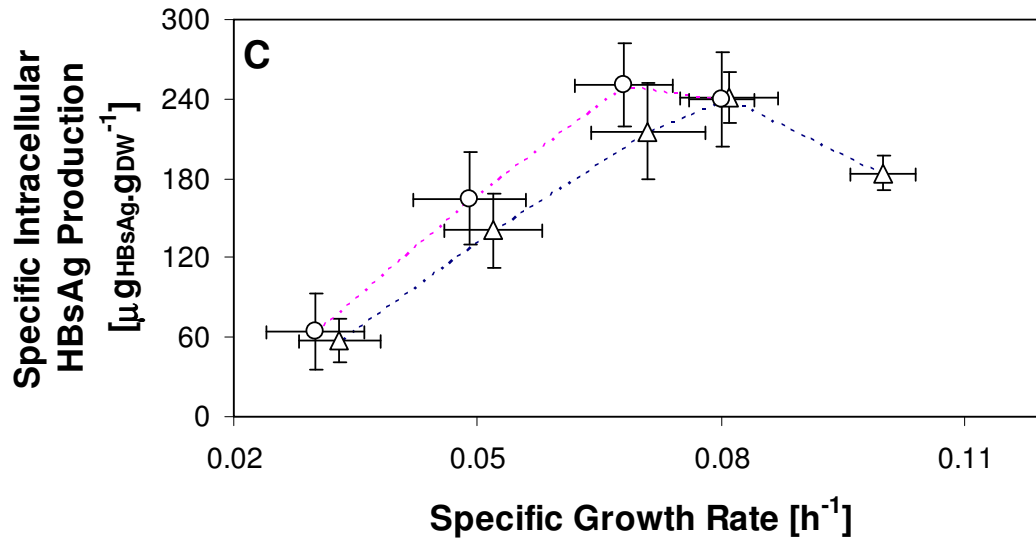
From Figure 1F, the following substrate utilization equations were obtained for ‘fast-growing high-biomass producing’ (Equation 5) and ‘slow-growing high-glucoamylase producing’ (Equation 6) transformant fermentations, respectively:

$$-q_s = 1.754\mu + 0.0195 \quad \text{Equation 5.}$$

$$-q_s = 1.929\mu + 0.0245 \quad \text{Equation 6.}$$

A maximum specific rate of substrate consumption (q_s^{max}) and m_s of $\sim 0.2 \text{ g}_{\text{glucose}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$ and $0.0195 \text{ g}_{\text{glucose}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$, respectively was obtained for the ‘fast-growing high-biomass producing’ transformant at 0.1 h⁻¹ (Figure 1F, Equation 5). A lower q_s^{max} and m_s of $\sim 0.18 \text{ g}_{\text{glucose}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$ and $0.0245 \text{ g}_{\text{Glucose}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$, respectively was obtained for the ‘slow-growing high-glucoamylase producing’ transformant cultivations at 0.08 h⁻¹ (Figure 1F, Equation 6). The calculated $Y_{\text{XS}}^{\text{max}}$ at μ of 0.05 h⁻¹ and above correlated with the values initially obtained in batch fermentations (Table 1) for both transformants. At lower μ of 0.03 h⁻¹, $Y_{\text{XS}}^{\text{max}}$ values were observed to decrease to $\sim 0.4 \text{ g}_{\text{DW}} \cdot \text{g}_{\text{Glucose}}^{-1}$ for both transformants (data not shown).





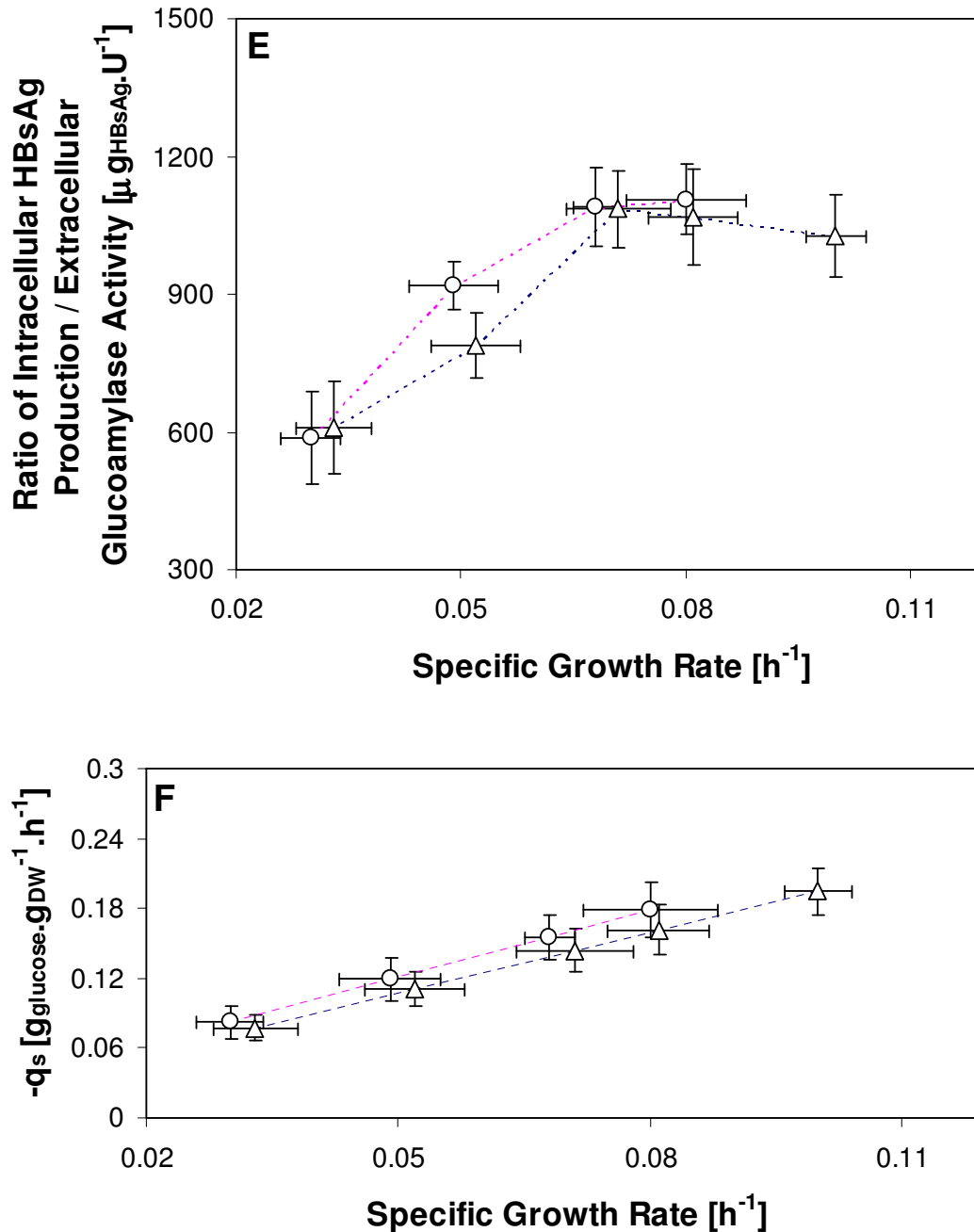


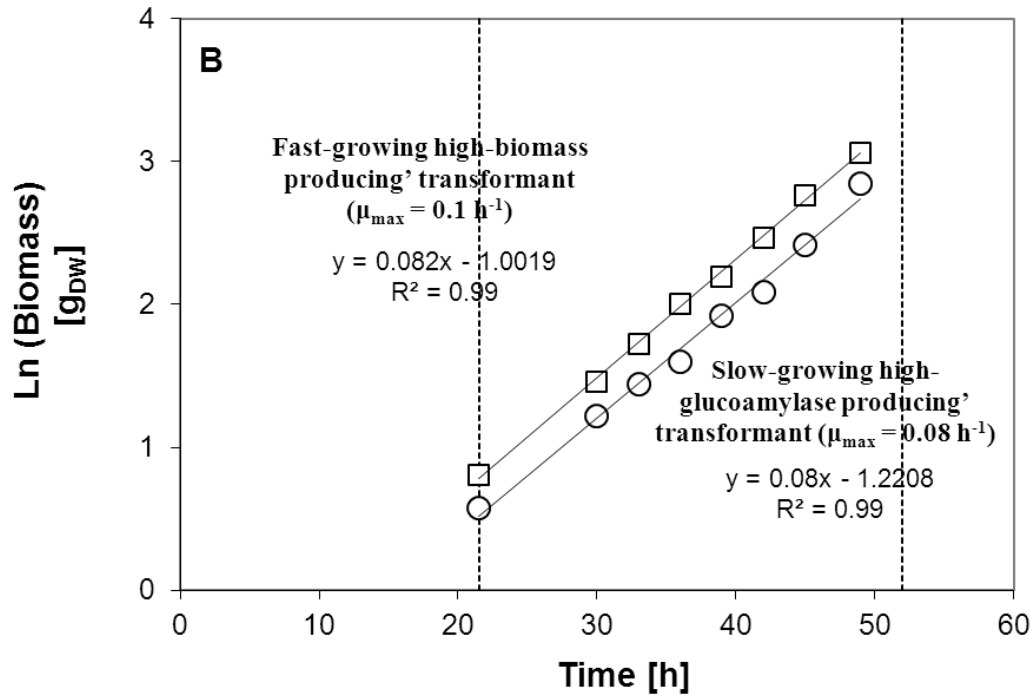
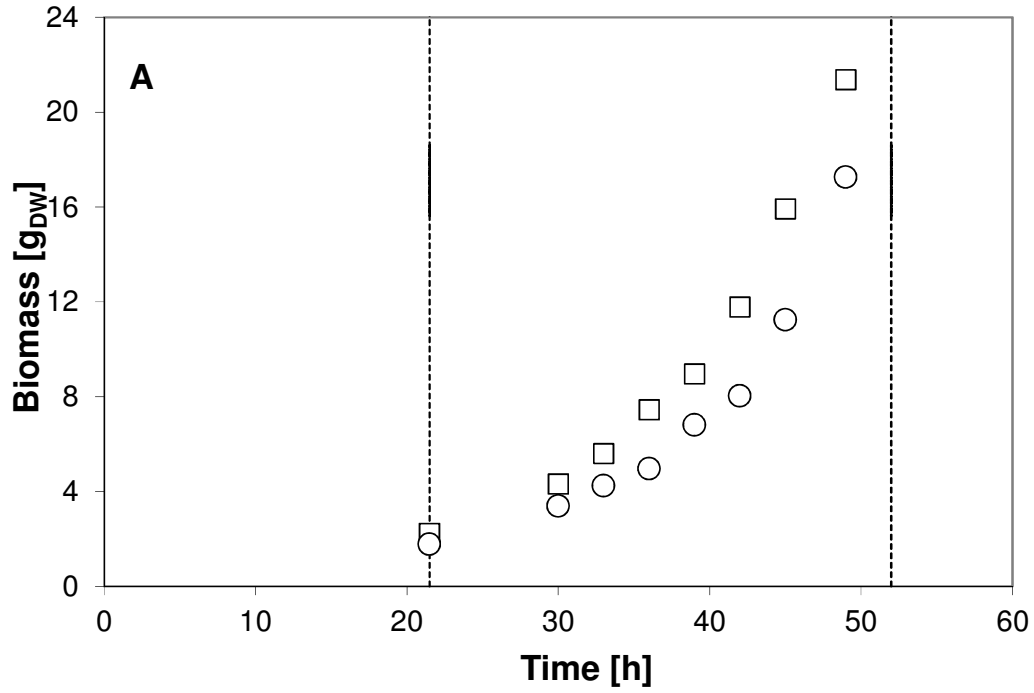
Figure 1. Glucose-limited exponential fed-batch fermentations of *A. niger* at different μ over a range of 0.03 to 0.1 h^{-1} : Shown is data regarding two HBsAg-producing *A. niger* transformants (carrying the $Gla_{G2}::S$ fusion cassette and controlled by the inducible Gla_p promoter); Δ , ‘fast-growing high-biomass producing’ transformant DAHB 21#20; and \circ , ‘slow-growing high-glucoamylase producing’ transformant DAHB 21#40. Depicted are the effects of μ data on: (A) Dry biomass concentration, (B) specific extracellular glucoamylase activity, (C) specific intracellular HBsAg production, (D) volumetric intracellular HBsAg production, (E) the ratio of intracellular HBsAg production and extracellular glucoamylase activity using the maximum values

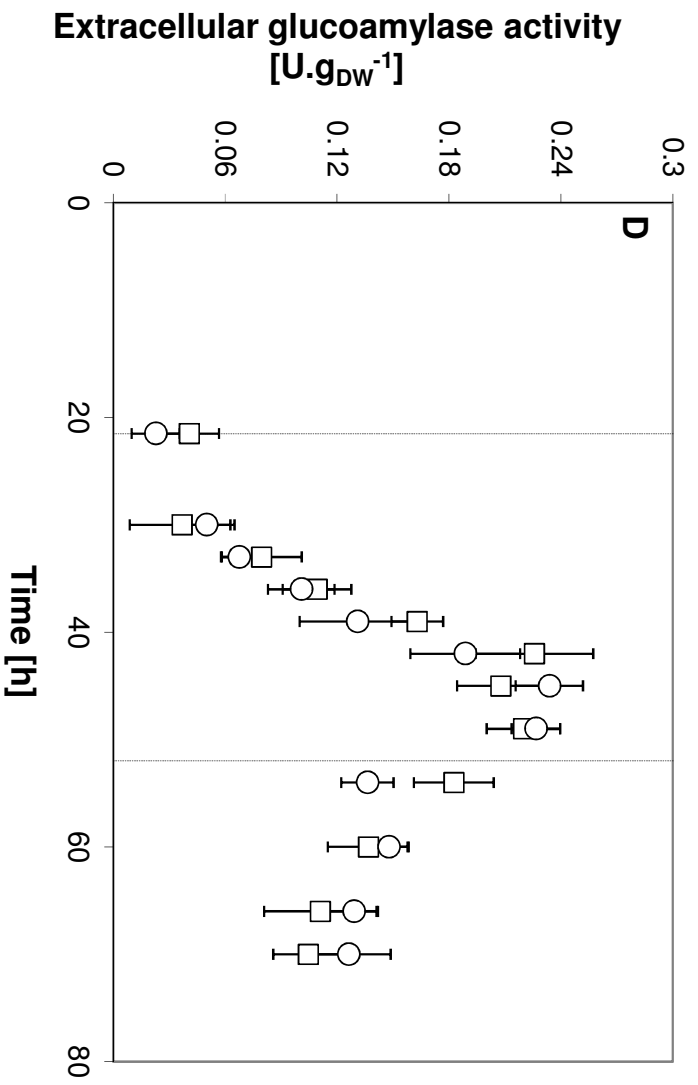
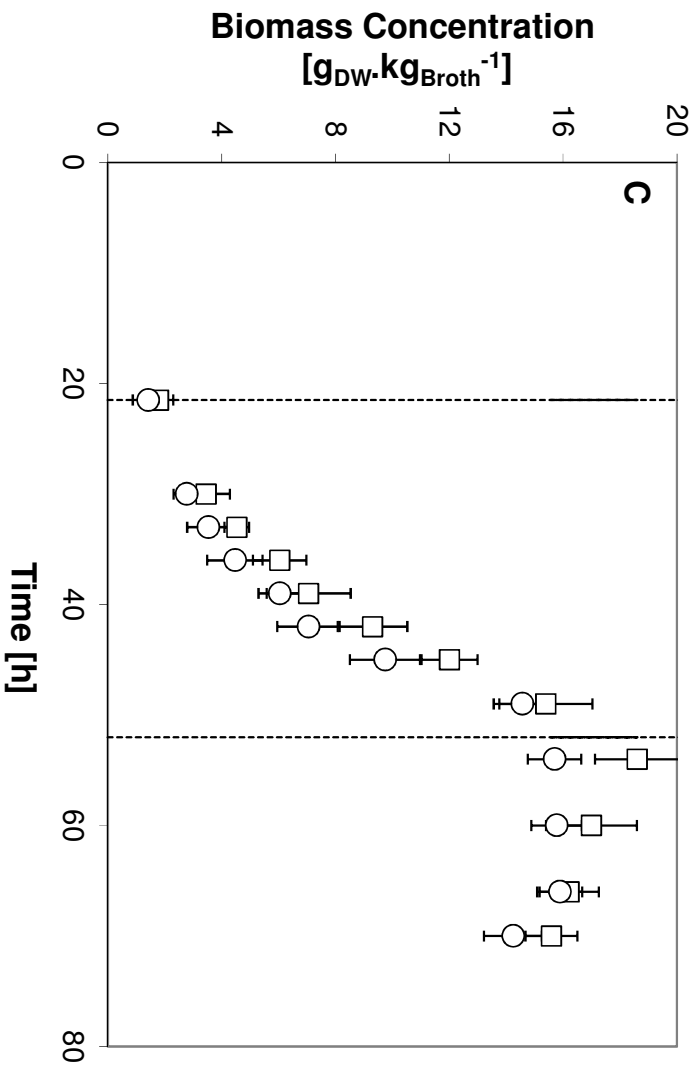
measured in the various glucose-limited exponential fed-batch fermentations, and (F) specific rate of substrate consumption (q_s). Error bars represent the standard deviation of the mean. X-axis error bars represent the standard deviation of the μ evaluated as calculated from biomass production in the exponential growth phase. Triplicate exponential fed-batch fermentations were performed for both transformants over a range of 0.07 to 0.1 h^{-1} . Data representing cultivations at 0.03 to 0.05 h^{-1} represent duplicate fermentations.

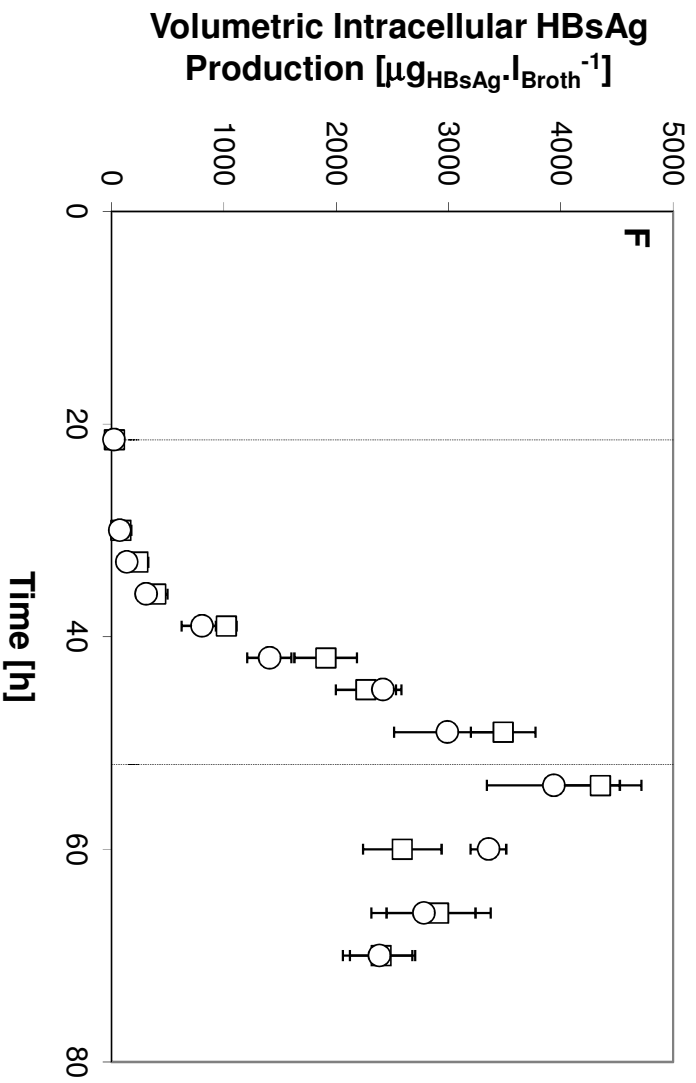
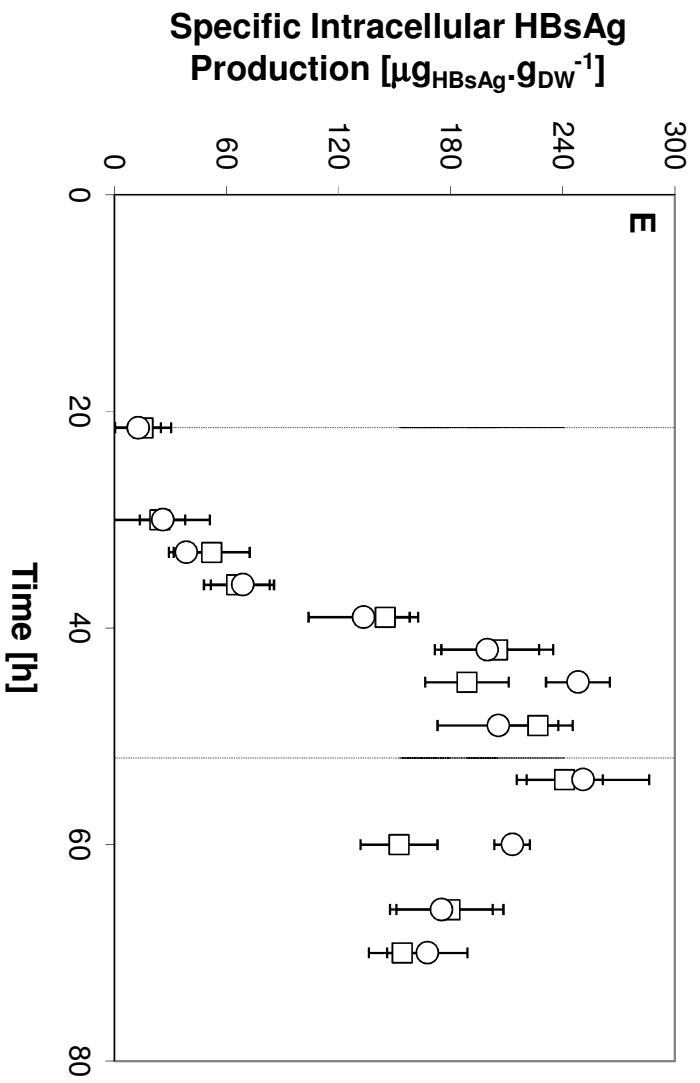
Carbon-limited exponential fed-batch production profiles controlled at $\mu = 0.08 \text{ h}^{-1}$

In Figure 2 the production profiles resulting from glucose-limited exponential fed-batch fermentations regulated at $\mu = 0.08 \text{ h}^{-1}$ of the ‘fast-growing high-biomass producing’ transformant DAHB 21#20; and the ‘slow-growing high-glucoamylase producing’ transformant DAHB 21#40 are shown. Production profiles of biomass (Figure 2A), \ln (biomass) (Figure 2B), biomass concentration (Figure 2C), specific extracellular glucoamylase activity (Figure 2D), specific intracellular HBsAg production (Figure 2E), and volumetric intracellular HBsAg production (Figure 2F) are shown.

Figure 2G depicts the correlations between specific intracellular HBsAg production and specific extracellular glucoamylase activity of the two transformants. These were shown to be similar to the findings in Manuscript 2 of this dissertation. The significance of the two correlations were confirmed through linear regression analysis, with $p_{\text{values}} < 0.05$ of the x variables and intercepts at a 95 % confidence level and R^2 values ≥ 0.96 explained more than 96 % of the total variation (Figure 2E). No significant difference was observed between the two correlations ($p > 0.05$).







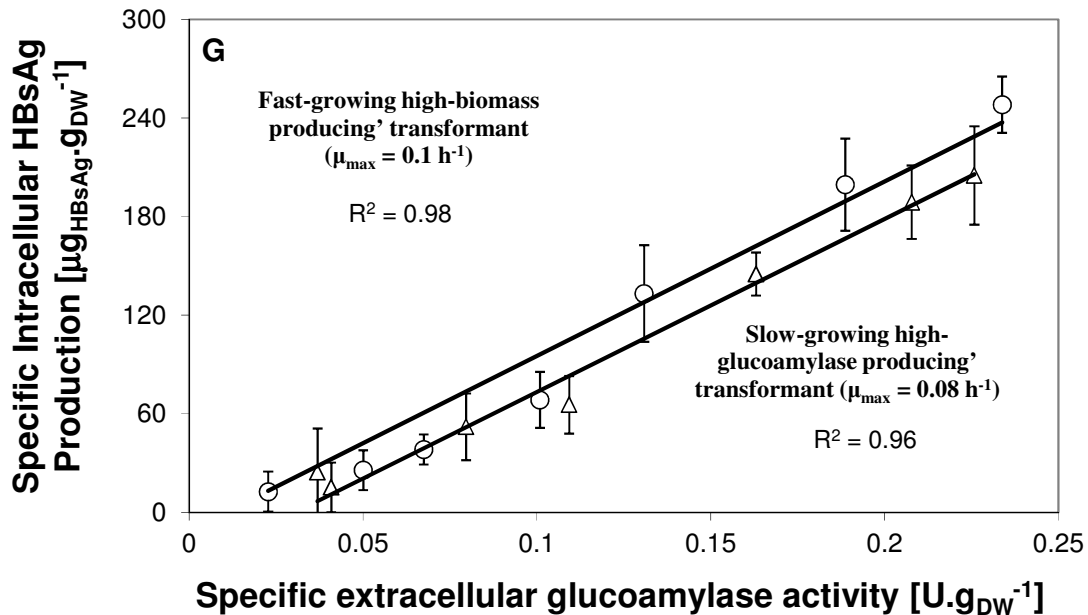


Figure 2. Glucose-limited exponential fed-batch fermentations regulated at $\mu = 0.08 \text{ h}^{-1}$ of two HBsAg-producing *A. niger* transformants (carrying the *GlaA_{G2}::S* fusion cassette and controlled by the inducible *GlaA_p* promoter); Δ , ‘fast-growing high-biomass producing’ transformant DAHB 21#20; and \circ , ‘slow-growing high-glucoamylase producing’ transformant DAHB 21#40. Depicted are the production profiles of: (A) biomass, (B) \ln (biomass), (C) biomass concentration, (D) specific extracellular glucoamylase activity, (E) specific intracellular HBsAg production, (F) volumetric intracellular HBsAg production. Figure 2 (G) depicts the correlation between specific intracellular HBsAg production and specific extracellular glucoamylase activity. Error bars represent the standard deviation of the mean. Triplicate exponential fed-batch fermentations were performed for both transformants.

DISCUSSION

In this study we assess glucose-limited exponential fed-batch fermentation as a strategy for the cultivation of HBsAg producing transformants in *A. niger*. The effects of μ on HBsAg production of two HBsAg producing transformants, previous identified (Manuscript 2 of this dissertation), were evaluated; (1) the ‘fast-growing high-biomass producing’ transformant, DAHB21#20, and (2) the ‘slow-growing high-glucoamylase producing’ transformant, DAHB21#40. Both transformants made use of the gene fusion approach, using the expression

vector containing the *GlaA_{G2}* carrier gene fused to the HBsAg *S* gene, which encouraged high expression of the fused cassette as well as passage of the HBsAg under the mask of a homologous gene product through the fungal secretory pathway (Manuscript 1 and 2 of this dissertation). Intracellular HBsAg production was detected in both transformants. In glucose batch fermentation highest intracellular HBsAg production was observed in a HBsAg-producing *A. niger* transformant, with an inherently reduced μ ('slow-growing high-glucoamylase producing' transformant, DAHB21#40) (manuscript 2 of this dissertation). Efficient processing of the heterologous protein of interest may be compromised by the saturation or overloading of the processing machinery caused by unfolded and/or misfolded proteins, which are prevented from leaving the ER (Lodish *et al.* 1983; Shuster 1991; Parekh *et al.* 1995; Shusta *et al.* 1998; Smith *et al.* 2004; Damasceno *et al.*, 2007). This has led to the hypothesis that a reduced μ may enhance the efficiency of *A. niger* to synthesize and produce HBsAg, resulting in improved production levels.

Detailed analyses of glucose-limited exponential fed-batch fermentations of the 'fast-growing high-biomass producing' and 'slow-growing high-glucoamylase producing' transformants were performed to elucidate the effects of μ on HBsAg production in *A. niger*. The μ studied (0.03–0.1 h⁻¹) covered the range from a low μ to a rate approaching μ_{\max} of each transformant. Glucose-limited exponential fed-batch fermentations were successfully implemented as a means of controlling the μ at a constant level. Continuous fermentations are conventionally implemented to acquire such control, however due to the long residence times, associated wall growth and difficulties in obtaining homogeneity in filamentous fungi cultivations, exponential fed-batch fermentations were preferred. The exponential feed allowed for accurate μ control and a homogenous cultivation allowed for accurate dry weight determinations for μ verification.

Results of this study indicate that glucose-limited exponential fed-batch fermentation had a significant effect on HBsAg production in *A. niger* using the *GlaA_{G2}::S* gene fusion approach and *GlaA_p* promoter. Higher intracellular HBsAg production levels were obtained in glucose-limited fed-batch fermentations cultivated at $\mu \approx \mu_{\max}$ in comparison to glucose batch fermentations of both transformants. At a $\mu \approx \mu_{\max}$, ‘slow-growing high-glucoamylase producing’ transformant fed-batch fermentations at 0.08 h^{-1} and ‘fast-growing high-biomass producing’ transformant fed-batch fermentations at 0.1 h^{-1} obtained increased volumetric HBsAg production levels of $\sim 17 \%$ and 34% , respectively, in comparison to that obtained in batch fermentations (Table 1 and Manuscript 2 of this dissertation). It has been previously reported that under control of the inducible *GlaA_p* promoter, glucose acts as a strong inducer at low concentrations but exhibits reduced induction efficiency or repression at higher concentrations, by carbon catabolite repression (Ruijter and Visser, 1997; Ganzlin and Rinas, 2008). Thus glucose-limited exponential fed-batch fermentation for the current *A. niger* HBsAg production system may provide a method of ensuring high *GlaA_p* induction efficiency while avoiding catabolic repression, resulting in increased HBsAg production levels.

Specific growth rate had a significant effect on the ‘fast-growing high-biomass producing’ transformant, using the *GlaA_{G2}::S* gene fusion approach, with regards to HBsAg production in *A. niger*. Maximum specific and volumetric HBsAg production levels were obtained by reducing the μ by 70% (0.08 h^{-1}) of the μ_{\max} in glucose-limited exponential fed-batch fermentation. Under these conditions, a 35% increase in volumetric HBsAg production was measured in comparison to cultivations controlled at $\mu = 0.1 \text{ h}^{-1}$ ($\mu \approx \mu_{\max}$). Specific production of proteins has been reported to increase consistently with increasing μ , as shown for Fab fragment production in *Pichia pastoris* (Zhang *et al.*, 2005) and β -galactosidase

production in *Escherichia coli* (Sanden *et al.*, 2003). This protein production characteristic is typically observed in growth-associated product formation. For other proteins, such as HBsAg in *P. pastoris* (Cregg *et al.*, 1987), cellobiohydrolase (Pakula *et al.*, 2005), and laccase (Rautio *et al.* 2007) in *Trichoderma reesei*, specific production is highest at lower μ . It has been suggested that during methanol-induced expression of HBsAg from the *AOX1* promoter in *P. pastoris*, a slower μ is essential for efficient HBsAg assembly (Cregg *et al.*, 1987). This is not evident in HBsAg production in *A. niger* using the *GlaA_{G2}::S* gene fusion approach, as a significant reduction in HBsAg production levels was observed for both transformants at lower μ ($\mu \leq 0.05 \text{ h}^{-1}$).

Growth rate regulation in glucose-limited fed-batch fermentations of the ‘slow-growing high-glucoamylase producing’ transformant did not result in similar % increases as observed in ‘fast-growing high-biomass producing’ transformant cultivations. An insignificant increase of 8 % ($p > 0.05$) in volumetric HBsAg production was observed in ‘slow-growing high-glucoamylase producing’ transformant fed-batch fermentations cultured at $\mu = 0.07 \text{ h}^{-1}$ in comparison to cultivations controlled at $\mu = 0.08 \text{ h}^{-1}$ ($\mu \approx \mu_{\text{max}}$). These findings indicate that HBsAg production was not efficient at the intrinsic growth rate ($\mu = 0.1 \text{ h}^{-1}$) of the ‘fast-growing high-biomass producing’ transformant, while HBsAg processing in ‘slow-growing high-glucoamylase producing’ transformant at $\mu = 0.08 \text{ h}^{-1}$ was closer to optimal.

HBsAg production in relation to extracellular glucoamylase activity was most efficient for both transformants at $0.07 < \mu < 0.08 \text{ h}^{-1}$, as reflected by the ratio between maximum intracellular HBsAg and maximum extracellular glucoamylase. This was a significant increase of ~ 20 % for both transformants to that obtained in glucose batch fermentation (Manuscript 2 of this dissertation). In glucose-limited exponential fed-batch fermentation, at lower μ ($\mu <$

0.07 h⁻¹), the ratio between maximum intracellular HBsAg and maximum extracellular glucoamylase was reduced. It is noteworthy that for HBsAg production in *A. niger*, using the *GlaA_{G2}::S* gene fusion approach under transcriptional control of *GlaA_p*, a directly proportional correlation existed between intracellular HBsAg production and extracellular glucoamylase in batch fermentation (Manuscript 2 of this dissertation). Similar correlations between intracellular HBsAg production and glucoamylase activity were observed for both transformants in glucose-limited exponential fed-batch fermentations as observed at $\mu = 0.08$ h⁻¹. With the decrease in μ and the associated decrease in extracellular glucoamylase, it appears that the processing and/ or processing efficiency of the fused glucoamylase-HBsAg product was reduced. However, HBsAg production was reduced at a higher rate than extracellular glucoamylase with the decrease in μ ($\mu < 0.07$ h⁻¹), as reflected by the ratio between maximum intracellular HBsAg and maximum extracellular glucoamylase. This could also suggest that at lower μ the maturation and assembly of HBsAg was affected after the fused protein was cleaved, resulting in a higher proportion of immature/ unassembled HBsAg products in relation to correctly folded glucoamylase. It is therefore concluded that through glucose-limited exponential fed-batch fermentation at an optimum μ using the *GlaA_{G2}::S* gene fusion approach, HBsAg production efficiency of both transformants was highest.

The reduction in biomass and protein production levels at low specific μ was attributed to the maintenance requirements of the cells. As μ was reduced, so was the specific glucose consumption rate. At a $\mu = 0.03$ h⁻¹, 0.077 g_{Glucose}·g_{DW}⁻¹·h⁻¹ and 0.083 g_{Glucose}·g_{DW}⁻¹·h⁻¹ was consumed for ‘fast-growing high-biomass producing’ and ‘slow-growing high-glucoamylase producing’ transformant fed-batch fermentations, respectively, of which the estimated m_s formed ~ 25 and 30 % respectively of the q_s . The high non-growth associated substrate consumption for maintenance under these conditions would potentially limit production of

both biomass and proteins. These limitations are reflected in the low biomass levels, extracellular glucoamylase activities and intracellular HBsAg production levels for both transformants at $\mu = 0.03 \text{ h}^{-1}$.

Intracellular HBsAg production levels were improved in glucose-limited fed-batch fermentation for both transformants. At optimum μ , specific HBsAg production were highest ($251 \pm 35 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$) in the ‘slow-growing high-glucoamylase producing’ transformant. Lower specific HBsAg production levels of $241 \pm 19 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ were obtained in ‘fast-growing high-biomass producing’ transformant fermentations at optimum μ . However, due to higher biomass levels, volumetric HBsAg production levels were highest ($4483 \pm 340 \mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$) in ‘fast-growing high-biomass producing’ transformant fermentations. This is approximately a 1.8 fold increase in comparison to HBsAg production levels obtained in glucose batch fermentations ($2500 \mu\text{g}_{\text{HBsAg}} \cdot \text{L}_{\text{Broth}}^{-1}$) (Manuscript 2 of this dissertation).

It is therefore concluded that due to the nature of the growth-associated HBsAg production through *GlaA_{G2}::S* gene fusion approach under transcriptional control of *GlaA_p*, production levels were highest when biomass production was optimal. Growth rate was observed to be a critical factor influencing HBsAg production and its efficiency in relation to extracellular glucoamylase activity, where highest levels were observed at a reduced and optimal μ ($0.07 < \mu < 0.08 \text{ h}^{-1}$). Thus glucose-limited exponential fed-batch fermentation at an optimum μ ($0.07 < \mu < 0.08 \text{ h}^{-1}$) for the current *A. niger* HBsAg production system provides a method for efficient HBsAg processing, resulting in increased HBsAg production levels.

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MANUSCRIPT 4

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IMPROVED HEPATITIS B SURFACE ANTIGEN PRODUCTION IN *ASPERGILLUS NIGER* THROUGH THE MODIFICATION OF CHAPERONE AND FOLDASE LEVELS

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Running Title: Improved Hepatitis B surface antigen production in *Aspergillus niger* through the modification of chaperone and foldase levels

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ABSTRACT

HBsAg production targeted through the secretory pathway in *A. niger* using glucoamylase-HBsAg gene fusion resulted in limitations/ bottlenecks at a (post)translational thereby negatively affecting HBsAg production. This was demonstrated through the increase in HBsAg production levels resulting from the over-production of chaperones/ foldases. The over-production of CLX and PDI resulted in the highest percentage increase (88 % and 107 %, respectively) and therefore reflected their critical involvement with regards to efficient HBsAg assembly using gene fusion. The combinations of BiP and associated combinations with CLX and PDI failed to improve HBsAg production levels. Reductions of up to 40 % were observed upon BiP over-production and therefore indicated unfavourable responses towards HBsAg assembly and production, which were triggered upon BiP over-expression using gene fusion. In glucose batch fermentations, transformants over-producing PDI obtained highest levels ($305 \pm 29.8 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$) in comparison to CLX transformants ($264 \pm 32.2 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$). In glucose-limited exponential fed-batch fermentations HBsAg production levels in transformants over-producing CLX and PDI were enhanced to similar levels in comparison to batch fermentations. Highest HBsAg production levels of $352 \pm 21.3 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ ($6237.2 \pm 437.2 \mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$) and $330.2 \pm 24.4 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ ($5760.4 \pm 531.9 \mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$) in CLX and PDI over-expressing transformants were observed. A significant increase in HBsAg production efficiency in relation to extracellular glucoamylase activity was observed in CLX cultivations at a reduced and optimized growth rate, reaching similar levels to PDI cultivations under similar conditions ($\sim 1330 \mu\text{g}_{\text{HBsAg}} \cdot \text{U}^{-1}$ and $1360 \mu\text{g}_{\text{HBsAg}} \cdot \text{U}^{-1}$, for the CLX and PDI carbon-limited exponential fed-batch fermentations, respectively), and indicated the associated positive affects on HBsAg production efficiency through CLX over-production and cultivation at a reduced and optimum growth rate. These findings, together with the potential application of the solid-state glucoamylase screen for the

identification of high HBsAg producers over-producing chaperones/ foldases, demonstrates its usefulness and application as a technology for other heterologous proteins and recombinant VLPs.

INTRODUCTION

Filamentous fungi have a large protein production capacity and have therefore been developed for the industrial production of both homologous and heterologous gene products (Archer and Peberdy, 1997). The fungal species, *Aspergillus niger*, has been investigated as an alternative option to the commonly employed yeasts for the production of the Hepatitis B (Hep B) vaccine. This system is based on recombinant fused-gene expression technology which facilitates the recombinant production and assembly of the Hepatitis B surface antigen (HBsAg). The native and well secreted catalytic domain of the glucoamylase gene was fused to the HBsAg *S* gene (Plüddemann, 2003), encouraging high expression of the fused cassette (Le Loir *et al.*, 2005) as well as potentially preventing intracellular proteolytic degradation by creating a passage through the secretory pathway for the production of the heterologous protein under the mask of a homologous gene product (Gouka *et al.*, 1997; Manuscript 1 & 2 of this dissertation). Expression of the *GlaA_{G2}::S* fusion gene in these transformants resulted in the secretion of glucoamylase into the extracellular medium of the production host, while HBsAg production was localized intracellularly and was observed to be associated with the membraneous fraction (Manuscript 1 of this dissertation). A substantial level of intracellular glucoamylase product degradation was also observed. A possible explanation for these findings can be attributed to the saturation or overloading of the processing machinery caused by unfolded and/or misfolded proteins, which are prevented from leaving the Endoplasmic reticulum (ER), as noted elsewhere in the literature (Lodish *et al.* 1983; Shuster 1991; Parekh *et al.* 1995; Shusta *et al.* 1998; Smith *et al.* 2004; Damasceno *et al.*, 2007).

The rate and efficiency of protein folding and assembly in the ER determines the overall rate of transport through the secretory pathway (Hammond and Helenius 1994). In *A. niger* using the *GlaA_{G2}::S* gene fusion approach, a reduced growth rate ($\sim 0.08 \text{ h}^{-1}$), in glucose-limited exponential fed-batch fermentations, resulted in improved HBsAg production levels (Manuscript 3 of this dissertation). Similar observations have been reported previously (Gu *et al.*, 1991; Kirk and Piper, 1994). It has been suggested that during methanol-induced expression of HBsAg from the *AOX1* promoter in *Pichia pastoris*, a significantly reduced growth rate is essential for efficient HBsAg assembly (Cregg *et al.*, 1987). This was not evident in HBsAg production in *A. niger* using the *GlaA_{G2}::S* gene fusion approach, as a significant reduction in HBsAg production levels was observed at lower growth rates ($\leq 0.05 \text{ h}^{-1}$). HBsAg production was therefore most efficient at a reduced and optimized growth rate (Manuscript 3 of this dissertation).

The over-production of certain helper proteins has become a plausible strategy in enhancing heterologous protein production. The genes for several ER chaperones and foldases have been isolated from filamentous fungi; namely immunoglobulin binding protein (BiP) (van Gemeren *et al.*, 1997); protein disulfide isomerase (PDI) (Jeenes *et al.*, 1997; Ngiam *et al.*, 2000); and a family of calnexins (CLX) (Conesa *et al.*, 2002). By direct physical interaction, these helpers promote the correct folding of newly synthesized polypeptides and ensure that misfolded chains or aggregates are removed from the ER (Ellgaard and Helenius, 2003; van Anken & Braakman, 2005). A number of studies have analyzed the effects of altering the levels of ER chaperone expression on heterologous protein secretion. Harmsen *et al.* (1996) reported a 20-fold increase in the amount of extracellular prochymosin when BiP was over-expressed in *Saccharomyces cerevisiae* and similarly the studies of Damasceno *et al.* (2007) demonstrated a 3-fold increase in a single-chain antibody fragment in *P. pastoris*. CLX over-expression

enhanced the levels of functional serotonin transporter produced using the baculovirus expression system (Tate *et al.*, 1999). Similarly, CLX over-expression increased manganese peroxidase levels in *A. niger* 5-fold (Conesa *et al.*, 2002). The over-expression of PDI resulted in the increase in yields of acid phosphatase (4-fold) (Robinson *et al.*, 1994), human platelet-derived growth factor B homodimer (10-fold) (Robinson *et al.*, 1994), human apolipoprotein(a) KV kringle fragment (Cha *et al.*, 2006) and beta-glucosidase in *S. cerevisiae* (Powers and Robinson, 2007), as well as an antibody Fab fragment against human immunodeficiency virus type 1 in *P. pastoris* (Gasser *et al.*, 2007). In addition, secreted levels of a single-chain antibody fragment increased 2-fold when either BiP or PDI were over-expressed, and 8-fold when both chaperones were over-expressed in yeast (Shusta *et al.* 1998).

In *A. niger* using the *GlaA_{G2}::S* gene fusion approach, the positive effect of a reduced and optimized growth rate on HBsAg production, together with the intracellular accumulation of HBsAg and intracellular glucoamylase degradation products, suggests that limitations/bottlenecks, at a (post)translational level in the secretory pathway, are negatively affecting production. In this paper we are testing the hypothesis that such limitations occur, and can be demonstrated by increases in HBsAg production through over-expression of chaperones/foldases. The effects of over-producing the chaperones BiP and CLX, the foldase PDI, and the combinations thereof on the production of recombinant HBsAg are evaluated in *A. niger* expressing the *GlaA_{G2}::S* gene. Three HBsAg-producing *A. niger* transformants, varying in maximum specific growth rate and specific HBsAg production, were used as hosts for expressing modified levels of *bipA*, *pdiA*, *clxA* genes and combinations thereof. Transformants expressing modified chaperone and foldase levels created from these three *A. niger* host strains were evaluated for intracellular HBsAg, extracellular glucoamylase activity,

and biomass production. To compare the strategies of growth regulated cultivations and modifying chaperone levels as a means of enhancing HBsAg production levels in *A. niger*, highest HBsAg producing transformants expressing modified chaperone and foldase levels were evaluated in batch and glucose-limited exponential fed batch fermentations. As an additional objective of this study the potential application of the solid-state glucoamylase screen using starch as a selection pressure (previously described in Manuscript 2 of this dissertation) for the identification of high HBsAg producers over-producing chaperones/foldases, was evaluated.

MATERIALS AND METHODS

Strains and plasmids

Three HBsAg-producing *A. niger* transformants previously identified (Manuscript 1 & 2 of this dissertation), using the gene fusion strategy, were evaluated; (1) the uridine auxotrophic (*pyrG*), protease-deficient (*pvtT*), nonacidifying (*phmA*) mutant *A. niger* D15, DAHB19#7 (Manuscript 1 of this dissertation), as well as (2) the ‘fast growing high biomass producing’ DAHB21#20 (Manuscript 2 of this dissertation), and (3) the ‘slow growing high glucoamylase producing’ DAHB21#40 both derived from the *A. niger* MGG029- $\Delta aamA$ strain (Manuscript 2 of this dissertation). To modify the levels of BiP, CLX, PDI, and combinations thereof, the following plasmids were used as transforming vectors: plasmid pAN7-1, containing the hygromycin selection marker (Punt *et al.*, 1987), *GlaA_p-bipA/hph*, carrying the *A. niger bipA* gene under the control of the *A. niger* glucoamylase promoter (*GlaA_p*) and the hygromycin selection marker, and *GlaA_p-clxA*, and *GlaA_p-pdiA*, under control of the *A. niger GlaA_p* promoter and carrying the *A. niger* CLX gene (*clxA*) and protein PDI gene (*pdiA*), respectively (Ngiam *et al.*, 2000; Conesa *et al.*, 2002).

Fungal transformations

Selected *A. niger* strains were transformed with *GlaA_p-bipA/hph*, *GlaA_p-clxA* (in combination with pAN7-1), and *GlaA_p-pdiA* (in combination with pAN7-1) (Punt *et al.*, 1992; Ngiam *et al.*, 2000; Conesa *et al.*, 2002), and followed the protoplasting method described by Punt and van den Hondel (1992), using Novozym 234 (Sigma-Aldrich). Resulting transformants were selected for resistance to hygromycin B (HmB) on minimal medium (MM) (Bennett and Lasure, 1991) containing 1.2 M sorbitol as osmotic stabiliser, 1.5 % agar (Oxoid) and 0.1 % (v.v⁻¹) HmB (Punt *et al.*, 1987).

Inoculum preparation, media and cultivations

Fungal spores were obtained from a densely conidiating culture grown on potato dextrose agar (PDA) at 30 °C for 120 h and harvested with physiological saline solution (NaCl, 0.9 % w.v⁻¹). For high-throughput screening of fungal transformants in submerged cultivations, pre-weighed 50 ml Falcon tubes (Falcon, Becton Dickinson, Lincoln Park, USA) were inoculated with spores amounting to 1×10⁵ spores.ml⁻¹, final volume. Cultivations were incubated at 30 °C on a rotary shaker (Innova 2300, New Brunswick Scientific, Edison, USA) at 300 rpm. Each Falcon tube represented one sample time, thus 15 replicates insured triplicate analysis in increments of 24 h from 48 to 144 hours. Shake flask cultures were inoculated with a spore concentration of 1×10⁶ spores.ml⁻¹. Batch fermentations were inoculated with a shake flask pre-culture amounting to 10 % of the fermentor's total working volume. All cultivations were grown on minimal medium (MM) (Plüddemann and van Zyl, 2003) containing 3 % (w.w⁻¹) glucose and supplemented with 0.1 % (w.w⁻¹) casamino acids (Difco), 0.8 % (w.w⁻¹) tryptone and 0.8 % (w.w⁻¹) yeast extract. Shake flask cultivations were performed in 1 L Erlenmeyer flasks (un-baffled) at 30 °C on a rotary shaker (Innova 2300, New Brunswick Scientific, Edison, USA) at 180 rpm. Fermentations were performed in 2 L

bench-top bioreactors (INFORS AG, Switzerland) with a working volume of 1.5 L. The fermentation temperature was maintained at 30 °C ($\pm 0.1^\circ\text{C}$), aeration at 0.8 vvm, dissolved oxygen was maintained above 25 % saturation by varying impeller speed (starting rate of 150 rpm), through a cascaded controller. The impeller speed was ramped by 200 rpm for 1 min every hour to reduce wall growth. pH was controlled above a value of 4 using NH_4OH (25 % w.v⁻¹).

Exponential feed

A growth rate of 0.08 h^{-1} was maintained by manipulating the exponential feed rate in glucose-limited exponential fed-batch fermentations. During the carbon limited exponential feeding phase, the mass of residual glucose in the fermentor (M_s) was maintained close to zero and μ_{set} was controlled by directly controlling the mass of concentrated glucose substrate added in the fed-batch cultivation. This approach of controlling the mass of concentrated glucose substrate added in the fed-batch cultivation (equation 3) while $M_s \approx 0$ was used to regulate the production of biomass in the fermentor (equation 1) through the control of glucose substrate consumption by the filamentous fungi. This results in the control of the μ_{set} at a constant value under both transient and steady state conditions for biomass concentration (Martinez *et al.*, 1998; Kim *et al.*, 2000; Cheng *et al.*, 2003; Sun *et al.*, 2007; Chen *et al.*, 2008).

Biomass calculations

The biomass produced at time 1 ($M_{x(t1)}$) was used to calculate a predicted biomass profile for a particular μ_{set} using the principals of equation 1 (refer to derivations in Appendix A), to predict the biomass production at time 2 ($M_{x(t2)}$).

$$M_{x(t_2)} = M_{x(t_1)} e^{\mu_{\text{set}}(t_2-t_1)} \quad \text{Equation 1.}$$

Assuming the growth yield, Y_{XS} is constant throughout the exponential feeding phase, and applying the principals of equation 2 to obtain equation 3 (refer to derivations in Appendix A), the amount of glucose at a specific time point required for the production of biomass at μ_{set} , was calculated;

$$Y_{xs} = \frac{\text{mass of new biomass formed}}{\text{mass of glucose substrate consumed to produce new biomass}} \quad \text{Equation 2.}$$

$$M_{sf(t_2)} = \frac{(M_{x(t_2)} - M_{x(t_1)})}{Y_{XS}} + M_{sf(t_1)} \quad \text{Equation 3.}$$

where $M_{sf(t_2)} - M_{sf(t_1)}$ is the predicted mass of glucose required at time 2 to produce new biomass, $M_{x(t_2)} - M_{x(t_1)}$ at μ_{set} . The mass of glucose in the fermentor (M_s) was maintained at approximately zero for the duration of the feeding phase, where it was assumed that M_s was consumed by the organism as fast as it entered the fermentor to control μ_{set} . The amount of glucose fed was controlled and logged by an algorithm on the fermentor digital control unit (DCU) which exponentially increased the pump dosage every 10 seconds according to the glucose profile calculated (Equation 1 & 3). This data was cross referenced gravimetrically. Calculations regarding the exponential feeding methodology are presented in Appendix B.

Sample preparation

For high-throughput screening of fungal transformants, samples were centrifuged (10 000 rpm), supernatant fractionated and cell wet mass calculated. Wet mycelia was then suspended

in protein extraction buffer with 0.1% Triton X-100 (Plüddemann and van Zyl 2003) and homogenized for 1½ min at 4°C.

Fermentation cultivations were sampled at intervals of 3 to 8 h. Samples containing ± 50 g of cultivation medium were withdrawn and biomass concentration was measured by vacuum filtering 25 g sample on dry and pre-weighed filters (Whatman no.1), followed by washing with 40 ml 0.9% (w.v⁻¹) NaCl solution and drying to a constant weight at 105 °C for 24 to 48 h. The remaining 25 g sample was ground to a fine powder with liquid nitrogen in a mortar and pestle, and an aliquot of 0.3 g mycelia powder was stored at -80 °C in a 2 ml micro-centrifuge tube. Ground samples were suspended in protein extraction buffer with 0.1 % Triton X-100 (Plüddemann and van Zyl, 2003) and homogenized for 1 min at 4 °C. This was followed by clarification at 13 000 rpm and 4 °C. Extraction of supernatant resulted in samples containing total intracellular proteins including membrane-associated proteins.

HBsAg concentration determination

A “sandwich” type enzyme immunoassay based on monoclonal anti-HBsAg (HBsAg 3.0 EIA, Bio-Rad, Hercules, CA) was used to quantify completely assembled and properly folded HBsAg as per manufacturers specification.

Glucoamylase Activity

The activity of glucoamylase was measured by the method of Withers *et al.* (1998) using freshly prepared 0.1 % (v.v⁻¹) 4-nitrophenyl- α -D-glucopyranoside (PNPG) (Sigma-Aldrich N-1377) as substrate in 0.1 M Sodium Acetate buffer (pH 4.3). To commence the reaction, 100 μ l PNPG was added to 50 μ l sample (or glucoamylase standard) in a flat bottom micro-titre plate and incubated for 20 min at 25 °C. The reaction was terminated by the addition of 150 μ l

Borax (0.1 M Na₂B₄O₇·10 H₂O) solution, and absorbance at 400 nm was measured using a BioTek Power wave^{HT} micro-titre plate reader (BioTek Instruments Inc., USA) referenced with distilled water. Background absorbance was normalized with the addition of 150 µl Borax solution to 50 µl sample prior to the addition of 100 µl PNPG, in a duplicate assay. The measured background absorbance was subtracted from the absorbance readings obtained from the incubated samples and converted into glucoamylase activity using a standard curve (Amyloglucosidase Fluka 10113).

Glucose Concentration

Glucose concentration was measured using high performance ion chromatography (HPIC) (CarboPacTM PA1 column, Dionex, MA, USA) and ACCUTREND Glucose strips (Roche Diagnostics Ltd, East Sussex, UK).

RESULTS

Transformation of chaperone and foldase constructs into *A. niger* strains

To study the effect of modifying the levels of BiP, Calnexin, PDI and combinations thereof on the production of HBsAg by *A. niger*, expression cassettes containing *bipA*, *clxA* and *pdiA* genes were placed under control of the strong and regulated inducible glucoamylase promoter (*GlaA_p*). Three HBsAg-producing strains expressing the *GlaA_{G2}::S* gene fusion cassette were used as hosts for transformation: (1) *A. niger* D15, DAHB19#7 (Manuscript 1 of this dissertation), as well as (2) the ‘fast growing high biomass producing’ DAHB21#20 (Manuscript 2 of this dissertation), and (3) the ‘slow growing high glucoamylase producing’ DAHB21#40 (Manuscript 2 of this dissertation). These three HBsAg-producing transformants were super-transformed with various combinations of *GlaA_p-bipA/hph*, *GlaA_p-clxA*, *GlaA_p-pdiA*, and pAN7-1 (Punt *et al.*, 1992; Ngiam *et al.*, 2000; Conesa *et al.*, 2002).

Screening of HBsAg producing *A. niger* transformants expressing modified levels of chaperone and foldase gene constructs

Transformants, 526 in number, were obtained from agar plates containing minimal medium and HmB. After three passages on starch, acrylamide and HmB solid state cultivations, 104 transformants were selected, based on improved growth using starch, acrylamide and HmB as selection pressures (data not shown).

Effect of chaperone and foldase over-expression on HBsAg production

The 104 transformants selected in solid state cultures were screened further for increased HBsAg production in liquid cultivations, containing starch as carbon source. To establish the effects of chaperone and foldase over-expression on HBsAg production in *A. niger*, the maximum specific HBsAg production levels for all transformants over-expressing the various chaperones and foldases were determined. Intracellular accumulation of HBsAg was observed in all transformants. No extracellular HBsAg above a detection limit of 0.32 pg.ml⁻¹ culture broth was detected (data not shown).

Results indicated that 67 % of transformants over-expressing the BiP chaperone had a negative influence on HBsAg production (Figure 1) with a highest reduction of up to 40 % (Table 3). 33 % of transformants suggested an improvement in HBsAg production (Figure 1), with the highest HBsAg production levels amounting to a 17.4 % increase (Figure 2). However, with an average standard deviation of ± 20 % amongst all samples analysed with the 'sandwich' ELISA method, it can be concluded that there was no significant difference in HBsAg production between the above mentioned BiP over-producing transformant and the control/ parent transformant.

A high percentage of transformants over-producing PDI and CLX (78 % and 70 %, respectively) obtained similar or increased HBsAg production levels in comparison to the HBsAg producing parent transformants (Figure 1). Of these transformants over-producing PDI and CLX, 33 % and 35 %, respectively obtained HBsAg production increases ≥ 25 % (Figure 1). Highest HBsAg production increases in transformants over-producing PDI and CLX were 107 % and 88 %, respectively (Figure 2). No significant difference was observed between the maximum HBsAg production levels in CLX and PDI over-producing transformants (DAHB 21#20 PDI 7, 107 ± 23.9 % and DAHB 21#20 CLX 2, 88 ± 19.8 %), as indicated by the t-test for two independent samples ($t_{\text{observed}}=0.949$, $p_{\text{value}} = 0.443$).

PDI/ CLX over-producing transformants obtained a maximum HBsAg percentage increase of 43 % (Figure 2), where 22 % of transformants over-producing PDI/CLX affected HBsAg production with an increase greater than 25 % (Figure 1). The co-expression of BiP/ PDI, BiP/ CLX and BiP/ PDI/ CLX resulted in maximum HBsAg production level increases of 19.6 %, 29.2 %, and 18.5 %, respectively (Figure 2). In many cases over-producing the abovementioned chaperones in these combinations resulted in the decrease in HBsAg production of up to 30 % (Figure 3).

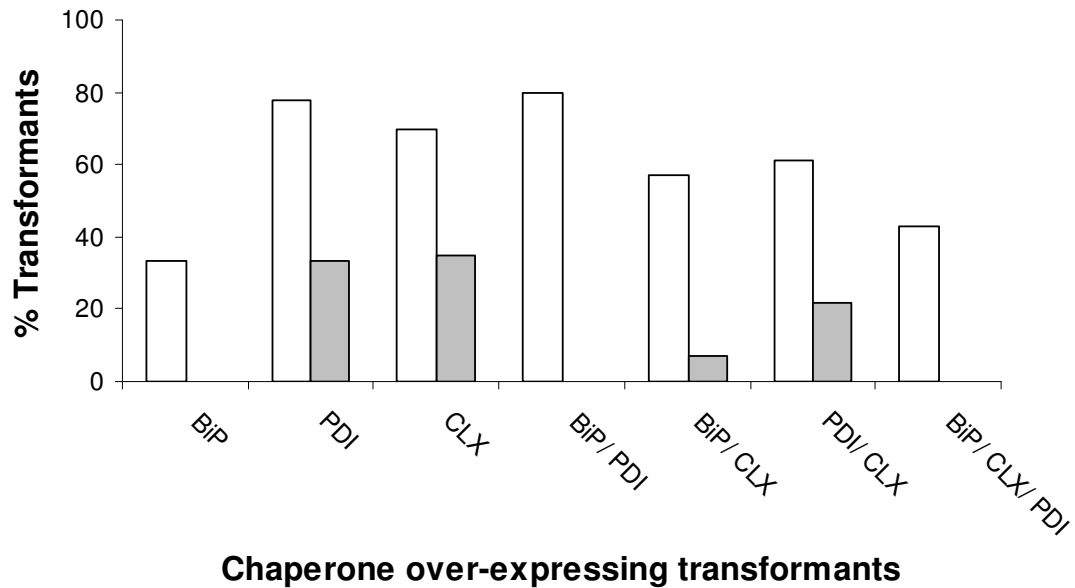


Figure 1: Percentage transformants, over-producing various chaperones and combinations thereof, showing; (in white bars) similar or increased HBsAg production levels in comparison to the parent HBsAg producing transformants, (in grey bars) an increase in HBsAg production levels $\geq 25\%$ in comparison to the parent HBsAg producing transformants. Reference points of HBsAg increase are calculated from the maximum HBSAg levels of parent transformants DAHB 19#7, DAHB 21#20 and DAHB 21#40. Cultures were grown in 50 ml Falcon tubes with a 25 ml working volume. Cultures were inoculated with $\pm 5 \times 10^5$ spore/ml, and incubated at 30 °C under shaking conditions of 300 rpm. HBsAg production was measured in increments of 24 hours from 48 to 144 hours. Data represents 6 replicated cultivations with standard deviations less than 20 %.

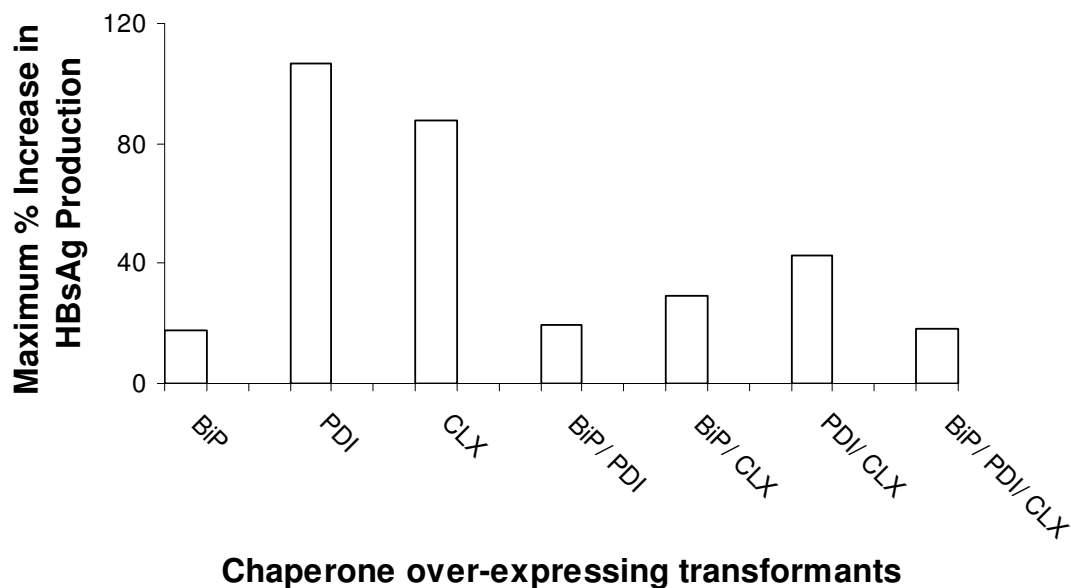


Figure 2: Maximum percentage HBsAg increases from transformants over-producing various chaperones and combinations thereof. Reference points of HBsAg increase are calculated from the maximum HBsAg production levels of parent transformants DAHB 19#7, DAHB 21#20 and DAHB 21#40. Data represents 6 replicated cultivations with standard deviations less than 20%.

To determine the effect of choice of *A. niger* expression host with regards to chaperone and foldase over-expression on HBsAg production, HBsAg production levels for each host strain were compared. Through the over-production of BiP, CLX, PDI and combinations thereof in *A. niger* D15 19#7, highest HBsAg production increases of 41 % over-producing CLX were measured (Figure 3A). In comparison, 16 % of the DAHB 21#20 and DAHB 21#40 strains over-producing BiP, CLX, PDI and combinations thereof, obtained HBsAg production levels equal or higher than 41 %. In the ‘slow growing high glucoamylase producing’ DAHB 21#40 strains over-producing BiP, CLX, PDI and combinations thereof, highest HBsAg production increases of 48 % over-producing PDI were measured (Figure 3B). In comparison, 18 % of the ‘fast growing high biomass producing’ DAHB 21#20 strains over-producing BiP, CLX, PDI and combinations thereof, obtained HBsAg production levels equal or higher than 48 %.

Highest HBsAg production increases of 107 % in DAHB 21#20 over-producing PDI were recorded (Figure 3B). Statistical analysis using the t-test for two independent samples comparing the maximum HBsAg percentage increases of each host strain revealed a insignificant difference between *A. niger* D15 19#7 and *A. niger* MGG029- $\Delta aamA$ DAHB 21#20 ($P_{\text{values}} > 0.05$), while a significant difference was observed between *A. niger* MGG029- $\Delta aamA$ DAHB 21#20 and MGG029- $\Delta aamA$ DAHB 21#40 ($P_{\text{values}} < 0.05$).

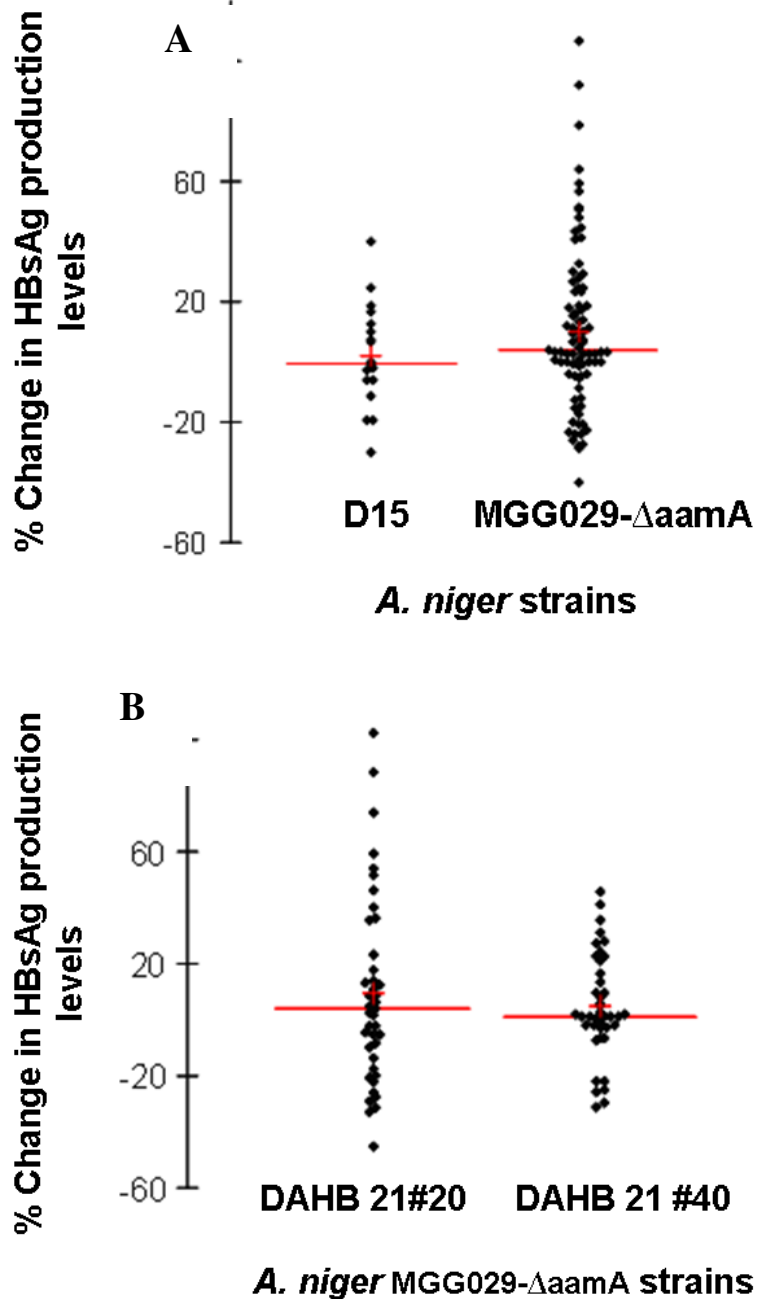


Figure 3: Comparison of percentage HBsAg increases between (A) chaperone over-producing transformants derived from *A. niger* D15 19#7 and *A. niger* MGG029- Δ aamA (DAHB 21#20 and DAHB 21#40), (B) chaperone over-producing transformants derived from *A. niger* MGG029- Δ aamA DAHB 21#20 and DAHB 21#40. Reference points of HBsAg increase are calculated from the maximum HBSAg levels of parent transformants DAHB 19#7, DAHB 21#20 and DAHB 21#40. Data represents 6 replicated cultivations with standard deviations less than 20 %.

Evaluation of intracellular HBsAg production in relation to extracellular glucoamylase

In Figure 4 data representing the percentage changes in intracellular HBsAg production levels and the percentage changes in extracellular glucoamylase activity between DAHB 21#20 and DAHB 21#40 over-producing BiP, CLX, PDI and combinations thereof, are evaluated. Of these transformants 24 % obtained a percentage increase in intracellular HBsAg production levels ≥ 25 %. 64 % of these transformants, representing a significant increase in intracellular HBsAg production were observed to have a percentage increase in extracellular glucoamylase activity ≥ 25 %. The significance of the correlation between the percentage changes in intracellular HBsAg production levels and the percentage changes in extracellular glucoamylase activity was confirmed through linear regression analysis ($p_{\text{value}} < 0.05$), where an R^2 value = 0.66 explained 66 % of the total variation (Figure 4).

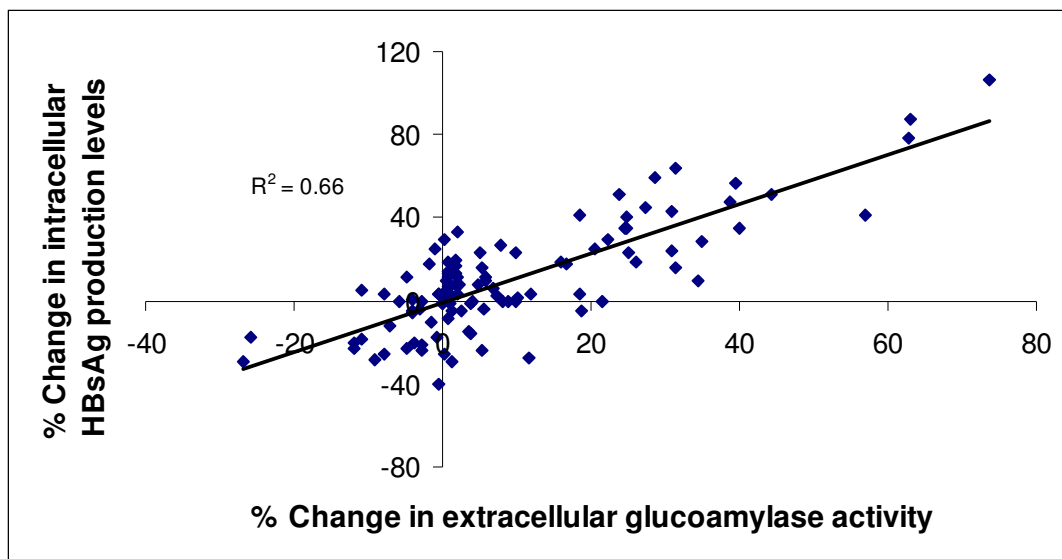


Figure 4: Percentage changes in intracellular HBsAg in relation to percentage changes in extracellular glucoamylase activity between DAHB 21#20 and DAHB 21#40 over-producing BiP, CLX, PDI and combinations thereof. Data represents 6 replicated cultivations with standard deviations less than 20 %.

Batch fermentation of two highest HBsAg producing transformants

From the liquid culture screen, two high HBsAg producing transformants were identified; the *A. niger* MGG029- Δ *aamA* DAHB 21#20 strain expressing CLX and PDI (DAHB 21#20 PDI 7 and DAHB 21#20 CLX 2). To evaluate and compare the performance of the two high HBsAg producing transformants, triplicate batch fermentations were conducted. Results are summarized in Table 1. A carbon substrate of 3 % glucose in a rich media was used for cultivation to ensure rapid offline analysis of carbon consumption. Similar biomass concentration profiles were observed between transformants, with the CLX obtaining the highest at $17.43 \pm 0.73 \text{ g}_{\text{DW}} \cdot \text{kg}_{\text{Broth}}^{-1}$ and a maximum growth rate of $0.098 \pm 0.009 \text{ h}^{-1}$, while PDI fermentations obtained maximum biomass levels of $16.6 \pm 1.3 \text{ g}_{\text{DW}} \cdot \text{kg}_{\text{Broth}}^{-1}$ and a maximum growth rate of $0.106 \pm 0.007 \text{ h}^{-1}$. Similar glucose consumption rates (data not shown) and biomass yield coefficients (Y_{XS}), were also observed ($Y_{\text{XS}} \text{ 'CLX'} = 0.55 \pm 0.025 \text{ g}_{\text{DW}} \cdot \text{g}_{\text{Glucose}}^{-1}$ and $Y_{\text{XS}} \text{ 'PDI'} = 0.56 \pm 0.018 \text{ g}_{\text{DW}} \cdot \text{g}_{\text{Glucose}}^{-1}$; Table 1). Glucoamylase for both cultures were observed to be secreted, and reached maximum levels of $0.21 \pm 0.024 \text{ U} \cdot \text{g}_{\text{DW}}^{-1}$ in CLX fermentations and $0.23 \pm 0.019 \text{ U} \cdot \text{g}_{\text{DW}}^{-1}$ in PDI fermentations. Highest HBsAg production levels were observed in PDI fermentations ($305 \pm 29.8 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$) while CLX fermentations levels obtained maximum levels of $264 \pm 32.2 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$. Similarly to the liquid screening cultivations, no extracellular HBsAg was detected. Linear correlations between intracellular HBsAg and extracellular glucoamylase activity, with $R^2_{\text{values}} \geq 0.87$ were observed in the two *GlaA_{G2}::S* transformants during the exponential growth phase in glucose batch fermentation (data not shown). The ratio between maximum intracellular specific HBsAg production and maximum extracellular specific glucoamylase activity was higher in PDI cultivations, where levels of $1326.1 \pm 117.9 \mu\text{g}_{\text{HBsAg}} \cdot \text{U}^{-1}$ were observed. In CLX cultivations a ratio of $1257.1 \pm 134.4 \mu\text{g}_{\text{HBsAg}} \cdot \text{U}^{-1}$ was recorded (Table 1).

Table 1: Performance of HBsAg-producing *A. niger* strains over-producing CLX and PDI in glucose based batch fermentations.

<i>A. niger</i> strains	Growth rate [h ⁻¹]	Maximum Biomass [g _{DW} ·kg _{Broth} ⁻¹]	Y _{xs} [g _{DW} ·g _{Glucose} ⁻¹]	Maximum Glucoamylase [U·g _{DW} ⁻¹]	Maximum HBsAg [μg _{HBsAg} ·g _{DW} ⁻¹]	Intracellular HBsAg/ extracellular glucoamylase ratio [μg _{HBsAg} ·U ⁻¹]
<i>A. niger</i> MGG029-ΔaamA DAHB 21#20 CLX 2	0.098 ± 0.009	17.43 ± 1.0	0.55 ± 0.025	0.21 ± 0.024	264 ± 32.2	1257.1 ± 134.4
<i>A. niger</i> MGG029-ΔaamA DAHB 21#20 PDI 7	0.106 ± 0.007	16.61 ± 1.3	0.56 ± 0.018	0.23 ± 0.019	305 ± 29.8	1326.1 ± 117.9

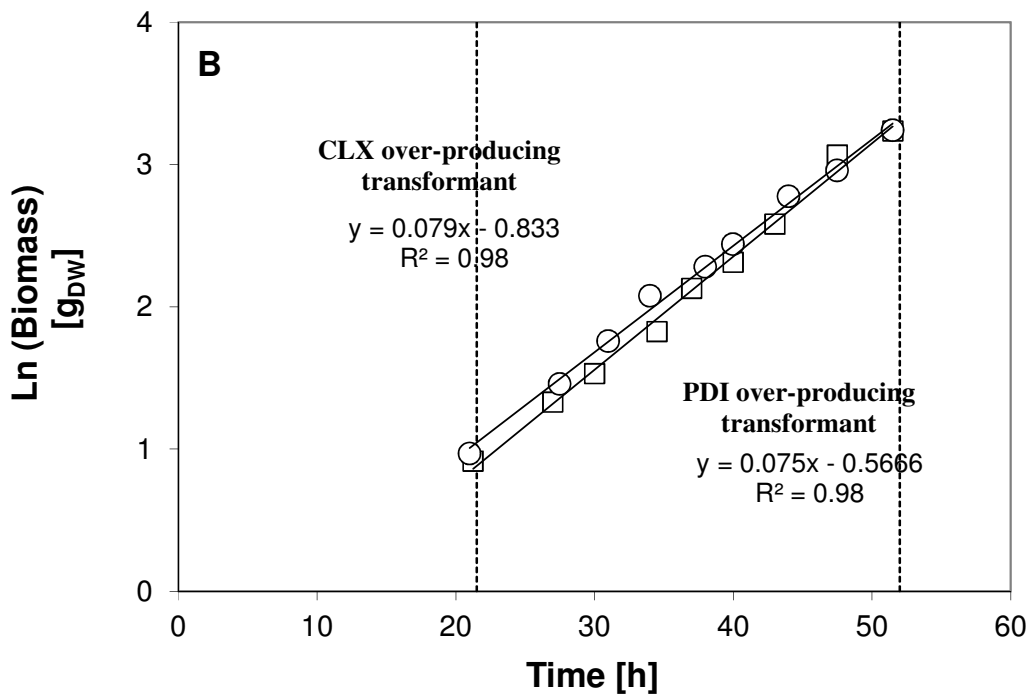
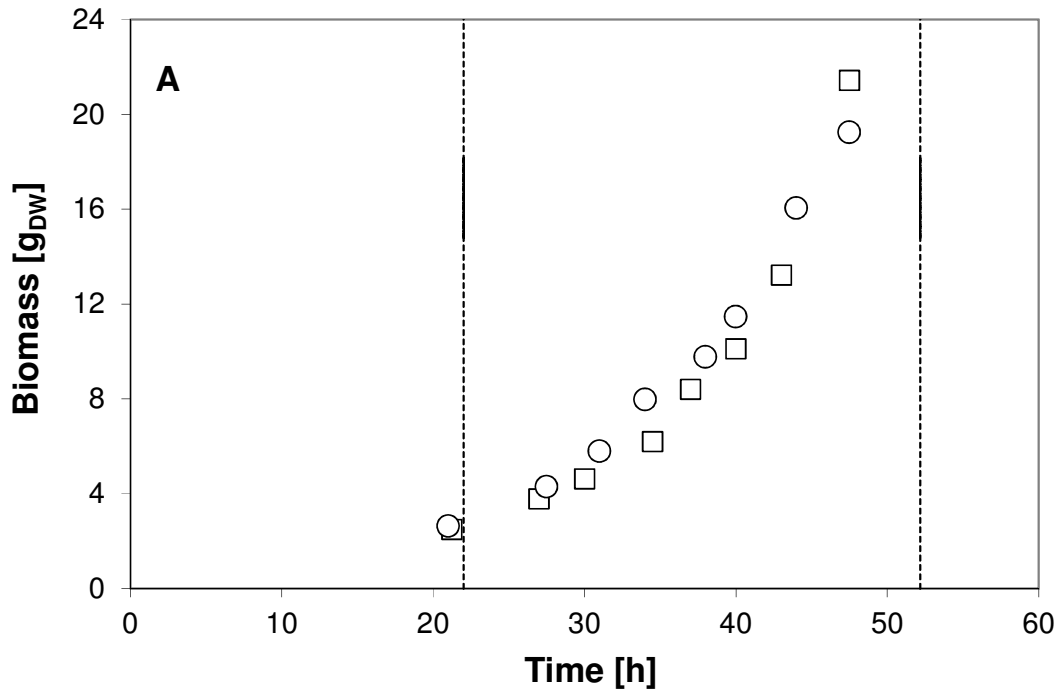
Growth regulated, glucose-limited exponential fed-batch fermentations of two highest HBsAg producing transformants

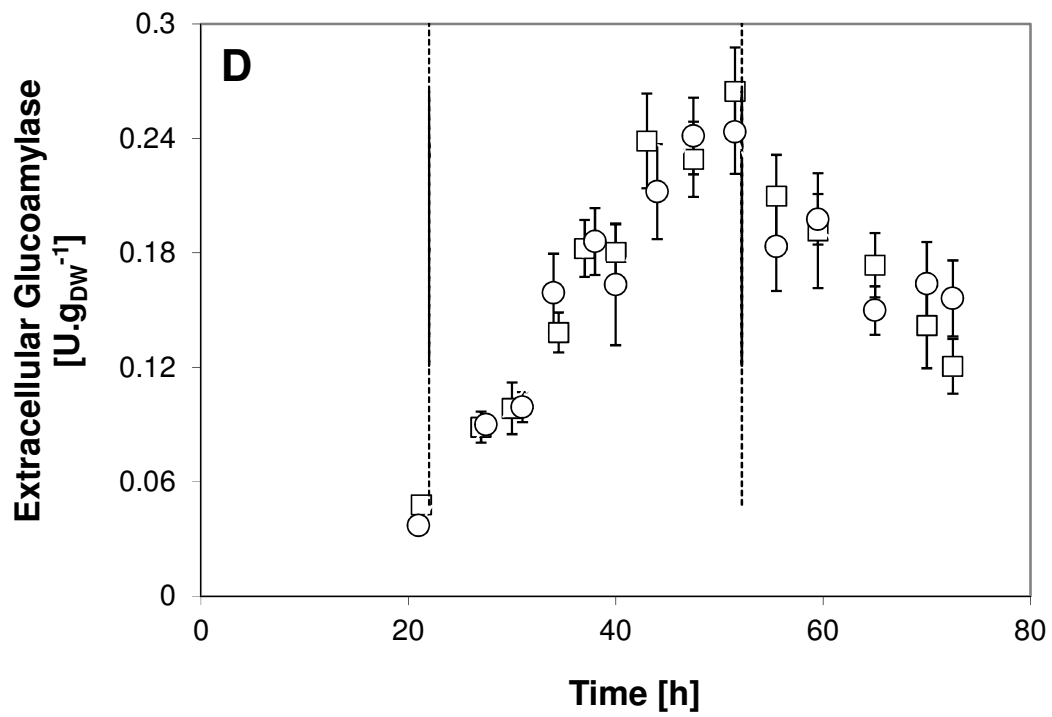
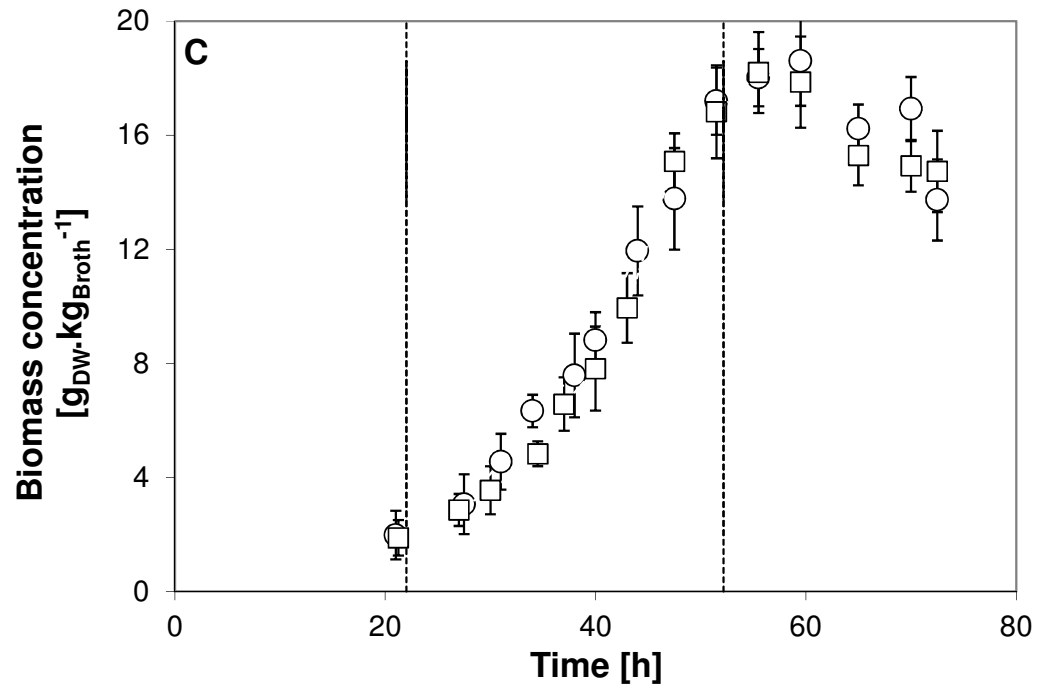
To determine the effects of growth rate on HBsAg production in transformants over-producing CLX and PDI, carbon-limited exponential fed batch fermentations were conducted. Triplicate fed-batch cultivations were performed at a growth rate of 0.08 h^{-1} . The exponential feed rate was calculated on the initial biomass produced after an initial glucose charge of 10 g.kg^{-1} culture medium was exhausted. Upon depletion (between 19 and 20 h) of the initial glucose the exponential feed was executed and was stopped after biomass levels ceased to increase and residual glucose accumulation was observed. Thereafter, fermentations were monitored and sampled for an additional 10 h before the run was stopped. In all cultivations during the exponential feeding phase the residual glucose concentration was controlled below the detection limit of $5 \text{ mmol.l}_{\text{broth}}^{-1}$.

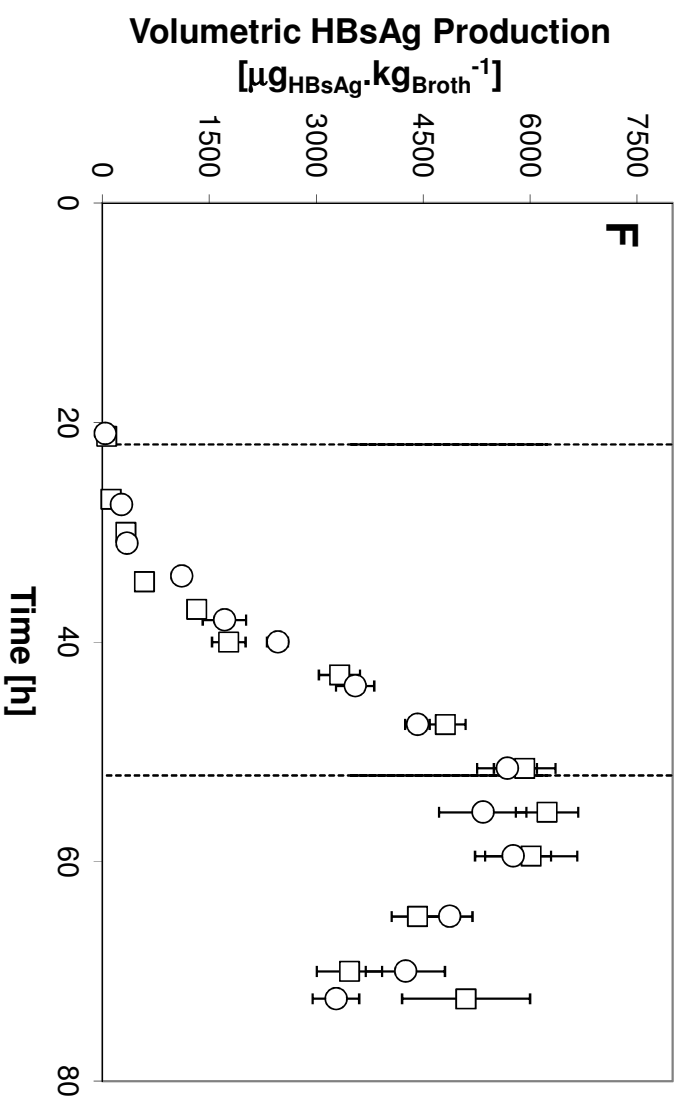
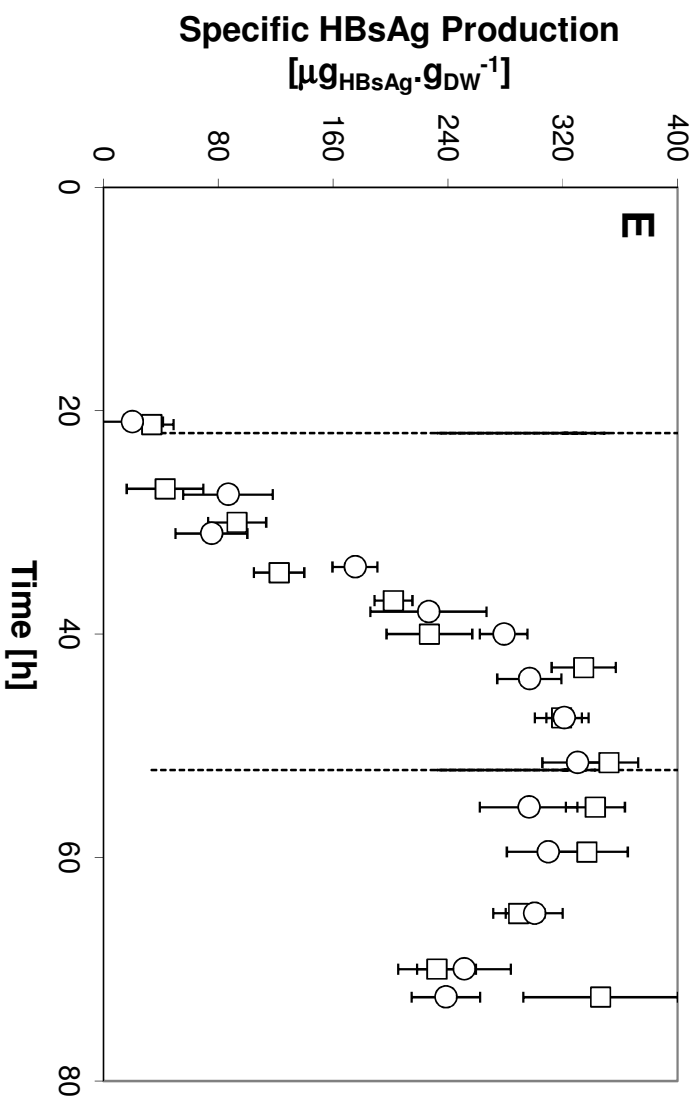
Similar biomass concentration profiles were observed between transformants, with the CLX obtaining the highest at $18.19 \pm 1.4 \text{ g}_{\text{DW}}.\text{kg}_{\text{Broth}}^{-1}$, while PDI fermentations obtained maximum biomass levels of $18.59 \pm 1.6 \text{ g}_{\text{DW}}.\text{kg}_{\text{Broth}}^{-1}$ (Figure 5C). Growth rates, calculated from total biomass data (Figure 5A), of 0.079 ± 0.02 and $0.075 \pm 0.01 \text{ h}^{-1}$ were calculated for CLX and PDI fermentations, respectively, and were noted to be similar to that of the required rate of 0.08 h^{-1} (Figure 5B). Similar biomass yield coefficients (Y_{XS}), were also observed ($\sim 0.56 \text{ g}_{\text{DW}}.\text{g}_{\text{Glucose}}^{-1}$) (data not shown). Extracellular glucoamylase activity profiles followed a growth associated trend (Figure 5D), with the highest levels obtained of $0.26 \pm 0.023 \text{ U.g}_{\text{DW}}^{-1}$ and $0.24 \pm 0.022 \text{ U.g}_{\text{DW}}^{-1}$ in the CLX and PDI over-expressing transformants, respectively. Similar to the extracellular glucoamylase activity profiles, intracellular HBsAg production levels also followed a growth associated trend with the highest levels occurring after $\sim 52 \text{ h}$ (Figure 5E). The CLX over-expressing transformant achieved highest specific levels of $352 \pm$

$21.3 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$, while the PDI over-expressing transformant obtained levels of $330.2 \pm 24.4 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$. Highest volumetric HBsAg production levels were $6237.2 \pm 437.2 \mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$ and $5760.4 \pm 531.9 \mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$ in CLX and PDI over-expressing transformants, respectively (Figure 5F). Similarly to batch fermentations, no extracellular HBsAg was detected (data not shown). A linear correlation between intracellular specific HBsAg production and extracellular specific glucoamylase activity was observed in both CLX and PDI over-expressing fermentations (Figure 5G).

The significance of the two correlations were confirmed through linear regression analysis, with $p_{\text{values}} < 0.05$ of the x variables and intercepts at a 95 % confidence level and R^2 values ≥ 0.90 explained more than 90 % of the total variation (Figure 5E). No significant difference was observed between the two correlations ($p > 0.05$). From Figures 5B and 5 C, the ratio between the maximum intracellular HBsAg production levels and maximum extracellular glucoamylase activity were similar, at $\sim 1330 \mu\text{g}_{\text{HBsAg}} \cdot \text{U}^{-1}$ and $1360 \mu\text{g}_{\text{HBsAg}} \cdot \text{U}^{-1}$, for the CLX and PDI carbon-limited exponential fed-batch fermentations, respectively.







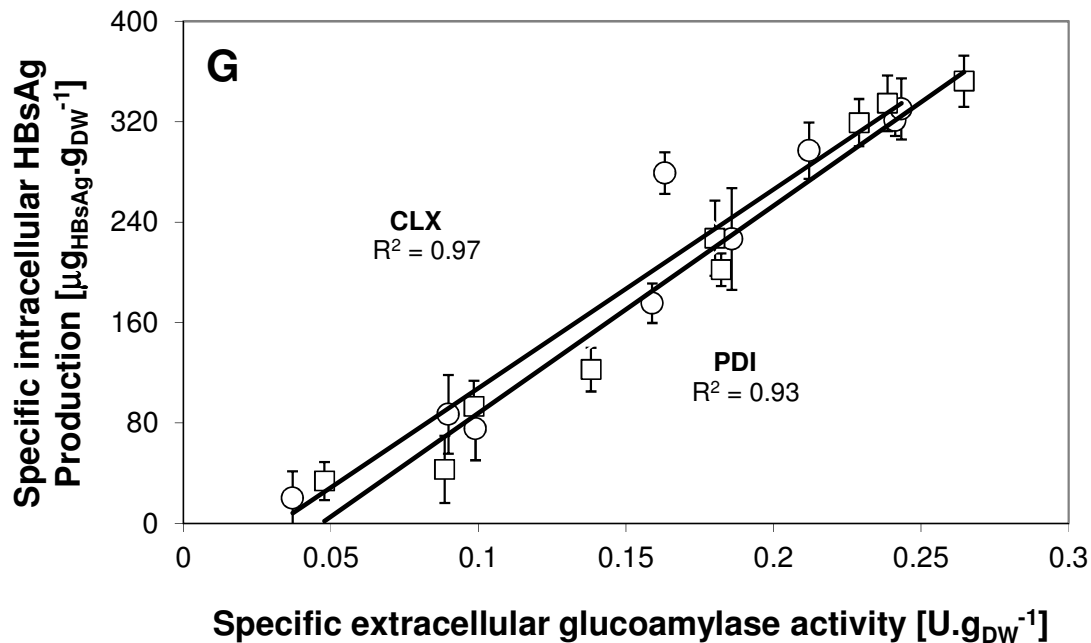


Figure 5. Glucose-limited exponential fed-batch fermentations of *A. niger* at a growth rate of 0.08 h^{-1} : Shown is data regarding two HBsAg-producing *A. niger* MGG029- $\Delta aamA$ transformants (carrying the *GlaA*::*S* fusion cassette and controlled by the inducible *GlaA_p* promoter); \square , CLX over-expressing transformant DAHB 21#20 CLX2; and \circ , PDI over-expressing transformant DAHB 21#20 PDI7. Depicted are the effects of specific μ data on: (A) Dry biomass, (B) \ln (biomass), (C) biomass concentration, (D) specific Glucoamylase activity, (E) specific HBsAg production, (F) volumetric HBsAg production, and (G) the ratio between intracellular HBsAg and extracellular glucoamylase activity. Error bars represent the standard deviation of the mean. Triplicate exponential fed-batch fermentations were performed for both transformants.

DISCUSSION

Previously described in this dissertation, HBsAg production was realized by targeting HBsAg production through the secretory pathway in *A. niger*, resulting in completely assembled and properly folded HBsAg (Manuscript 1 of this dissertation). This was achieved by implementing a gene fusion strategy using the highly expressed catalytic domain of the glucoamylase gene (*GlaA_{G2}*) fused to the HBsAg *S* gene. This has the advantage of

encouraging high expression of the fused cassette (Le Loir *et al.*, 2005) as well as passage of the target protein under the mask of a homologous gene product through the fungal secretory pathway (Gouka *et al.*, 1997). Expression of the *GlaA_{G2}::S* fusion gene in *A. niger* transformants resulted in the intracellular accumulation of HBsAg associated with the membranous fraction. Glucoamylase was secreted from the production host, although with a substantial level of intracellular glucoamylase product degradation (Manuscript 1 of this dissertation). These findings together with the positive effect of a reduced and optimized growth rate on HBsAg production as observed in Manuscript 3 of this dissertation, suggests that limitations/ bottlenecks, at a (post)translational level in the secretory pathway, are negatively affecting production. This has led to the hypothesis that such limitations occur in the current *A. niger* system, and can be demonstrated by increases in HBsAg production through the over-production of BiP, CLX, PDI, and the combinations thereof. In this study we explore the effects of over-production of BiP, CLX, PDI, and the combinations thereof as a means of reducing the speculated bottlenecks in the secretory pathway and therefore enhance HBsAg production.

The over-production of BiP, CLX, PDI, and the combinations thereof significantly influenced HBsAg production efficiency through the expression of the *GlaA_{G2}::S* fusion gene. HBsAg production levels were enhanced by up to 107 %, and in other cases were even reduced by 40 %. These findings reflect the varied responses in HBsAg production resulting through the over-production of these chaperones/ foldases. Furthermore, significant variance in HBsAg production levels was observed amongst chaperones over-expressing transformants carrying similar chaperone gene products. This was attributed to vector copy number (Verdoes *et al.*, 1994) and/ or site of integration (Verdoes *et al.*, 1993). However, the maximum percentage increase in HBsAg production levels over-expressing the various chaperones/ foldases reflects

the capability of these chaperones/ foldases in facilitating HBsAg production through the secretory pathway when over-produced correctly and at an optimum amount.

The over-production of CLX, PDI, and combinations thereof significantly increased HBsAg levels by 88 %, 107 %, and 43 %, respectively. These findings suggest that the functions of CLX and PDI in the secretory pathway may be critical for efficient HBsAg production using the *GlaA_{G2}::S* fusion gene.

CLX is glycan-dependent molecular chaperone and is a major component of the ER quality control system. CLX has several intracellular functions; (1) facilitates the folding of glycosylated protein by slowing folding events and allowing them to occur in a controlled manner (Helenius *et al.*, 1997; Conesa *et al.*, 2002), (2) retains immature or unassembled structures in the ER, (3) prevents aggregation, and (4) potentially aids in the sorting of malformed substrates for degradation (Pearse and Hebert, 2009). The positive effect of CLX over-expression on HBsAg production suggests its participation in the maturation of HBsAg . The fact that CLX potentially stabilizes protein folding by slowing folding events, allowing them to occur in a controlled manner, may be a significant attribute which may also explain the increase in HBsAg production levels at a reduced and optimized growth rate as observed in Manuscript 2 of this dissertation.

The foldase, PDI, from the thioredoxin superfamily, is known to be an essential protein that catalyzes the oxidation, reduction, and isomerization of disulfide bonds (Laboissiere *et al.*, 1995; Wilkinson and Gilbert 2004). PDI behaves as a chaperone as it inhibits the aggregation of misfolded proteins (Gilbert, 1997; Wang, 1998; Ferrari and Soling, 1999). HBsAg is a conformational antigen which is critically dependent on disulfide bonds between cysteine groups in HBsAg (Mangold *et al.*, 1997). Thus, the increase in HBsAg production levels

caused by the over-expression of PDI could induce increased levels of disulfide bond formation activity between inter or intra-molecules of HBsAg (Kim *et al.* 2009).

HBsAg-producing transformants over-expressing the *bipA* gene did not significantly improve HBsAg levels. In fact, HBsAg production was reduced in a high percentage of transformants (67 %) where a reduction in HBsAg production of up to 40 % was observed. Similar results have been reported, where over-expression of the *bipA* gene failed to improve production levels of fungal cutinase in *A. niger* (van Gemeren *et al.*, 1998), and significantly reduced manganese peroxidase levels in *A. niger* (Conesa *et al.*, 2002). These reports are contradictory to previously reported studies (Harmsen *et al.*, 1996; Shusta *et al.* 1998; Damasceno *et al.*, 2007). The variety in results could be attributed to the multi-functionality of the BiP chaperone as well as the inherent characteristics of the recombinant gene product of interest. BiP is classified as an Hsp70 class heat shock protein and is involved in several functions in the ER: (1) assists in protein folding, (2) is involved in translocation, and (3) assists in the ER-associated degradation (ERAD) pathway (Brodsky *et al.*, 1999; Gething, 1999; Matlack *et al.*, 1999). The inherent characteristics of the recombinant *GlaA_{G2}::S* fused gene product can potentially be sensitive or unresponsive to the above mentioned functions.

Molinari *et al.* (2002) observed a sequential involvement of the CLX and BiP/PDI chaperone systems in mammalian cells. CLX bound to newly synthesized proteins and thus facilitated folding as well as prevented aggregation and premature degradation. BiP and PDI were also involved in this phase, but were also essential in directing terminally misfolded proteins, after their release from CLX, for degradation in the ERAD pathway (Molinari *et al.*, 2002). A similar synergistic effect between BiP and PDI was reported by (Shusta *et al.*, 1998). However, the joint over-expression of *bipA*, *clxA*, *pdiA* and combinations thereof did not

result in a significant increase in HBsAg production levels. Similar results were observed in the study of Damasceno *et al.* (2007) where the joint over-expression of BiP and PDI failed to improve the production of a single-chain antibody fragment in *P. pastoris* (Damasceno *et al.* 2007). Assuming that BiP, PDI and CLX have similar functions in *A. niger* as in mammalian cells, together, with the fact that the joint over-expression of *bipA*, *pdiA*, *clxA* and combinations thereof resulted in an insignificant increase in HBsAg production levels, while the joint over-expression of *pdiA* and *clxA* genes resulted in a 43 % increase, suggests that unfavorable responses are triggered upon BiP over-expression resulting in the failure to improve HBsAg production levels. Furthermore, the fact that in some cases a reduction in HBsAg production levels was observed upon BiP over-expression may indicate that BiP has a pronounced function in directing premature HBsAg for degradation in the ERAD pathway.

The over-production of the chaperones/ foldases improved the production efficiency of glucoamylase-HBsAg product by either facilitating the expression and production of glucoamylase-HBsAg, glucoamylase, or HBsAg. Highest percentage increases in HBsAg were observed when both intracellular HBsAg and extracellular glucoamylase activity were improved significantly. These findings together with the substantial level of intracellular glucoamylase product degradation observed (Manuscript 1 of this dissertation) and high expression levels of the *GlaA_{G2}::S* gene observed in the study of Plüddemann, (2003) suggest that limitations in the secretory pathway occur at a posttranslational level before the cleavage of the fused glucoamylase-HBsAg product.

The choice of strain together with the inherent characteristics of the product of interest are critical factors contributing towards product yield, as noted by Punt *et al.*, 1998; Tate *et al.*, 1999; Ngiam *et al.*, 2000; Wang and Ward 2000; Conesa *et al.*, 2001. In this study, highest

percentage increases in HBsAg production levels through the over-production of chaperones/ foldases were obtained from transformants produced from the ‘fast growing, high biomass producing’ transformant (DAHB 21#20) derived from *A. niger* strain, MGG029- $\Delta aamA$. These findings together with the improved HBsAg production levels obtained through a reduced and optimized growth rate in carbon-limited exponential fed-batch fermentations, it is speculated that limitations/ bottlenecks, in the secretory pathway, were significant in the ‘fast growing, high biomass producing’ transformant (DAHB 21#20) in comparison the ‘slow growing high glucoamylase producing’ (DAHB 21#40) and *A. niger* D15 transformants. However, further studies are required to confirm this by define the processing mechanisms of HBsAg using gene fusion, the limitations/ bottlenecks in the secretory pathway, and other complex processes influencing production efficiency, including the UPR/ ERAD responses and chaperone interactions. The use of fluorescent proteins as a carrier protein for HBsAg production, together with microscopy and ‘omics’ technologies may provide the necessary information required to unravel these mysteries of the *A. niger* secretory pathway.

It has previously been reported that a reduced and optimized growth rate has a positive affect on HBsAg production levels (Manuscript 3 of this dissertation). The positive affect of CLX on HBsAg production levels is possibly explained in the ability to stabilize protein folding by slowing folding events (Helenius *et al.*, 1997; Conesa *et al.*, 2002) and therefore a synergistic effect between growth rate and CLX facilitation on the fused *GlaA_{G2}::S* gene may be apparent. In glucose batch fermentations, transformants over-producing PDI obtained highest levels ($305 \pm 29.8 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$) in comparison to CLX transformants $264 \pm 32.2 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$). The intracellular HBsAg production efficiency in relation to extracellular glucoamylase activity was highest in PDI batch fermentations as reflected by the ratio between maximum intracellular HBsAg production levels and maximum extracellular

glucoamylase activities. In glucose-limited exponential fed-batch fermentations HBsAg production levels in transformants over-producing CLX and PDI were enhanced to similar levels in comparison to batch fermentations. Highest HBsAg production levels of $352 \pm 21.3 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ ($6237.2 \pm 437.2 \mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$) and $330.2 \pm 24.4 \mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$ ($5760.4 \pm 531.9 \mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$) in CLX and PDI over-expressing transformants were observed. A significant increase in HBsAg production efficiency in relation to extracellular glucoamylase activity was observed in CLX cultivations at a reduced and optimized growth rate in comparison to batch fermentations, reaching similar levels to PDI fed-batch fermentations ($\sim 1330 \mu\text{g}_{\text{HBsAg}} \cdot \text{U}^{-1}$ and $1360 \mu\text{g}_{\text{HBsAg}} \cdot \text{U}^{-1}$, for the CLX and PDI carbon-limited exponential fed-batch fermentations, respectively). These findings signify the associated positive affects on HBsAg production efficiency through CLX over-production and cultivation at a reduced and optimum growth rate.

As an additional objective of this study we evaluate the potential use of the solid state screening method selecting for high extracellular glucoamylase previously described in Manuscript 2 of this dissertation. In this dissertation (Manuscript 2), selecting for high extracellular glucoamylase producing transformants in solid state cultivation using starch as a selection pressure resulted in the selection of high intracellular HBsAg producing transformants. This method of selection was not implemented in this study as a starch selection pressure was less sensitive to the *A. niger* D15 transformants producing native amylases. This strategy may have been implemented with the *A. niger* MGG029- ΔaamA transformants lacking native glucoamylase and acid amylase. However, it was hypothesized that in cases where only glucoamylase or HBsAg production was improved through the over-production of chaperones/ foldases, the use of the glucoamylase screen would result in either false positive or masked results, thereby reducing the overall efficiency of the screen.

However, results indicate that by selecting for a percentage increase in extracellular glucoamylase activity $\geq 25\%$, a high efficiency of transformants (72 %) manifesting improved HBsAg production levels of $\geq 25\%$ was obtained. These findings, together with the acceptable correlation between solid state and liquid cultures (Manuscript 2 of this dissertation), suggests the suitable application of the solid state screening method selecting for high extracellular glucoamylase for the identification of high HBsAg producers resulting from the over-production of chaperones/ foldases.

It can therefore be concluded that by targeting HBsAg production through the secretory pathway in *A. niger* using the gene fusion approach limitations/ bottlenecks at a (post)translational level become evident, negatively affecting HBsAg production. This was demonstrated through the increase in HBsAg production levels resulting from the over-production of chaperones/ foldases. The over-production of CLX and PDI resulted in the highest percentage increase and therefore reflects their critical involvement with regards to efficient HBsAg assembly using gene fusion. Further investigation is required to determine optimum copy number and site of integration of these chaperones/ foldases, where site directed integration of these genes may result in further improved production levels. The kinetic and specificity of these interactions may also contribute significantly towards the knowledge of the overall mechanism of HBsAg expression and assembly using gene fusion. The associated positive effects of CLX over-production and cultivation at a reduced and optimized growth rate also requires further investigation, where a continuous cultivation using starch as a selection pressure at a reduced and optimum dilution rate may provide a platform for enhanced extracellular activity and associated intracellular HBsAg production levels. The potential application of the glucoamylase screen using starch as a selection pressure for the identification of high HBsAg producers over-producing chaperones/ foldases, together with

the positive affects of PDI, CLX and growth rate on HBsAg production, demonstrates its usefulness and application as a technology for other heterologous proteins and recombinant VLPs.

Acknowledgements

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CONCLUSIONS & FUTURE PROSPECTS

This study serves as a proof-of-concept that by targeting HBsAg production through the secretory pathway in *A. niger* through gene fusion, completely assembled and properly folded HBsAg, is obtained. Gene fusion of the highly expressed catalytic domain of glucoamylase fused to the HBsAg *S* gene, resulted in the secretion of glucoamylase, while, similar to other recombinant microbial systems, HBsAg accumulated intracellularly in membrane associated fractions (Manuscript 1 of this dissertation). These findings together with (1) the intracellular accumulation of intracellular glucoamylase degradation products (2) the positive affects of a reduced and optimized growth rate on HBsAg production and (3) the increases in HBsAg production through over-expression of chaperones/ foldases, suggests that limitations/ bottlenecks in the secretory pathway using gene fusion as a 'stand alone' technology, are negatively affecting production. These limitations or inefficiencies in HBsAg production using the gene fusion approach can be reduced, thereby enhancing HBsAg production, by incorporating the following technologies described in this dissertation;

1. Improved selection of high HBsAg producing transformants using starch as a selection pressure and extracellular glucoamylase to indirectly quantify HBsAg production levels (Manuscript 2 of this dissertation)

The successful enhancement of HBsAg production levels was largely based on the development of effective transformants. This was achieved through the development of an improved methodology using the gene fusion technology to identify and select high HBsAg producing transformants (Manuscript 2 of this dissertation). The high efficiency obtained through selecting for high extracellular glucoamylase transformants and the associated correlation between intracellular HBsAg production and secreted glucoamylase in batch fermentation reflects the ability of the glucoamylase screen to indirectly quantify high HBsAg

producing transformants through extracellular glucoamylase determination. This system was shown to be robust and allowed for high-through put screening of several hundred transformants. In principle, this method can be applied to any heterologous protein that is secreted as a fusion with the glucoamylase protein in *A. niger* MGG029- $\Delta aamA$ transformants lacking native glucoamylase and acid amylase.

2. A reduced and optimum growth rate through glucose-limited exponential fed-batch fermentations (Manuscript 3 of this dissertation)

The development of glucose-limited exponential fed-batch fermentations for the controlled culturing of HBsAg producing *A. niger* transformants had a positive affect on HBsAg production (Manuscript 3 of this dissertation). HBsAg production levels using this approach were increased 1.8 fold in comparison to batch fermentations. Thus glucose-limited exponential fed-batch fermentation at an optimum μ ($0.07 < \mu < 0.08 \text{ h}^{-1}$) for the current *A. niger* HBsAg production system provides a method for efficient HBsAg processing, resulting in increased HBsAg production levels.

3. Modifying the chaperone/ foldase levels

By modifying the levels of chaperones/ foldases in HBsAg producing transformants, HBsAg production was affected (Manuscript 4 of this dissertation). The over-production of CLX and PDI resulted in the highest percentage increase (88 % and 107 %, respectively) and therefore reflected their critical involvement with regards to efficient HBsAg assembly using gene fusion. The combinations of BiP and associated combinations with CLX and PDI failed to improve HBsAg production levels. Reductions of up to 40 % were observed upon BiP over-production and therefore indicated unfavorable responses towards HBsAg assembly and production, which were triggered upon BiP over-expression using gene fusion.

The integration of these approaches together with the gene fusion strategy into *A. niger* resulted in the significant increase in HBsAg production as summarized in Table 1.

Table 1: Approaches and associated intracellular HBsAg production levels in *A. niger*. Values represent the maximum volumetric intracellular HBsAg production levels obtained in each approach evaluated.

Strategy	Strain	Specific intracellular HBsAg production [$\mu\text{g}_{\text{HBsAg}} \cdot \text{g}_{\text{DW}}^{-1}$]	Volumetric intracellular HBsAg production [$\mu\text{g}_{\text{HBsAg}} \cdot \text{kg}_{\text{Broth}}^{-1}$]
Gene fusion*	<i>A. niger</i> D15	34	297
Strain selection*	<i>A. niger</i> MGG029- <i>aamA</i> strain (slow-growing high glucoamylase producing transformant)	77	712
Strain selection incorporating modified chaperone/ foldase levels*	<i>A. niger</i> MGG029- <i>aamA</i> strain (fast-growing high biomass producing transformant) over-producing PDI	108	1049
Batch fermentation	<i>A. niger</i> MGG029- <i>aamA</i> strain (slow-growing high glucoamylase producing transformant)	219	3140
Carbon-limited exponential fed-batch fermentation	<i>A. niger</i> MGG029- <i>aamA</i> strain (fast-growing high biomass producing transformant)	241	4483
Batch fermentation incorporating modified chaperone/ foldase levels	<i>A. niger</i> MGG029- <i>aamA</i> strain (fast-growing high biomass producing transformant) over-producing PDI	305	5102
Carbon-limited exponential fed-batch fermentation incorporating modified chaperone/ foldase levels	<i>A. niger</i> MGG029- <i>aamA</i> strain (fast-growing high biomass producing transformant) over-producing CLX	352	6237

* Shake flask cultivation using starch as carbon source

Further improvement in HBsAg production yields may be obtained by implementing the following approaches:

Holistic approaches that will define the processing mechanisms of HBsAg using gene fusion, the limitations/ bottlenecks in the secretory pathway, and other complex processes influencing production efficiency, including the UPR/ ERAD responses and chaperone interactions. The use of fluorescent proteins as a carrier protein for HBsAg production may provide the necessary information required to unravel the mysteries of the *A. niger* secretory pathway. Not only can these technologies be used to study protein trafficking under different conditions, but they have also been of use in elucidating the relationships between different organelles in a noninvasive manner. The use of multiple different fluorochromes allows the observation of interactions between organelles and between proteins. The ‘omics’ technologies will also provide a platform through the overall analysis of gene expression (transcriptomics), protein (proteomics) and metabolite (metabolomics) production at the level of the complete organism. Together with advanced microscopy technologies, and fluorescent proteins as a carrier protein for HBsAg production, these technologies will add value in visualizing metabolic pathways and protein–protein interactions in living systems and hence direct research towards further strategies to improve HBsAg protein production levels.

Investigations regarding the optimum copy number and site of integration of the HBsAg fused gene and chaperones/ foldases may also provide significant information contributing towards site directed gene transfer techniques, which may lead to improve HBsAg protein production levels.

A mutagenesis-based selection program with classical genetic strain improvement coupled to the extracellular glucoamylase screen may provide a selection of host strains manifesting increased HBsAg production and efficient processing.

As already mentioned, glucose-limited exponential fed-batch proved to be a suitable cultivation strategy inducing high HBsAg production levels. However in Manuscript 2 it was shown that through the transcriptional control of the *GlaA_p* promoter, starch as a carbon substrate and its degradation products induced highest specific HBsAg production levels. Despite highest HBsAg production levels in starch based fermentation, the accumulation of residual glucose remains a challenge as this coincided with the reduction in extracellular glucoamylase activity and HBsAg production mid-way through the exponential phase. The phenomenon of residual glucose accumulation indicates that the rate of starch hydrolysis is greater than glucose consumption of the host organism. In order to further enhance HBsAg production levels of this system in starch based cultivations, a possible solution would be to implement a continuous or fed batch fermentation, where the rate of starch hydrolysis can be equilibrated with glucose consumption. Another possible solution would be to use the constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter to control transcription. However, if the rate of starch hydrolysis and glucose consumption by the organism is not maintained using starch as a carbon substrate, the residual glucose concentration will accumulate and limitations in the form of catabolic repression may appear.

To determine the effectiveness of this technology and the HBsAg production levels required for this process to be economically viable, the following studies are required:

Immunogenic studies in animals to benchmark results to those acquired in other microbial systems.

Conceptual process designs and scale-up studies as well as cost assessments to determine the production costs and product prices.

This study has clearly shown that the filamentous fungus *Aspergillus* is an effective alternative host for the production of HBsAg. The knowledge gained from this dissertation has contributed not only towards further developing *A. niger* as an expression host for heterologous protein production, but also to the VLP technology in which it is expressed. With further knowledge of the processing mechanisms of HBsAg as well as studies investigating HBsAg production efficiency, HBsAg production levels will potentially improve further. This is an exciting venture, as this work paves the way for the investigation of *Aspergillus* as a potential host for the heterologous production of proteins from viruses such as HIV-1, HPV and rotavirus for the development of subunit vaccines to address the increasing health risk posed by these viruses, especially in Africa.

APPENDIX A**EXPONENTIAL FED-BATCH BIOMASS AND FEED EQUATION DERIVATIONS**

In the exponential fed-batch cultivations the concentration of residual glucose in the fermentor, during the carbon limited exponential feeding phase, was maintained close to zero and the set growth rate (μ_{set}) was controlled by directly controlling the amount of concentrated glucose substrate added in the fed-batch cultivation. This approach of controlling the amount of concentrated glucose substrate added in the fed-batch cultivation (equation 3), while glucose residual concentration was maintained at zero, was used to regulate the production of biomass in the fermentor through the control of glucose substrate consumption by the filamentous fungi. This results in the control of the μ_{set} at a constant value under both transient and steady state conditions for biomass concentration (Martinez *et al.*, 1998; Kim *et al.*, 2000; Cheng *et al.*, 2003; Sun *et al.*, 2007; Chen *et al.*, 2008).

The equations for the carbon limited exponential feeding phase are derived from first principal mass balances.

Biomass balance

A biomass balance over the fed-batch bioreactor yields:

$$\frac{d(VC_x)}{dt} = \frac{(C_x)dV}{dt} + \frac{VdC_x}{dt} \quad \text{Equation 1.}$$

$$\frac{d(VC_x)}{dt} = \mu VC_x \quad \text{Equation 2.}$$

$$\mu VC_x = \frac{(C_x)dV}{dt} + \frac{VdC_x}{dt} \quad \text{Equation 3.}$$

$$\mu = \frac{1}{V} \frac{dV}{dt} + \frac{1}{C_x} \frac{dC_x}{dt} \quad \text{Equation 4.}$$

which upon rearrangement using the principal that $1/X = d(\ln X)/dX$ results in:

$$\mu = \frac{d(\ln V)}{dt} + \frac{d(\ln C_x)}{dt} \quad \text{Equation 5.}$$

where μ is the specific growth rate (h^{-1}), V is the fed-batch reactor culture volume (L), and C_x is the biomass concentration ($\text{g}_{\text{DW}} \cdot \text{L}^{-1}$).

The dilution rate D (h^{-1}) therefore equates to:

$$D = \frac{d(\ln V)}{dt} \quad \text{Equation 6.}$$

And upon rearrangement of Equation 5 & 6, Equation 7 describes the general condition for μ using (Ramirez *et al.*, 1994).

$$\mu = D + \frac{d(\ln C_x)}{dt} \quad \text{Equation 7.}$$

Equation 7 can be simplified to Equation 12 by applying the following derivation:

$$\frac{d(V C_x)}{dt} = \frac{(V C_x)_{t_2} - (V C_x)_{t_1}}{t_2 - t_1} \quad \text{Equation 8.}$$

However, $M_{x(t)} = (V C_x)_{(t)}$ Equation 9.

Where M_x is the total biomass (g_{DW}) in the fed-batch reactor. Therefore from Equation 8 and Equation 9;

$$\frac{d(M_x)}{dt} = \frac{(M_x)_{t_2} - (M_x)_{t_1}}{t_2 - t_1} \quad \text{Equation 10.}$$

And from equation 9, 10 and 2, Equation 11 is obtained.

$$\frac{d(M_x)}{dt} = \mu M_x \quad \text{Equation 11.}$$

Upon re-arrangement of Equation 11, Equation 12 describes the general condition for μ using M_x .

$$\mu = \frac{d(\ln M_x)}{dt} \quad \text{Equation 12.}$$

Equation 12 is therefore a simplified equivalence to Equation 7, where $\frac{d(\ln M_x)}{dt}$ in Equation 12 incorporates both D and $\frac{d(\ln C_x)}{dt}$ term in Equation 7 (refer to Equation 13) and is the general equation used to measure μ at μ_{set} .

$$\mu = \frac{d(\ln M_x)}{dt} = D + \frac{d(\ln C_x)}{dt} \quad \text{Equation 13.}$$

Glucose substrate balance

The glucose substrate balance yields;

$$\frac{d(V C_s)}{dt} = \frac{d(V_{sf} C_{sf})}{dt} - q_s V C_x \quad \text{Equation 14.}$$

Where C_s is the glucose substrate concentration in the reactor, V_{sf} is the volume of the feed added to the reactor, C_{sf} is the concentrated glucose substrate concentration in the feed and q_s is the specific glucose consumption rate ($\text{g}_{\text{Glucose}} \cdot \text{g}_{\text{DW}}^{-1} \cdot \text{h}^{-1}$).

Using the principals of Equation 9;

$$\frac{d(M_s)}{dt} = \frac{d(M_{sf})}{dt} - q_s M_x \quad \text{Equation 15.}$$

Where M_s is the glucose substrate mass in the reactor, and M_{sf} is the mass of the feed added to the reactor.

In glucose-limited exponential fed batch cultivations when a variable glucose exponential feed (concentrated) is implemented to maintain a $\mu_{\text{set}} \leq \mu_{\text{max}}$, the mass of the glucose substrate in the reactor must be equal to zero upon commencement of the feed and must remain so for the entire feeding phase, with the assumption that M_s is consumed by the organism as fast as it enters the reactor. This was confirmed for all cultivations (Dissertation, Manuscript 3, line 18 & 19, pg 148). Therefore,

$$\frac{d(M_s)}{dt} = \frac{d(V C_s)}{dt} = 0 \quad \text{Equation 16.}$$

And from Equation 14, Equation 17 and 18 is achieved;

$$\frac{d(V_{sf} C_{sf})}{dt} = q_s V C_x \quad \text{and} \quad \frac{d(M_{sf})}{dt} = q_s M_x \quad \text{Equation 16. and Equation 17.}$$

Therefore to determine the amount of glucose required for the exponential growth of M_x , dividing Equation 11 by Equation 17 yields;

$$\frac{d(M_x)}{d(M_{sf})} = \frac{\mu}{q_s} = y_{xs} \quad \text{Equation 18.}$$

Where Y_{xs} ($\text{g}_{\text{DW}} \cdot \text{g}_{\text{glucose}}^{-1}$) is the biomass yield coefficient. Therefore the amount of M_{sf} at time t_2 required for M_x at time t_2 at a controlled μ is;

$$M_{sf(t_2)} = \frac{(M_{x(t_2)} - M_{x(t_1)})}{y_{xs}} + M_{sf(t_1)} \quad \text{Equation 19.}$$

And $M_{x(t_2)}$ is derived from equation 12;

$$M_{x(t_2)} = M_{x(t_1)} e^{\mu_{\text{set}}(t_2 - t_1)} \quad \text{Equation 20.}$$

Where $M_{x(t_2)}$ can be predicted assuming biomass production at μ_{set} .

And by combining equation 20 into equation 19 yields;

$$M_{sf(t_2)} = \frac{M_{x(t_1)}(e^{\mu_{\text{set}}(t_2 - t_1)} - 1)}{y_{xs}} + M_{sf(t_1)} \quad \text{Equation 21.}$$

Equation 21 is the general equation used for determining the mass of concentrated glucose feed required for consumption to produce the desired mass of biomass and therefore control μ at μ_{set} and Equation 12 is the general equation used to measure μ at μ_{set} .

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APPENDIX B

EXPONENTIAL FED-BATCH METHODOLOGY AND TYPICAL CALCULATIONS

In this section an account of the application of the methodology described in Appendix A, to determine the exponential glucose feed so as to control μ_{set} , to experimental fed-batch cultures is provided. Experimental data discussed here represents data shown in Manuscript 3 regarding glucose limited exponential fed batch cultivations of the fast growing high biomass producing transformant DAHB21#20 at the set specific growth rate ($\mu_{\text{set}} = 0.08 \text{ h}^{-1}$).

The exponential fed-batch cultivations comprised of two phases; firstly a batch phase, followed by an exponential feeding phase. The batch phase was terminated after an initial charge of glucose ($10 \text{ g}_{\text{glucose}}/\text{kg}_{\text{broth}}$) was depleted as estimated by the sudden increase in dissolved oxygen. This was the signal used to initiate the exponential feed as described previously (Yamane and Shimizu, 1984; Paalme et al., 1990; Riesenberget al., 1991; Yamane et al., 1992; Yang et al., 1992 and Ramfirez et al., 1997). The feed profile was terminated when residual glucose accumulation was observed in the fermentor, at the end of the fed-batch phase. The feed profile was predetermined using data from the sources in Table 1.

Table 1: Parameters associated with the fast growing high biomass producing transformant DAHB21#20 used for exponential feed determination

Parameters		Source
Biomass yield coefficient, Y_{xs} ($\text{g}_{\text{DW}} \cdot \text{g}_{\text{glucose}}^{-1}$)	0.56	Batch data
Glucose feed concentration, C_{sf} ($\text{g}_{\text{glucose}} \cdot \text{L}^{-1}$)	150	Specified
Biomass concentration at start of fed-batch phase, $C_{x(1)}$ ($\text{g}_{\text{DW}} \cdot \text{L}_{\text{broth}}^{-1}$)	2.2	Batch data
Reactor volume at start of fed-batch phase, $V_{(1)}$ (L_{broth})	1.3	Specified
Set specific growth rate during fed-batch phase, μ_{set} (h^{-1})	0.08	Specified

The estimated biomass concentration (determined from previous batch fermentations) at the start of the fed batch phase, $C_{x(1)} = 2.2 \text{ g}_{\text{DW}} \cdot \text{L}_{\text{broth}}^{-1}$, was used to determine the total amount of biomass $M_{x(1)}$ in the reactor at the start of the fed batch phase by multiplying by the reactor volume at start of fed-batch phase, $V_{(1)} = 1.3 \text{ L}_{\text{broth}}$ to obtain $M_{x(1)} = 2.86 \text{ g}_{\text{DW}}$. $M_{x(1)}$ and the specific growth rate to be maintained during fed-batch phase, μ_{set} , together with Equation 20 in Appendix A were used to obtain a predetermined biomass profile (Figure 1).

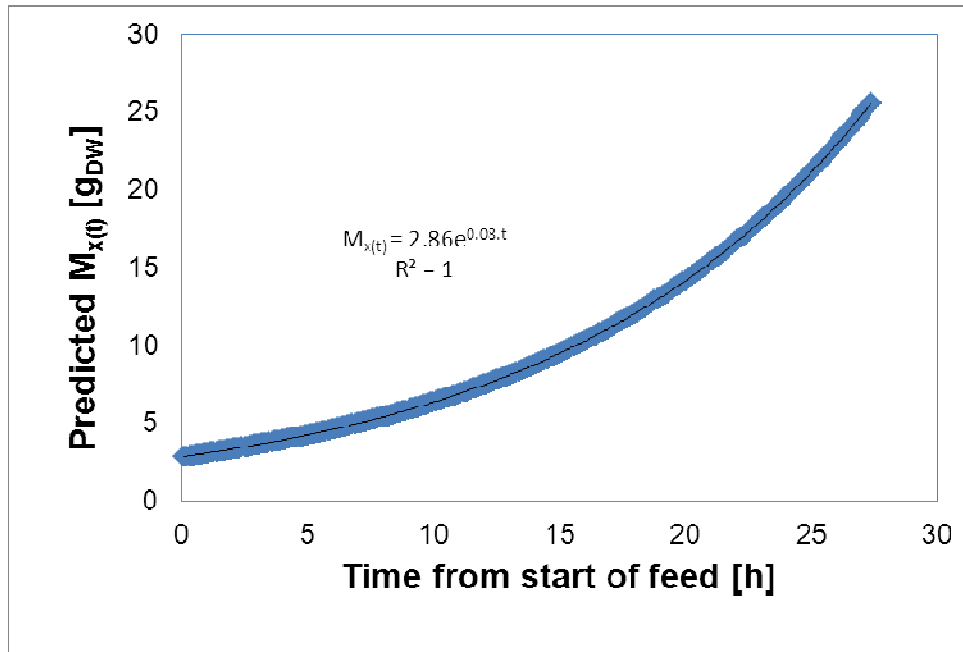


Figure 1: Predicted biomass production $M_{x(t)}$ during exponential feeding phase.

A typical calculation, considering time point 1 (21.5 h) and time point 2 (30 h), is as follows:

$$M_{x(t_2)} = M_{x(t_1)} e^{\mu_{set}(t_2 - t_1)} \quad (\text{Appendix A, Equation 20}) \quad \text{Equation 1}$$

$$M_{x(t_2)} = 2.86 e^{0.08(30 - 21.5)} = 5.6 \text{ g}_{\text{DW}} \quad \text{Equation 2}$$

And similarly, to determine $M_{x(t_3)}$ considering time point 2 (30 h) and time point 3 (33 h), is as follows:

$$M_{x(t_3)} = 5.6 e^{0.08(33 - 30)} = 7.12 \text{ g}_{\text{DW}} \quad \text{Equation 3}$$

The general equation for the predicted total amount of biomass at time point $i+1$ ($M_{x(i+1)}$) can then be described as in Equation 4.

$$M_{x(t_{i+1})} = M_{x(t_i)} e^{\mu_{set}(t_{i+1}-t_i)} = M_{x(t_i)} e^{0.08(t_{i+1}-t_i)} \quad \text{Equation 4}$$

And to predict the amount of biomass in the fermentor in equal increments of 10 seconds (Equation 5) an identical profile to Figure 1 is obtained:

$$M_{x(t_{i+1})} = M_{x(t_i)} \cdot e^{0.08 \cdot 10 \left[\frac{\text{sec}}{\text{sec}} \cdot \frac{1[\text{min}]}{60[\text{sec}]} \cdot \frac{1[\text{h}]}{60[\text{min}]} \right]} = M_{x(t_i)} \cdot e^{0.000222} = M_{x(t_i)} \cdot 1.000222 \quad \text{Equation 5}$$

Using the predicted $M_{x(t)}$ data (Figure 1) and the biomass yield coefficient, Y_{xs} ($\text{g}_{\text{DW}} \cdot \text{g}_{\text{glucose}}^{-1}$) (Table1), together with Equation 21 from Appendix A, a profile of the mass of glucose required for consumption ($M_{sf(t)}$), from the start to the end of the feed, to produce the desired mass of biomass at μ_{set} was determined (Figure 2).

A typical calculation to determine $M_{sf(t_2)}$, considering time point 1 (21.5 h) and time point 2 (30 h), is as follows:

$$M_{sf(t_2)} = \frac{M_{x(t_1)} (e^{\mu(t_2-t_1)} - 1)}{y_{xs}} + M_{sf(t_1)} \quad (\text{Appendix A, Equation 21}) \quad \text{Equation 6}$$

$$M_{sf(t_2)} = \frac{2.86 \cdot (e^{0.08(30-21.5)} - 1)}{0.56} + 0 = 4.97 \text{ g}_{\text{glucose}} \quad \text{Equation 7}$$

And similarly, to determine $M_{sf(t_3)}$ considering time point 2 (30 h) and time point 3 (33 h), is as follows:

$$M_{sf(t_3)} = \frac{5.6 \cdot (e^{0.08(33-30)} - 1)}{0.56} + 4.97 = 7.71 \text{ g}_{\text{glucose}} \quad \text{Equation 8}$$

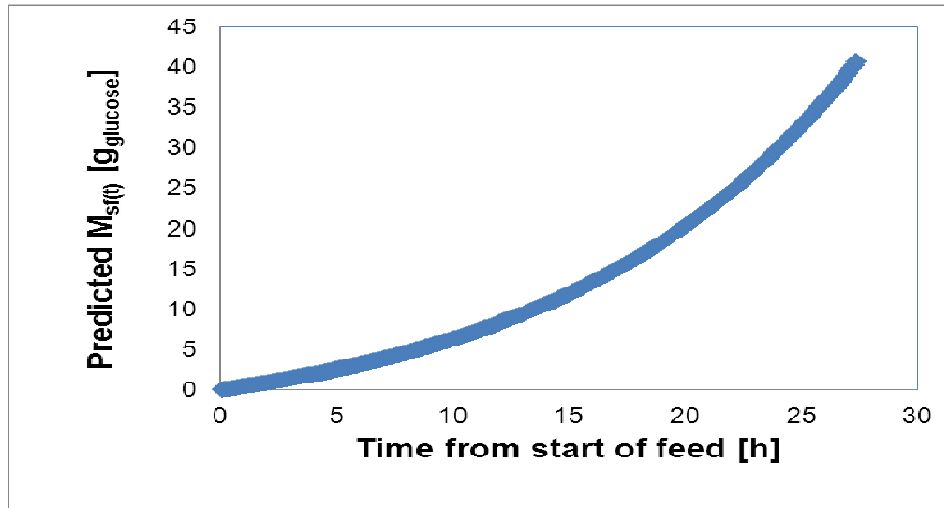


Figure 2: Predicted mass of glucose $M_{sf(t)}$ required during exponential feeding phase to control μ at μ_{set} .

Thus the mass of glucose required to be pumped in to the vessel is determined. For example, considering time point 2 (30 h) and time point 3 (33 h), the mass of new biomass generated between time point 3 and time point 2 ($\Delta M_{x(3-2)}$) at the $\mu_{set} = 0.08 \text{ h}^{-1}$ is described as Equation 9.

$$\Delta M_{x(3-2)} = M_{x3} - M_{x2} = 7.12 - 5.6 = 1.62 \text{ g}_{\text{DW}} \quad \text{Equation 9}$$

And the mass of glucose required to maintain the new biomass generation between time point 3 and time point 2 ($\Delta M_{sf(3-2)}$) at the $\mu_{set} = 0.08 \text{ h}^{-1}$ is described as Equation 10.

$$\Delta M_{sf(t_{3-2})} = M_{sf(t_3)} - M_{sf(t_2)} = 7.71 - 4.97 = 2.74 \text{ g}_{\text{glucose}} \quad \text{Equation 10}$$

Using the method of Equation 10, a profile of the mass of glucose required to be pumped in every 10 seconds during the feeding phase is then determined (Figure 3) i.e. $\Delta M_{sf(i+1)} = M_{sf(i+1)} - M_{sf(i)}$, and can be defined as the predicted mass of glucose required to be pumped in the fermentor in 10 second intervals (Equation 11):

$$\Delta M_{sf(t_{i+1})} = \Delta M_{sf(t_i)} \cdot e^{0.08 \cdot 10 [\text{sec}] \cdot \frac{1 [\text{min}]}{60 [\text{sec}]} \cdot \frac{1 [\text{h}]}{60 [\text{min}]}} = \Delta M_{sf(t_i)} \cdot e^{0.000222} = \Delta M_{sf(t_i)} \cdot 1.000222 \quad \text{Equation 11}$$

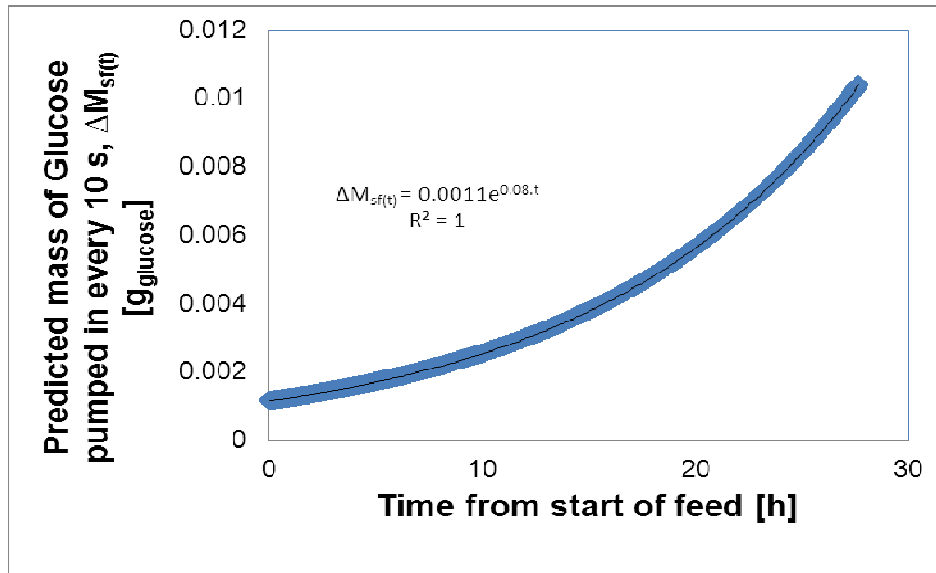


Figure 3: Predicted mass of glucose $\Delta M_{sf(t)}$ required to be pumped in every 10 seconds during exponential feeding phase to control biomass growth ($\Delta M_{x(t)}$) at μ_{set} .

With a 150 g.L^{-1} concentrated glucose feed reservoir (Table 1) a profile of the volume of glucose to be added to the reactor, $\Delta V_{sf(t)}$ is determined. A typical calculation, considering the mass of glucose to be added between time point 2 and time point 3, $\Delta M_{sf(3-2)} = 2.74$, to determine $\Delta V_{sf(3-2)}$, is as follows:

$$\Delta V_{sf(t_{3-2})} = \frac{\Delta M_{sf(t_{3-2})}}{C_{sf}} \quad \text{Equation 12}$$

$$\Delta V_{sf(t_{3-2})} = \frac{2.74 \left[\frac{g_{glucose}}{L} \right]}{150 \left[\frac{g_{glucose}}{L} \right]} \cdot \frac{1000 [ml]}{1 [L]} = 18.3 \text{ ml}_{glucose} \quad \text{Equation 13}$$

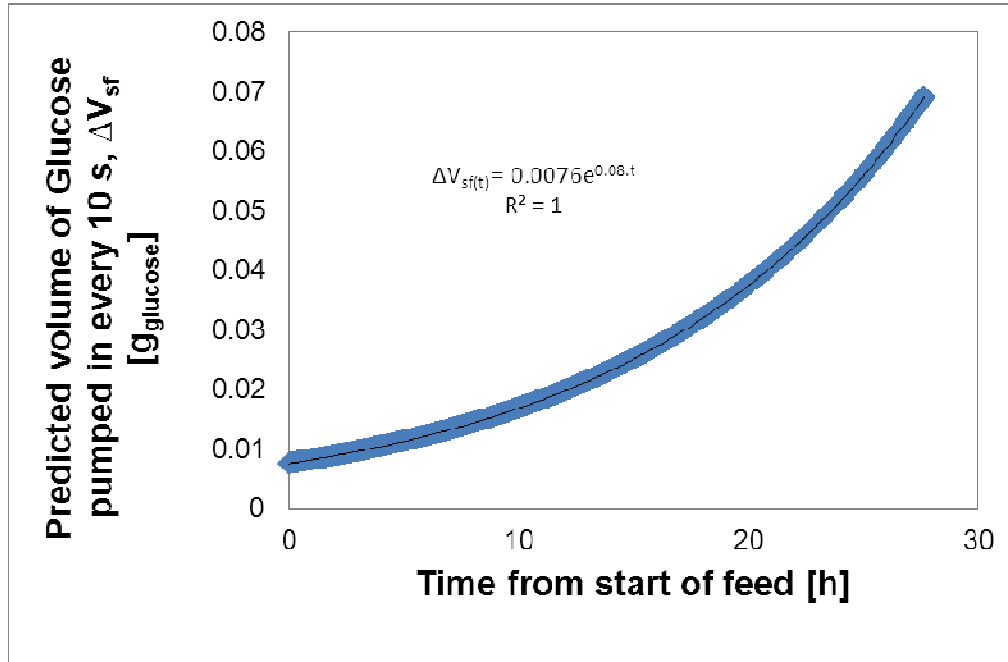


Figure 4: Predicted volume of glucose $\Delta V_{sf(t)}$ (glucose concentration of feed reservoir at $C_{sf} = 150 \text{ g}_{glucose} \cdot L^{-1}$) to be pumped in every 10 seconds during exponential feeding phase to control biomass growth ($\Delta M_{x(t)}$) at μ_{set} .

The predicted volumetric feed profile $\Delta V_{sf(t)}$ as a function of time in 10 second increments shown in Figure 4 is described in Equation 14, where 0,0076 is the initial volume $\Delta V_{sf(1)}$ in ml to be pumped into the vessel at the start of the feeding phase.

$$\Delta V_{sf(t)} = 0.0076 \cdot e^{0.08 \cdot t} \quad \text{Equation 14}$$

And similarly to Equation 11 for the predicted mass of glucose required to be pumped in the fermentor ($\Delta M_{sf(i+1)}$), the predicted volume of glucose required to be pumped in the fermentor in 10 second intervals ($\Delta V_{sf(i+1)}$) can be defined as in Equation 15.

$$\Delta V_{sf(t_{i+1})} = \Delta V_{sf(t_i)} \cdot e^{0.08 \cdot 10 \frac{[\text{sec}]}{60} \cdot \frac{1[\text{min}]}{60} \cdot \frac{1[\text{h}]}{60}}{60}} = \Delta V_{sf(t_i)} \cdot e^{0.000222} = \Delta V_{sf(t_i)} \cdot 1.000222 \quad \text{Equation 15}$$

Once the predicted volumetric glucose feed profile to be added to the fermentor every 10 seconds $\Delta V_{sf(t)}$ is determined (Figure 4), the Infors fermentor substrate pump is calibrated with respect to a particular silicon tube diameter (Figure 5).

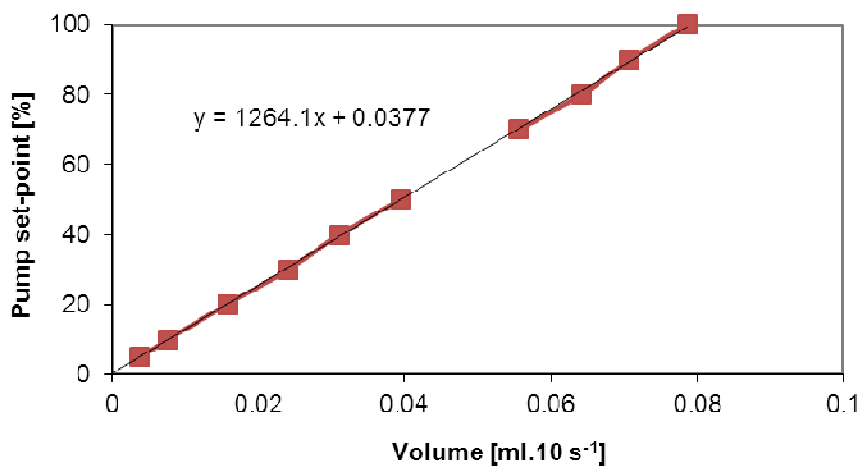


Figure 5: Typical Infors fermentor substrate pump calibration curve.

The required volumetric glucose feed profile ($\Delta V_{sf(t)}$) (Figure 4) is then converted into the required pump set-point $P_{sf(t)}$ using the standard curve in Figure 5 (Figure 6). A typical calculation is as follows: consider the initial volume $\Delta V_{sf(1)}$ in ml to be pumped into the vessel at the start of the feeding phase, 0.0076 ml (Figure 4). Using the pump calibration curve in Figure 5 the pump set point is determined (Equation 14).

$$P_{sf(t_i)} = (1264.1 \cdot \Delta V_{sf(t_i)} + 0.0377) \quad \text{Equation 16}$$

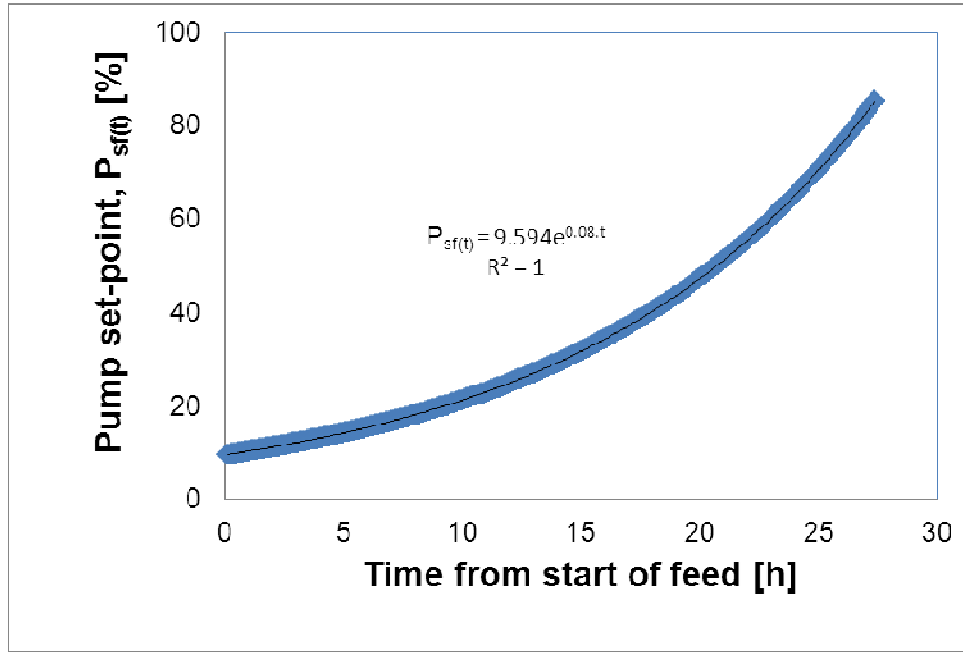


Figure 6: Predicted Infors substrate pump set-point profile ($P_{sf(t)}$) required during exponential feeding phase to control μ at μ_{set} .

An algorithm was then programmed into the Infors Fermentor software batch recipe using the data calculated from Figure 7, where the pump set-point profile was calculated to be $9.594e^{0.08t}$. The term, 9.594 is the initial pump set-point at the beginning of the exponential phase ($P_{sf(1)}$) and was programmed to increase by a factor of 1.000222 every 10 seconds (Equation 15). The term 1.000222 is calculated from the $e^{0.08t}$ term whereby $1.000222 = e^{0.08(10/60/60)}$ to obtain 't' with respect to 10 second increments.

$$P_{sf(t_{i+1})} = P_{sf(t_i)} \cdot e^{0.08 \cdot 10 \left[\frac{\text{sec}}{\text{sec}} \cdot \frac{1[\text{min}]}{60[\text{sec}]} \cdot \frac{1[\text{h}]}{60[\text{min}]} \right]} = P_{sf(t_i)} \cdot e^{0.000222} = P_{sf(t_i)} \cdot 1.000222 \quad \text{Equation 17}$$

Thus, to determine the pump set-point (P_{sf}) as a function of $M_X(t)$ Equation 18 and 19 are derived from Equations 16, 12, and 6, where the term $e^{\mu \cdot (t_{i+1} - t_i)}$ is a function of 10 second increments.

$$P_{sf(t_{i+1})} = \left[1264.1 \cdot \frac{M_{x(t_i)} \cdot (e^{\mu(t_{i+1}-t_i)} - 1)}{Y_{sx} \cdot C_{sf}} + 0.0377 \right] \quad \text{Equation 18}$$

$$P_{sf(t_{i+1})} = \left[3.345 \cdot M_{x(t_i)} + 0.0377 \right] \quad \text{Equation 19}$$

$M_{x(t)}$ profile Correction Calculations

Therefore, as already mentioned (Exponential Fed-batch Methodology and Typical Calculations), once the initial charge of glucose (10 g_{glucose}/kg_{broth}) was depleted in the batch phase, as estimated by the sudden increase in dissolved oxygen, the exponential feed was initiated. Samples were taken in increments ranging between 3 and 8 hours. Due to the size of the fermentor, together with the high concentration of the glucose feed and the associated small dosage requirements of the feed to control μ at μ_{set} , the loss of volume in the reactor due to sampling was not assumed negligible and was accounted for. The following calculations are a typical account of the methods used to adjust the pump set-point, in response to reactor volume changes by sampling. Consider time point 2, where according to Equation 20 in Appendix A and $M_{x1} = 2.86$ g_{DW} in Figure 1, $(M_{x2})_{before\ sampling} = 5.6$ g_{DW} is the predicted biomass after 8.4 hours from the commencement of the exponential feed, before sampling, with an associated biomass concentration $(C_{x2})_{before\ sampling} = 5.6$ g_{DW}/1.3 L = 4.3 g_{DW}·L_{broth}⁻¹. After sampling, ~49 ml of culture broth was removed from the fermentor. Assuming $(C_{x2})_{before\ sampling} = (C_{x2})_{after\ sampling} = (C_{x2})_{sample}$, biomass after sampling can be adjusted, $(M_{x2})_{after\ sampling} = (M_{x2})_{before\ sampling} - (C_{x2})_{sample} \cdot V_{sample} = 5.4$ g_{DW}. The adjusted $(M_{x2})_{after\ sampling}$ is then used to correct the required feed ΔV_{sf} by adjusting the associated pump set-point P_{sf} (Equation 19). Thus prior to sampling, M_{x2} was predicted to be 5.6 g_{DW} with an associated feed pump setting $P_{sf} = 18,769\%$. With the associated loss of biomass due to sampling, $(M_{x2})_{after\ sampling}$ was

predicted to be 5.4 g_{DW} with an associated feed pump setting (as calculated by Equation 19) $P_{sf} = (3.345) \cdot (5.4) + 0.0377 = 18.100\%$. Thus, from this time point onwards, a revised profile for the predicted $M_{x(t)}$, $\Delta V_{sf(t)}$, and $P_{sf(t)}$ was determined to control μ at μ_{set} . This profile was maintained until the next sampling time was performed, where upon, similarly to the procedures described above, the associated loss of biomass due to sampling was again revised and the P_{sf} adjusted.

Thus Figure 7 illustrates the comparison between; (1) the predicted $M_{x(t)}$ profile **without** sample volume adjustment, (2) the predicted $M_{x(t)}$ profile **with** sample volume adjustment, and (3) the $M_{x(t)}$ profile as determined from the dry weight analysis.

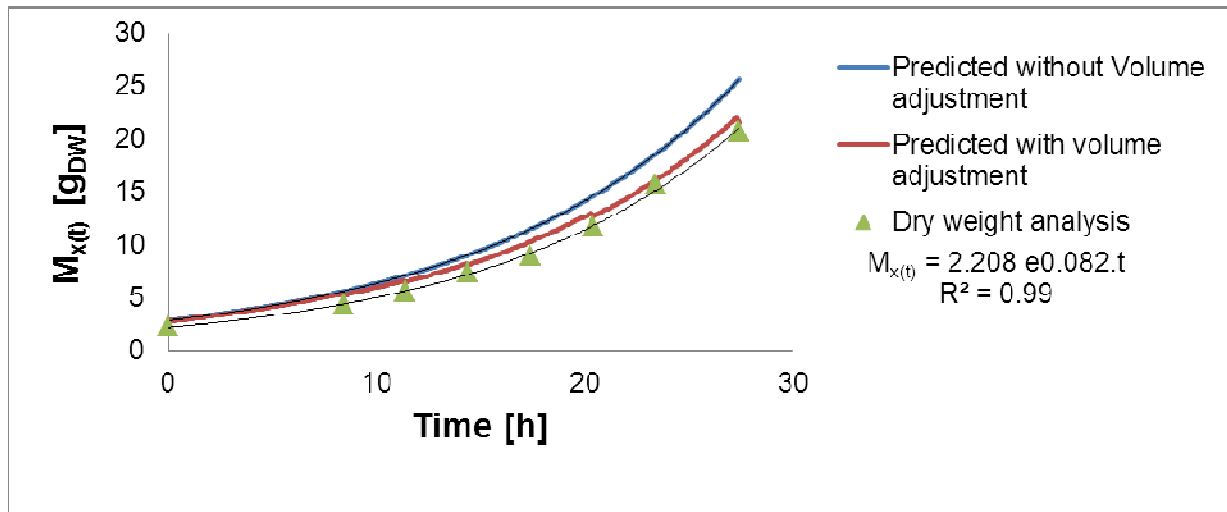


Figure 7: $M_{x(t)}$ profile during exponential feeding phase to control μ at μ_{set} of; (1) the predicted $M_{x(t)}$ profile **without** sample volume adjustment, (2) the predicted $M_{x(t)}$ profile **with** sample volume adjustment, and (3) the $M_{x(t)}$ profile as determined from the dry weight analysis.

Figure 8 portrays the $\Delta V_{sf(t)}$ profile during exponential feeding phase to control μ at μ_{set} of; (1) the predicted $\Delta V_{sf(t)}$ profile **without** sample volume adjustment, and (2) the predicted $\Delta V_{sf(t)}$ profile **with** sample volume adjustment.

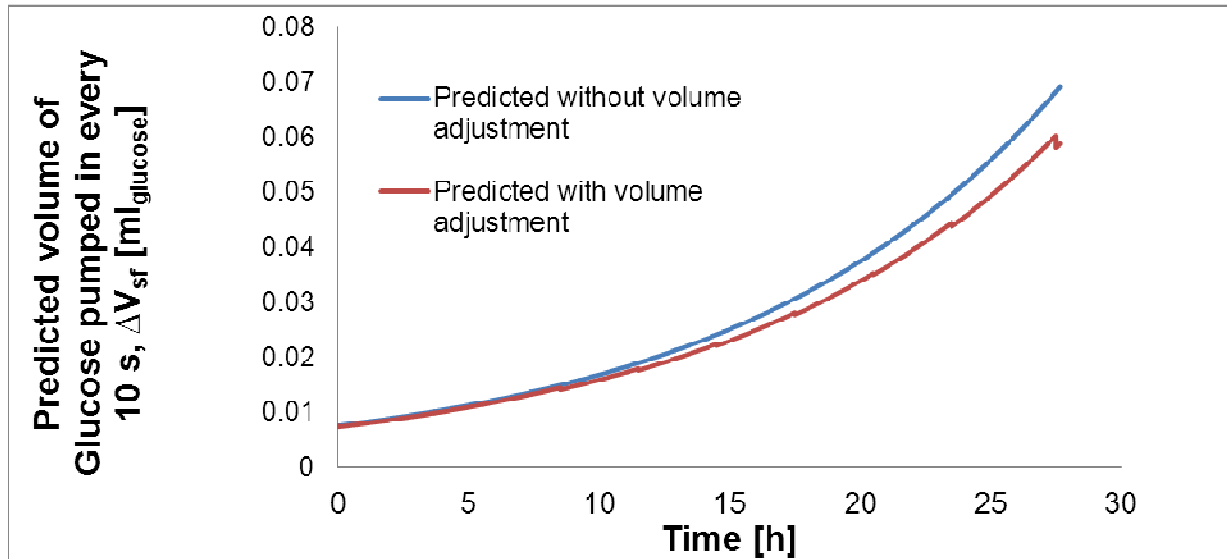


Figure 8: $\Delta V_{sf(t)}$ profile during exponential feeding phase to control μ at μ_{set} of; (1) the predicted $\Delta V_{sf(t)}$ profile **without** sample volume adjustment, and (2) the predicted $\Delta V_{sf(t)}$ profile **with** sample volume adjustment.

Figure 9 portrays the $M_{sf(t)}$ profile during exponential feeding phase to control μ at μ_{set} of; (1) the predicted $M_{sf(t)}$ profile **without** sample volume adjustment, and (2) the predicted $M_{sf(t)}$ profile **with** sample volume adjustment.

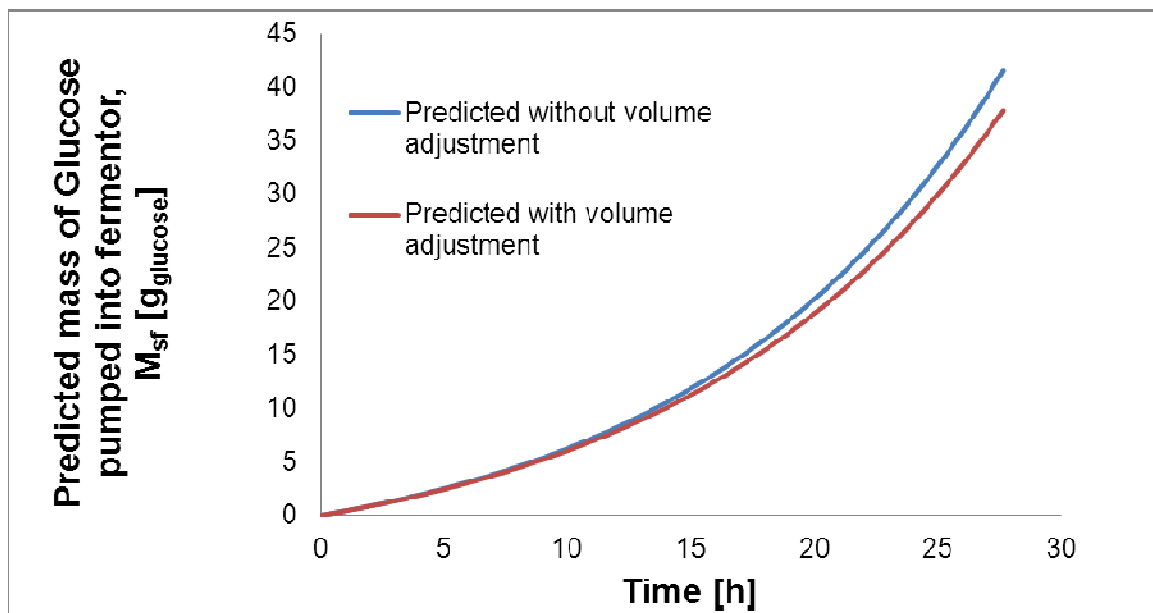


Figure 9: $M_{sf(t)}$ profile during exponential feeding phase to control μ at μ_{set} of; (1) the predicted $M_{sf(t)}$ profile **without** sample volume adjustment, and (2) the predicted $M_{sf(t)}$ profile **with** sample volume adjustment.

Figure 10 portrays the $P_{sf(t)}$ profile during exponential feeding phase to control μ at μ_{set} of; (1) the predicted $P_{sf(t)}$ profile **without** sample volume adjustment, and (2) the predicted $P_{sf(t)}$ profile **with** sample volume adjustment.

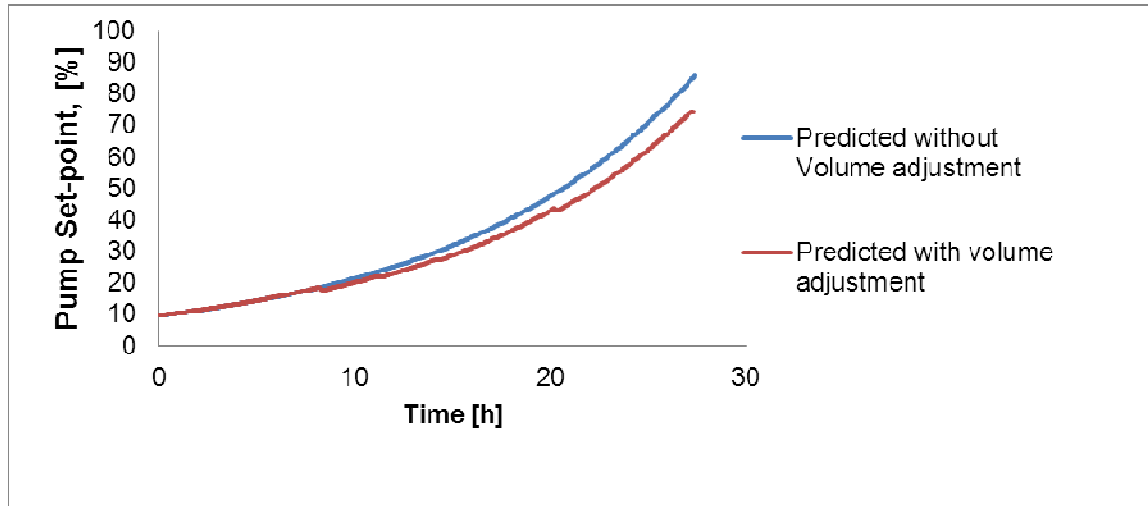


Figure 10: $P_{sf(t)}$ profile during exponential feeding phase to control μ at μ_{set} of; (1) the predicted $P_{sf(t)}$ profile **without** sample volume adjustment, and (2) the predicted $P_{sf(t)}$ profile **with** sample volume adjustment.

To verify μ , the $\ln(M_{x(t)})$ profiles of Figure 7 are determined (Figure 11). From Figure 11 it is evident that the predicted $\ln(M_{x(t)})$, predicted without sample volume adjustment, obtained a controlled $\mu = 0.08 \text{ h}^{-1}$ with an associated initial $\ln(M_{x(1)})$ of 1.051. The predicted $\ln(M_{x(t)})$, taking into account volume adjustment due to sampling, was also controlled at $\mu = 0.08 \text{ h}^{-1}$ with an associated initial $\ln(M_{x(1)})$, which ranged between 0,926 and 1,051 and changed every time the $M_{x(t)}$ data was adjusted to account for the loss of biomass from sampling. In comparison, the $M_{x(t)}$ data resulting from the dry weight analysis obtained a controlled $\mu = 0.082 \text{ h}^{-1}$ with an associated initial $\ln(M_{x(1)})$ of 0.789 and an acceptable deviation of 0.02 off the set-point of $\mu_{set} = 0.08 \text{ h}^{-1}$.

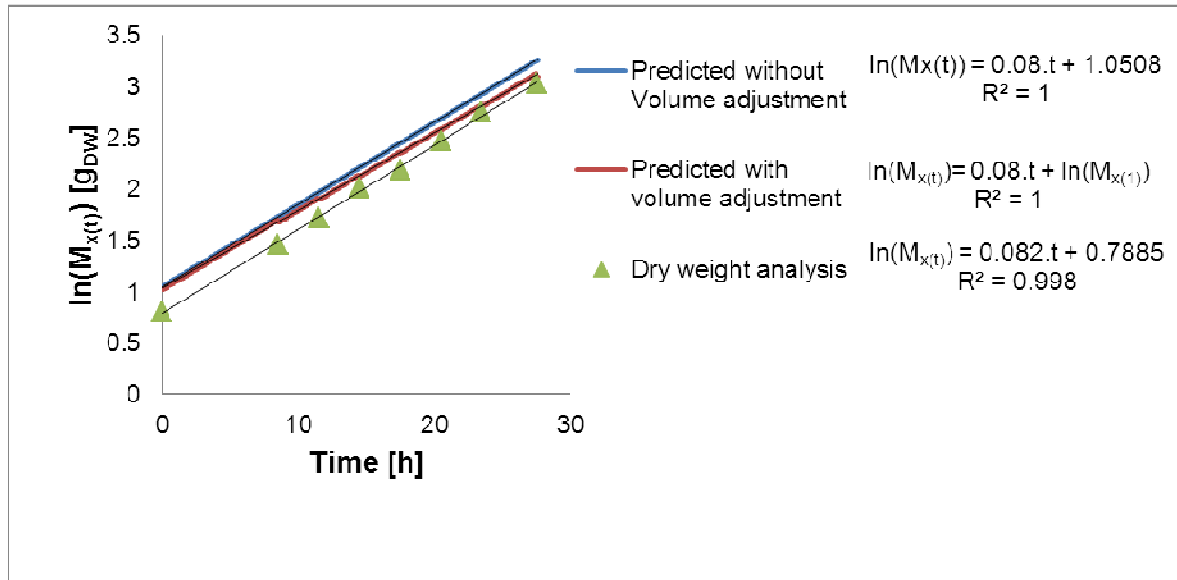


Figure 11: $\ln(M_{x(t)})$ profile during exponential feeding phase to control μ at μ_{set} of; (1) the predicted $\ln(M_{x(t)})$ profile **without** sample volume adjustment, (2) the predicted $\ln(M_{x(t)})$ profile **with** sample volume adjustment, and (3) the $\ln(M_{x(t)})$ profile as determined from the dry weight analysis.

The approach of controlling the amount of concentrated glucose substrate added in the fed-batch cultivation (equation 3), while glucose residual concentration was maintained at zero, results in the control of the μ_{set} at a constant value under both transient and steady state conditions for biomass concentration (Martinez *et al.*, 1998; Kim *et al.*, 2000; Cheng *et al.*, 2003; Sun *et al.*, 2007; Chen *et al.*, 2008). The equations derived in Appendix A together with the typical calculations described in this section of the fast growing high biomass producing transformant DAHB21#20 to control $\mu_{set} = 0.08 \text{ h}^{-1}$, gives an account of the implementation of the Exponential Fed-batch methodology to execute the exponential fed-batch cultivations described in Manuscript 3 and Manuscript 4 of this dissertation.

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