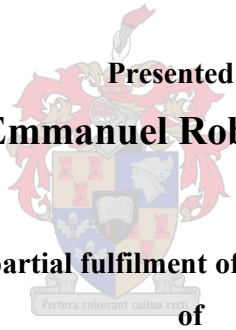
***UNIVERSITY OF STELLENBOSCH***

# OPTIMISATION OF A RECOMBINANT HEPATITIS B VACCINE THROUGH THE CULTIVATION AND FERMENTATION OF *ASPERGILLUS NIGER*

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**Supervised by**

**Dr J. Görgens and Prof. W. H. van Zyl**

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**DECLARATION**

I , the undersigned hereby declare that the work contained in this document is of my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

The experiments in this thesis constitute work carried out by the candidate unless otherwise stated and complies with the stipulations set out for the degree of Masters of Science in Process Engineering, by the University of Stellenbosch.

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## This Work Is Dedicated To

My Mother and My Father

and

All my Teachers

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# I SYNOPSIS

The development of non-replicating vaccines is an emerging option for safe, effective vaccines, several of which contain virus-like particles (VLPs). Many recombinant expression systems have been evaluated as hosts for VLP production for the prevention of infectious diseases. The filamentous fungi *Aspergillus niger* has emerged as a potential alternative expression system for cost effective VLP vaccine production. Hepatitis B surface antigen (HBsAg) was used as a model VLP product to benchmark *A. niger's* production capacity with those of *Saccharomyces cerevisiae*, *Pichia pastoris* and *Hansenula polymorpha*. Bioprocessing strategies were used to optimise VLP production by recombinant *A. niger* in batch culture. In particular, the effect of the parameters culture temperature, inoculum concentration, agitation intensity, dissolved oxygen ($dO_2$) concentration and culture pH on biomass formation, morphology and VLP (HBsAg) production concentration was quantified. At an optimum agitation of 100 rpm and optimum $dO_2$ concentration of 50 %, HBsAg production levels were increased 9-fold compared to yields obtained in shakeflask cultivation. Highest HBsAg production levels of 3.6 $mg.\ell_{culture}^{-1}$ and 350 $\mu g.g_{DW}^{-1}$ were recorded, at a biomass concentration of 10.5 $g_{DW}.\ell_{culture}^{-1}$. These production levels compare favourable with those obtained by other production systems under similar conditions. HBsAg VLPs mostly accumulated intracellularly, although under optimum bioreactor conditions significant HBsAg accumulation in the cytoplasm and culture supernatant was also observed. The impact of these process parameters on VLP production and cell morphology was attributed to environmental stress conditions. Volumetric biomass and HBsAg production levels were maximised under conditions of lowest environmental stress, resulting in the most optimal small-pelleted morphology. These results indicate a substantial potential for further engineering of the *A. niger* production system for the high level of intracellular and extracellular VLP production.

## OPSOMMING

Die ontwikkeling van nie-repliserende vaksienes is tans ‘n ontluikende opsie vir die produksie van veilig en effektiewe vaksienes, waarvan meeste pseudovirale partikels (VLPs) bevat. ‘n Groot aantal potensiele gasheer organismes is reeds ge-evalueer om te dien as rekombinante uitdrukkings sisteme vir die produksie van psuedovirale partikels. ‘n Filamentagtige fungi, *Aspergillus niger*, toon groot potensiaal om as ‘n alternatiewe gasheer te dien vir die produksie van pseudovirale partikels. ‘n Rekombinante stam van die fungi, wat die Hepatitis B oppervlak antigeen (HBsAg) uitdruk, is gebruik om die produksie kapasiteit van *A. niger* te evalueer en te vergelyk met standaard vlakke van produksie sisteme wat gegrond is op *Saccharomyces cerevisiae*, *Pichia pastoris* en *Hansenula polymorpha*. Die produksie van die Hepatitis B pseudovirale partikels, deur die rekombinante *A. niger* stam in ‘n lot kultuur, is geoptimaliseer deur middel van bioproseserings strategïe. Die effek van bioproseserings parameters insluitend temperatuur, inokulum konsentrasie, meng temp, $dO_2$ konsentrasie en pH, op biomassa konsentrasie, selmorfologie en pseudovirale partikel konsentrasie, is geanaliseer. Daar is vasgestel dat ‘n 9-voud verhoging, in vergelyking met skudfles kulture, in die HBsAg produksie vlakke bereik is by ‘n optimale meng tempo van 100 rpm en $dO_2$ konsentrasie van 50 %. Die hoogste gedokumenteerde HBsAg produksie vlakke was 3.6 $mg.\ell_{kultuur}^{-1}$ en 350 $\mu g.g_{DW}^{-1}$ by ‘n biomassa konsentrasie van 10.5 $g_{DW}.\ell_{kultuur}^{-1}$. In vergelyking met produksie vlakke gedokumenteer uit ander produksie sisteme, was die waardes verkry tydens hierdie studie goed vergelykbaar. Die HBsAg pseudovirale partikels het meestal intrasellulêr geakkumuleer, maar tydens optimale bioreaktor kondisies is daar ‘n noemswaardige hoeveelheid HBsAg in die sitoplasma en die kultuur bostand waargeneem. Die impak van die bioproseserings parameters op die produksie van pseudovirale partikels kan toegeskryf word aan omgewings druk kondisies. Biomassa en HBsAg produksie vlakke kon dus verhoog word deur die vermindering van omgewings druk, wat terselfde tyd korrel-agtige morfologie teweë gebring het. Hierdie studie het bewys dat daar aansienlike potensiaal is om hoë vlakke van beide intrasellulêre en ekstrasellulêre pseudovirale partikel produksie te bereik deur verdere ontwikkeling van die *A. niger* produksie sisteem.

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# III LIST OF ABBREVIATIONS

**TABLE III.1:** List of Abbreviations.

| Abbreviation | Full Text |
|---|---|
| ADH | Alcohol dehydrogenase |
| AIDS | Acquired Immune deficiency syndrome |
| AOX | Alcohol Oxidase |
| Au | Australian antigen |
| BSA | Bovine Serum Albumin |
| CCRD | Central Composite Rotary Design |
| CHO | Chinese Hamster Ovary |
| CMIA | Chemiluminescent Micoparticle |
| DST | Department of Science and Technology |
| DW | Dry weight |
| EPI | Expanded Programme of Immunisation |
| ER | endoplasmic reticulum |
| FCCC | Fox Chase Cancer Centre |
| FDA | Food and Drug Administration |
| GPD | Glyceraldehyde-3-phosphate |
| GRAS | Generally Regarded As Safe |
| HBsAg | Hepatitis B surface Antigen |

**TABLE III.1**: List of Abbreviations (Continued).

| Abbreviation | Full Text |
|---|---|
| HBV | Hepatitis B Virus |
| HEWL | Hen Egg White Lysozyme |
| HBV | Hepatitis B Virus |
| HEWL | Hen Egg White Lysozyme |
| HPLC | High Pressure Liquid Chromatography |
| HIV | Human Immunodeficiency virus |
| L protein | Large protein |
| M protein | Middle protein |
| MOX | Methanol oxidase |
| NBS | New Brunswick Scientific |
| OTR | Oxygen Transfer Rate |
| OUR | Oxygen Uptake Rate |
| PGK | 3-phosphoglycerate kinase |
| ROS | Reactive oxygen species |
| S protein | Major protein |
| VLP | Virus-like particle |
| vvm | Volume of gas inlet flow rate per volume of |
| WHO | World Health Organisation |

# IV LIST OF SYMBOLS

**TABLE IV.1:** List of Symbols

| Symbols | Units |
|---|---|
| $a_w$ | Water activity, % |
| $C_L$ | Dissolved oxygen concentration ($mol.m^{-3}$) |
| $C_L^o$ | Psuedo-steady-state dissolved oxygen concentration at 0.242 $mol.m^{-3}$ |
| $C_L^*$ | Dissolved oxygen concentration in equilibrium with gaseous oxygen concentration ($mol.m^{-3}$) |
| $k_La$ | Volumetric mass transfer coefficient |
| P | Pressure (Pa) |
| $Q_G$ | Gas flow rate ($m^3.s^{-1}$) |
| $Q_{O_2}X$ | Oxygen uptake rate ($mol.m^{-3}.s^{-1}$) |
| OTR | Oxygen transfer rate ($mol.m^{-3}.s^{-1}$) |
| OUR | Oxygen uptake rate ($mol.m^{-3}.s^{-1}$) |
| R | Gas constant (8.306 $Pa.m^3.(mol.K)^{-1}$) |
| Specific OUR | Specific oxygen uptake rate ($mol.g_{DW}^{-1}.s^{-1}$) |
| T | Temperature (K) |
| $V_L$ | Liquid volume in bioreactor ($m^3$) |
| y | gaseous mole fraction |

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# 1 INTRODUCTION

Besides malaria and HIV/AIDS, Hepatitis B represents one of the major diseases facing humanity in Africa and elsewhere in the world (WHO, 2004). An estimated 2 billion people have been infected by the hepatitis B virus (HBV) and 15-17 % of those infected become chronic carriers, with the highest endemicity occurring in developing countries (Beutel, 1998; Kane, 1998).

The development of safe and efficient vaccines have been one of the most far-reaching and important public health initiatives of the 20$^{th}$ century. Considerable attention is being paid to the development of subunit vaccines as a means of curbing the spread of infectious disease (Hansson *et al.*, 2000).Vaccines for the hepatitis B virus (HBV), produced by recombinant yeast and mammalian cultures with excellent safety and efficacy profiles, have been commercially available for the last 20 years, and have proved to be an example of a very successful production process based on the application of modern techniques in biotechnology. The vaccine has been successfully incorporated into universal infant immunization programs in a 100 countries and from an economic standpoint compare favourably to other EPI (Expanded Program of Immunization) vaccines (Kane, 1998).

Despite this success, the purchasing price of the vaccine still represents the principal barrier to the vaccine's use in many developing countries, especially Africa and Asia where virus endemicity are highest and government health care is lowest (Beutel, 1998; Kane, 1998). However, with the recent expiration of the original patents on the production of this vaccine, the opportunity for the development of a cheaper, generic production process has become available. In an effort to develop such a generic process, investigations have been carried out to identify alternative production hosts capable of succeeding current recombinant based systems. There are five major microbial and eukaryotic expression hosts that are commonly used to produce recombinant vaccine antigens; bacterial, yeast, insect, mammalian and transgenic plant expression systems.

However, each has manifested limitations, such as absence of phosphorylation and posttranslational processing in bacteria, hyperglycosylation with the yeast *Saccharomyces cerevisiae*, expensive media demands for insect and animal cells, and long development times and low production levels for transgenic plant cells.

## 1.1 Background

Within the last decade, the cultivation of filamentous fungi has brought much attention upon itself. Filamentous fungi are eukaryotic microorganisms that influence our everyday lives in diverse areas such as medicine, agriculture, and basic science. The use of filamentous fungi for the production of commercial products is not a recent discovery, but an ancient practise, which has increased rapidly over the last 50 years. With the advent of gene technology, this capability has been further expanded and now fungi are employed to produce not only homologous, but heterologous proteins as well (Davies, 1994; Kinghorn and Unkles, 1994; van den Hondel *et al.,* 1991). They have been extensively used in industrial processes and have been harnessed for the production of antibiotics, enzymes, fermented foods, vitamins, pharmaceuticals and organic acids, to name just a few, and therefore large-scale fermentation technology and downstream processing are already well established (Bodie *et al.,* 1994).

Traditionally, bacteria, yeast and mammalian cells have been the workhorses for recombinant heterologous protein production in the biopharmaceutical industry. However, each has manifested limitations, such as hyperglycosylation with yeast, absence of glycosylation and phosphorylation in bacteria, and expensive media demands for animal cells. Compared to the above-mentioned expression systems, several characteristics of filamentous fungi as expression hosts present potential advantages, particularly their efficient secretion of endogenous proteins. The issue of hyperglycosylation is less of a problem in filamentous fungi compared to *S. cerevisiae*, as their glycosylation patterns closely resemble those found in mammalian cells. Filamentous fungi can also be described as robust and can grow on various substrates

and organic compounds (Jeenes *et al.*, 1991; van den Hondel *et al.*, 1991). They are known to produce and secrete enzymes in large quantities and through their use in the food and food processing industry, the species *Aspergillus oryzae* and *Aspergillus niger* have gained GRAS (Generally Regarded As Safe) status. Several heterologous proteins of pharmaceutical importance have also been successfully produced in *Aspergillus*; tissue plasminogen activator (1 $mg.\ell_{culture}^{-1}$) (Upshall *et al.*, 1987), human interleukin-6 (5-10 $mg.\ell_{culture}^{-1}$) (Gouka and van den Hondel, 1997), human lactoferrin (2 $g.\ell_{culture}^{-1}$) (Ward *et al.*, 1995), human interferon α-2 (1 $mg.\ell_{culture}^{-1}$) (Gwynne *et al.*, 1987) and an industrial process for human superoxide dismutase via intracellular expression in *Aspergillus nidulans* has been developed (Davies, 1994).

With the increasing demand for vaccines as a means to control and eradicate disease and the inherent advantages of filamentous fungi as a heterologous protein production system, the evaluation of *Aspergillus* for the production of complex immunogenic viral proteins has emerged as a promising alternative to current subunit vaccine production technologies. For HBsAg virus-like particle (VLP) production, *A. niger* was transformed with the Hepatitis B virus *S* gene encoding the major viral envelope protein (Plüddemann and van Zyl, 2003). Production of the 24 kDa S protein, as well as a 48 kDa S protein dimer, in the membrane-associated protein fraction of the recombinant *A. niger* strain was observed. The yield of Hepatitis B pseudoviral particles, with a diameter of 22 nm, from mycelium of the recombinant *Aspergillus* strain was estimated at 0.4 $mg.\ell_{culture}^{-1}$ and 200 $\mu g.g_{DW}^{-1}$ during shakeflask cultivation (Plüddemann and van Zyl, 2003). These results compared favourably with the reported levels initially obtained in yeasts (Valenzuela, 1982; Hsieh *et al.,* 1988; Gu *et al.,* 1991; Cregg *et al*, 1987) indicating the potential of the *Aspergillus* expression system as an alternative cost-effective vaccine production system.

In order to develop a successful, cost effective vaccine production system, bioreactor development and design are of paramount importance. This is not a simple taskdue 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 growth medium and physical environment. Many genes and physiological mechanisms are involved in the process of morphogenesis. In submerged culture, a large number of factors contribute to the development of any particular morphological form. Particular morphological forms achieve maximum performance and thus it is a very difficult task to deduce unequivocal general relationships between process variables, product formation, biomass concentration and fungal morphology. Too many parameters influence these interrelationships and the role of many of them is still not fully understood. Biochemical engineers are trying to elucidate these factors, in order to optimise operating conditions in bioreactors for product and process improvement.

## 1.2 Aim of research project

The inherent advantages of filamentous fungi as a heterologous protein production system merit the quantification of the capacity of *A niger* to produce and assemble complex immunogenic viral proteins into VLPs. This study addresses the production of HBsAg as a model VLP by recombinant *A. niger* by analysing the influence of the bioprocessing parameters, culture temperature, incoculum concentration, agitation intensity, dissolved oxygen ($dO_2$) concentration and culture pH, on VLP production during micro-pelleted growth in batch cultivation. The complex and dynamic relationships existing between the environmental conditions, VLP production and microbial behaviour are assessed. Benchmark comparisons set by alternative recombinant production systems *S. cerevisiae*, *P. pastoris* and *H. polymorpha* are used to determine the feasibility of the current *Aspergillus* system as a VLP production system.

Thus, in order to optimise the HBsAg VLP production, the following aspects will be the focus of attention:

1) The effect and impact the bioprocess parameters, temperature, inoculum concentration, pH, dissolved oxygen ($dO_2$) concentration and agitation intensity have on the following:
   a) Intracellular HBsAg production.
   b) Total cellular protein production.
   c) Macro-morphological properties.
   d) Rheological properties as well as the specific oxygen uptake rate (SOUR).
   e) Growth characteristics as well as biomass concentrations.

2) Plüddemann and van Zyl (2003) discovered that the HBsAg produced in the *A. niger* strain remains within intracellular sites associated with the membrane organelles. An objective of this study is to determine whether, under certain process conditions, HBsAg production can be associated not only intracellularly but released extracellularly as well.

3) The effect and impact cell fragmentation have on mycelial morphology and HBsAg production levels in terms of the following:
   a) Stability and robustness of HBsAg pseudoparticles assembly, by the protease-deficient *A. niger* strain, during the latter stages of the cultivation.

4) Compare optimum HBsAg production levels with other benchmark production systems based on the yeasts *S. cerevisiae, H. polymorpha* and *P. pastoris.*

Successful optimisation of the Hepatitis B vaccine on laboratory-scale will be complimented by further scale-up and bioprocess development in future investigations.

## 1.3 Implications of such a study

The present research project is a critical step towards the development of a South African production process for a recombinant Hepatitis B vaccine, which may have significant benefits to the African continent as a whole. The proposed local commercial process has the potential to significantly reduce the cost of the commercial vaccine by obtaining lower production and purification costs. Besides the economic benefits to the local government (South African intellectual property), South Africa can also help to improve the Hepatitis B vaccination status of the rest of Africa.

The present project is also in line with recent investments by the Department of Science and Technology (DST) in the development of a local biotechnology industry, which is considered as a major economic growth area for the future. The development of a biotechnology industry is dependent on the availability of chemical engineers with suitable training. The present project will directly contribute to this need.

The successful completion of the present research project will give South Africa First World recognition with regards to Biotechnology, contribute to the development of an engineering work force in biotechnology and advance the cause of lower Hepatitis B vaccination costs for Africa.

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# 2 LITERATURE REVIEW

## 2.1 Filamentous fungi, *Aspergillus niger*: Vaccine system of the future

### 2.1.1 Introduction

The use of filamentous fungi for the production of commercial products is an ancient practise, which has increased rapidly over the last 50 years. The fermentation of alcoholic beverages, practiced in the days of the Pharaohs, is one of the earliest known examples of the exploitation of the biochemical activities of a fungus by humans. The use of yeast to leaven bread also dates back to biblical times. The production of alcoholic beverages, biomass and the manufacture of therapeutic compounds, together with the production of simple organic compounds, remain the major fields in which fungi are used. Apart from the rather unsophisticated techniques used for the production and maintenance of yeast in the brewing and baking industries, the deliberate growth of fungi for commercial purposes did not commence until well into the twentieth century. The development of the sulfite process for the production of glycerol by a yeast fermentation, which was widely used during World War I, probably marks the beginning of industrial mycology (Papagianni, 2004).

However, it is since the advent of recombinant DNA technology that this capability has been further expanded and now fungi are employed to produce not only homologous, but heterologous proteins as well (Davies, 1994; Kinghorn and Unkles, 1994; van den Hondel *et al.*, 1991). Today, filamentous fungi, in particular *Aspergilli*, are emerging as another important eukaryotic host for the cost-effective production of functional proteins from a variety of sources (Davies, 1994; van den Hondel *et al.*, 1991). They have been extensively used in industrial processes and have been harnessed for the production of antibiotics, enzymes, fermented foods, vitamins, pharmaceutical products and organic acids, to name just a few (Alexopoulos and Mims, 1979), and therefore

large-scale fermentation technology and downstream processing are already well established (Bodie *et al.*, 1994).

Therefore, with the increasing demand for vaccines as a means to control and eradicate disease, the answer lies in the abilities of the recombinant host. Thus, does the filamentous fungi, *A. niger*, offer sufficient advantages to be viewed as a prominent heterologous protein production host, and furthermore, an effective alternative for current vaccine production technologies?

### 2.1.2 *Aspergillus*, a potential host for heterologous protein production

When the new age of recombinant DNA technology dawned, scientists were able to manipulate biological host genomes in order to produce heterologous proteins. The organism of choice for the host of foreign genes was, for a long period of time, the bacterium *Escherichia coli*. *E. coli* is a single-celled organism that reproduces mainly through asexual reproduction. The simplicity of the organism makes it easy and cheap to work with. The food source is simple, and it does not require elaborate facilities for growth and maintenance. Its rapid growth cycle allows for a quick increase in the population size of a particular strain, as an *E. coli* population can double in less than an hour. However, while the simplicity of *E. coli* makes it a desirable host for the production of heterologous proteins, it also has its disadvantages as a host. *E. coli* is a prokaryote, and thus does not have any of the membrane bound organelles found in eukaryotes. In eukaryotes, a protein is often post-translationally modified in different organelles, such as the ER or the Golgi apparatus. These modifications are often necessary to convert the protein into a functional form, and often involve addition of different forms of glycolation. Without these organelles heterologous proteins expressed in *E. coli* are incorrectly folded and thus non-functional (Miyanohara *et al.*, 1983; Baneyx, 1999). Due to these shortcomings, other microorganisms, such as mammalian cells, plant cells and yeasts, have been studied as suitable replacements for *E. coli*.

In order to produce complex proteins that are identical to the human version, (a main prerequisite for approval by the drug-regulatory authorities), scientists often resort to mammalian hosts (Michel *et al.*, 1984; MacNab *et al.*, 1976). The mammalian cell lines have been successfully cultured in bioreactors. However, such production systems require complex and costly equipment, methodology, culture media, and poses problems in the scaling up of production. Furthermore, heterologous protein production in mammalian cells is time consuming and the risk of contamination is high. There are also fears relating to the safety of the protein products derived from mammalian cells, because these cells may become tumorigenic as well as the possible presence of retroviruses. Thus, even though mammalian cultures have many advantages, these constraints have made this method unfavourable for other heterologous protein production systems (Moir and Mao, 1990).

Yeast cells have been found to be most favourable hosts in many heterologous protein production systems. It combines the ease of genetic manipulation and rapid growth characteristics of prokaryotic cells, with the ability to perform post-translational modifications of eukaryotic cells (Cregg *et al.*, 1993). Traditionally, *S. cerevisiae* has been used as a heterologous host for the production of pharmaceutical products, but the use of *S. cerevisiae* too has several limitations. Firstly, the heterologous product yields have been found to be generally low (1 to 5 percent of the total protein) (Cregg *et al.*, 1985). In addition to difficulties with scaling up the protein production to increase yield, hyperglycosylation of secreted glycoproteins not only diminishes activity of the foreign protein but it has been shown that these proteins tend to be antigenic when introduced into mammals. Another negative aspect of *S. cerevisiae* is that it does not have a strong inducible promoter. Finally, some heterologous proteins produced by *S. cerevisiae* are not secreted, but found in the periplasmic space. This leads to problems with purification and ultimately decreases production yields (Buckholz and Gleeson, 1991). This has therefore led to further investigations for alternative host cells.

Several characteristics of the yeast, *P. pastoris*, as expression hosts give them potential advantages over *S. cerevisiae*. Firstly, hyperglycosylation of proteins produced by

*P. pastoris* is less, and differs from that of *S. cerevisiae*, in that *P. pastoris* glycans do not have α 1,3-linked mannose residues. These have been found to be responsible for the highly antigenic nature of the glycoproteins produced by *S. cerevisiae* (Cregg *et al.*, 1993). Thus, the post-translational modifications made by *P. pastoris* are more suitable for use in humans. *P. pastoris* has a strong inducible promoter, which is related to the fact that it is a methylotropic yeast. The first step in methanol utilisation is the oxidation of methanol to formaldehyde and hydrogen peroxide (Ledeboer *et al.*, 1985), which is catalysed by the enzyme alcohol oxidase (*AOX*). When grown on methanol, *AOX* can make up to thirty-five percent of the total cellular protein (Cregg *et al.*, 1985). Thus, favourable production levels can be obtained if a heterologous protein were to be expressed under control of the *AOX* gene promoter. Furthermore, since transcription of the promoter is highly inducible, the production of the foreign protein can be strictly controlled. Lastly, *P. pastoris* grows on a simple medium and does not secrete high amounts of endogenous protein. Thus, the heterologous protein secreted into the culture is relatively pure and purification is easier to accomplish (Faber *et al.*, 1995).

During the past few years, numerous studies have been presented on *A. niger*, potentially one of the most important fungi host cells for production and secretion of heterologous proteins. The employment of *A. niger* as a host organism for production and secretion of homologous and heterologous proteins demonstrates many advantages over *E. coli, S. cerevisiae,* and *P. pastoris* (Saunders *et al.*, 1989). *A. niger* is a prodigious exporter species of homologous proteins and is able to produce certain enzymes in quantities of kilograms per cubic meter. Filamentous fungi are also rather robust and can grow on many organic compounds (Jeenes *et al.*, 1991; van den Hondel *et al.*, 1991). *A. niger* has a long history of usage within the fermentation industry and has gained GRAS status. This often facilitates the path toward regulatory improval of the production system. Even though empirical optimisation is required for each new production system, the fermentation industries are familiar with the baseline conditions required to maximize production of homologous proteins in *Aspergillus* (Bodie *et al.*, 1994). Thus, it provides a good foundation for the identification of

physicochemical influences that are likely to be of greatest importance to heterologous protein production and secretion using a similar strain. *Aspergillus* is capable of carrying out efficient post-translational modifications of products. Hyperglycosylation is less of a problem in filamentous fungi than in *S. cerevisiae,* as their glycosylation patterns more closely resemble those found in mammalian cells (Upshall *et al.*, 1987). This is especially important for some proteins derived from higher eukaryotes. Several proteins of pharmaceutical importance have also been successfully produced in *Aspergillus,* including tissue plasminogen activator (1 $mg.\ell_{culture}^{-1}$) (Upshall *et al.*, 1987), human interleukin-6 (5-10 $mg.\ell_{culture}^{-1}$) (Gouka *et al.*, 1997), human lactoferrin (2 $g.\ell_{culture}^{-1}$) (Ward *et al.*, 1995), human interferon α-2 (1 $mg.\ell_{culture}^{-1}$) (Gwynne *et al.*, 1987), and an industrial process for human superoxide dismutase through intracellular expression in *Aspergillus nidulans* has been developed (Davies, 1994). Furthermore, *Aspergillus* species are effective secretors of proteins, often in a native, correctly folded form. They tend not to accumulate large quantities of the protein intracellularly, in the form of inclusion bodies, as some bacteria and yeast do. Finally, transformation stability is relatively high; therefore, the threat of revertants is less pronounced (Saunders *et al.*, 1989).

### 2.1.3 General Information on filamentous fungi

Filamentous fungi are eukaryotic microorganisms. As the name implies, most filamentous fungi grow as hyphae (branched filaments of cells joined together) which are collectively called mycelia. Filamentous fungi account for virtually all the fungal kingdom, excluding that of yeast, which represent a group of essentially unicellular fungi. Most fungi grow as septate hyphae (the hyphae contain cross-walls called septa) which divide them into distinct uninucleate cell-like units.

Filamentous fungi are morphologically complex microorganisms, exhibiting different structural forms throughout their life cycles. They can reproduce asexually by fragmentation of their hyphae as well as by forming spores both asexually and, less frequently, sexually (Tortora *et al.*, 1998). The basic vegetative structure of growth

consists of a tubular filament known as hypha that originates from the germination of a single reproductive spore. Hyphae grow by the elongation of the tips. As the hypha continues to grow, it frequently branches repeatedly to form a mass of hyphal filaments referred to as mycelium. When grown in submerged culture, these fungi exhibit different morphological forms, ranging from dispersed mycelial filaments to densely interwoven mycelial masses referred to as pellets. The particular form exhibited, is determined not only by the genetic material of the fungal species but also by the nature of the inoculum as well as the chemical (medium constituents) and by physical (temperature, pH, mechanical forces) culturing conditions (Atkinson and Daoud, 1976; Kossen, 2000).

### 2.1.4 The fungus *Aspergillus*

The first person to study the *Aspergilli* was a priest by the name of Micheli in 1729, who published Nova Plantarum Genera in which his microscopical research on fungi was included, distinguishing stalks and spore heads (Alexopoulos and Mims, 1979; Raper and Fennell, 1965). He noted that the spore chains or columns radiated from a central structure to produce a pattern that suggested the aspergillum (a device used to sprinkle holy water) and thus he applied the name *Aspergillus* to the moulds he observed. Species of *Aspergillus* belong to the first fungal organisms that were cultivated on artificial media and studied for their biochemical properties and they are one of the most common fungi found in man's environment (Samson, 1994). Since ancient times, *Aspergillus* species have been used in fermentation of food in Japan and other Asian countries and the early discovery of their ability to produce organic acids was made at the turn of the century. By 1928 more than 2000 papers had been published which in some way concerned *Aspergilli* (Raper and Fennell, 1965).

*A. niger* is a member of the genus *Aspergillus*, asexual (anamorphic) filamentous fungi. The genus is widely distributed, and has been observed in a broad range of habitats because they can colonize on a wide variety of substrates. *A. niger* is commonly found as a saprophyte growing on dead leaves, stored grain, compost piles, and other decaying

vegetation. The spores are widespread, and are often associated with organic materials and soil.

### 2.1.5 Homologous and Heterologous protein production in *Aspergillus*

The fungus *Aspergillus* is the basis of a number of industrial processes involving fermentation, such as the fermentation of the cacao bean and the preparation of certain cheeses. It is employed in the commercial production of many organic acids, plant growth regulators, mycotoxins, some drugs such as ergometrine and cortisone and some vitamin preparations, and is responsible for the manufacture of a number of antibiotics, particularly penicillin and griseofulvin (Harvey and McNiel, 1994).

Citric acid is the most extensively produced organic acid by means of modern biotechnological means (Bodie *et al.*, 1994). At the advent of the previous century, citric acid could only be obtained commercially from citrus fruits (Johnson, 1954). Today, however, the product is obtained by submerged fungal fermentation. Advantages of submerged cultures include lower labour cost, higher yield, shorter time cycle, simpler operation, and easier maintenance of asepsis. TABLE 2.1 presents the major classes of *Aspergilli* organic acids of commercial importance and some of their sources.

**TABLE 2.1:** Major classes of *Aspergilli* organic acids of commercial importance and some of their sources (Papagianni, 2004).

| Organic Acid | Sources |
|---|---|
| Citric Acid | *A. niger* |
| Gluconic acid | *A. niger, A. oryzae* |
| Kojic acid | *A. flavus, A. oryzae* |
| L-Malic acid | *A. citricus, A. niger, A. ochraceus, A. oryzae* |

The major applications for industrial enzymes are in the manufacture of foods and beverages, wastewater treatment and the manufacturing of fine chemicals (Bodie *et al.*, 1994). The current world market for industrial enzymes is estimated at

US $1.6 billion a year and a significant growth is predicted for the future (refer to TABLE 2.2. Filamentous fungi are sources of about 40% of available enzymes (Archer and Peberdy, 1997; Maister, 2001).

**TABLE 2.2:** Global sales (in millions of US $) of industrial enzymes from 1992 to 1998 and projected to 2009 (Godfrey, 2001).

| Year | Global Sales (in millions of US $) | Change (%) |
|---|---|---|
| 1992 | 600 | |
| 1993 | 720 | +20 |
| 1994 | 864 | +20 |
| 1995 | 933 | +8 |
| 1996 | 1138 | +22 |
| 1997 | 1434 | +26 |
| 1998 | 1550 | +8 |
| 2005 predicted | 1700 | +5 average |
| 2009 predicted | 2250 | +8.1 average |

As new and improved molecular-biological techniques have developed, new methods and ideas to use filamentous fungi for the production of homologous and heterologous proteins have emerged. Many fungal and non-fungal proteins have been expressed with varying success (Gouka *et al.*, 1997b; Kinghorn and Unkles, 1994; Verdoes *et al.*, 1995). A large market exists for recombinant enzymes in various industries, including animal feed, glucose syrup, alcohol, wine, brewing, baking, fruit juice and dairy industries (Archer, 2000; Archer and Peberdy, 1997; Bennett, 1998; Bodie *et al.*, 1994). Some bacterial and fungal enzymes of commercial importance that have been produced by recombinant *Aspergilli* are summarised in TABLE 2.3. For extensive lists of industrial products and the preferred names of the filamentous fungi that produce them, the reader is referred to the ‘‘ATCC names of industrial fungi’’ (Jong *et al.*, 1994).

**TABLE 2.3:** Enzymes expressed in *Aspergillus* (Adapted from Plüddemann and van Zyl, 2003).

| Enzyme | Source | Expression host | Production levels | Reference |
|---|---|---|---|---|
| Acid phosphatase | *Aspergillus awamori* | *A. awamori* | 24.2 U | Piddington *et al.*, 1993 |
| Acid protease | *Aspergillus oryzae* | *A. oryzae* | 5236 U.g dry mycelium$^{-1}$ | Gomi *et al.*, 1993 |
| Alkaline protease | *A. oryzae* | *A. oryzae* | 41529 U.g substrate$^{-1}$ | Cheevadhanarak *et al.*, 1991 |
| α -Amylase | Barley | *A. niger* | 60 mg.$\ell_{culture}^{-1}$ | Juge *et al.*, 1998 |
| scFv Antibody fragments | Human | *A. awamori* | 200mg.$\ell_{culture}^{-1}$ | Frenken *et al.*, 1998 |
| α -L-Arabino-furanosidase | *A. niger* | *A. niger* | 2.48 U.m$\ell^{-1}$ | Flipphi *et al.*, 1993 |
| | | *A. nidulans* | 2.64 U.m$\ell^{-1}$ | |
| Aspartic proteinase | *Rhizomucor miehei* | *A. oryzae* | 3.3 g.$\ell^{-1}$ | Christensen *et al.*, 1988 |
| | | *A. awamori* | 1.97 g.$\ell^{-1}$ | Ward *et al.*, 1993 |
| | *Mucor pusillus* | *A. oryzae* | 20 mg.$\ell^{-1}$ | Murakami *et al.*, 1993 |
| Bovine enterokinase | Cattle | *A. niger* | 5 mg.$\ell^{-1}$ | Svetina *et al.*, 2000 |
| Bovine pancreatic trypsin inhibitor | Cattle | *A. niger* | 10 - 20 mg.$\ell^{-1}$ | MacKenzie *et al.*, 1998 |
| Catalase | *A. niger* | *A. niger* | 11 U.mg protein$^{-1}$ | Fowler *et al.*, 1993 |
| Cell surface glycoprotein Bm86 | Cattle tick (*Boophilus microplus*) | *A. nidulans* | 1.8 mg.$\ell^{-1}$ | Turnbull *et al.*, 1990 |
| | | *A. niger* | NR | |
| Chloroperoxidase | *Caldariomyces fumago* | *A. niger* | 10 mg.$\ell^{-1}$ | Conesa *et al.*, 2001 |
| Chymosin | Calf | *A. oryzae* | 0.16 mg.$\ell^{-1}$ | Tsuchiya *et al.*, 1993 |

**TABLE 2.3:** Enzymes expressed in *Aspergillus* (Adapted from Plüddemann and van Zyl, 2003) (continued).

| Enzyme | Source | Expression host | Production levels | Reference |
|---|---|---|---|---|
| Cutinase | *Fusarium solani pisi* | *A. awamori* | 30 – 70 mg.ℓ$^{-1}$ | van Gemeren *et al.*, 1996 |
| Endoglucanase | *Cellulomonas fimi* | *A. nidulans* | ~20 mg.ℓ$^{-1}$ | Gwynne *et al.*, 1987 |
| 1,4-β-Endoxylanase | *A. awamori* | *A. awamori* | 58 kU.mℓ$^{-1}$ | Hessing *et al.*, 1994 |
| Δ6-Fatty acid desaturase | *Mortierella alpina* | *A. oryzae* | NR | Sakuradani *et al.*, 1999 |
| α -Galactosidase | *A. niger* | *A. niger* | NR | Den Herder *et al.*, 1992 |
| Glucoamylase | | *A. awamori* | 4.6 g.ℓ$^{-1}$ | Finkelstein *et al.*, 1989 |
| | Plants | *A. awamori* | 0.4mg.ℓ$^{-1}$ | Gouka *et al.*, 1996 |
| | *A. niger* | *A. niger* | 7.5 g.ℓ$^{-1}$ | Finkelstein *et al.*, 1989 |
| | | *A. nidulans* | 1.2 g.ℓ$^{-1}$ | Devchand *et al.*, 1989 |
| | | *A. awamori* | 4.6g.ℓ$^{-1}$ | Finkelstein *et al.*, 1989 |
| Glucose oxidase | *A. oryzae* | *A. oryzae* | 29.4 U.mℓ$^{-1}$ | Hata *et al.*, 1991 |
| | *A. niger* | *A. niger* | 14 U.mg protein$^{-1}$ | Whittington *et al.*, 1990 |
| | | *A. nidulans* | 5 U.mg protein$^{-1}$ | |
| α -Glucosidase | *A. niger* | *A. nidulans* | 18 U.g protein$^{-1}$ | Nakamura *et al.*, 1997 |
| β-Glucosidase<br>Cellobiohydrolase<br>Endoglucanase | *Trichoderma reesei* | *A. oryzae* | Co-expressed, several hundred mg.ℓ$^{-1}$ cellulases | Takashima *et al.*, 1998 |

**TABLE 2.3:** Enzymes expressed in *Aspergillus* (Adapted from Plüddemann and van Zyl, 2003) (continued).

| Enzyme | Source | Expression host | Production levels | Reference |
|---|---|---|---|---|
| β -1,4-Endoglucanase | *T. reesei* | *A. niger* | 138 U.mℓ$^{-1}$ | Rose and van Zyl, 2002 |
| Insulin | Human | *A. niger* | 776 mU.ℓ$^{-1}$ | Mestric *et al.*, 1996 |
| Interferon alpha-2 | Human | *A. nidulans* | 0.2 mg.ℓ$^{-1}$ | MacRae *et al.*, 1993 |
| | | | 1 mg.ℓ$^{-1}$ | Gwynne *et al.*, 1989 |
| Interleukin-6 | Human | *A. nidulans* | 4.8 mg.ℓ$^{-1}$ | Contreras *et al.*, 1991 |
| Laccase | *Coprinus cinereus* | *A. oryzae* | 8 – 135 mg.ℓ$^{-1}$ | Yaver *et al.*, 1999 |
| | *Myceliophthora thermophila* | *A. oryzae* | 11 – 19 mg.ℓ$^{-1}$ | Berka *et al.*, 1997 |
| | *Pycnoporus cinnabarinus* | *A. niger* | 70 mg.ℓ$^{-1}$ | Record *et al.*, 2002 |
| | *Trametes villosa* | *A. oryzae* | NR | Yaver *et al.*, 1996 |
| Lactoferrin | Human | *A. oryzae* | 2 g.ℓ$^{-1}$ | Ward *et al.*, 1995 |
| Lignin peroxidase | *Phanerochaete chrysosporium* | *A. niger* | Enzyme inactive | Conesa *et al.*, 2000 |
| Lipase | *Thermomyces lanuginosa* | *A. oryzae* | 2.1 relative lipase units.mℓ$^{-1}$ | Yaver *et al.*, 2000 |
| Lysozyme | Hen egg white | *A. niger* | 1 mg.ℓ$^{-1}$ | Archer *et al.*, 1990 |
| Manganese peroxidase | *P. chrysosporium* | *A. niger* | 100 mg.ℓ$^{-1}$ | Conesa *et al.*, 2000 |
| | | *A. oryzae* | 5 mg.ℓ$^{-1}$ | Stewart *et al.*, 1996 |
| | *Pleurotus eryngii* | *A. nidulans* | 148.5 U.mg protein$^{-1}$ | Ruiz-Duenas *et al.*, 1999 |
| | | *A. oryzae* | 5 mg.ℓ$^{-1}$ | Stewart *et al.*, 1996 |
| β2-microglobulin | Human | *A. nidulans* | 117μg.ℓ$^{-1}$ | O'Herrin *et al.*, 1996 |
| Mucus proteinase inhibitor | Human | *A. niger* | 3 mg.ℓ$^{-1}$ | Mikosch *et al.*, 1996 |

**TABLE 2.3:** Enzymes expressed in *Aspergillus* (Adapted from Plüddemann and van Zyl, 2003) (continued).

| Enzyme | Source | Expression host | Production levels | Reference |
|---|---|---|---|---|
| Pectate lyase | *Erwinia carotovora* | *A. niger* | 0.4 mg.ℓ$^{-1}$ | Bartling *et al.*, 1996 |
| | | *A. nidulans* | 2.0 mg.ℓ$^{-1}$ | |
| | | *A. awamori* | 0.8 mg.ℓ$^{-1}$ | |
| Pectin lyase A | *A. niger* | *A. niger* | NR | Harmsen *et al.*, 1990 |
| Pectin methyl esterase | *A. niger* | *A. niger* | 45 U.mg protein$^{-1}$ | Khanh *et al.*, 1991 |
| Phenol oxidase | *Acremonium murorum* | *A. awamori* | 0.6 g.ℓ$^{-1}$ | Gouka *et al.*, 2001 |
| Phytase | *A. awamori* | *A. awamori* | 328.9 U | Piddington *et al.*, 1993 |
| Polygalacturonase I | *A. niger* | *A. nidulans* | 510 U.mℓ$^{-1}$ | Bussink *et al.*, 1992a |
| Polygalacturonase II | *A. niger* | *A. niger* | 88 U.mℓ$^{-1}$ | Bussink *et al.*, 1990; Bussink *et al.*, 1992b |
| Polygalacturonase C | *A. niger* | *A. nidulans* | 33 U.mℓ$^{-1}$ | Bussink *et al.*, 1992a |
| Porcine pancreatic phospholipase $A_2$ | Human | *A. niger* | 10 mg.ℓ$^{-1}$ | Roberts *et al.*, 1992 |
| Taka amylase | *A. oryzae* | *A. oryzae* | 12 g.ℓ$^{-1}$ | Christensen *et al.*, 1988 |
| Thaumatin | Plant (*Thaumatococcus danielli*) | *A. oryzae* | 50 ng.ℓ$^{-1}$ | Hahm and Batt, 1990 |
| | | *A. awamori* | 5 - 7 mg.ℓ$^{-1}$ | Faus *et al.*, 1998 |
| | | | 100 mg.ℓ$^{-1}$ | Moralejo *et al.*, 1999 |
| Pancreatic phospholipase | Pig | *A. niger* | 10 mg.ℓ$^{-1}$ | Roberts *et al.*, 1992 |

**TABLE 3.2:** Enzymes expressed in *Aspergillus* (Adapted from Plüddemann and van Zyl, 2003) (continued).

| Enzyme | Source | Expression host | Production levels | Reference |
|---|---|---|---|---|
| Tissue plasminogen activator | Human | *A. nidulans* | 1 mg.$\ell^{-1}$ | Upshall *et al.*, 1987 |
| | | *A. niger* | 12 - 25 mg.$\ell^{-1}$ | Wiebe *et al.*, 2001 |
| Triglyceride lipase | *R. miehei* | *A. oryzae* | 8810 U.mg protein$^{-1}$ | Huge-Jensen *et al.*, 1989 |
| Vanillyl-alcohol oxidase | *Penicillium simplicissimum* | *A. niger* | NR | Benen *et al.*, 1998 |
| Xylanase | *A. awamori* | *A. awamori* | 58 kU.m$\ell^{-1}$ | Hessing *et al.*, 1994 |
| | *A. awamori* | *A. niger* | 140 kU.m$\ell^{-1}$ | |
| | *T. reesei* | *A. niger* | 480 U.m$\ell^{-1}$ | Rose and van Zyl, 2002 |

NR = Not Reported

In addition to the expression of fungal enzymes, many studies on heterologous gene expression have considered the genes of higher eukaryotes using the expression hosts *A. nidulans, A. niger* and *Aspergillus awamori* for commercial reasons (van den Hondel *et al.*, 1991; Radzio and Kuck, 1997).

High-level heterologous protein production facilitates the study of the structure and/or biological function of a protein. Where the availability of a certain protein may be erratic, expression in a microbial host can fulfil demand and pharmaceutically important proteins that were previously only obtained from biological samples such as serum can be obtained more easily with less risk of contaminating biological agents, such as viruses (Plüddemann and van Zyl, 2003). The success of *A. niger* for industrial production of biotechnological products is largely due to the metabolic versatility of this strain. The industrial importance of *A. niger* is not limited on its more than 35 native products but also on the development and commercialisation of the new products which are derived by modern molecular biology techniques.

### 2.1.6 Future directions for the *Aspergillus* expression system

The results described in the previous section indicate that although considerable progress has been made in the development of *Aspergillus* for the production of complex mammalian proteins, not all problems have been resolved. However, the utility of this host for effective expression of mammalian proteins has been proven with the numerous successes that have been achieved. However, despite the apparent success of expressing higher eukaryotic proteins in *Aspergillus*, the potential of this host to produce another pharmaceutically important group of proteins, namely viral proteins, is largely unknown. Only one report has been published in a Chinese journal of the production of Hepatitis B surface antigen in *Aspergillus foetidus* (Liu *et al.*, 1990). No other reports have been found to date detailing the production of viral proteins in *Aspergillus*.

### 2.1.7 Conclusion

Therefore, with the increasing demand for vaccines as a means to control and eradicate disease and the inherent advantages of filamentous fungi as a protein production host, the evaluation of *Aspergillus* for the production of immunogenic viral proteins has emerged as a promising and exciting alternative to the current vaccine production technologies.

## 2.2 Hepatitis B

### 2.2.1 Introduction

Hepatitis B, caused by the infectious Hepatitis B virus (HBV), is a major public health concern. It is deemed as one of the most widespread and infectious diseases in the world today, causing serious liver disease. Between one-third and one-quarter of people infected chronically with HBV are expected to develop progressive liver disease, which includes cirrhosis and primary liver cancer. In fact, The World Health Organisation (WHO) ranks Hepatitis B as the ninth leading cause of death worldwide. An estimated 400 million people worldwide are diagnosed with chronic HBV infection, of which 1 million carriers are estimated to die annually due to Hepatitis B and its consequences. HBV is second only to tobacco as a known human carcinogen (WHO, 2004).

There is no effective cure or treatment in current existence for individuals already infected. Much more research is required before completely understanding and controlling the spread of this infectious agent (Kassianides *et al.*, 1988). Furthermore, safe and effective vaccines against HBV have been available since 1982. Since 1991, WHO has recommended that a Hepatitis B vaccine be included in routine immunization schedules for all children in all countries (Vryheid *et al.*, 2001).

### 2.2.2 Global distribution

Even though HBVs have been found in other primates, humans remain the principal reservoir (Mosley, 1975). Many individuals are affected worldwide; approximately 6% of the world's population is infected with the HBV and 45% of the world population occupy areas in which chronic HBV infection is highly endemic. The problem is most prominent in developing countries with 95% of chronic carriers residing in the developing world (Ayoola, 1988). In Southeast Asia and tropical Africa, chronic carriers of the virus represent 10% or more of the population, whereas they make up less

than one percent in North America and Western Europe. It is estimated that there are about 110 million HBV carriers in Africa alone with 60% to 99% of healthy African adults, from rural areas, showing evidence of exposure to HBV. The estimated data of the world prevalence of the HBV carrier state in 2003 is tabulated in TABLE 2.4.

**TABLE 2.4:** Estimated world prevalence of HBV carrier state, (WHO, 2004).

| Region | Estimated population (in millions) | Carrier prevalence (%) | Number of carriers (in millions) |
|---|---|---|---|
| Asia | 3200 | 8 | 254 |
| Africa | 680 | 16 | 108 |
| Former USSR | 296 | 4 | 9.8 |
| Latin and South America | 430 | 3 | 12.6 |
| Europe | 503 | 1 | 6 |
| Japan | 135 | 2 | 2.7 |
| North America | 360 | 0.5 | 1.8 |
| Oceania | 26 | 2-4 | 0.6 |
| **TOTAL** | **5630** | **5.55** | **395.5** |

Hepatitis B researchers have divided the world into areas of "high", "intermediate", and "low" HBV endemicity, basing this division on markers and on the primary modes of HBV transmission.

In the western industrialised countries (developed countries) such as the United States, the prevalence of chronic HBV infection is low (<2%). This is a result of immunisation programmes, where children and adolescents are routinely vaccinated against Hepatitis B. The highest incidence of disease is in persons in direct contact with chronic carriers or with their blood samples (nurses, doctors, dentists), recipients of blood or blood products (haemophiliacs, patients receiving blood transfusions or dialysis treatments), prison inmates, intravenous drug abusers, homosexuals and persons with multiple sex partners.

Regions of intermediate chronic HBV infection (2%–7%) include the developing countries such as South Central and Southwest Asia, Israel, Japan, Eastern and Southern Europe, Russia, most areas surrounding the Amazon River basin, Honduras, and Guatemala. Viral transmission in these areas occurs most frequently from an infected mother to her infant (Hino *et al.*, 2001), and to a lesser extent, adult transmission.

The prevalence of chronic HBV infection is high (>8%) in all third world countries and some developing countries such as; all of Africa; Southeast Asia, including China, Korea, Indonesia, and the Philippines; the Middle East, except Israel; south and Western Pacific islands; the interior Amazon River basin; and certain parts of the Caribbean (Haiti and the Dominican Republic). With regard to southern Africa specifically, in a study performed in Swaziland in 1983 and in Namibia in 1985, 82.6% and 98.9%, amongst those tested showed exposure to HBV, respectively (Ayoola, 1988). In South Africa, more than 70% of the population has been exposed to HBV, with an estimated 10% being carriers (i.e. HBsAg positive) of the virus (Tsebe *et al.*, 2001). The carrier rate differs regionally, with higher rates of infection in rural areas (5-25%) than urban areas (<5%). In Africa all susceptible groups (i.e. those who are negative for HBV markers) are at high risk of infection, with the highest risk occurring among children (Ayoola, 1988; Garrison and Baker, 1991; Tiollais and Buendia, 1991). A pre-immunisation survey conducted in South Africa in 1999 indicated HBsAg carriage of 8.9% in 1-year-olds, increasing to 12.8% in 3-year-olds and rising to 15.7% by the age of 6 years (Vardas *et al.*, 1999). Amongst African adults, blood scarification in tribal rituals, tattooing, blood-sucking vectors, sexual intercourse and uncontrolled injections have been incriminated in transmitting HBV (Ayoola, 1988). FIG. 2.1 illustrates the geographical distribution of the Hepatitis B prevalence in 2002 and categorises the areas in terms of high, intermediate and low infection.

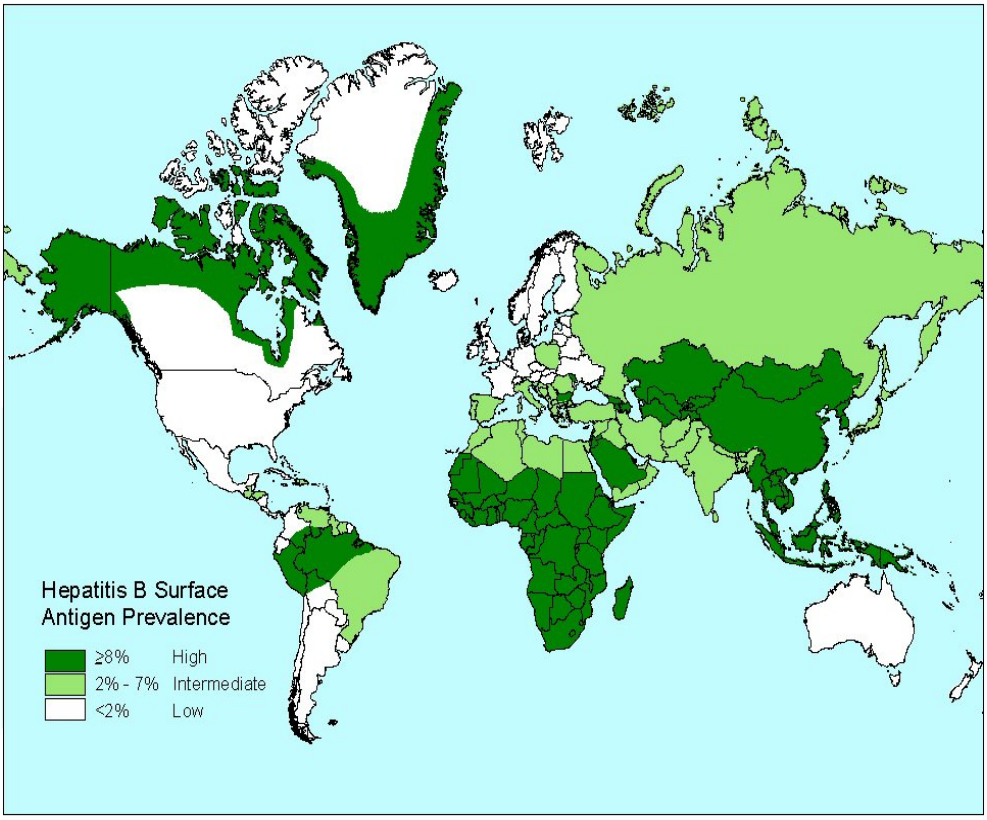


**FIGURE 2.1:** Geographic Distribution of Hepatitis B Prevalence, 2004, (WHO, 2004).

HBV is therefore primarily a disease of infants in third world and developing nations, whereas in Western countries it is mostly confined to adults, due to the availability of various Hepatitis B vaccines and the increasing knowledge of how the virus is spread. Due the burden of HBV disease in the developing world, it is essential that these populations are vaccinated against the Hepatitis B virus. WHO has recommended, since 1991 that Hepatitis B vaccine be included in routine immunization schedules for all children in all countries (Vryheid *et al.*, 2001). However, these same populations have the least access to the vaccine. The primary reason for limited access to vaccines is due to cost. One dose of the vaccine can cost approximately US $30-$55, bringing the three-dose cost to upwards of US $100-$150. Unfortunately, this is far too expensive for

many countries in the developing world that spend about US $12 or less per capita per year for all organized health care. Therefore, even though the WHO recommends that the HBV vaccine is added to the Expanded Programme of Immunization (EPI), many countries have been unable to do so. FIG. 2.2 represents a geographical representation of the countries using a Hepatitis B vaccine in their national immunisation programme in the year 2001.

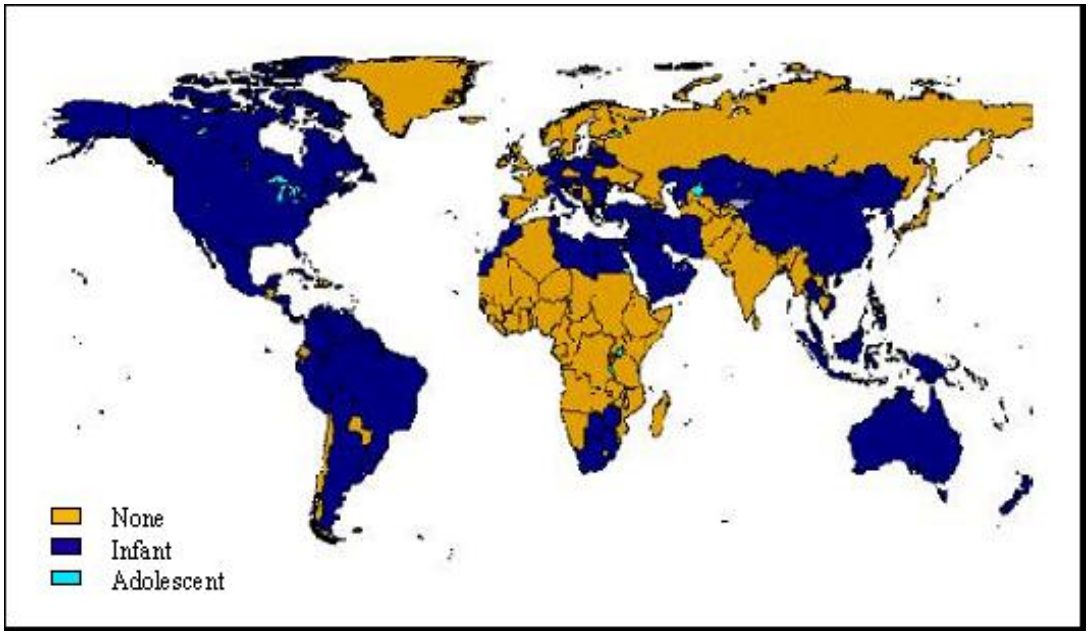


**FIGURE 2.2**: Countries using Hepatitis B vaccine in their national immunisation programme, 2003 (WHO, 2004).

In order to curb the spread of the disease a mass vaccination strategy must be made feasible, where large amounts of safe, affordable vaccine must be produced and made available to the public.

### 2.2.3 Origin and History of the Hepatitis B virus

The origin of the Hepatitis B virus is unclear. With human HBV as the archetype, the members of the *hepadnaviridae* family include the Eastern woodchuck Hepatitis B

virus, the Beechy ground squirrel Hepatitis B virus, and the Beijing duck Hepatitis B virus. The various strains of this virus have been found to be species specific. They all target the liver as the primary site of infection and have the ability to cause persistent infection.

In 1883, Lürman reported an epidemic of hepatitis that occurred in shipyard workers in Bremen. This was the first possible report of a Hepatitis B epidemic (Purcell, 1994; Zuckerman, 1975). The concept of Hepatitis B was only introduced in 1947, when MacCallum categorized infectious (epidemic) and serum hepatitis (MacCallum, 1947), and was only accepted by the World Health Organisation in 1973 (WHO, 1973).

### 2.2.4 The Hepatitis B virus life cycle

The Hepatitis B virus is primarily found in the blood of infected individuals. However, HBV has also been detected in other bodily fluids including urine, saliva/nasopharyngeal fluids, semen, and menstrual fluids (Alter *et al.*, 1977 and Davison *et al.*, 1987). This virus has not been detected in faeces, perhaps due to inactivation and degradation within the intestinal mucosa or by the bacterial flora (Grabow *et al.*, 1975).

Transmission of HBV is done most efficiently via percutaneous introduction (i.e. needle stick injury). Sexual transmission is also possible, though inefficient. There are other potential routes of transmission, but their efficiency is not easily measured. Children of mothers with active HBV are also at risk of acquiring HBV. Uninfected individuals living with an HBV carrier are at greater risk of contracting HBV than those not living with a carrier. This is likely because HBV can survive even on a dry surface for over a week (Bond *et al.*, 1981). However, it should be noted that for HBV to infect, it still must gain entry into the bloodstream of an uninfected individual. Once the virus invades the body, it binds to the cell surface and penetrates it with the help of its envelope proteins. Inside the plasma membrane of the cell, the virus is not degraded but is transported to the nucleus where the partially circular DNA is made into covalently

closed circular DNA (cccDNA). cccDNA functions as the template for RNA synthesis. This RNA is then reverse transcribed into an open, circular DNA molecule. The new, circular DNA is subsequently packaged into viral envelopes in the endoplasmic reticulum and then transported out of the cell. Unlike other viruses, HBV does not integrate itself onto the host genome but retains a core of cccDNA in the nucleus of the cell by transporting some of the newly synthesized HBV DNA back to the nucleus. Thus, the HBV continues to replicate itself inside the host's liver cells (Plotkin and Orenstein, 1994 and Mahoney *et al.*, 1999).

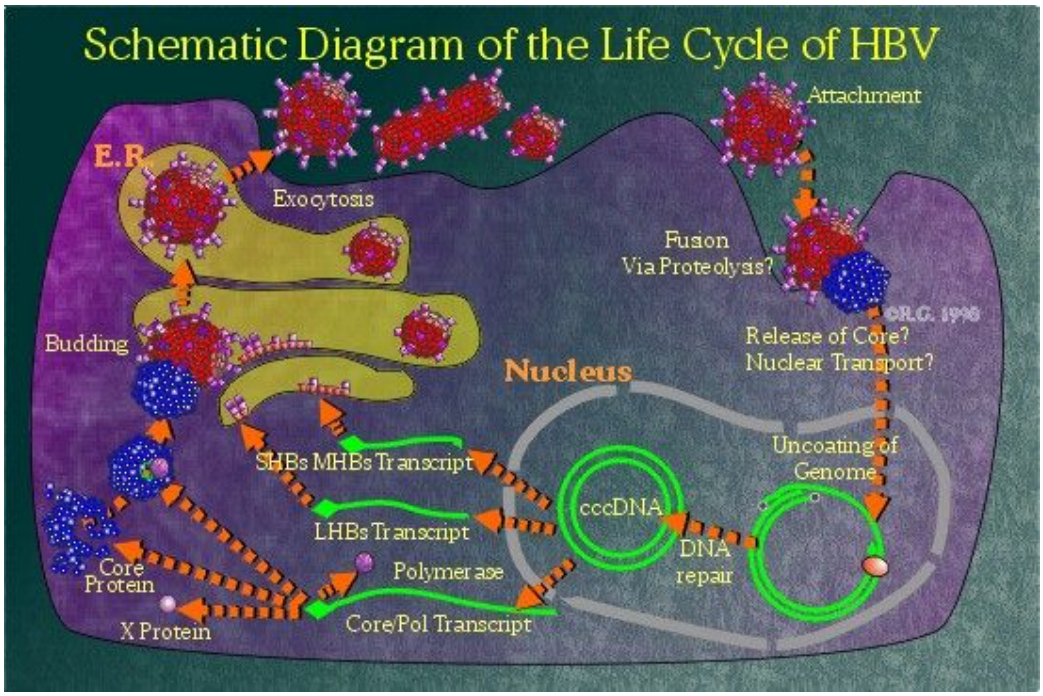


**FIGURE 2.3:** The Life Cycle of the Hepatitis B Virus. (http://www.globalserve.net/HBV.htm).

### 2.2.5 Structure of the Hepatitis B Virus

The Hepatitis B virus is a small (440Å), enveloped, double-stranded DNA virus classified in the family *Hepadnaviridae* (hepatropic DNA viruses), genus *Orthohepadnavirus* (Huovila *et al.*, 1992; Purcell, 1994). The HBV comprises of a

double-walled structure consisting of two concentric protein coats (Ganem and Varmus, 1987; Huovila *et al.*, 1992; Purcell, 1994).

The envelope, or outer coat, contains three surface proteins that stimulate the production of antibodies in the infected host and are known as the Hepatitis B surface antigens (HBsAgs). Due to the multiple start codons, polypeptides of three different sizes called the major (S) (24 kDa), middle (M) (33 kDa) and large (L) (39 kDa) protein can all be produced by the same HBsAg coding gene. All three-envelope components are glycosylated, type II transmembrane proteins that form multimers. These multimers are stabilised by disulphide bridges, which are formed by cysteine residues present in the S domain (Seeger and Mason, 2000). The viral particle also has a host-derived lipid composition consisting of phospholipids (principally phosphatidyl choline), free and esterified cholesterol, and small quantities of triglycerides (Ganem and Varmus, 1987). The capsid, or inner coat also known as the core, comprises of a single core protein species that surrounds and interacts with the viral DNA.

The Hepatitis B virion, known as the Dane particle, has a diameter around 42 nm and is deemed as an infectious particle. The envelope surrounds the inner nucleocapsid which is comprised of 180 Hepatitis B core proteins arranged in an icosahedral arrangement with T=3 and T=4 symmetry (Crowther *et al.*, 1994; Nassal and Schaller, 1993; Onodera *et al.*, 1982). The nucleocapsid also contains at least one Hepatitis B polymerase protein as well as the infectious HBV genome (Gerlich and Robinson, 1980). One Dane particle contains 300 to 400 major S protein molecules and 40 to 80 middle and large protein molecules (Tiollais *et al.*, 1985), which together with the capsid form the 42 nm diameter infectious viral particle (virion) (Ganem and Varmus, 1987).

The S protein together with the larger envelope proteins spontaneously assemble into filamentous and spherical particles 22 nm in diameter. These particles are secreted from infected cells in a ratio of $10^3$ to $10^6$ for every virion present. These two particles are

termed pseudo viral particles; the sphere is composed of the small and middle Hepatitis B surface proteins, whereas the filament also includes the large Hepatitis B surface protein.

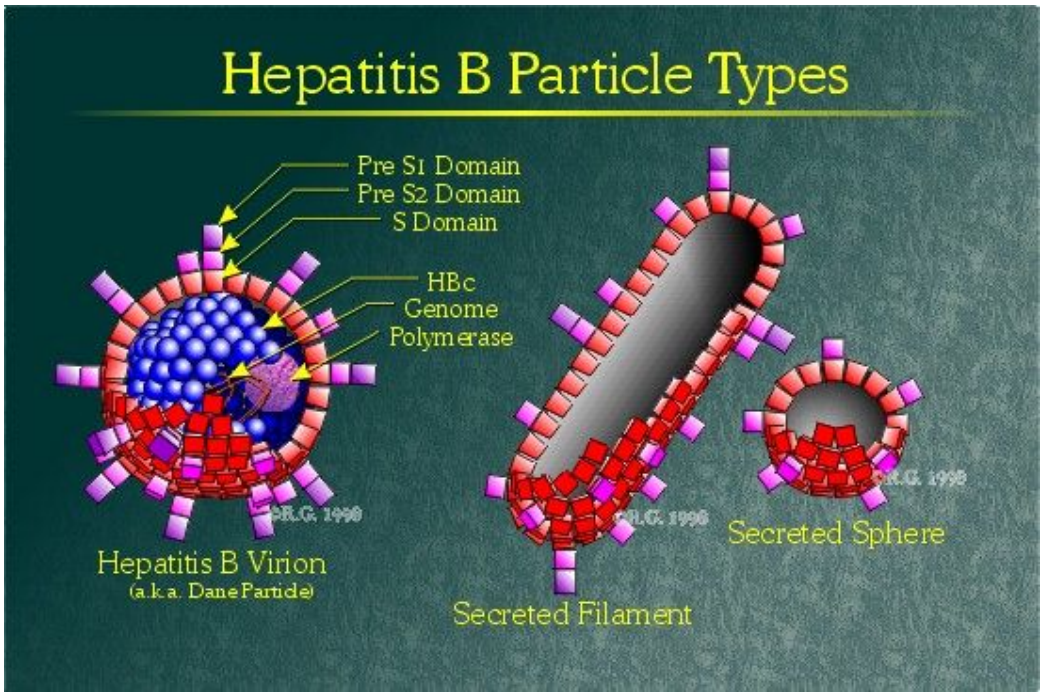


**FIGURE 2.4:** Hepatitis B particle Types (http://www.globalserve.net /HBV.htm).

These pseudoviral particles, termed the Hepatitis B surface antigen (HBsAg), are absent of viral DNA, namely the Hepatitis B core, polymerase, and genome, which confers a non-infectious nature to these particles. In terms of the HBV vaccine, the most important part of the genome is the HBsAg gene. It is particularly important because the S protein product contains an epitope that generates a protective host immune response against the virus, and is thus the key in the current HBV vaccine. High levels of these non-infectious particles can be found during the early and acute phases of infection (Hoofnagle, 1981) on the surface of the viral envelope as well as in excess in the blood. As a result, these S domain proteins are believed to allow the infectious viral particles to traverse the blood stream undetected by neutralizing host antibodies (Thomas, *et al.*, 1988). In addition, non-infectious particles have been reported to

enhance HBV infection in the duck Hepatitis B system, having important functions in the viral life cycle, such as binding and penetration, envelopment and virus maturation. Binding of non-infectious particles to potential host cells appears to activate the cells, possibly to prime the cells for infection (Gerlich, 1991). Whether or not this is also true for the human Hepatitis B virus remains to be proven.

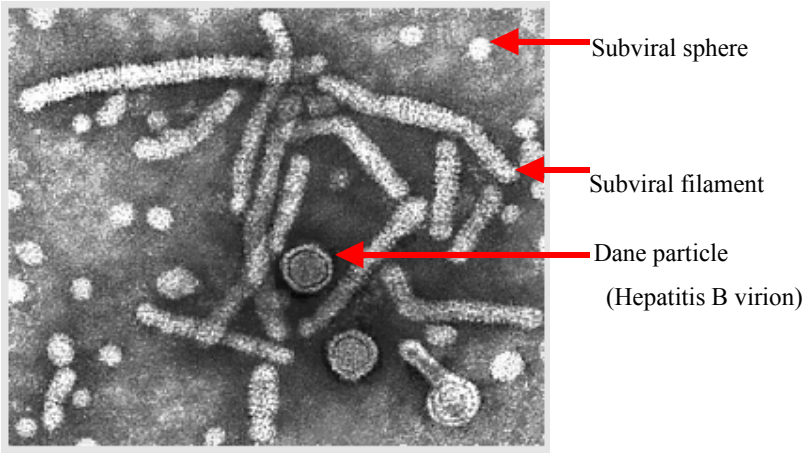


**FIGURE 2.5:** Electron micrograph of serum containing the three distinct morphological forms of the Hepatitis B viral particles. Magnification: 252 000x (Zuckerman, 1975).

These pseudoviral particles have thus been targeted for heterologous expression to produce recombinant vaccines. Recombinant DNA methods have provided an alternative to the blood-origin vaccine obtained from plasma of chronic carriers. To date, yeasts (Dehoux *et al.*, 1986; Hardy *et al.*, 2000; Valenzuela *et al.*, 1982), plants (Domansky *et al.*, 1995; Mason *et al.*, 1992) and mammalian cells (Michel *et al.*, 1984) transformed with appropriate expression vectors are able to synthesise HBsAg for immunisation purposes.

### 2.2.6 Hepatitis B vaccine

#### 2.2.6.1 Background

While searching for polymorphic serum proteins in 1963, Blumberg accidentally discovered a previously unknown protein in the blood of an Australian aborigine (Blumberg, *et al.*, 1967). This protein was denoted as the Australia (Au) antigen, and it

became apparent that this protein was related to the Hepatitis B virus. Later, Prince, Okohi, and Murakami established that the Au antigen (now known as the HBsAg) was only found in the serum of Hepatitis B infected patients, thus confirming the relation between the antigen and the HBV (Prince, 1968; Okochi and Murakami, 1968). The following year, Blumberg isolated small particles from HBsAg-positive blood, and with further investigation revealed that some of the particles were whole viruses, whereas the genes responsible for causing infection and disease were absent from some of the other isolated particles (Blumberg, 1997). Similarly, in 1970, Dane and co-workers found virus-like particles (VLPs) in the serum of patients suffering from Hepatitis B, and designated these particles as the HBV (Dane *et al.*, 1970). Kaplan detected an endogenous DNA-dependent DNA polymerase within the core of these VLPs (Kaplan *et al.*, 1973). This discovery allowed for the detection and characterisation of the HBV genome (Robinson and Greenman, 1974).

Consequent experiments revealed that certain Hepatitis B particles could induce protective immunity against HBV infection. In 1971, Krugman discovered that injections of Hepatitis B-contaminated blood containing heat-killed HBVs, gave some protection against Hepatitis B (Krugman, 1985). It was also suggested that the nucleic-acid-free particles, isolated by Blumberg, could be used to stimulate immunity against the infectious virus (Blumberg, 1997). Okochi and Murakami discovered that patients, who received blood containing HBsAg antibodies during blood transfusions, were less likely to develop post-transfusion hepatitis than patients who received blood from which the antibodies were absent (Okochi and Murakami, 1968).

2.2.6.2 Development

Blumberg and Millman were intrigued by the fact that HBsAgs initiated an immune response that protected people from Hepatitis B. Thus, they proposed the development of a vaccine from HBsAg particles obtained from the blood of Hepatitis B carriers.

At first, vaccine production strategies comprised of live attenuated forms of the virus to stimulate an immune response. However, the concept of producing a subunit vaccine from purified human blood infected with the virus soon became apparent as a safer and more efficient method for vaccine production. Although the Fox Chase Cancer Centre (FCCC) filed a patent concerning the proof of concept in 1969, many years of subsequent research and development was dedicated to this production strategy, and only in 1981 a serum, derived vaccine was made available for general use. This vaccine provided more than 90% protection against Hepatitis B and had no adverse side effects (Szmuness *et al.*, 1981).

With the availability of this new plasma-derived vaccine, a Hepatitis B immunisation strategy was developed in the United States in the 1980s, targeting groups at high-risk of HBV infection (Katkov and Dienstag, 1995). This serum-derived vaccine production system contained a few unforeseen obstacles (Prince and Vnek, 1982). One of these obstacles was that large quantities of blood were required from Hepatitis B carriers, and that the extensive use of this subunit vaccine was affected by the limited availability of acceptable carrier plasma. Furthermore, there was a need for strict procedures for the purification and inactivation of the Hepatitis B virus, as well as prolonged safety tests necessary for clearance of the batches of vaccines. Finally, the probability that other infectious agents, which cause blood-transmissible diseases, may be present in the sera and may escape the process of inactivation during vaccine production, raised great concern. Consequently, the supply of the antigen was so expensive that the vaccine was generally only available to particular high-risk groups, thus not being a solution to the widespread problem in third world countries (Barr *et al.*, 1989; Kitano *et al.*, 1987).

Furthermore, the prevailing strategy of targeting the Hepatitis B vaccine to specific high-risk groups was shown to be ineffective in curbing the HBV epidemic in the U.S., suggesting the necessity for more widespread immunisation programs (Katkov and Dienstag, 1995). On account of these obstacles, there was a general tendency to develop and replace these blood-derived vaccines with vaccines obtained through genetic engineering techniques, which requires being safe and inexpensive.

Safer and more cost effective production strategies were considered with the advent of recombinant DNA technology. In 1977, Rutter and co-workers proposed the development of a Hepatitis B vaccine by producing HBsAg particles through recombinant technology. This production process would ensure no contamination from other sources as well as allow production of large quantities of the vaccine. Once the HBV genome was successfully sequenced (Valenzuela *et al.*, 1979), genes encoding non-infectious particles could be determined. Thus, various non-infectious particle producing biological systems could be explored using the new recombinant techniques. The first of these systems was carried out using bacterial cells, but these experiments were unsuccessful (Valenzuela *et al.*, 1979). Following this, Hall developed a model system using yeast cells, and in 1982 Rutter and Hall successfully produced pure HBsAg particles from genetically altered yeast cells (Valenzuela *et al.*, 1982). Finally, in 1986, after nine years of research, this recombinant vaccine was the first of its kind for use in humans and was licensed by the U.S. Food and Drug Administration (FDA) for general use.

Shortly after the yeast vector was created, French researchers created a similar particle, which was able to induce the correlate of immunity. In this system, the recombinant plasmid was transfected into Chinese hamster ovary (CHO) cells. The plasmid varied slightly from the recombinant yeast plasmid, since it contained an additional piece of the HBV genome. While the yeast plasmid included only the *S* gene of HBV, the CHO clones used the *S* gene plus the pre-S2 portion of the genome, creating a vector that began one start codon upstream in the genome. Although this system was somewhat different, the same 22 nm particle containing the immunogenic HBsAg was produced.

The efficacy of this vaccine was tested against the efficacy of HBsAg molecules purified from human systems, and the efficacies were found to be identical (Crosnier *et al.*, 1980). This confirmed the fact that the artificial immunogens, or better known as virus-like particles (VLPs), produced in CHO cells, could be used as an alternative production system concerning a Hepatitis B vaccine. Until this day, research has continued in this field, exploring alternative and genetically improved expression systems.

#### 2.2.6.3 Development of the Recombinant Hepatitis B Virus Vaccine

Since the advent of recombinant DNA technology, the Hepatitis B virus surface antigen gene has been cloned and expressed in prokaryotic and eukaryotic cells. TABLE 2.5 is a tabulated summary of the HBsAg production yields from some of the recombinant hosts.

**TABLE 2.5:** Summary of HBsAg yields from various microbial hosts.

| Heterologous Host | Initial Yield [mg.ℓ$^{-1}$] | Reference | Optimised Yield [mg.ℓ$^{-1}$] | Reference |
|---|---|---|---|---|
| *E. coli* | - | (Burrell *et al.*, 1979) | - | (Miyanohara *et al.*, 1983) |
| *S. cerevisiae* | 0.0025 | (Valenzuela *et al.*, 1982) | 25 | (Gu *et al.*, 1991) |
| *P. pastoris* | 0.14 | (Hardy *et al.*, 2000) | 1300 | (Hardy *et al.*, 2000) |
| *A. niger* | 0.4 | (Plüddemann and van Zyl, 2003) | ? | - |

The first non-infectious particle producing biological system used was the prokaryotic cell, *E. coli*. However, the levels of expression obtained in bacteria were low, and efficient incorporation of the antigen in immunogenic 22 nm particles was not complete and hence, non-functional. Furthermore, the HBsAg gene product produced by these cells seemed to be either unstable or caused effects deleterious to the host, or both (Valenzuela *et al.*, 1979; Miyanohara *et al.*, 1983). Due to this, this system was not

utilised as an antigen source for the production of vaccines (Valenzuela *et al.*, 1979; Burrell *et al.*, 1979).

The recombinant vaccines against Hepatitis B, which are available commercially, are fundamentally based on the production of HBsAg by genetically manipulated yeasts. The yeast *S. cerevisiae* has been widely used for the production of biologically active heterologous proteins for wide use (Kingsman *et al.*, 1987) and for obtaining HBsAg in large quantities (McAleer *et al.*, 1984; Bitter *et al.*, 1988). The first plasmid transformed into *S. cerevisiae*, was constructed by Valenzuela and co-workers, and allowed for the expression of the *S* gene, which codes for HBsAg, under the control of the ADH1 promoter. This system yielded 10 to 25 $\mu g_{HBsAg}.\ell_{culture}^{-1}$ (Valenzuela *et al.*, 1982). Subsequently, the expression of the *S* gene, under control of the constitutive *S. cerevisiae* 3-phosphoglycerate kinase (*PGK*) gene promoter, yielded 50 $\mu g_{HBsAg}.\ell_{culture}^{-1}$ (Hitzeman *et al.*, 1983). Miyanohara and co-workers managed a yield of 2.8 $mg_{HBsAg}.\ell_{culture}^{-1}$ by expression of the S gene under control of the repressible yeast acid phosphatase gene promoter in *S. cerevisiae* (Miyanohara *et al.*, 1983). Under control of the glyceraldehyde-3-phosphate (*GPD*) gene promoter in *S. cerevisiae*, *S* gene expression levels of 3 $mg_{HBsAg}.\ell_{culture}^{-1}$ were measured (Hsieh *et al.*, 1988), in batch cultivations. Under fed batch conditions, the HBsAg production was enhanced almost six times to 20 $mg.\ell_{culture}^{-1}$ or 700 $\mu g.g^{-1}$ dry weight (DW). This phenomenon can be explained by the fact that the HBsAg production is expressed as an exponential function of time when the specific growth rate is controlled to a constant value in growth associated product formation (Gu *et al.*, 1991).

It is important to note that the antigen produced by *S. cerevisiae*, remains intracellularly. Chen and co-workers attempted to express HBsAg in the secretion pathway of *S. cerevisiae*, but the yeast was still unable to secrete the protein extracellularly (Chen *et al.*, 1995). The intracellular product is extracted by different methods of cellular disruption and purified by various physicochemical methods. These methods have made it possible to achieve levels of purity greater than 97%, and the product has an

antigenic behaviour similar to the plasma antigen as demonstrated in animals and humans (Hauser *et al.*, 1988).

M and L proteins have also been targeted for expression in *S. cerevisiae*, since the preS regions contain important antigenic determinants. These antigenic properties became apparent when it was noted that during HBV infections, antibodies were produced against preS regions. The immunogenicity of S sequences in normally non-responding animals appear to be increased by preS sequences (Peterson, 1987; Yap *et al.*, 1995).

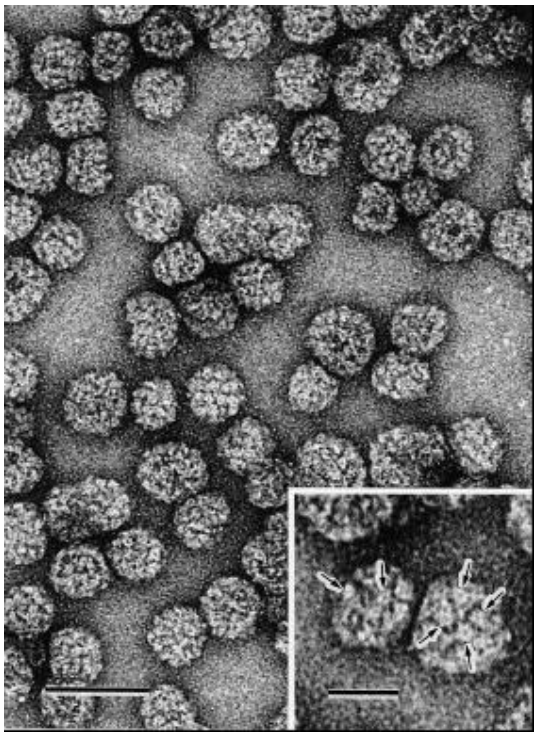

**FIGURE 2.6:** Negatively stained HBsAg particles isolated from recombinant yeasts. ×280000. Bar=50 nm. Inset: ×465000. Bar=20 nm. Note subunit structures with pores (arrows) (Yamaguchi *et al.*, 1998).

The methylotropic yeasts, *P. pastoris* and *Hansenula polymorpha* were also investigated as a HBsAg vaccine production system and were proved to be very effective HBsAg producing hosts. Initially *P. pastoris* was found to produce high levels of protein, with HBsAg comprising 2-3% of the soluble protein of the cell, reaching concentration levels of 48 $mg_{HBsAg}.\ell_{culture}^{-1}$ or 6.6 $mg_{HBsAg}.g_{DW}^{-1}$ (Cregg *et al.*, 1987).

During industrial scale production, a genetic construct is utilised in which the *S* gene is cloned under the regulation signals of the promoter of the enzyme Alcohol Oxidase I, and levels of 1.3±0.2 $g_{HBsAg}.\ell^{-1}$ purified sample or 17 $ml.g_{DW}^{-1}$ was obtained, using a polyclonal HBsAg assay, under fed batch conditions (Hardy *et al.*, 2000). To date this is the highest published HBsAg expression rate achieved, and consequently this *P. pastoris*-derived HBsAg has been employed as a safe, efficacious vaccine (HEBERBIOVAC HB, Heberbiotec S. A., Cuba). A recombinant *P. pastoris* strain expressing the *S* gene under control of the constitutive *P. pastoris* glyceraldehye-3-phosphate dehydrogenase promoter has also been constructed.

Production with this strain reduces fermentation time for peak production levels, since this strain, unlike those described above, does not need methanol induction (Vassileva *et al.*, 2001). An important advantage the *P. pastoris* system offers, in comparison to that of *S. cerevisiae*, is the very efficient incorporation of the antigen into particles of 22 nm. Virtually all the antigen produced is in particulated form, unlike that in *S. cerevisiae*, in which only a small proportion of the monomer of 24 kDa is incorporated into the antigen particles (Valenzuela *et al.*, 1982, Hitzeman *et al.*, 1983, Miyanohara *et al.*, 1983).

*H. polymorpha* also produced high levels of antigen (5% to 8% of total cell protein) containing both S and L surface antigens and has also been shown to successfully express M protein (Gellissen, 2000; Janowicz *et al.*, 1991; Shen *et al.*, 1989). The proteins were also found to be glycosylated far less than in *S. cerevisiae,* making this an attractive alternative host (Janowicz *et al.*, 1991). A production strain was generated by introducing a plasmid with the *S* gene fused to the *H. polymorpha* methanol oxidase (*MOX*) promoter (Gellissen, 2000). The transformed strain is able to grow on glycerol and methanol as carbon sources. Currently, two *H. polymorpha*-derived Hepatitis B vaccines are commercially available: Hepavax-Gene, produced by KGCC (Korea) and AgB, produced by LPC (Argentina). No publicised HBsAg production levels have been found in the literature. The two major producers of HBV vaccine containing the HBsAg that are available in the United States are Merck and SmithKline Beecham. In other

parts of the world, there are several companies producing recombinant vaccines (such as Pasteur Merieux Connaught in France) as well as a few that are still selling plasma derived vaccines. The two vaccines produced by Merck contain only the S protein, whereas the vaccine produced in France, GENHEVAC B, contains the pre-S2 peptide as well. Yeast-based vaccines are most widely used.

**TABLE 2.6:** Major producers of HBV vaccine containing the HBsAg psuedo-viral particles (Mahoney and Kane, 1999; Plotkin and Orenstein, 1994; http://www.sb.com; http://www.merck.com; http://www.aventispasteur.com/).

| Manufacturer | Brand Name | Type of Vaccine |
|---|---|---|
| Centro de Ingeniera Genetica Y Biolotecnologia | Enivac-HB | Recombinant DNA |
| Chiel Jedang | Hepaccine-B | Plasma derived |
| Korea Green Cross | Hepavax B | Plasma derived |
| Korea Green Cross | Hepavax-Gene | Recombinant DNA |
| LG Chemical | Euvax B | Recombinant DNA |
| Merck Sharpe and Dohme | Recombivax H-B | Recombinant DNA |
| Merck Sharpe and Dohme | Comvax | Combined Hib and recombinant DNA |
| Pasteur Merieux Connaught | Genhevac B | Recombinant DNA (mammalian cell) |
| SmithKilne Beecham | Engerix-B | Recombinant DNA |
| SmithKilne Beecham | Twinrix | Combined hepatitis A and B recomb.) |
| Heberbiotec S. A, Cuba | Heberbiovac HB | Recombinant DNA |
| LPC, Argentinia | AgB | Recombinant DNA |
| SmithKilne Beecham | Tritanrix-HB | Combined DTP and recombinant |
| Swiss Serum and Vaccines Institute | Heprecombe | Recombinant DNA (mammalian cell) |

#### 2.2.6.4 Alternative hosts for the Recombinant Hepatitis B Virus Vaccine

Alternative hosts that have previously been used for the expression of Hepatitis B antigens include; mammalian cells, vaccinia virus, the yeast *Kluyveromyces lactis*, plant hosts and the filamentous fungi, *A. niger* (Barr *et al.*, 1989; Gellissen, 2000; Gellissen

and Hollenberg, 1997; Gellissen and Veenhuis, 2001; Michel *et al.*, 1984; Rutter *et al.*, 1983; MacNab *et al.*, 1976; Cheng *et al.*, 1986; Hadfield *et al.*, 1993; Hollenberg and Gellissen, 1997; Martinez *et al.*, 1992; Plüddemann and van Zyl, 2003; Thanavala, 1996).

Eukaryotic mammalian cells have also been used in the production of HBsAg (Michel *et al.*, 1984; Rutter *et al.*, 1983; MacNab *et al.*, 1976). However, the production of vaccines using this method requires both complex and costly equipment, methodology and culture media. Furthermore, the scale-up of the production system poses a great problem. There are also fears relating to the safety of vaccines derived from mammalian cell lines, which are tumorigenic due to the risk of the presence of retrovirus. Due to these complications mammalian cell cultures are not the preferred choice for the HBsAg production system.

The genetically manipulated vaccinia virus makes it possible to obtain recombinant viruses, which express HBsAg alone or in combination with other antigens for the production of live vaccines against infectious agents (Cheng *et al.*, 1986). The vaccines obtained in this way have not been approved for large-scale use in humans because of numerous technical and ethical considerations.

The HBsAg has also been expressed in tobacco plants and potato plants as a means to produce edible vaccines (Domansky *et al.*, 1995; Thanavala, 1996). The transgenic tobacco-derived HBsAg was shown to retain the immunological characteristics needed to generate HBsAg-specific B and T cell responses. However, the maximal level of HBsAg obtained was not sufficient to use the plants for the production of a vaccine (Domansky *et al.*, 1995).

Recently, the filamentous fungus *A. niger* has been considered as a heterologous host for the expression of the HBV *S* gene (Plüddemann and van Zyl, 2003). The *A. niger* strain was transformed with the *S* gene under control of the constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter. Approximately seven

copies of the expression cassette were integrated on the genome, resulting in high level transcription of the *S* gene. Production of the 24 kDa S protein and a 48 kDa S protein dimer in the membrane-associated protein fraction of the recombinant *A. niger* strain was obtained (Plüddemann and van Zyl, 2003). It was found that this fungus produces 22 nm pseudoviral particles similar to that isolated from human serum as well as that produced by yeast cells. Initial production levels of 0.4 $mg_{HBsAg}.\ell_{culture}^{-1}$ (200 $\mu g.g_{DW}^{-1}$) were obtained during shake-flask experiments. Furthermore, by implementing a well-optimised fermentation, yields at least five to ten times greater than shake-flask yields should theoretically be achieved, thus indicating the potential of such an *Aspergillus* system (Davies, 1994).

### 2.2.7 Conclusion

The recombinant fungal HBsAg producing system (Plüddemann and van Zyl, 2003) could allow for the production of a cheaper generic Hepatitis B vaccine. Thus, the already firmly established base not only demands for continued research and development in this area, but also a detailed investigation on other potential vaccines and pharmaceutical proteins that can be expressed in this expression system. Thus, with the inherent advantages of filamentous fungi as a protein production system, one is encouraged to recommend *A. niger*, as a "microbial factory", having the potential to effectively and efficiently produce immunogenic viral proteins. And therefore, emerging as a promising alternative to the current vaccine production technologies.

## 2.3 Submerged Bioprocessing of the filamentous fungi, *A. niger*.

### 2.3.1 Introduction

Industrial bioprocesses with filamentous fungi embrace the production of a majority of commercially important products of biotechnology, in the sense of quality as well as the diversity of metabolites. The development of the bioreactor has been an essential factor in this success, where its purpose is to provide optimum environmental conditions that allow for the formation of the desired fungal product to proceed at the desired rate.

A dynamic relationship exists between these environmental conditions and the growth pattern of the fungal microorganisms. Distinct cultivation conditions result in different morphological and physico-chemical characteristics of the fungal elements and thereby affecting the production levels of native as well as heterologous proteins.

Filamentous fungi are morphologically complex organisms, differing in structure at different times in their life cycle, differing in form between surface and submerged growth, differing also with the nature of the growth medium and physical environment. Many genes and physiological mechanisms are involved in the process of morphogenesis and product formation. And thus, from a biochemical engineering viewpoint, a concerted effort must be made in order to identify parameters and variables that have the potential of affecting product yield. Optimisation of each parameter is then necessary to achieve process improvement and process optimisation.

### 2.3.2 Factors influencing heterologous protein production

Cultivation of filamentous fungi can be complex in order to produce reproducible and optimal product yields. Sentiments that must be taken into account to obtain these criteria, include various interdependent process parameters and variables, (type of cultivation, modes of operation, broth rheology, fungal morphology and biomass

concentration) that will influence the optimum levels of the desired product (heterologous protein) within the bioreactor process system. As a bioprocessing engineer, it is of vital importance to quantitatively identify the influential process parameters in the bioprocess and determine its quantitative interaction with the other process parameters. FIG. 2.7 illustrates these interdependent process parameters and variables that may be of fundamental importance to process optimization.

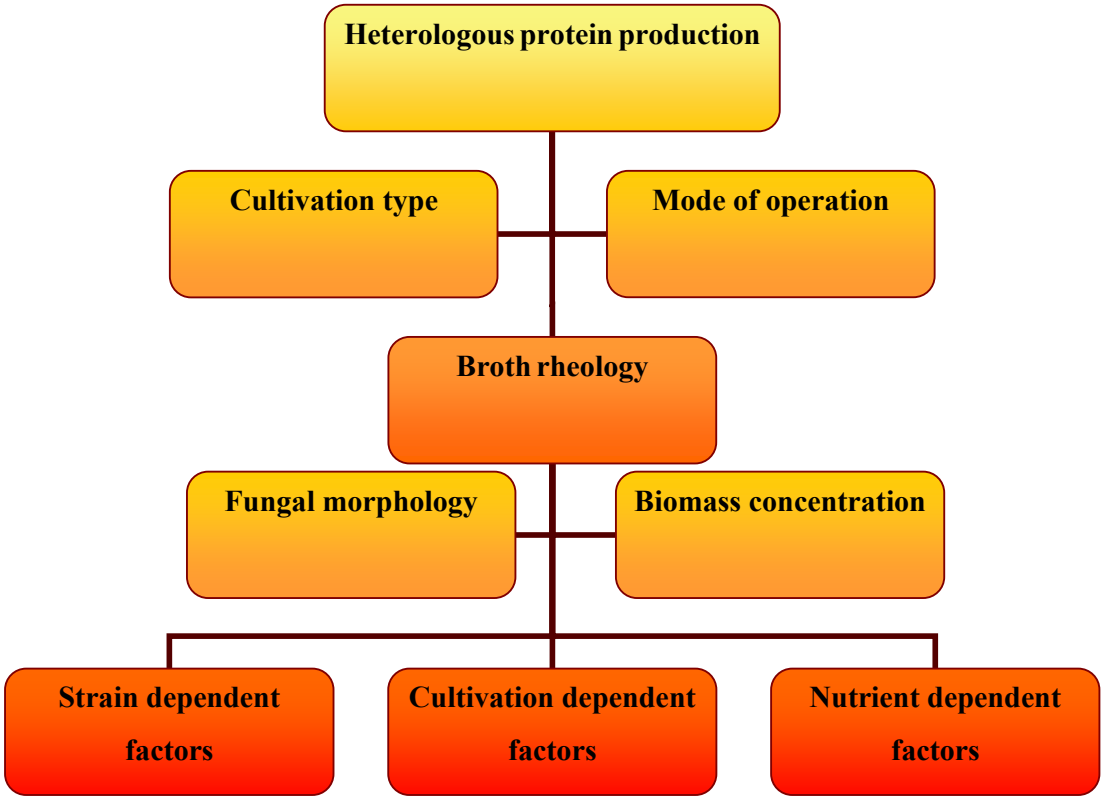


**FIGURE 2.7:** A simplistic illustration of the interdependent process factors that influence product yield within a submerged bioreactor process system.

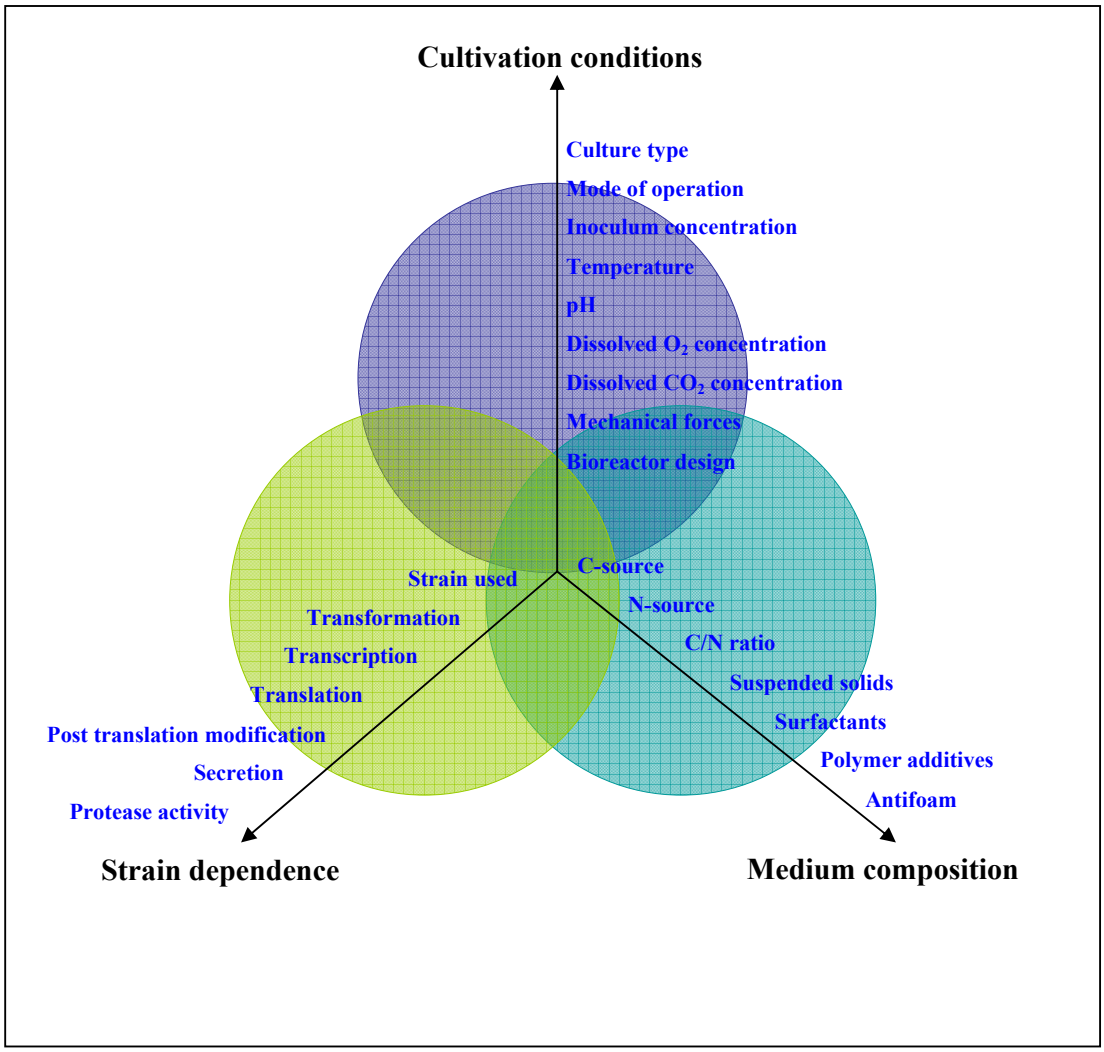


**FIGURE 2.8:** Factors affecting fungal biomass concentration and morphology and product yields.

### 2.3.3 Cultivation type: Submerged vs. Solid-State Cultures

'Bioprocessing/fermentation industry' usually implies an emphasis on submerged liquid cultivation systems. But solid-state cultivations have also been developed with success, especially in the Japanese traditional food and beverage industry. Industrial enzymes and vitamins have also been produced in filamentous solid-state cultures and at times have exceeded the amount of homologous as well as heterologous proteins secreted in submerged cultures (Elinbaum *et al.*, 2002; Ashokkumar *et al.*, 2001; Diaz-

Godinez *et al.*, 2001; Maldonado and Strasser de Saad, 1998; Acuna-Arguelles *et al.*, 1995; Iwashita, 2002).

The major difference between liquid fermentations and solid-state fermentations is the mixing property. Liquid fermentations are in theory perfectly mixed reactions, and so each part of the reactor contains at any time the same quantity of microorganisms, nutrients and metabolites (Gervias and Bensoussan, 1994). Solid-state fermentations, which are characterized by a reduced liquid phase, present a high viscosity. Thus mixing is very limited and if done so, leads to great shear forces that drastically injure the microbial cells (Gervias and Bensoussan, 1994). Therefore, each system, solid-state and submerged has its advantages. TABLE 2.7 illustrates some of the features commonly attributed to each system.

**TABLE 2.7:** Some features of solid-state and submerged cultivation of the fungus *Aspergillus* (Harvey and McNiel, 1994).

| Feature | Growth on solid surface | Growth within liquid nutrient media |
|---|---|---|
| Shear forces | Low | Often high |
| $O_2$ transfer | Gas phase to cell | Gas to liquid to cell |
| Nutrient source | Substratum | Bulk liquid |
| Water activity ($a_w$) | Low | High |
| Degree of differentiation | Often pronounced and usually sporulating | Usually mycelial or pellet form and rarely sporulating |

Despite the success fungi based solid-state cultivations have achieved, fungi-based submerged cultures are the preferred choice of culture system. It is surprising that the filamentous fungi genus *Aspergillus,* with the type of morphology that has enabled it to successfully colonize on surfaces (i.e. soil, plants, grain animals, etc.), should be a success in the comparatively 'unnatural' environment found in submerged cultures. Besides this, submerged cultures attribute of preference in comparison to solid-state cultivations is justified by the allowable ease of handling and control (Iwashita, 2002).

### 2.3.4 Modes of operation: Batch, continuous and fed-batch

#### 2.3.4.1 Batch Cultivation

Batch cultures have been used widely and with much success in citric acid as well as enzyme production systems. A batch culture is inoculated with a microorganism, in various forms, into complete medium required for the entire fermentation process. No additional media or inoculum is added during the fermentation process unless a titrant is added for pH control and, if necessary, antifoaming agent (Harvey and McNiel, 1994). Batch growth is typically divided into a lag phase, exponential phase and stationary phase. The lag phase represents a period during which the spores germinate and the fungal cells adapt to a new environment. This is followed by the exponential phase, which is characterized by a significant increase in cell mass and the growth rate reaches its maximum. A reduction in the specific growth rate occurs when the fungus begins to experience an unfavourable growth environment, such as the limitation of required nutrients and $O_2$, the development of an adverse culture pH or the accumulation of end products of the metabolism that are inhibitory. If the latter two are not reducing growth, the effect of a limiting substrate concentration is a more than a probable cause. This leads the culture into the stationary phase, where secondary metabolite production generally begins. The stationary phase may be defined simplistically as the balance between hyphal mass increase and decrease. However, if the hyphal mass accumulates intracellular storage material during the reduced growth phase, a slight increase in hyphal mass may be observed during the endogenous metabolism of these storage materials. In addition, if the hyphae begin to autolyse, new growth could be expected from the products of autolysis, e.g., release of a limited nutrient (Papagianni and Moo-Young, 2002). Thus, batch cultivations have the disadvantage of being unsuitable for fermentation involving substrate inhibition. Once the cells enter this phase of growth and before the organism enters the stationary phase, there may not be sufficient time or nutrients available to allow formation of the product at an economically viable rate. These organisms are more suited to a fed-batch process.

2.3.4.2 Fed Batch

The majority of large-scale industrial fungal fermentations involve fed-batch cultures in which a batch culture is supplied either with additional nutrients or with additives required for product formation, on a continuous or intermittent basis (pulse feeding), with the aim of avoiding inhibition by the substrate, or $O_2$ limitation. Fed-batch cultivations have proved to obtain increased biomass levels when compared to a batch culture. This is primarily due to an increased amount of carbon/nitrogen source that is able to accumulate within a fed-batch culture. Fed-batch cultures have also proved to produce greater production levels, particularly in fermentations regarding growth associated products i.e. greater biomass yields. It has also been noted that fed-batch cultivations can be used as a manner of reducing broth viscosity. This allows improved mass and heat transfer, particularly in fermentations in which high viscosity of the broth is due to the filamentous morphology of the microorganism (Harvey and McNiel, 1994).

If one were to compare fed-batch to continuous cultivations, there is one major difference in that specific growth rate is not constant in the former, at a constant dilution rate. It rather decreases as the culture volume increases. In order to obtain steady state (constant specific growth rate) the dilution rate must be varied exponentially. This action will increase the time constant within the exponential phase and ultimately a biomass concentration will be achieved greater than in a batch mode. This strategy can only be maintained until the maximum working volume within the bioreactor is reached. Thus, the fed-batch mode of culture represents a useful tool in maximising product formation in growth-associated systems and in studies of substrate limitations and various inhibition phenomena (Papagianni, 1995; Papagianni *et al.,* 1999c).

2.3.4.3 Continuous Culture

To date continuous cultivations have been successfully employed on an industrial scale for the treatment of wastewater, sludge, etc. Industry has been reluctant to use

*Aspergillus* as the primary organism in a continuous culture, but it has been well documented on a laboratory scale, where they have been used to determine various growth parameters as well as controlling the specific growth rate of filamentous fungi (Withers *et al.*, 1994; Schickx *et al.*, 1993; Smith and Robertson, 1980).

Continuous culture is usually preceded by growth of the fungus to the stationary phase in a batch culture. When supply of fresh medium is initiated, growth proceeds and material from the vessel is washed out, until the concentration of the medium is reduced to a level, at which it limits the specific growth rate. The main advantage of this type of system is that the biomass and product formation proceed under steady state and the operator's optimal conditions, thus making the process more economical from both the financial and labour saving point of view (Harvey and McNiel, 1994).

The problems of filamentous growth in submerged batch culture are exacerbated in continuous culture. Wall growth increases continuously and maintenance of steady state is difficult. Breakage of mycelia to provide new centres for growth may be a solution. Another problem of great importance is the maintenance of stability of filamentous fungi in prolonged culture. The importance of stability maintenance has increased recently because of the use of such organisms as hosts for the expression and secretion of heterologous proteins (Peberdy, 1994).

### 2.3.5 Broth rheology, fungal morphology and protein production

The two major factors influencing the rheology of the bulk fluid are biomass concentration and fungal cell morphology and can be related directly to the production of various microbial proteins and metabolites. As the biomass concentration increases, the number of fungal particles in the fermentation fluid increases. This leads to an increased number of potential interactions between these particles that could cause a possible increase in broth viscosity. In submerged cultivation involving filamentous organisms, the morphology can vary from discrete compact pellets of hyphae to homogeneous suspension of dispersed mycelia. These morphological differences are

associated with significant differences in growth kinetics and physiology. Growth of dispersed mycelia is effectively equivalent to that of a unicellular organism, with homogeneous distribution of biomass, substrate and products, and exponential growth at a constant specific rate in batch culture, where substrates are in excess. The filamentous form of mycelial hyphae easily causes entanglement, and the cultivation broth becomes very viscous. The rheological behaviour is usually non-Newtonian, leading to relatively low viscosities in regions of high shear rate (near the impeller) and very high viscosities in region with low shear rate (near the bioreactor wall). The high viscosity and pseudo-plasticity of the suspension cause many problems during the cultivation, decreasing the mass transfer, heat transfer and requiring more power input for mixing.

In this case, only a small part of the bioreactor, around the impeller, is maintained at the optimal condition. Increasing the agitation rate improves the overall homogeneity, but this also raises the power consumption and often damages the cells due to the high shearing (van Suijdam and Metz, 1981; Braun and Vecht-Lifshitz, 1991).

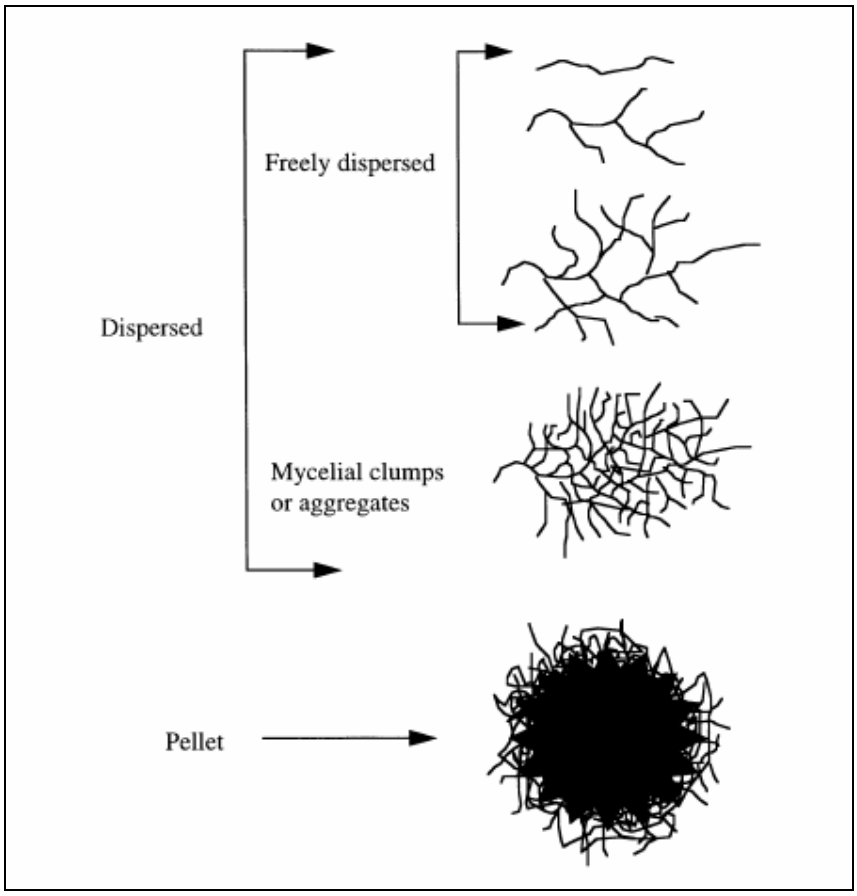


**FIGURE 2.9:** Schematic representation of fungal morphologies in suspension cultures.

In comparison to dispersed mycelia, the pellet form, in the macroscopic feature, can be an attractive growth form for cultivation of fungi. The most important advantages are the decrease of the viscosity of the cultivation broth and the rheological properties become Newtonian. The Newtonian fluid is characterized by good mass and heat transfer properties. Moreover, the pellet formation facilitates the separation of fluid in down stream processes (van Suijdam *et al.*, 1980). The macroscopic features of cultivation medium containing fungal pellets show homogeneity in their rheological properties. In comparison, the microscopic scale of pellet shows some heterogeneity due to the zonation in accordance with the different hyphal densities inside the pellet. The zonation process has been discussed by many authors (Kobayashi *et al.*,1973; König *et al.,* 1982; Lawton *et al.,* 1989; Buschulte *et al.,* 1991; Pottel and Bellgardt, 1992). As

long as there is sufficient supply of oxygen to all cells within the pellet, it grows in density as well as in size. After some time, the oxygen concentration in the centre of the pellet drops to almost zero, restricting cell growth to a zone next to the pellet surface. The limitation of oxygen supply and the removal of metabolic products can lead to an alteration of the cellular metabolism and enzyme excretion kinetics (Hermersdörfer *et al.*, 1987). In studies of penicillin production using *Penicillium chrysogenum* pellets, the transfer resistance inside the fungal pellet is high in large pellets. This causes an oxygen deficiency and autolysis of the cells at the centre of the pellet. However, the thickness of the layer which contains the living cells remains constant, regardless of the pellet size (Schügerl *et al.,* 1983; Wittler *et al.,* 1986).

On the other hand, if the pellets consist of a loose, open, more filamentous mycelium, agitation of the cultures allows nutrients and oxygen to reach all constituent hyphae and supports exponential growth of the entire biomass. The latter type of growth is also more easily controlled because, in the ideal state, all of the hyphae are growing exponentially and all are in contact with well-stirred medium, so all can respond rapidly to manipulation of the medium. These advantages have to be traded off against increased viscosity caused by the filamentous growth. Thus, the ability to control the morphology of a fungus in submerged culture is vitally important, in order to manipulate and optimise product yield.

### 2.3.6 Process Problems associated with submerged fungal fermentations

Special focus is given to the growth and methods of growth of suitable microorganisms on various substrates, in order to optimise the synthesis of useful products, (including heterologous proteins). This is not always possible due to problem factors associated with morphology and broth rheology within the bioreactor. Many of these problem factors are particularly pronounced in fermentations involving filamentous fungi, such as the genus *Aspergillus*.

Process problem factors associated with fungal fermentations are difficult to isolate and correct. These problem factors have complex interactions with each other and the solution to one problem factor may often lead to the activation of another process problem. Biochemical engineers are trying to elucidate these factors, in order to optimise operating conditions in bioreactors for product and process improvement.

Attachment and growth on bioreactors' walls, agitators, probes and baffles (surface growth), can also lead to a degree of heterogeneity within the biomass which is more pronounced in the case of pelleted growth. Areas of growing and non-growing biomass inside the bioreactor influence the overall growth kinetics.

The viscous nature of some submerged filamentous fungi cultivations causes several difficulties. Generally, the more viscous a fermentation fluid, the harder and more expensive it is to achieve sufficient momentum transfer to generate a homogenous, well-mixed cell suspension. Therefore, mixing is of vital importance to ensure that the system is not heterogenous and that there is no formation of stagnant non-mixed zones. If mixing is not sufficient, nutrient gradients become established with $O_2$ limitations particularly becoming a problem with aerobic fermentations (Charles, 1978, Metz *et al.*, 1979 and McNiel and Harvey, 1993).

The sufficient $O_2$ transfer to active cells is critical in order to ensure an aerobic fermentation. Oxygen mass-transfer is even more complex in relation to viscous broths as it is transferred from the sparged gas into the bulk liquid and then only into the $O_2$ demanding metabolic sites within the cell. The dispersal of the sparged gas has been noted to be restricted in viscous broths resulting in coalescence and increasing bubble volume (Oosterhuis and Kossen, 1984). These phenomena drastically affect the $O_2$ transfer rate within the reactor, resulting in rapid depletion of dissolved $O_2$ in the outer stagnant regions of the vessel (Charles, 1978). The strong negative influence of broth viscosity on $O_2$ transfer has been clearly demonstrated by Moo-Young and co-workers (Moo-Young *et al.*, 1987).

Oxygen is not the only variable that may become limiting due to high viscosities and poor mixing. Other nutrients, such as carbon- and nitrogen sources as well as vital trace elements may also become limiting. This has catastrophic implications in the bioreaction, as these limitations may result in the suppression or even total inhibition of metabolite and native as well as heterologous protein production. Poor mixing in bioreactors can result in problems other than those associated with nutrient limitations. Heat transfer, pH control and sampling also pose problems due to poor mixing (Metz, 1979). These potential effects of non-homogenous mixing, together with nutritional limitations give rise to a level of cellular stress which when elevated can result in strain degradation (Humphrey, 1998). Down-stream processing problems may also occur with viscous fermentations, with regards to maintaining fluid flow in pipelines used to transport the broth from the bioreactor to the product recovery plant (Hyde and Scott, 1992; Oolman and Lui, 1991).

It is important to understand the causes and consequences of these problem factors and their effects on product yield and broth rheology. By further identifying influential factors, classified in the categories of strain dependent factors, nutritional dependent factors and cultivation dependent factors, these difficulties in fungal cultivations can either be avoided or rectified.

### 2.3.7 Strain dependent factors

Strategies for optimising the production of proteins have to ensure that strain dependent factors do not limit protein yields. Several strain dependent factors that may negatively affect the production levels of heterologous protein production include, transformation, transcription, translation, post translation.

#### 2.3.7.1 Transformation, transcription, translation and post translation

The transformation frequency in *Aspergillus* is low (Fincham, 1989), but conversely, the mitotic stability of transformants is high (Cullen *et al.*, 1987). Thus, once

transformants have been isolated, the loss of transforming DNA is unlikely. Despite this, problems may arise due to persistence and replication of untransformed nuclei. In order to avoid this, the necessary precautions must be undertaken (Buxton and Radford, 1984). Furthermore, there seems to be no simple relationship between copy number of the transforming gene and protein production in some species. This ambiguity can be explained by the consequence of the often random nature of the integration process (Harvey and McNeil, 1994). Finally, the specific area or areas of the genome into which the foreign DNA is introduced may also affect the expression level (Saunders *et al.*, 1989).

Several strong promoters from highly expressed genes are currently being exploited for the expression of recombinant genes. Highly inducible promoters (e.g., the promoter of the *glaA* gene encoding glucoamylase from *A. niger*) and strong constitutive promoters (glyceralaldehyde-3-phosphate-dehydrogenase from *A. nidulans*) are available and have been described by Jeenes *et al.* (1991) and Saunders *et al.* (1989). Due to the strength of these promoters, Jeenes *et al.* (1991) have implied that the messenger RNA levels of the heterologous genes are relatively stable in the cytoplasm and the pre-heterologous protein secretion process is not restricted in the transcriptional level (Harvey and McNeil, 1994). An approach to transcriptional enhancement has been to alter gene dosage (Verdoes *et al.*, 1993; Verdoes *et al.*, 1994a,b). For a fermentation process involving heterologous protein production, an inducible promoter that can be switched on at any point in the fermentation is usually favoured.

Kozak (1986) has demonstrated the influence of a specific sequence upstream of the initiation code that may influence translation. It is yet unclear whether protein production is markedly influenced in the filamentous fungi at the stage of translation (Jeenes *et al.*, 1991).

One of most important events associated with the post-translational modification of proteins, is glycosylation. Glycosylation plays an important role in protein folding, stability and function (Sorensen *et al.*, 1996; Wang *et al.*, 1996). It is perceived that

there are limitations during this process at several levels, which affects biosynthesis and secretion of homologous and heterologous glycoproteins. An understanding of the glycosylation of proteins is important when using filamentous fungi as hosts for heterologous protein production as the diversity in the composition of glycans associated with proteins could have major consequences with respect to their antigenicity and functionality (Speake *et al.*, 1980).

2.3.7.2 Protein secretion

Few studies of protein secretion have been done with filamentous fungi. This is primarily due to the lack of knowledge, on a molecular basis, with regards to protein secretion in filamentous fungi. This led Peberdy to refer to protein secretion in filamentous fungi as "a highly productive black box" (Peberdy, 1994). It is known that the secretory pathway has the functions of protein folding, glycosylation, processing, and targeting either to the outside of the cell or to intracellular sites. The cell wall acts as a barrier to all secreted proteins to some extent and many fungal enzymes are partially cell wall-associated (Gordon *et al.*, 2000b; Archer and Peberdy, 1997). The secretory pathway may be limited in capacity/ability in the production of many secreted proteins.

To identify possible limitations in the secretory pathway, the processes involved in secretion is required to be elucidated. The protein secretion pathway processes in filamentous fungi do not essentially differ from those in yeast and mammalian cells (Moir and Mao, 1990) and are reviewed by Gouka *et al.* (1997b).

Essentially, co-translational entry of a protein into the lumen of the ER with concomitant processing of the N-terminal signal peptide is followed by translocation through the ER lumen to membrane-bound vesicles and targeting to the Golgi compartment. In the Golgi compartment processes such as glycosylation and processing occur, and finally the protein is transported to the cell wall, again in vesicles, and is

secreted into the extracellular medium through the fusion of the vesicles with the hyphal membrane. The precise location of protein secretion is under debate, but is suggested to

occur primarily at the hyphal tips (Gordon *et al.*, 2000a; Gordon *et al.*, 2000b). The wall at the hyphal tips is newly synthesised and may be more porous than elsewhere, thereby facilitating secretion. Therefore, those factors that increase the number of active tips may improve yield (Pluschkell *et al.*, 1996; Juge *et al.*, 1998).

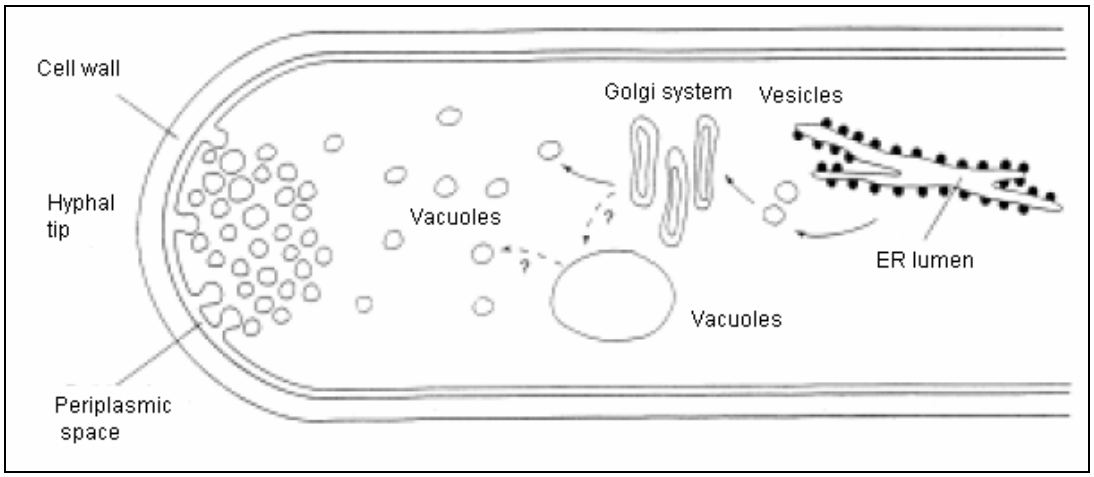


**FIGURE 2.10**: A hypothetical secretory pathway in filamentous fungi (Peberdy, 1994).

Heterologous proteins of biotechnological importance produced in filamentous fungi either remain within the cell (i.e. bound to membrane organelles, cytoplasm, or periplasm) or are secreted extracellularly into the medium. In many heterologous protein production systems extracellular secretion is favourable, because it makes the product easier and cheaper to purify (Archer and Peberdy, 1997). Continuous cultivation types are a favourable option reducing product loss which can occur due to inefficient cell lysis or internal proteolysis during intracellular product recovery (Jeenes *et al.*, 1991; Smart, 1991). Furthermore, possible toxic effects of intracellular product build-up are reduced and, by passing through the secretory pathway, processing events such as glycosylation and proteolytic maturation can occur to yield correctly folded fully active molecules. However, there are some disadvantages to secreting

heterologous proteins into the growth medium. Even with the successful secretion of heterologous proteins, some considerable fraction of the yield could be lost due to extracellular protease degradation (see next section). Furthermore, many fungi also produce large amounts of organic acid, resulting in a low pH (pH 1.5-2.0 in some cases), which can alter the structure of certain proteins or lead to increased sensitivity to acid proteases active in the culture broth (Jeenes *et al.*, 1991).

2.3.7.3 Proteases

Proteolytic degradation by fungal proteases is recognised as one of the major problems interfering with efficient heterologous protein production (van den Hombergh *et al.*, 1997c; Archer and Peberdy, 1997). *Aspergilli* can secrete a diversity of proteases and it has been shown that proteases are responsible for the degradation of many heterologous proteins. Degradation can occur intracellularly during the secretion process and after secretion in the culture fluid as a result of host cell proteases (Jarai, 1997). Hyphal disruption in older cultures and a decrease in culture pH are major factors that influence the enhanced synthesis causing intracellular degradation (Gouka *et al.*, 1996) and release of proteases into the broth culture (Archer *et al.*, 1992). Many filamentous fungi secrete considerable amounts of proteolytic enzymes, some of which are of interest in industrial applications, such as soy sauce production, *sake* brewing and milk coagulation in cheese manufacturing (Cheevadhanarak *et al.*, 1991; Gomi *et al.*, 1993; Thompson, 1991).

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 alkalinisation of the medium with concomitant protease induction (Bartling *et al.*, 1996). Some problems with proteolysis may also be overcome by the inclusion of protease inhibitors in the growth media, but this is unlikely to be a cost-effective measure (Archer and Peberdy, 1997).

2.3.7.4 Strategies to improve heterologous protein production

Other strategies that should be considered for the improvement of protein production include the following genetic strategies:

1) The introduction of multicopies of genes,
2) random mutagenesis (MacKenzie *et al.*, 2000),
3) DNA-tagged mutagenesis involving restriction enzyme-mediated integration as a mutagen (Yaver *et al.*, 2000),
4) The use of strong promoters and efficient secretion signals,
5) Gene fusion with a gene encoding part or whole of a well-expressed and secreted protein,
6) The construction and use of protease-deficient host strains, and
7) Physiological optimisation through temperature or substrate adjustments (MacKenzie *et al.*, 1994).

Although considerable progress has been made concerning the understanding of protein overproduction, it should be considered that overcoming limitations at, for example, the level of transcription would probably reveal new limitations at other levels concerning cell physiology and protein secretion. In addition, protein secretion in filamentous fungi has proven to be very expensive in relation to energy consumption. Therefore extensive protein overproduction may affect cell physiology, which could in turn affect gene expression and genetic stability (Verdoes *et al.*, 1995).

Gene fusion strategies have been successful in the past few years. Fusion has resulted in increases in heterologous protein production levels up to a 1000-fold. Along the secretory pathway, in most cases the fusion protein is cleaved, resulting in the secretion of separate proteins. Cleavage occurs either by autocatalytic processing of the heterologous protein by an unknown fungal protease or by a *KEX2*-like protease, for which a recognition site has been introduced specifically into the fusion gene (van den Hombergh *et al.*, 1997).

Various strategies have been employed to address the problem of proteolytic degradation of heterologous protein products. Many protease-encoding genes have been cloned and used to provide specific protease deletion strains and protease-deficient mutants have also been produced (Archer and Peberdy, 1997). Several protease-deficient UV mutants of *A. niger* have also been isolated and characterised (Mattern *et al.*, 1992; van den Hombergh *et al.*, 1995; van den Hombergh *et al.*, 1997a). Another strategy to construct protease-deficient mutants is the antisense control strategy (Zheng *et al.*, 1998). 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.*, 2000b). This kind of protease-deficient, non-acidifying strain is commonly used in the expression of heterologous proteins, particularly non-fungal proteins, and was also utilised for the expression of viral proteins (Plüddemann and van Zyl, 2003; Gordon *et al.*, 2000b; Wiebe *et al.*, 2001). Down regulation of protease expression could also help resolve the problem. The extracellular fungal proteases are also strictly pH regulated, e.g., 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. Unfortunately, little work concerning this has been done.

In conclusion, the factors influencing heterologous protein yield are complex and include biochemical and biophysical properties of the protein itself. Therefore, optimisation strategies must be individually designed and assessed for each heterologous protein production system.

### 2.3.8 Nutritient dependent factors

The media used in fungal submerged industrial fermentations influence growth, morphology and product formation and therefore must contain all the required elements in a suitable form: conidial fungi require water, molecular oxygen, an organic source of carbon and energy, a source of nitrogen other than molecular nitrogen and several other

elements. Besides the elements mentioned above, at least 10 are essential for growth namely; hydrogen, phosphorus, potassium, sulphur, magnesium, manganese, iron, zinc, copper and molybdenum. The first five are needed in relatively large quantities (macronutrients). The latter five are required in small amounts (micronutrients).

In laboratory research with microorganisms, pure defined chemicals (synthetic) may be used in the production of culture media; however, in the industry complex substrates are frequently used for economic reasons. These complex substrates may comprise of various forms of carbon and nitrogen sources. Trace elements are also required to deliver various metal ions and salts to the microorganism. Within these complex substrates, additives are often included. These additives may be in the form of complex organic material, suspended solids, surfactants, polymer additives, antifoam, etc. Collectively these media components are classified as the ingredients for the fermentation media; and due to various component forms, concentration, and ability to interact with other components, heterologous protein production, broth rheology, biomass concentration, and fungal morphology may be influenced. Thus in order to obtain an optimally balanced culture media for maximal process production, a thorough evaluation of media component form, concentration, and interactiveness must be performed. TABLE 2.8 highlights various media components that are necessary in certain fungal cultivations and may have the ability to manipulate product yield.

**TABLE 2.8:** Some nutritional dependent factors affecting heterologous protein production.

| Nutritional dependent factors | Microorganism | Reference |
|---|---|---|
| Carbon source | *A. niger* | Hermersdörfer *et al.*, 1987 |
| Nitrogen source | *A. niger* | Hermersdörfer *et al.*, 1987 |
| C/N ratio | Various | Braun and Vecht-Lifshitz, 1991 |
| Complex organic material | *Rhizopus arrhizus* | Byrne and Ward, 1987 |
| Polymer | *A. niger*<br>*Pe. chrysogenum* | Elmayergi, 1975<br>Pedersen *et al.*, 1994 |
| Surfactants | *A. niger* | Metz and Kossen, 1977 |
| Metal ions | Various | Clark and Lentz, 1961 |
| Solid particles | *A. niger* | Metz and Kossen, 1977 |

Both the type and concentration of the carbon source appear particularly influential with regards to process optimisation (Papagianni, 2004). Filamentous fungi are heterotrophic. Organic compounds supporting most growth are usually sugars (e.g., D-glucose, D-fructose, sucrose), which are rapidly taken up. Other carbon sources include, molasses, malt extract, starch, dextrin, sulfite waste liquors, polysaccharides, amino acids, lipids, organic acids, proteins, alcohols and hydrocarbons are also used. A small amount of exogenous carbon may be required to maintain the fungus even when it is not growing. According to the work done on *Penicillium chrysogenum* by Cundell *et al.* (1976), protein production, rheology, morphology and specific growth rate are all influenced by the type of carbon source. Experiments performed by Papagianni *et al.* (1999c) confirmed that initial glucose concentration in the fermentation medium affected both the rate of citric acid production by *A. niger* and the morphology of the producer organism. It was also noted that the level of glucose had a marked effect on production rates; the specific growth rate of citric acid formation increased with increasing initial glucose concentration from 1% to 14%, and the specific growth rate increased with decreasing glucose concentration (Papagianni, 2004). In addition, carbon

limitation contributes greatly to the process of cell aging and autolysis in fungal cultures, resulting in heavily vacuolated hyphae and fragmentation (Papagianni, 2004).

Nitrogen sources have also been recorded to have a large influence regarding process optimisation. Nitrogen may be supplied as ammonia, as nitrate or in organic compounds, such as amino acids or proteins. Beet or cane molasses, corn-steep liquor, Pharmamedia, whey powder, soy flour, yeast extract and others are used as industrial raw materials, rich in nitrogen. Phosphate is a convenient and readily used source of phosphorus, while organic forms are also used in industrial processes. Both play an important role in metabolite overproduction and affect fungal morphology. Factors favouring increased growth rates, including excess phosphate concentrations, and have been shown to reduce pellet formation (Katz *et al.*, 1972). Contradictions exist however, where in the work of Znidarsic *et al.* (2000), increased nitrogen concentrations led to larger and denser pellet structures of *Rhizopus nigricans*, while at the low nitrogen levels, pellets appeared small, very light and fluffy, and the fungus showed an increased tendency to form clumps. Du *et al.* (2002) studied the effect of the nitrogen source on morphology and antibiotic production with *Rhizopus chinesis*. In media containing different nitrogen compounds, the morphology, in terms of hyphal length and degree of branching, varied significantly. The activity of the antibiotic also varied with the nitrogen source, where the highest antibiotic production, accompanied by pellet growth, was achieved in a medium containing corn-steep liquor (CSL). The pellets were fluffy with a compact core and a loose outer zone. When ammonium sulfate was used as nitrogen source, the pellets were larger, compact, with a smooth surface and antibiotic productivity was lower. The same was the effect with *Pe. chrysogenum* in the study of Pirt and Callow (1959); ammonium sulfate in the medium caused compact pellet growth, while CSL produced looser structures. Omission of nitrogen in the medium greatly affects fungal growth and metabolite production. Schrickx *et al.* (1995) studied the growth behaviour and protein production by *A. niger* without a nitrogen source, where low metabolic activity was observed and fragmentation became the dominant characteristic in the culture.

Certain trace elements and metals are essential for fungal growth, and at various concentrations influence process production levels. Much work has been done on trace metals with *A. niger* concerning the growth and citric acid production. Zinc, manganese, copper, iron, heavy metals and alkaline metals have been shown to affect both fungal morphology and citric acid production (Shu and Johnson,1984; Mattey, 1992; Wold and Suzuki, 1976; Haq *et al.*, 2002). The influence of $Mn^{2+}$ ions on protein synthesis was considered to be of major importance since cycloheximide, an inhibitor of *de novo* protein synthesis, was found to antagonize the effect of manganese addition (Clark, 1961 and Kisser *et al.*, 1980). Cellular anabolism of *A. niger* is impaired under manganese deficiency and/or nitrogen and phosphate limitation. The protein breakdown under $Mn^{2+}$ deficiency results in a high intracellular $NH_4^+$ concentration, which causes inhibition of the enzyme phosphofructokinase (an essential enzyme in the conversion of glucose and fructose to pyruvate), leading to a flux through glycolysis and the formation of citric acid (Kubicek and Röhr, 1977; Habison *et al.*, 1979).

Manganese ions are also known to be specifically involved in many cellular processes, such as cell wall synthesis and sporulation. Kisser *et al.* (1980) studied the development of morphology and cell wall composition of *A. niger* under conditions of manganese deficient and sufficient cultivations in an otherwise citric acid producing medium. Omission of manganese ions from the nutrient medium resulted in abnormal morphological development, characterized by increased spore swelling and squat, bulbous hyphae. The inhibition of glucoprotein turnover caused by the absence of manganese ions in the medium led to the loss of hyphal polarity and increased branching and chitin synthesis.

Often medium formulations for pilot- and industrial-scale fungal fermentations contain solid or semi-solid components because of economic considerations. These components can provide attachment sites for fungal growth and hence encourage hyphal aggregates of no distinct form as opposed to the regularly shaped pellets or mycelia commonly seen in synthetic media.

The presence of surfactants (surface-active agents) in the growth medium has also been found to have a profound effect on fungal morphology and broth rheology (Metz and Kossen, 1977). The addition of polymers such as, carboxypolymethylene, polyacrylic acid and polyacrylate reduce spore agglomeration and hence decrease pellet formation in a number of filamentous fungi (van Suijdam *et al.*, 1980; Takahashi *et al.*, 1960; Elmayergi *et al.*, 1973; Jones *et al.*, 1988b; Trinci, 1983). It has also been suggested that these polymers act by adsorbing to the spore surface and prevent aggregation through electrostatic repulsion (Seviour and Read, 1985; Elmayergi *et al.*, 1973; Jones *et al.*, 1988a). Using antifoaming agents have also been found to affect morphology and protein production. Van Suijdam *et al.* (1980) observed in *Pe. chrysogenum*, uneven spore germination, irregular pellet formation and hence a drop in metabolite production, when droplets of antifoam (polypropylene glycerol 2000) were added.

From the above mentioned paragraphs concerning nutrient dependent factors, there is a point of criticism concerning some of the published work regarding this topic. Many of the experiments published in the literature have been designed to isolate, improve and optimise a single causative factor and ignore other process factors that are also likely to be affected by a change in media composition. A typical example of this is culture pH. By increasing or decreasing a component source concentration, the question is whether it alters the culture pH significantly to affect biomass concentration, cell morphology, product formation, etc. It is recommended that media components be evaluated at various constant pH levels in order to determine optimum process control.

### 2.3.9 Cultivation condition dependent factors

From an engineering point of view, the cultivation condition parameters used in submerged fungal cultivations are of paramount importance regarding bioprocess design, as their influence on growth, morphology and product formation can be used to maximise product yield. As illustrated in FIG. 2.11, complex interactions exist between the process conditions, the productivity and the morphology in fermentations of filamentous microorganisms. The cultivation condition dependent factors have a central

role, together with product formation, and as a bioprocessing engineer, considerable efforts must be undertaken to optimise each process parameter in order to maximise product yield for a particular bioprocess system.

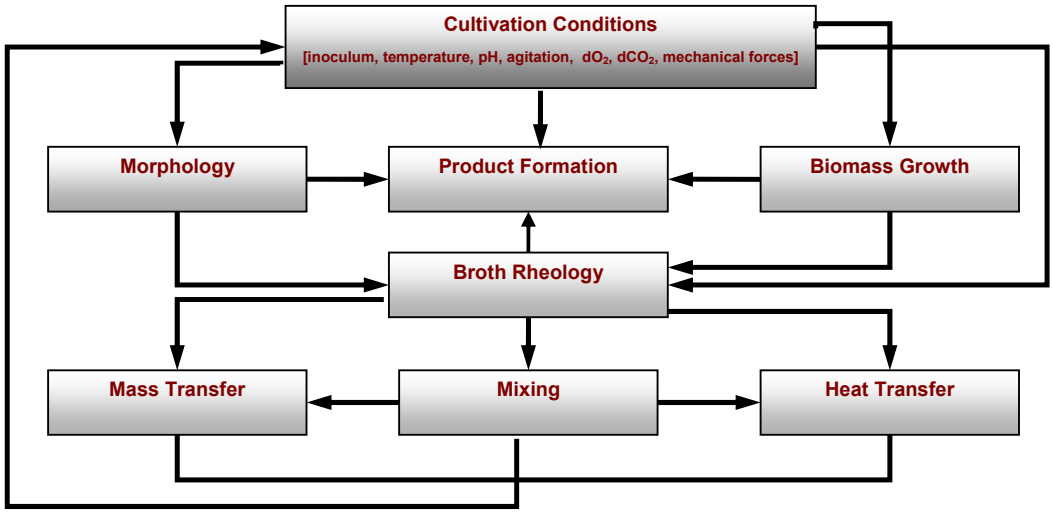


**FIGURE 2.11:** Complex interactions between process conditions, productivity, and morphology in submerged fermentations of filamentous microorganisms (adapted from Metz, 1976).

### 2.3.9.1 Inoculum

Among the factors that define process optimisation, the amount, type (spore or vegetative) and age of the inoculum are of prime importance. In early studies, the effect of the inoculum was assessed mainly by the presence or absence of pellets and their characteristics (Vecht-Lifshitz *et al.*, 1989; Smith and Calam, 1980). This was due to a lack of an adequate method to monitor mycelial morphology during fermentations. During the last decade, the application of image analysis techniques for quantification of morphology in fungal fermentations resulted in more systematic studies on the inoculum effects, and since then many relevant studies have contributed to the understanding of inoculum effects on fungal fermentations. TABLE 2.9 highlights the inoculum related effects on filamentous fungi cultivations found in the literature.

**TABLE 2.9**: Inoculum related effects on filamentous fungi cultivations.

| Factor | Fungus | Reference |
|---|---|---|
| Coagulation of spores favours pellet formation. | *A. niger* | Metz and Kossen, 1977 |
| Low spore inoculum favours pellet formation. | *A. niger*<br>*A. oryzae*<br>*Pe. chrysogenum* | Hermersodofer *et al.*, 1989<br>Calam, 1976<br>Whitaker and Long, 1973 |
| Low inoculum concentration favours pellet formation. | *Ca. fumago* | Carmichael and Pickard, 1989 |
| Inoculum levels at $5x10^5$ spores.m$\ell^{-1}$ favours pellet formation. | *Pe. chrysogenum* | Tucker and Thomas, 1992 |
| Low spore inoculum produces dense pellets, high spore inoculum produces open pellets. | *Penicillium patulum* | Smith and Calam, 1980 |
| Type of inocula (spore or vegetative) affects pellet size and clump formation. | *A. niger* | Papagianni *et al.*, 1999d; Papagianni *et al.*, 2001; Papagianni and Moo-Young, 2002 |
| Mycelial pellets formed from a fragmented hyphae inocula. | *Cunninghamella echinulate* | Chen and Liu, 1997 |

#### 2.3.9.2 Temperature

In theory, temperature can definitely have an effect on metabolite production and broth rheology. This process parameter has not been investigated thoroughly in filamentous fungi cultivations, but it is a known fact that a change in temperature brings about simultaneous changes in other culture variables, such as growth rate and morphology (Bull and Bushell, 1976; Miles and Trinci 1983; Papagianni, 2004). Dissolved oxygen tension is also temperature dependent and varies inversely with increasing temperature (refer to TABLE 2.10). Similarly, nutritional and pH requirements for growth may be influenced by the temperature as well as the rate of medium evaporation. This is seen to have its greatest effect in continuous cultures and could lead to erroneous results concerning growth kinetic interpretations. TABLE 2.10 highlights temperature related effects on filamentous fungi cultivations found in the literature.

**TABLE 2.10:** Temperature related effects on filamentous fungi cultivations.

| Factor affected by temperature increase | Fungus | Reference |
|---|---|---|
| An increase in specific growth rate, (27 $^{o}$C to 35 $^{o}$C), with a max. at 35 $^{o}$C. | *A. oryzae* | Carlsen *et al.*, 1995 |
| Increase of the hyphal growth unit length and wall thickness, (15 $^{o}$C –30 $^{o}$C). | *Pe. chrysogenum* | Miles and Trinci 1983 |
| Oxygen supply becomes inadequate | Various | Schügerl *et al.*, 1998 |
| Morphology manipulation (pelleted growth) | *A. awamori* | Braun and Vecht-Lifshitz, 1991<br>Schügerl *et al.*, 1998 |
| Metabolite and protein production enhancement. Optimum between 25 $^{o}$C to 30 $^{o}$C | *A. awamori*<br>Various | Schügerl *et al.*, 1998<br>Papagianni, 2004 |
| Autolysis enhancement. | Various | Papagianni, 2004 |

2.3.9.3 pH

The pH of the medium is an environmental factor that is often neglected and can often affect productivity most significantly. pH profiles may be interpreted in terms of effects on transport of nutrients, nutrient solubilities, enzyme reactions or surface phenomena. The composition of the medium generally contributes significantly towards initial pH and the extent and direction of pH drifts during growth of the fungus. Poorly buffered media containing ammonium salts are likely to become more acidic during growth, while media containing nitrate are likely to become alkaline. A desired objective that is not always possible is to minimize the pH drifts. High concentrations of ions such as phosphate are often used to stabilise the pH, but to the detriment of biological activity,

(e.g., growth and enzyme activity) (Papagianni, 2004). TABLE 2.11 highlights the pH related effects on filamentous fungi cultivations found in the literature.

**TABLE 2.10:** pH related effects on filamentous fungi cultivations.

| Factor affected by pH | Fungus | Reference |
|---|---|---|
| Optimum biomass yield under glucose limitations @ pH 6.9 and 30 $^{o}$C. | *A. nidulans* | Bull and Bushell, 1976 |
| Growth inhibition @ pH 3 and lower. | *A. nidulans* | Rowley and Pirt, 1972 |
| Hyphal length reduction as pH increases between 6 and 7.4. | *Pe. chrysogenum* | Pirt and Callow, 1959 |
| Maximum hyphal length @ pH 6 | *Pe. chrysogenum* | Miles and Trinci, 1983 |
| A tendency to form pellets as pH increases. | Various | Whitaker and Long, 1973<br>Gerlach *et al.*, 1998<br>Braun and Vecht-Lifshitz, 1991 |
| pH affects surface properties of spores thus affecting morphology. | *A. niger*<br>*A. oryzae* | Galbraith and Smith 1969<br>Carlsen *et al.*, 1995 |
| Optimum α-amylase production @ pH 6 | *A. oryzae* | Carlsen *et al.*, 1995 |
| Optimum pH 4.5 in HEWL production | *A. niger* | Mainwaring *et al.*, 1999 |
| Biomass decreases and heterologous protein increases as pH levels rise between pH 6 and 7 | *A. niger* | Punt *et al.*, 2002 |

#### 2.3.9.4 Dissolved oxygen concentration

Present information suggests that most fungi require molecular oxygen to grow and can grow over very wide ranges of dissolved oxygen ($dO_2$) concentrations. Changes in $dO_2$ concentrations in the growth media have been reported to dramatically influence heterologous protein synthesis in fungi (Varder and Lilly, 1982; Zhou *et al.*, 1992; Gomez *et al.*, 1988; Gibbs and Seviqur, 1996). However, from studies found in the literature, $dO_2$ concentration appears to exert little or no influence on mycelial macro-morphology (van Suijdam and Metz, 1981 and Carter and Bull, 1971), but a significant influence towards the micro-morphology, (pellet size, hyphal length, diameter and number) and rheological properties of the fermentation broth (Olsvik and Kristiansen,

1992a). Some of these reports in the literature seem to contradict each other so special thought must be undertaken in determining optimum $dO_2$ levels for a start up operation. A possible explanation for the so-called change in micro-morphology and broth rheology phenomenon is that the variations in $dO_2$ concentration induce changes in hyphal tensile strength and flexibility (Zetelaki and Vas, 1968). The work done by Zetelaki and Vas (1968) suggest that $O_2$ enriched *A. niger* cultures have thinner cell walls than normal aerated cultures. This leads to the hypothesis that if the cell walls become thinner, protein secretion through the cell wall is enhanced. At this point, no reports in the literature have been found to support this claim.

Furthermore, events such as hyphal tip formation have been noted to be the main visible expression of the differentiation processes of *A. niger* in an $O_2$ enriched environment, which have been shown to play an important role in the process of extracellular enzyme production. Under different oxygen enrichment conditions, *A. niger* seems to develop different growth strategies by undergoing both physiological and micro-morphological changes. *A. niger* has been noted to adapt to high $dO_2$ concentrations in a number of ways. These include; an increased specific growth rate, a decreased size of hyphal elements and an increase in the proportion of active cytoplasm, i.e., in the active compartment ("active length") by switching from mostly inactive vacuolated, long filaments with few branches, to a shorter, active cytoplasm abundant branch form. Enzyme production has been found to be best with short mycelia with high active length when the $O_2$ supply was in excess. Biomass concentration has not been reported to affect the enzyme concentrations (either native or heterologous) directly (Wongwicharn *et al.*, 1999).

#### 2.3.9.5 Dissolved carbon dioxide concentration

The production of carbon dioxide ($CO_2$) in large amounts is common in many aerobic fermentations as a consequence of cell respiratory activity. For filamentous fungi, pronounced effects on both morphology and product formation have been associated with elevated levels of dissolved $CO_2$ ($dCO_2$) and its effects have been reviewed

(McIntyre and McNeil, 1998). McIntyre and McNeil (1997a) studied the effects of $dCO_2$ on morphology, growth and citric acid production by *A. niger* in batch cultures and reported quantitative information on morphological changes by using image analysis. In processes where the inlet gas stream contained more that 3% $dCO_2$, raised $dCO_2$ concentration was associated with decreased biomass and citrate concentrations, decreased substrate consumption, and morphological changes. An increase in the value of the hyphal growth unit, mean hyphal length, and mean branch length was observed as influent $CO_2$ was increased above 5%.

Studies with *Pe. chrysogenum* (Pirt and Mancini, 1975; Smith and Ho, 1985; Ho and Smith, 1986) showed in *Pe. chrysogenum* that levels lower than 3% saturation of $dCO_2$ concentration in the inlet gas stream had no marked effects on morphology and production. Between 3% and 5% $dCO_2$ concentration the hyphal branching frequency was increased. Further increases in $dCO_2$ concentration (15% to 20%) gave rise to a decrease in penicillin production levels and the development of an aberrant morphology was noted. Light microscopy led to the observation that $dCO_2$ affected the morphology of *Pe. chrysogenum* with the normal, healthy hyphae becoming long, thin and diffuse when exposed to increased concentrations (Smith and Ho, 1985). It was proposed that the effects observed on *Pe. chrysogenum* were due to the fact that high levels of $CO_2$ could affect membrane transport properties of the cell or that the cells were subject to osmotic swelling (Smith and Ho, 1985; Ho and Smith, 1986).

#### 2.3.9.6 Mechanical forces

In submerged fermentations, the purpose of agitation is to mix the cultivation broth in such a way that a uniform suspension of microbes is achieved. Good mixing is of vital importance in submerged cultivations, ensuring good mass (oxygen and nutrient) and heat transfer and thus providing a homogenous, well-mixed cell suspension. In the case of a conventional stirred tank bioreactor, especially in fungal cultivations, the vessel can be divided into two regions. The region around the impeller represents a zone of high energy dissipation and, hence, good mass and heat transfer. The remainder of the

fermentation volume may be subject to an inadequate supply of oxygen and nutrients, due to poor mixing. In large fermenters, mass transfer gradients across the vessel become significant and this can affect mycelial growth and product formation. Agitation applied during fermentation creates shear forces, which can affect microorganisms in several ways, e.g., damage to cell structure, morphological changes, as well as variations in growth rate and product formation. The magnitude and variation of shear and other forces in submerged bioreactors have been discussed extensively in the literature (Chisti 1999a).

Exposure to a high shear zone may not cause instantaneous damage to the cells of the microorganism, but damage may occur gradually due to shear as well as hydrodynamic stresses. This implies that the microorganism has the ability to adapt to a certain level of mechanical stress. On the other hand, these effects may depend on the age of the cell. Potential damage to microorganisms can limit the impeller speed or power input. Consequently, the oxygen and nutrient transfer capability of a fermenter can be limited, ultimately reducing the volumetric productivity. Furthermore, changes in morphology can alter the viscosity of filamentous fermentation broths, with additional effects on mixing and mass transfer. For each culture, optimum conditions of agitation will exist that will partly depend on the resistance of the hyphae to mechanical forces and also on their physiological state (Moo-Young *et al.*, 1992; Paul *et al.*, 1994a; Papagianni *et al.*, 1999b). TABLE 2.12 highlights the mechanical force related effects on filamentous fungi cultivations found in the literature. The studies described in TABLE 2.12 have demonstrated the effects of mechanical forces on protein as well as metabolite production, biomass concentration, broth rheology, and morphology of filamentous microorganisms. However, it must be emphasised that the superimposed effects of agitation on these variables are sometimes difficult to quantify and compare to one another. For instance, bioreactor geometry and impellor type, diameter and area, (the greater the impellor area the greater the shear force), differ from experiment to experiment. This data, which is obviously required for benchmark comparisons, is often left out of research articles. Another point of criticism refers to many research reports,

where improved mycelial growth, due to increased oxygen transfer rates, is claimed as an agitation effect, possibly counteracting shear damage.

**TABLE 2.12:** Mechanical force related effects on filamentous fungi cultivations.

| Results in an increase in agitation | Fungus | Reference |
|---|---|---|
| Decrease in biomass yield as a result of high agitation. | *A. oryzae* | Li *et al.*, 2002 |
| Increase in biomass yield as a result of high agitation. | *Pe. chrysogenum* | König *et al.*, 1981 |
| Decrease in hyphal length; increase in hyphal diameter, increase in hyphal branching frequency. | *Pe. chrysogenum*<br>*A. niger* | Smith *et al.*, 1990; Metz *et al.*, 1981; Nielsen *et al.*, 1995; Papagianni *et al.*, 1998; Papagianni *et al.*, 1999b |
| Reduction in clump or pellet size at high agitation. | *Pe. chrysogenum* | Nielsen *et al.*, 1995 |
| A decrease in specific growth rate at high agitation. | *A. niger* | Mitard and Riba, 1988 |
| An increase in specific growth rate at high agitation. | *A. niger* | Papagianni *et al.*, 1999b |
| Compactness of pellets increase. | *A. niger* | Gomez *et al.*, 1988 |
| Increased fragmentation. | *A. oryzae*<br>*A. niger* | Li *et al.*, 2002<br>Papagianni, 1995; Papagianni *et al.*, 1998 |
| Increased viscosity. | *A. oryzae* | Li *et al.*, 2002 |
| Decrease in metabolite including heterologous as well as native protein production yield as a result of high agitation. | *A. clavayus*<br>*A. fumigatus*<br>*A. niger*<br>*A. terreus*<br>*Pe. chrysogenum*<br>*A. oryzae* | Manolov, 1992<br>Wase *et al.*, 1985<br>Ujacova, 1980; Roukas, 1991<br>Park *et al.*, 1993<br>Smith *et al.*, 1990; Makagiansar *et al.*, 1993;<br>Li *et al.*, 2002 |
| Protein production independent of agitation. | *A. oryzae* | Amanullah *et al.,* 1999; Amanullah *et al.*, 2000 |
| Increase in protein production yield as a result of high agitation. | *A. niger* | Gomez *et al.*, 1988; Papagianni, 1995; Papagianni *et al.*, 1994; Papagianni *et al.*, 1998 |

### 2.3.10 Bioreactor design considerations

An efficient large-scale bioprocess relies very much on optimisation of the abiotic phase, *i.e.* bioreactor design and performance. A primary step in optimising the bioprocess is obviously the choice of a suitable bioreactor type. The proper scale-up and design should ensure growth in the appropriate morphology.

#### 2.3.10.1 Choice of bioreactor

The viscosity of the culture broth, appropriate morphology, shear sensitivity of the organism, oxygen demand and therefore the potential oxygen supply, as well as heat evolution and the necessary cooling, are the main parameters, which dictate the choice of the bioreactor. However, product price, productivity, reactor size, the necessary control of process parameters, and the mode of operation also influence this choice. Generally, the stirred tank reactor (STR) is better for viscous broths, but is impractical at volumes greater than 500 $m^3$ because the agitation power becomes too high when good mixing is required. These high agitation rates obviously enforce great shear stress on filamentous fungi and in some cases cause a decrease in metabolite production including heterologous protein production. This problem can be solved with the bubble column or airlift bioreactors, which can reach volumes of several thousands of cubic meters, but are not suitable for viscous broths. Here, a significant reduction of energy consumption and consequently of operating costs can be achieved. Since only gas sparging provides the agitation, the mechanical complexity of the system and the risk of contamination is reduced. Compared to stirred tanks, airlift bioreactors are considered low-shear systems with more evenly distributed energy dissipation and shear stress throughout the reactor. In addition, the efficiency of oxygen transfer in these reactors is comparable with stirred tanks. Theoretically, airlift reactors are preferred when operating conditions demand a minimum shear rate without loosing out on a well-mixed cell suspension. Considering these advantages, there are numerous successful industrial applications regarding these reactors. TABLE 2.13 compares protein yields, in fungi

cultivations of various production systems, in STRs with Ruston turbines compared to bioreactors with other configurations.

**TABLE 2.13:** Comparing metabolite production, including heterologous as well as native protein production, in fungi cultivations in STRs with Rushton turbines compared to bioreactors with other configurations.

| Reactor type and configuration | Fungus | Influence on yield compared to STR (protein) | Reference |
|---|---|---|---|
| Airlift | *Acremonium persicinum* | Increase (*B*-Glucan) | Stasinopoulos and Seviour, 1992 |
| | *A. fumigatus* | Increase (Cellulase) | Wase *et al.*, 1985 |
| | *A. niger* | No difference (Gluconic acid) | Träger *et al.*, 1989 |
| | *Aureobasidium pullulans* | No difference (Pullulans) | Gibbs and Seviour, 1992 |
| | *Au. pullulans* | Decrease (Pullulans) | Gibbs and Seviour, 1998 |
| | *Fusariun graminearum* | Increase (Quorn acid) | Trinci, 1994 |
| Airlift (stainless steal sieves in draught tube) | *A. terreus* | Increase (Itaconic acid) | Okabe *et al.*, 1993 |
| Airlift tower loop reactor | *Ce. acremonium* | Decrease ( Cephalosporin C) | Bayer *et al.*, 1989 |
| Assisted airlift base | *Au. pullulans* | Increase (Pullulans) | Gibbs and Seviour, 1998 |
| Airlift with external loop | *Sc. glucanicum* | Comparable (Scleroglucan) | Wang and McNeil, 1995 |
| Tower loop reactor | *Pe. chrysogenum* | Decrease (Penicillin) | König *et al.*, 1981 |

2.3.10.2 Choice of impellor

Generally, high power inputs are required to obtain high transfer rates. Moreover, the more viscous a culture broth becomes, the higher the power input required. This is one

of the reasons why the stirred tank bioreactor is the most frequently used reactors for submerged fungal cultivations. Oxygen and heat transfer rates, which are a function of rheological properties of the fermentation broth, shear rate and the power input, also depend on the type and size of impellor. Most STRs are fitted with standard radial flow Rushton impellors. However, several alternative impellor designs appear to be more effective at simultaneously reducing shear as well as achieving bulk mixing in viscous filamentous fermentation fluids (Nienow, 1990; Humphrey, 1998), as well as affecting protein production. TABLE 2.14 compares fungal protein yields in STRs fitted with rushton turbines to STRs with other impellor designs.

**TABLE 2.14:** Comparing metabolite yields, including heterologous as well as native protein yields, in fungi cultivations in STRs fitted with rushton turbines to STRs with other impellor designs.

| **Impellor type and configuration** | **Fungus** | **Influence on yield compared to radial flow Rushton impellor (product yield)** | **Reference** |
|---|---|---|---|
| Variable pitch axial flow | *Au. pullulans* | Little difference (Pullulans) | Gibbs and Seviour, 1998 |
| Helical ribbon | *Au. pullulans* | Little difference (Pullulans) | Gibbs and Seviour, 1998 |
| | *Sc. commune* | Decrease (B-glucan) | Rau *et al.*, 1992 |
| Axial flow impellors positioned to create counterflow mixing | *F. monilliformis* | Decrease ( Gibberellic) | Priede *et al.*, 1995 |
| Draft tube/propeller system | *Sc. commune* | Increase (B-glucan) | Rau *et al.*, 1992 |
| Fan impellor | *Sc. commune* | Increase (B-glucan) | Rau *et al.*, 1992 |
| Fundaspin | *Sc. commune* | Decrease (B-glucan) | Rau *et al.*, 1992 |
| Intermig | *Sc. commune* | Increase (B-glucan) | Rau *et al.*, 1992 |

### 2.3.11 Conclusions

The literature review confirms that rheological properties, including fungal morphology and biomass yield are the key variables, necessary to explain and predict the bioreactor performance and to design a bioprocess capable of maximising product yield, (heterologous protein production). These key variables have an interactive relationship with a number of environmental parameters, including strain, nutritional and cultivation dependent factors that have the ability, when manipulated, to dramatically alter morphology, biomass yield, rheology and ultimately HBsAg product yield.

# 3 EXPERIMENTAL PROCEDURES

## 3.1 Experimental Material and Methods

This section provides the information on the filamentous fungi strain and other materials used in the experiments. In addition, this section also outlines the fungal culture materials as well as describes the various protocols utilised.

### 3.1.1 Recombinant fungal strain

The recombinant microorganism used in this study was the protease-deficient *Aspergillus niger* DAHB11#8 strain (Plüddemann and van Zyl, 2003). This strain carries the Hepatitis B *S* gene under the transcriptional control of the constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter. The host (A. niger D 15) strain was originally developed at the TNO Nutrition & Food Research Institute in the Netherlands as the *Aspergillus niger* D15 strain and was generously donated by Prof. C.A.M.J.J van den Hondel (TNO, Zeist, The Netherlands).

### 3.1.2 Inoculum preparation

As a stock culture, a spore suspension stored at -80$^{o}$C in 15% glycerol solution was used. Using this method of strain preservation, neither change in cell productivity nor morphology was observed throughout this study. Unless otherwise stated, inoculation was carried out for both shake flask and bioreactor cultures at $1\times10^{6}$ spores.m$\ell^{-1}$ obtained from a densely conidiating culture grown on rich agar medium for 96 to 120 hours. The spores were harvested with a sterile physiological saline solution, NaCl (0.9 % w/v) at high concentrations of approximately $1\times10^{9}$ spores.m$\ell^{-1}$ and stored at 4 $^{o}$C for less than 2 months. The inoculum was prepared in two stages, which included an inoculum and a preculture. This method was performed in order to ensure reproducible growth rates and morphological properties, nullifying any effects caused by spore age.

The preculture consisted of exactly 10% of the primary culture's total volume, and was inoculated with a predetermined concentration of spores to allow for the exact amount of required spores in the primary culture ($1\times10^{6}$ spores.m$\ell^{-1}$). The preculture was then incubated on a shaker at 30°C and 120 rpm for 12 hours.

### 3.1.3 Spore counting

Spore concentration for inoculation was controlled by counting the number of spores on a haemocytometer. The haemocytometer has a grid pattern that indicates a standard volume under a cover slip. In order to determine the spore count, it is common practice to make a 1/10 dilution of the spore suspension in water. 10µl of the diluted suspension was placed on a counting chamber (depth 0.1 mm, Area 0.0025 mm$^2$), covered with a cover slide and placed under 10 x magnification. The spores in 5 of the blocks (4 corners and centre) were then counted, refer to FIG. 3.1. Equation 3.1 was used as the standard equation for the type and make of haemocytometer to determine the spore concentration of spores per mℓ.

Spore concentration (spores/ ℓ) = (Total no. of spores from 5 block) × (Chamber factor of 50 000) × (dilution factor of 10) **Eq. 3.1**

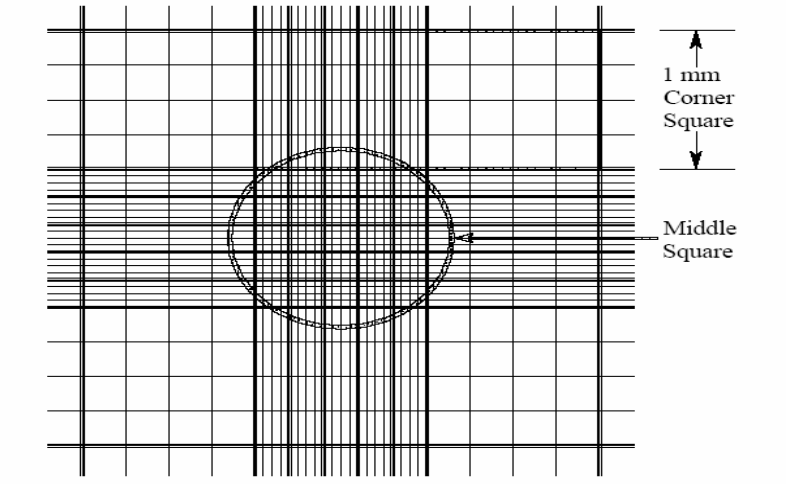


**FIGURE 3.1:** Standard haemocytometer chamber.

### 3.1.4 Cultivation media

The cultivation medium was based on the work of Plüddemann and van Zyl (2003). Modifications were made according to experimental requirements, and the basic compositions of the medium are tabulated in Tables 3.1, 3.2 and 3.3.

A rich agar plate medium was used for the sporulation of the recombinant strain *A. niger* and composed of the following:

**TABLE 3.1:** Rich medium composition of sporulation agar plates.

| **Ingredients of medium** | **Concentration [grams.ℓitre$^{-1}$]** |
|---|---|
| Glucose | 10 g.ℓ$^{-1}$ |
| Neopeptone | 2 g.ℓ$^{-1}$ |
| Yeast extract | 1 g.ℓ$^{-1}$ |
| Casamino acids | 1 g.ℓ$^{-1}$ |
| Agar | 18 g.ℓ$^{-1}$ |
| 50x AspA | 20 mℓ.ℓ$^{-1}$ |
| 1M $MgSO_4$ | 2 mℓ.ℓ$^{-1}$ |
| 1000x trace elements | 1 mℓ.ℓ$^{-1}$ |
| Distilled $H_2O$ | ± 900 mℓ.ℓ$^{-1}$ to add up to 1000 mℓ |

The liquid culture medium, used for cultivation of the recombinant *A. niger* strain was composed of the following:

**TABLE 3.2:** The composition of basic rich media used for the liquid batch cultivations.

| **Ingredients of medium** | **Concentration[grams.ℓitre$^{-1}$]** |
|---|---|
| Glucose | 35 g.ℓ$^{-1}$ |
| Yeast extract | 5 g.ℓ$^{-1}$ |
| Casamino acids | 1 g.ℓ$^{-1}$ |
| 50x AspA | 20 mℓ.ℓ$^{-1}$ |
| 1M $MgSO_4$ | 2 mℓ.ℓ$^{-1}$ |
| 1000x trace elements | 1 mℓ.ℓ$^{-1}$ |
| Distilled $H_2O$ | ± 900 mℓ.ℓ$^{-1}$ to add up to 1000 mℓ |

The final pH was adjusted to pH 5.5 before sterilization (121$^{o}$C, 15 min). The trace element solution was stored at 1000x concentrated sterile-filtered stock solution and comprised of the following:

**TABLE 3.3:** The composition of the 1000 x concentrated trace elements stock solutions.

| **Ingredients of medium** | **Composition [grams]** |
|---|---|
| Distilled $H_2O$ | 80 mℓ |
| $ZnSO_4.7H_20$ | 2.2 g |
| $H_3BO_3$ | 1.1 g |
| $MnCl_2.4H_20$ | 0.5 g |
| $FeSO_4.7H_20$ | 0.5 g |
| $CoCl_2.6H_20$ | 0.17 g |
| $CuSO_4.5H_20$ | 0.16 g |
| $Na_2\ MoO_4.2H_20$ | 0.15 g |
| EDTA | 5.0 g |
| Total volume | 100 mℓ |

Compounds were added one by one in the order given, boiled, allowed to cool to 60 $^{o}$C, adjusted to pH 6.5 with KOH, cooled to room temperature and thereafter adjusted to 100 mℓ with $dH_20$ and autoclaved at 121 $^{o}$C for 15 minutes. The sugar carbon source (glucose) and the antifoam reagent, antifoam 289 (Sigma-Aldrich) were sterilized separately at 121$^{o}$C for 15 minutes at 15 psig and added to the medium before inoculation.

### 3.1.5 Cultivation conditions

#### 3.1.5.1 Shake flask cultivation

Shake flask experiments were carried out in 2 ℓ Erlenmeyer flasks. Cultivation was carried out on a rotary shaker (New Brunswick Scientific, Edison, W. J., U.S.A.) for 216 hours, at 120 rpm, 30 $^{o}$C and $1\times10^{6}$ spores.m$\ell^{-1}$ unless otherwise stated, and 300 mℓ working volume.

#### 3.1.5.2 Bioreactor cultivation

Two 10 ℓ bioreactors were used in this study, (BioFlo 110 Non-Jacketed Vessels, New Brunswick Scientific (NBS) Co., USA) with a working volume of 8 ℓ. In both bioreactors a single 30×20 mm 3-bladed down-flow pitched blade impeller was used and agitation speeds ranged between 100-600 rpm. The agitation rate was carried out at 100 rpm for the first 4 hours of the cultivation and increased up to the various agitation rates for the rest of cultivation. Similarly, aeration, performed by filtered sterile air (0.2 μ absolute filters, resterilizable), entered the bioreactor at 0.25 vvm (volume of gas inlet flow rate per volume of culture per minute), during the first four hours of cultivation and increased to 0.5 vvm afterwards, where it would remain for the duration of the cultivation. The decreased agitation and aeration in the first hours was performed to prevent spore flotation and adhesion to the walls of the culture vessels.

The air supply was passed through a rotameter to control the aeration rate (0.5 vvm). In oxygen enriched cultures or in cultivations where the culture $dO_2$ concentration required control independent from the agitation and aeration rate, NBS gas mixers were used. Pure oxygen was mixed with air and $N_2$ gas. Dissolved oxygen ($dO_2$) concentrations were analysed by an online NBS polarographic electrode, an online NBS pH electrode measured the acidity of the culture broth and the temperature was controlled at a constant $30^oC$ ($\pm 0.1\ ^oC$) by a heating blanket as well as a cooling water stream (0.7 $\ell.min^{-1}$) that passed through a stainless steel heat transfer coil.

The exhaust gases left the top of the reactor through a condenser cooled with water circulating at $-4^oC$ and at 1.1 $\ell.min^{-1}$. The exhaust gases then passed through a sterile filter (0.2 $\mu$ absolute filters, resterilizable) to prevent contaminating the laboratory with *A. niger* spores. In the exhaust gas of bioreactor cultures, the concentration of $O_2$ and $CO_2$ were determined by infrared gas analysis systems (INNOVA AirTech Instruments 1301 Gas Analyser). Aeration rate and off-gas analysis data were used to calculate the specific oxygen uptake rate (SOUR). The pH, when it was controlled, was controlled by the addition of a 2 $mol.\ell^{-1}$ KOH and 2 $mol.\ell^{-1}$ $H_2SO_4$ titrant. Foam was suppressed by the addition of antifoam reagent, with the aid of a level sensor. A foam trap was also present as an extra precaution.

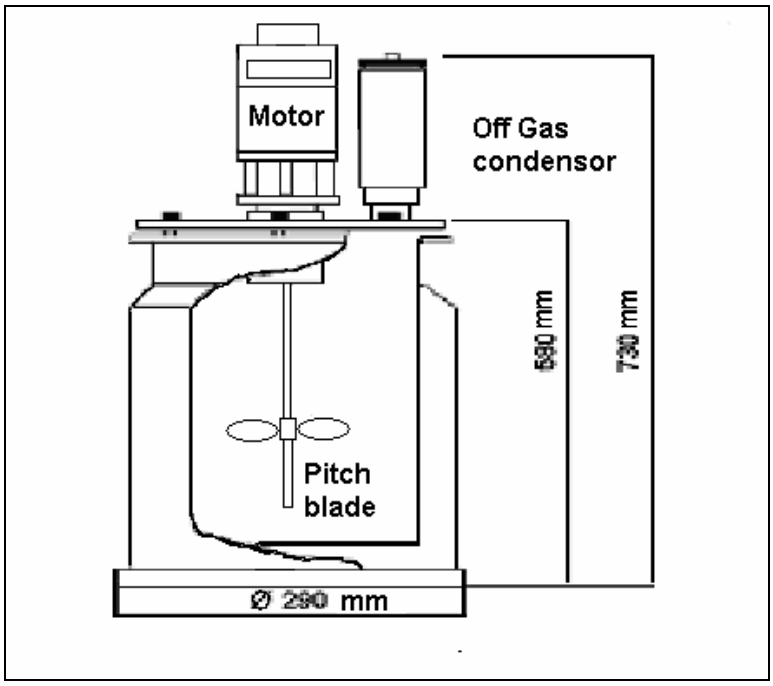


**FIGURE 3.2:** Schematic structure of this 10 ℓ bioreactor and its geometric dimensions.

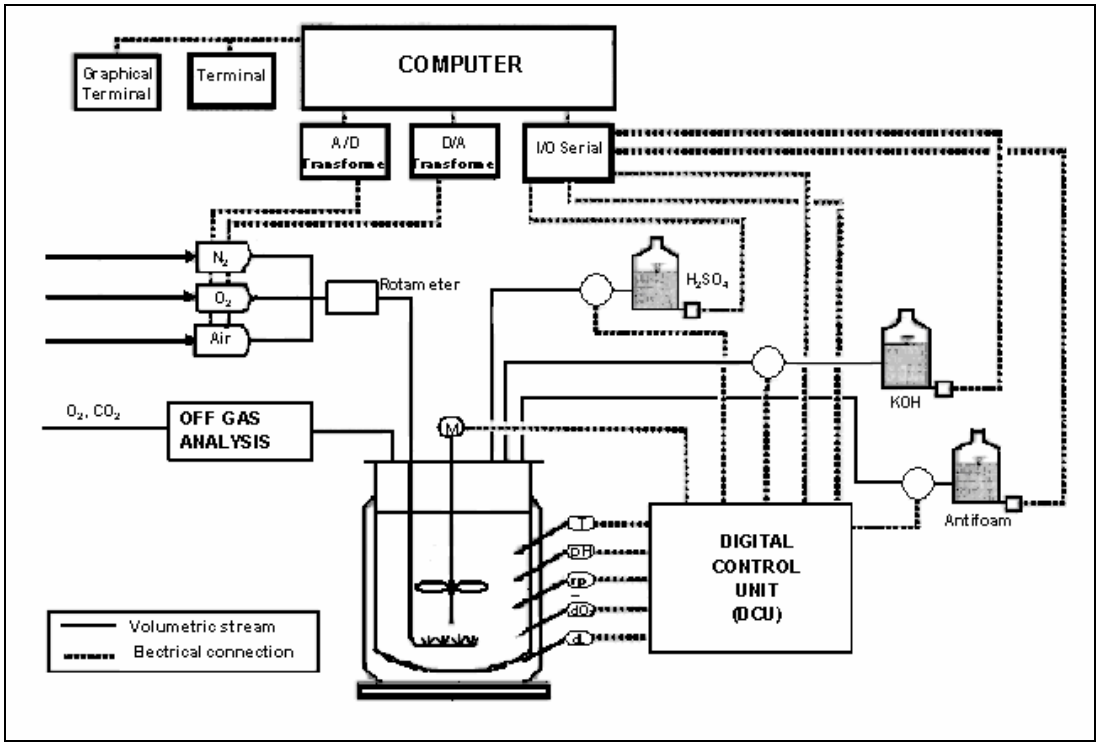


**FIGURE 3.3:** A schematic of the NBS bioreactor with all connected instruments as used in this study.

### 3.1.6 Sample preparation

Samples within the shake flask cultivations were taken in the form of 25 mℓ samples every 24 hours. Samples were taken by manually agitating the flask to ensure mycelia were in a well mixed suspension. Thereafter 25 mℓ was poured into a pre-weighed sterile falcon tube. During bioreactor cultivation, aliquots of the culture were removed from the vessel through a sampling tube of 4 mm diameter. The sample (30 mℓ of broth) was withdrawn using a syringe acting through an in-line air filter. Samples were collected in pre-weighed sterile falcon tube every six to twelve hours. Mycelia were separated from the sample liquid broth by filtering the sample through sterile Miracloth. Mycelia were then squeezed well to remove excess liquid. The filtered medium was collected into a sterile tube, snap frozen in liquid nitrogen and stored at –80 $^{o}$C (this is the extracellular fraction). Mycelia were collected into pre-weighed foil, wrapped up, weighed, snap frozen in liquid nitrogen, and stored at –80 $^{o}$C.

### 3.1.7 Assay preparation

In the shake flask cultivations 15 mℓ culture broth was used for assay preparation and in the bioreactor cultivations, 40 mℓ culture broth was required. After the filtering and freezing process was performed (see above), mycelium samples were ground in a mortar and pestle under liquid nitrogen to a fine powder and an aliquot of 0.3 g mycelium powder was placed into 2 mℓ Eppendorf microfuge tubes. These were also stored at –80 $^{o}$C. Two 0.3 g samples were prepared, one for intracellular protein extraction and analysis and the other for dry weight determination. Once the samples were ground, two extraction buffers were prepared, one with Triton X-100 for the extraction of both the intracellular membrane and cytoplasmic associated proteins and one without the Triton for the extraction of the cytoplasmic fraction. The buffer composition included: 50mM NaP (pH7) / 1mM EDTA / 20µM PMSF / 0.1% SDS (or 0.1% Triton X-100). The extraction of the intracellular cytoplasmic fraction was performed first, followed by the membrane fraction and was performed according to the following protocol:

1) 1 mℓ extraction buffer was added to the 0.3g ground mycelia and vortexed to ensure good mixing.
2) Sample placed on ice for 10 min.
3) Centrifuged at 14 000 rpm for 15 min at 4°C to remove debris.
4) Extraction of upper aqueous layer to clean Eppendorf microfuge tube. This is the crude intracellular total protein extract.

With regards to extracellular protein determination, samples were concentrated using size exclusion membranes in the Amicon.

### 3.1.8 Dry weight

In the shake flask cultivations 10 mℓ culture broth was used for biomass determination and in the bioreactor cultivations 40 mℓ culture broth was required. A biomass growth curve was determined by measuring dry cell weight. Samples were filtered through dried, sterile Miracloth, followed by washing with distilled water (two to five mℓ). The wet mycelia were then placed in pre-weighed foil and weighed. After this, mycelia samples were placed in an oven for drying at 60 °C for 168 hours. Consequently, average dry weight (DW) was determined, divided by broth sample volume to give biomass DW as $mg_{DW}.m\ell^{-1}$ or $g_{DW}.\ell_{culture}^{-1}$.

### 3.1.9 HBsAg concentration determination

The HBsAg analysis was performed at Dr Davies Pathologists, Cape Town. The ARCHITECT HBsAg assay was used (Abbott labotatories), and can be described as a Chemiluminescent Micoparticle Immunoassay (CMIA). It allows for the quantitative determination of fully assembled HBsAg particles (VLPs) by using microparticles coated with monoclonal anti-HBs, derived from human serum, to detect the HBsAg present in the sample. Results from the ARCHITECT were obtained in $IU.m\ell^{-1}$ and the concentration of the specimen was determined using a previously generated ARCHITECT HBsAg calibration curve.

### 3.1.10 Total protein concentration determination

Intracellular total protein was determined using the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as a standard.

### 3.1.11 Calculation of SOUR

The specific oxygen uptake rate (SOUR) was calculated according to the stationary method depicted in equations 3.2-3.6, as performed by Patel and Thibault, (2004). The

basis of the calculations is stated in equation 3.1, which states that the rate of change of the dissolved oxygen concentration in the fermentation medium is equal to the rate of the oxygen mass transfer from the gas to the liquid-phase minus the rate of the oxygen utilization rate by the microorganism.

$$\frac{dC_L}{dt} = OTR - OUR \qquad \textbf{Eq. 3.2}$$

$$\frac{dC_L}{dt} = k_L a\left(C_L^* - C_L\right) - OUR \qquad \textbf{Eq. 3.3}$$

$$\frac{dC_L}{dt} = k_L a\left(C_L^* - C_L\right) - Q_{O_2} X \qquad \textbf{Eq. 3.4}$$

Under steady state conditions it is assumed $\frac{dC}{dt} \to 0$; OTR = OUR

Therefore under psuedo-steady-state conditions, equation 3.4 can be rearranged to calculated the mass transfer coefficient ($k_La$) as follows:

$$k_L a = \frac{Q_{O_2} X}{C_L^* - C_L^0} \qquad \textbf{Eq. 3.5}$$

And similarly, the OUR is calculated in equation 3.5 as follows:

$$OUR = Q_{O_2} X \qquad \textbf{Eq. 3.6}$$

Equation 3.5 requires an estimate of $Q_{O_2}X$ that can be obtained using the difference in oxygen concentration between the inlet and the outlet gas streams as it represents the oxygen consumption by microorganisms. A total inlet and outlet gas balance is required and can be done with the data obtained by the gas mixer and the off gas analyser.

$$OUR = \frac{1}{V_L}\left(\frac{P_1}{RT_1}Q_{1,G}y_{IN,O_2} - \frac{P_2}{RT_2}Q_{2,G}y_{OUT,O_2}\right) \qquad \textbf{Eq. 3.7}$$

The SOUR is then determined by dividing the OUR by the fungal biomass concentration (fungal dry weight per liter culture.

$$SOUR = \frac{OUR}{DW} \qquad \textbf{Eq. 3.8}$$

Where:

| | |
|---|---|
| $C_L$ | Dissolved oxygen concentration ($mol.m^{-3}$) |
| $C_L^o$ | Psuedo-steady-state dissolved oxygen concentration at 0.242 $mol.m^{-3}$ |
| $C_L^*$ | Dissolved oxygen concentration in equilibrium with gaseous oxygen concentration ($mol.m^{-3}$) |
| DW | Gram dry weight biomass per liter culture |
| $k_La$ | Volumetric oxygen mass transfer coefficient ($s^{-1}$) |
| P | Pressure (Pa) |
| $Q_G$ | Gas flow rate ($m^3.s^{-1}$) |
| $Q_{O_2}X$ | Oxygen uptake rate ($mol.m^{-3}.s^{-1}$) |
| OTR | Oxygen transfer rate ($mol.m^{-3}.s^{-1}$) |
| OUR | Oxygen uptake rate ($mol.m^{-3}.s^{-1}$) |

| | |
|---|---|
| R | Gas constant (8.306 $Pa.m^3.(mol.K)^{-1}$) |
| SOUR | Specific oxygen uptake rate ($mol.g_{DW}^{-1}.s^{-1}$) |
| T | Temperature (K) |
| $V_L$ | Liquid volume in bioreactor ($m^3$) |
| y | gaseous mole fraction |

### 3.1.12 High Performance Liquid Chromatography (HPLC) method

The quantitative determinations of carbohydrates (glucose, fructose and xylose), organic acids (gluconic acid, pyruvate, formate, acetate, acetoin, succin, citric acid and oxalic acid), etc. were determined by HPLC analysis using an Aminex HXP-87H column (BioRad) for separation (T = 25 $^oC$) and ultraviolet (UV) and refractive index detector (Techlab, Germany) for detection. Sulfuric acid (0.01 N) was used as the mobile phase (flow rate = 0.5 mℓ $min^{-1}$). It must be mentioned that even though all this data was gathered using the HPLC, only the glucose concentration during the fermentation was analysed and discussed in this study.

## 3.2 Experimental Philosophy

From the literature review it was evident that the cultivation of the filamentous fungi is indeed a complex operation, especially with regards to achieving reproducible and optimal product yields. As described in the literature review, various interdependent process parameters have been identified to influence the production levels of the desired product. From a bioprocess-engineering point of view, a methodical and deliberate process of elimination quantitatively isolated the most significant process parameters. Hence, this section discusses the development of the project, the thought process behind the scenes, concept justification, as well as experimental design and analysis.

### 3.2.1 Type of cultivation

Solid-state cultivations have many advantages and have been used with success in terms of protein production systems. Despite this, submerged cultivations have been the preferred choice of cultivation type in this study. This approach was justified by the fact that submerged cultivations allow for a high level of control, equipment and maintenance is relatively cost efficient, and the infrastructure of submerged cultivations is well established in the laboratories in which the experiments were performed (Biotechnology laboratory, University of Stellenbosch).

### 3.2.2 Mode of operation

The choice of operation mode was a difficult concept to reason in relation to this study. Each mode, as noted in the literature review, either batch, fed-batch or continuous systems, have advantages. In terms of a primary step in the development of the VLP production process, a batch mode of operation was selected. Factors that justify the mode of operation include the following:

1) Batch systems are preferred for the cultivation of non-growth associated product formation.
2) Due to the reduced culturing period, batch systems have a lower risk of contamination.
3) Batch systems have an increased genetic stability due to a freshly produced inoculum used for each batch.
4) Batch systems are more flexible compared to continuous cultures. A continuous system is dedicated to one product, while the batch system can be used to produce different products at different periods of the cultivation.

### 3.2.3 Process factors influencing product yield

As discussed in detail in the literature review, many variables have the potential to influence product yields. These factors are classified in three categories, namely; strain dependent factors, nutrition dependent factors and cultivation dependent factors. For a successful bioprocess design, all three of these categories must be investigated thoroughly. However, with regards to the aim of the present study, cultivation dependent factors (bioprocessing parameters) were investigated in detail. The strain dependent factors were initially investigated by Plüddemann and van Zyl (2003). A few new ideas have been suggested in the literature review, but have been set aside for the time being. Nutrition dependent factors are currently being investigated under a different project title and hence will not be discussed in this study.

### 3.2.4 Experimental design of cultivation dependent factors

The cultivation dependent factors require a labour intensive effort in order to obtain optimised levels. From the literature review, the following factors were identified to theoretically have the most significant impact on VLP production yields on the bioprocess of this study.

1) Temperature,
2) Inoculum concentration,
3) pH,
4) Agitation, and
5) $dO_2$

#### 3.2.4.1 Shake flask cultivations

Shake flask cultivations are the simplest and least labour intensive cultivation. In addition, shake flask cultures are cost efficient and large amounts of simultaneous experimental runs can be executed. A disadvantage in shake flask cultivations is the low

quality of control when compared to bioreactor cultivations. As already discussed in the literature review, complex interactions exist between process conditions. These interactions differ quantitatively from process factor to process factor. In direct relation to the process parameters, temperature and inoculum concentration, no direct interaction was suspected to occur between these two parameters. Thus, the high quality of control obtained in bioreactor cultivation was not necessary. Hence, temperature optimisation as well as inoculum concentration investigations was performed in shake flask cultures, and the experimental design of these cultivations is tabulated in TABLE 3.4.

**TABLE 3.4:** Experimental design in shake flask cultivations.

| **Optimisation** | **Temperature [$^{o}$C]** | **Inoculum concentration [spores.m$\ell^{-1}$]** | **Agitation [rpm]** | **pH** | **$dO_2$ [%]** |
|---|---|---|---|---|---|
| Temperature | 16 | | | | |
| | 23 | | | | |
| | 30 | $1\times10^{6}$ | 120 | Uncontrolled | Oxygen limited |
| | 37 | | | | |
| Inoculum concentration | 30 | $1\times10^{3}$ | | | |
| | | $1\times10^{5}$ | | | |
| | | $1\times10^{6}$ | 120 | Uncontrolled | Oxygen limited |
| | | $1\times10^{8}$ | | | |

Each experimental run was performed in triplicate. 40 mℓ Samples were taken every 24 hours for a total time of 216 hours. These samples were processed and analysed according to the following forms tabulated in TABLE 3.5. For more precise information on shake flask cultivation methodology, refer to section 3.1.5.1.

**TABLE 3.5:** Experimental analysis format of processed samples from shake flask cultivations.

| Shake Flask Processed Data | Shake Flask Processed Data Units |
|---|---|
| Biomass growth curve | $g_{DW}.\ell_{culture}^{-1}$ , $h^{-1}$ |
| Total intracellular protein | $mg.m\ell^{-1}$ and $mg.g_{DW}^{-1}$ , $mg.g^{-1}{}_{DW}.h^{-1}$ |
| Intracellular HBsAg | $\mu g.m\ell^{-1}$ and $\mu g.g_{DW}^{-1}$ , $\mu g.g^{-1}{}_{DW}.h^{-1}$ |
| pH | pH profile with regards to time |
| Broth morphology | Photographs of cultivation liquid |

### 3.2.4.2 Bioreactor cultivations

Bioreactor based cultures accommodate processes that require a high level of control. In order to determine the production effects of certain process parameters, a high level of control was required, especially when complex interactions between the process parameters were suspected. The cultivation dependent factors agitation, $dO_2$ concentration and pH, require this high level of control, which justifies the execution of these related experimental runs in a bioreactor. With regards to experimental design, it has been noted from the literature review that the relationship between $dO_2$ and agitation is significant. Agitation within a culture ensures a well mixed cell suspension. As the rate of agitation increases, so does the efficacy of the mixing, thereby causing the increase in $dO_2$ concentration. This complex interaction between these two parameters has caused much debate on the credibility and accuracy of many studies published in the literature. The question being, is product yield directly affected by the variance in agitation, or indirectly affected by $dO_2$ concentration as a result of a change in agitation? Therefore, it was decided that the experimental design would have to consist of a method where both the $dO_2$ concentration as well as the agitation effects could be analysed simultaneously. To achieve this, the plan was to maintain the $dO_2$ concentration as well as the agitation rate constant, during each experiment, and only varying the two parameters between experimental runs (see FIG. 3.4). For more precise information on bioreactor cultivation methodology, refer to section 3.1.5.2

Several experimental designs were considered (Akhnazarova and Kafarov, 1982; Box and Wilson, 1951; Cochran and Cox, 1992; Deshayes, 1980; Fannin *et al.*, 1981; Khuri and Cornell, 1987). A $2^2$ factorial design and central composite rotary design (CCRD), as described by Box *et al.* (1978) and Box and Draper (1959), was eventually selected, and utilised not only to analyse the individual effects of agitation rate and $dO_2$ concentration, but also the response surface contour plots of the process interaction of these two parameters (see FIG. 3.4). The advantages of these techniques include a larger operating window in which accurate experimental data may be processed with a lesser number of experiments. The basic CCRD comprises of 6 star points with an added central point. The number of repetitions per experiment depends on the reproducibility of the results and thus is directly responsible for the number of experimental runs required (see TABLE 3.6).

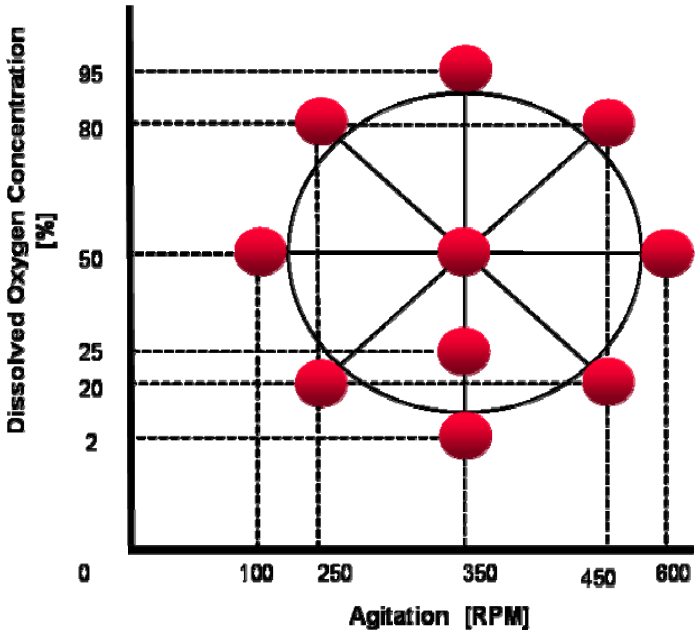


**FIGURE 3.4:** Illustration of central composite rotary design set-up.

According to this study (see FIG. 3.4), the Y-axis ($dO_2$ concentration) and X-axis (agitation) comprise of the basic CCRD structure with an additional 2 points. On the Y-axis a full range of $dO_2$ concentrations were investigated from high oxygen enrichment

levels of 95% and 80% to the oxygen limited levels of 2% and <2% cultures (surface culture aeration). On the X-axis a full range of agitation rates were also investigated from the low agitation rate of 100 rpm to the high agitation of 600 rpm. The additional point on the CCRD was characterised as critical: The point labelled (25%; 350 rpm) was thought in theory to be the point of optimum volumetric HBsAg product yield. The thought process behind this theory was that under these conditions, the microorganism would obtain sufficient $O_2$ transfer to the active cells, with minimal injury due to shear (Pappagianni, 2004) and minimal $O_2$ toxicity which could occur due to high $O_2$ concentrations within the cultivation (Bai *et al*., 2003). A control was also required to determine the effect of product yield without the control of the $dO_2$ concentration. The agitation was fixed at 350 rpm, while the $dO_2$ concentration was allowed to fluctuate according to the oxygen transfer rate of the broth and the oxygen utilisation rate of the microorganism.

The final parameter to be investigated was pH. From the literature review, it was evident that pH had a significant impact on production yield (Punt *et al.*, 2002). It was shown that by using a similar *A. niger* D15 strain, production yield of a heterologous protein hen egg-white lysozyme (HEWL) reached a maximum at pH 7 with a low biomass yield, while at pH 6 a maximum biomass yield was achieved with a lower protein yield. This valuable piece of research induced the curiosity as to whether similar observations would be noted in the HBsAg fungal production system. Hence, pH 6 and pH 7 culture conditions were investigated. Experiments were performed in a bioreactor, where the $dO_2$ concentration and agitation rate remained constant during and between experiments (350 rpm and 50% $dO_2$ concentration), while the pH conditions were controlled during experiments and altered between experiments. A control was required to determine the effect of product yield without pH control. Therefore the agitation and $dO_2$ concentration was fixed (350 rpm and 50% $dO_2$ concentration), while the pH was allowed to fluctuate accordingly.

Thus, an experimental design was set-up and a total of 22 fermentations were performed. The number of fermentation runs performed, including repetitions in the 10ℓ NBS bioreactor is illustrated in TABLE 3.6.

**TABLE 3.6:** Experimental design in bioreactor cultivations (inoculum concentration of $1\times10^6$ and a temperature of 30°C).

| **$dO_2$ [%]** | **Agitation [rpm]** | **pH** | **Repetitions** |
|---|---|---|---|
| 50 | 350 | uncontrolled | 3 |
| 50 | 100 | uncontrolled | 2 |
| 50 | 600 | uncontrolled | 2 |
| 95 | 350 | uncontrolled | 2 |
| 2 | 350 | uncontrolled | 2 |
| 80 | 250 | uncontrolled | 1 |
| 20 | 250 | uncontrolled | 1 |
| 80 | 450 | uncontrolled | 1 |
| 20 | 450 | uncontrolled | 1 |
| 25 | 350 | uncontrolled | 1 |
| 50 | 350 | 6 | 2 |
| 50 | 350 | 7 | 2 |
| Control | 350 | uncontrolled | 2 |

Samples were taken every 6 to 12 hours in accurate 40 ml quantities. These samples were then processed according to the following forms tabulated TABLE 3.7.

**TABLE 3.7:** Experimental analysis format of processed samples from bioreactor cultivations.

| Bioreactor Processed Data | Bioreactor Processed Data Units |
|---|---|
| Biomass growth | $g_{DW}.\ell_{culture}^{-1}$ , $h^{-1}$ |
| Total intracellular protein | $mg.m\ell^{-1}$ , $mg.g_{DW}^{-1}$ , $mg.g^{-1}_{DW}.h^{-1}$ |
| Intracellular HBsAg | $\mu g.m\ell^{-1}$ and $\mu g.g_{DW}^{-1}$ , $\mu g.g^{-1}_{DW}.h^{-1}$ |
| Glucose utilisation rate | $g.g^{-1}_{DW}.h^{-1}$ |
| pH | pH profile with regards to time |
| Broth morphology | Photographs of cultivation liquid |
| SOUR | $mmolO_2.g_{DW}^{-1}.h^{-1}$ |

### 3.2.5 Experimental analysis

As already described, two types of cultivation vessels were used to determine the effects various process parameters have on the HBsAg production system, shake flask cultivations and bioreactor cultivations. TABLE 3.8 illustrates the type of vessel used for the investigation of the following effects on the production system of each process parameter:

1) Biomass growth curve,
2) Total intracellular protein,
3) Intracellular HBsAg,
4) Broth glucose concentration profile,
5) pH, and
6) Broth rheology.

**TABLE 3.8:** Vessel type used for the analysis of each process parameter.

| Cultivation vessel | Process Parameters | Parameter range |
|---|---|---|
| Shake flask | Inoculum concentration | $1\times10^3$, $1\times10^5$, $1\times10^6$, $1\times10^8$ spores.m$\ell^{-1}$ |
| | Temperature | 16, 23, 30, 37 $^oC$ |
| Bioreactor | Agitation | Central composite rotary design<br>100, 250, 350, 450, 600 rpm |
| | $dO_2$ concentration | Central composite rotary design<br>2, 20, 25, 50, 80, 95 %<br>control: 350 rpm without $dO_2$ control |
| | pH | 6 and 7<br>control: 350 rpm 50% $dO_2$ without pH control |

In both, shake flask and bioreactor cultivations, the results illustrating biomass growth curve concentrations are processed in terms of $g_{DW}.\ell_{culture}^{-1}$ and portray the four distinct growth phases in a typical microbial cultivation; lag phase, exponential phase, stationary phase and the death phase. The analysis of these growth phases is vital in terms of determining optimum growth conditions and supplies crucial information as to the most effective harvesting period.

Protein concentration (total protein and HBsAg production) was processed volumetrically (mg.$\ell^{-1}$) and specifically (mg.$g_{DW}^{-1}$). The importance of processing both these formats are fundamentally significant with regards to processes optimisation, as specific yields define the amount of protein concentration per an equivalent mass, while volumetric yields define protein concentration per biomass yield in a litre of culture. It must be mentioned that, in relation to protein extraction, Plüddemann and van Zyl (2003), demonstrated that heterologous protein concentration was not secreted extracellularly and that the protein assembly was membrane associated. One of the aims of this study was determine whether in fact, under certain growth conditions,

intracellular cytoplasmic as well as extracellular HBsAg concentration could be observed.

The costs involved in determining the HBsAg concentration within samples was extremely high and thus certain HBsAg analysis was prioritised. Due to a lower expected product yield in shake flask experiments, it was decided that the cytoplasmic fraction of the intracellular composition would be ignored in both HBsAg and total protein analysis. In bioreactor cultivations, the protein production was expected to be considerably higher than shake flask cultivations and thus justified the investigation of HBsAg and total protein concentration in the cytoplasmic fraction. As a result, cytoplasmic analysis on HBsAg and total protein concentration was performed at (50% $dO_2$ and 100 rpm; 50% $dO_2$ and 350 rpm; 50% $dO_2$ and 600 rpm; 95% $dO_2$ and 350 rpm; 2% $dO_2$ and 350 rpm).

The primary carbon source (glucose) was measured (HPLC analysis) in intervals during bioreactor cultivations only. pH profiles were constructed in both shake flask and bioreactor cultivations. This allowed for the analysis of the pH fluctuations in the various cultures and as a result, any correlations that existed between the culture pH and other process variables were investigated. Broth morphology was analysed by observations made by the process operator during various intervals of the cultivation. In shake flask cultures photographs were taken of the fungal broth in the Erlenmeyer flasks, while in bioreactor cultivations photographs were taken of 7 mℓ broth samples poured into petri dishes.

### 3.2.6 Experimental development

Shake flask experiments were performed without any problems. Initially, bioreactor experiments, were performed in 1.3 ℓ NBS reactors, and could not boast of similar success. The required high level of control was unattainable. Biomass levels became uncontrollably high. The cells showed signs of stress causing large amount of foam formation (FIG. 3.5A). Relatively igh viscosities were observed (FIG. 3.5D). Wall

growth became a critical issue and well-mixed cell suspensions became impossible (FIG. 3.5A, B, C and D). Sample ports and probes became blocked, rendering it impossible to obtain periodic samples and online measurements became erroneous (FIG. 3.5C). The Rushton type impellors also caused problems at high agitation rates causing irregular cell growth and pellet shape (FIG. 3.5B and C). This was attributed to an excessive shear rate that was considered to increase cellular stress within the culture. Repetitions were not reproducible and were ascribed to the inappropriate scaling of the 1.3 ℓ bioreactor and thus an alternate experimental approach had to be formulated. FIG. 3.5 illustrates process problems obtained in the 1.3 l bioreactors.

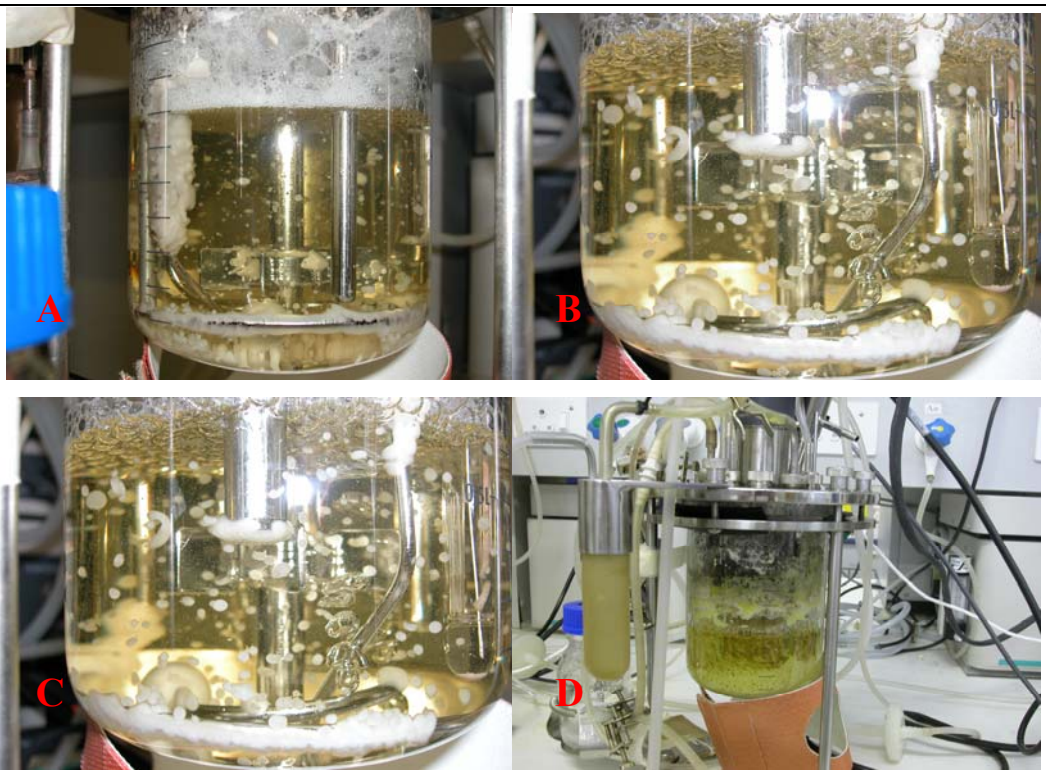


**FIGURE 3.5:** Photograph of NBS 1.3 ℓ bioreactor illustrating operating problems caused by high viscosities, irregular pellet shape, and wall growth.

Two adaptations were made to the process design:

1) A larger cultivation vessel (10ℓ NBS bioreactor) of geometric similarity replaced the 1.3 ℓ reactor in an effort to reduce the percentage wall growth.
   i.e. surface/volume ratio decreases as the size of a geometrically similar vessel increases, thus decreasing the percentage wall growth.
2) The introduction of a down-flow pitch-blade impellor to reduce the shear rate on the apparent shear sensitive microorganism.

The introduction of these process modifications proved successful. Cell wall growth percentage was reduced. Foam control, morphology control, increased HBsAg concentration and most importantly reproducible results were achieved. FIG. 3.6 illustrates the laboratory set-up of the 10ℓ NBS bioreactors.


**FIGURE 3.6:** Photograph of 10 ℓ NBS bioreactor set up.

# 4 RESULTS

## 4.1 Introduction

This work reports on the effects of various process parameters on the VLP (HBsAg) production, biomass formation and the percentage (%) of cellular protein consisting of HBsAg , as well as the mycelial morphology of recombinant *A. niger* under submerged culturing conditions. The outline of this section is divided into two sub-sections, namely shake flask and bioreactor cultivations. The aim of this study was the optimisation of the bioprocessing parameters in order to maximise HBsAg production levels within the *Aspergillus* production process. Cell morphology was tightly monitored and the effects of the bioprocessing parameters (temperature, inoculum concentration, agitation, $dO_2$ concentration, and pH) on biomass concentration as well as homologous and VLP (HBsAg) production were assessed.

## 4.2 Shake flask cultivations

### 4.2.1 Introduction

The aim of the shake flask cultivations was to determine the effects of culture temperature and inoculum concentration on mycelial morphology, culture growth, and homologous as well as VLP (HBsAg) production. All processed data is available in a tabulated form in Appendix A & B.

### 4.2.2 Temperature optimisation

#### 4.2.2.1 Effect of temperature on biomass production

The analysis of biomass production over varying temperature, illustrated in FIG. 4.1A, demonstrates a similar lag phase of approximately 24 hours in cultures grown at 16 and 23 $^{o}$C, while cultivations grown at 30 and 37 $^{o}$C were observed to have a reduced lag phase that only

lasted 12 and 8 hours, respectively. The exponential growth phase was dependent on the culture temperature. Specific growth rates were observed to increase with the increase in culture temperature from 0.02 $h^{-1}$ at 16 $^{o}$C to 0.029 $h^{-1}$ at 37 $^{o}$C. A maximum peak was observed to form in all cultivations followed by a temporary stationary phase and finally a death phase. An optimum biomass concentration of 20.5 $g_{DW}.\ell_{culture}^{-1}$ at 120 hours was observed in cultures grown at 30 $^{o}$C.

4.2.2.2 Effect of temperature on protein production yield

- **Total protein production**

The analysis of total cellular protein concentration demonstrated variance in terms of production profiles. Specific total cellular protein concentration (FIG. 4.2B) obtained maximum levels at a culture temperature of 30 $^{o}$C of 106 $mg.g_{DW}^{-1}$ at 96 hours with a intracellular specific production rate of 0.063 $mg.g^{-1}_{DW}.h^{-1}$. Cultures grown at 37 $^{o}$C obtained similar specific levels of 100 $mg.g_{DW}^{-1}$ at 120 hours, however the intracellular specific total protein production rate was significantly lower (0.012 $mg.g^{-1}_{DW}.h^{-1}$). Cultures grown at 16 and 23 $^{o}$C obtained very low production levels below 65 $mg.g_{DW}^{-1}$ and specific production rates. In comparison, volumetric total intracellular protein concentration (FIG. 4.2A) obtained maximum levels of 2000 $mg.\ell_{culture}^{-1}$ at 120 hours and a culture temperature of 30 $^{o}$C, while cultures grown at 16, 23 and 30 $^{o}$C obtained very low levels below 1000 $mg.\ell_{culture}^{-1}$.

- **HBsAg production**

The analysis of intracellular HBsAg concentration demonstrated the suppression of production at the cultivation temperatures of 16 and 37 $^{o}$C, where production levels below 20 $\mu g.g_{DW}^{-1}$ (FIG. 4.1D) and 200 $\mu g.\ell_{culture}^{-1}$ (FIG. 4.1C) were obtained, respectively. Specific and volumetric HBsAg production was enhanced in culture temperatures of 23 and 30 $^{o}$C. In these cultures specific HBsAg production was most efficient during the latter stages of the cultivation. Cultures grown at 30 $^{o}$C obtained the optimum specific HBsAg concentration of 57 $\mu g.g_{DW}^{-1}$ at 216 hours,

while optimum volumetric HBsAg concentration was measured at 30 $^{o}$C in the range of 1000 $\mu g.\ell_{culture}^{-1}$ at 144 hours.

- **Percentage cellular protein that HBsAg represents**

The significance of the percentage cellular protein that HBsAg represents (FIG. 4.1B) demonstrated the stability and robustness of the HBsAg particles during the latter stages of the cultivation grown at 23 and 30 $^{o}$C. Cultures grown at 16 $^{o}$C remained constant during the latter periods of the cultivation at values of approximately 0.03%, while cultivations grown at 37 $^{o}$C increased gradually to 0.1% at 192 hours, followed by a rapid decrease until 216 hours. Cultures grown at 30 $^{o}$C obtained the highest percentage cellular protein that HBsAg represents of 0.42% at 216 hours.

#### 4.2.2.3 Effect of temperature on culture pH

The analysis of the culture pH profile in relation to varying temperature cultures, illustrated in FIG. 4.2C, clearly demonstrated a sudden decrease in pH (initial pH 6.35) within the first 24 hours of culture growth. The largest decrease in pH during this period was observed in cultures grown at 37 $^{o}$C (pH 5.65), followed by 30 $^{o}$C cultures (pH 5.76), 23 $^{o}$C cultures (pH 5.85) and finally 16 $^{o}$C cultures (pH 5.94). For cultures grown at 37 $^{o}$C, a decrease in pH was followed by a gradual increase to a final pH of 7.56 at 216 hours. A similar trend was observed in 30 $^{o}$C cultures, where a final pH of 7.4 was reached. Conversely, the pH for the 23 $^{o}$C cultures increased gradually to a pH of 6.54 at 72 hours, which was followed by a gradual decrease in pH until a final value of pH 5.42 was reached at 216 hours. After 24 hours, cultures grown at 16 $^{o}$C were not observed to undergo a pH increase, but decreased gradually in pH to a final value of pH 3.82 at 216 hours.

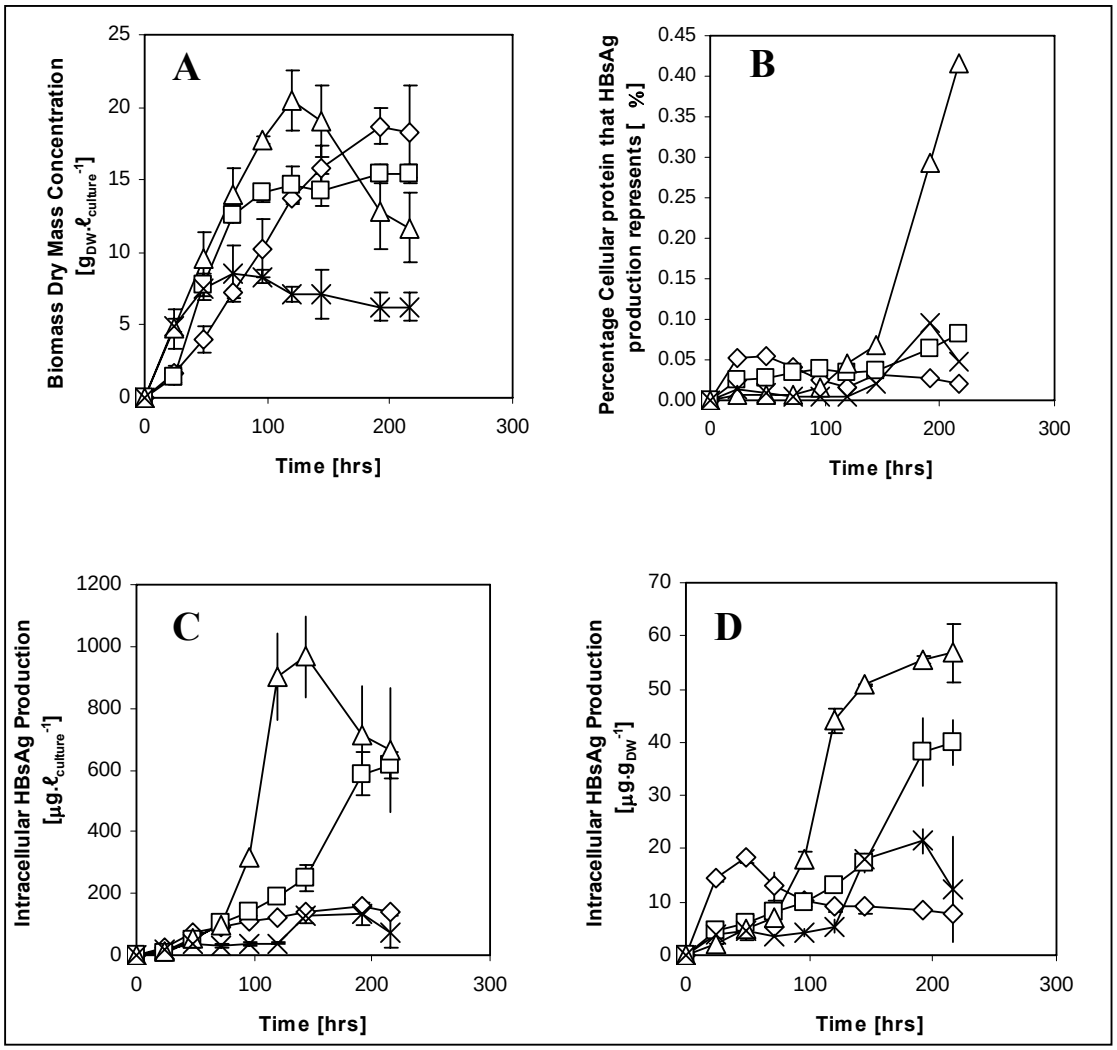


**FIGURE 4.1:** Effect of the bioprocessing parameter temperature ($^{o}$C) in a batch culture (shake flask) on (A) Biomass dry mass concentration. Results are given in grams dry weight $liter_{culture}^{-1}$. (B) Percentage cellular protein that HBsAg represents. Results are given in % HBsAg. (C) Intracellular HBsAg production. Results are given in micro grams $liter_{culture}^{-1}$ and (D) micro grams per $gram_{dry\ weight}^{-1}$ .Shown are data for (◇) 16 $^{o}$C, (□) 23 $^{o}$C, (△) 30 $^{o}$C and (×) 37 $^{o}$C. Results are based on three replications over a cultivation time of 220 hours.

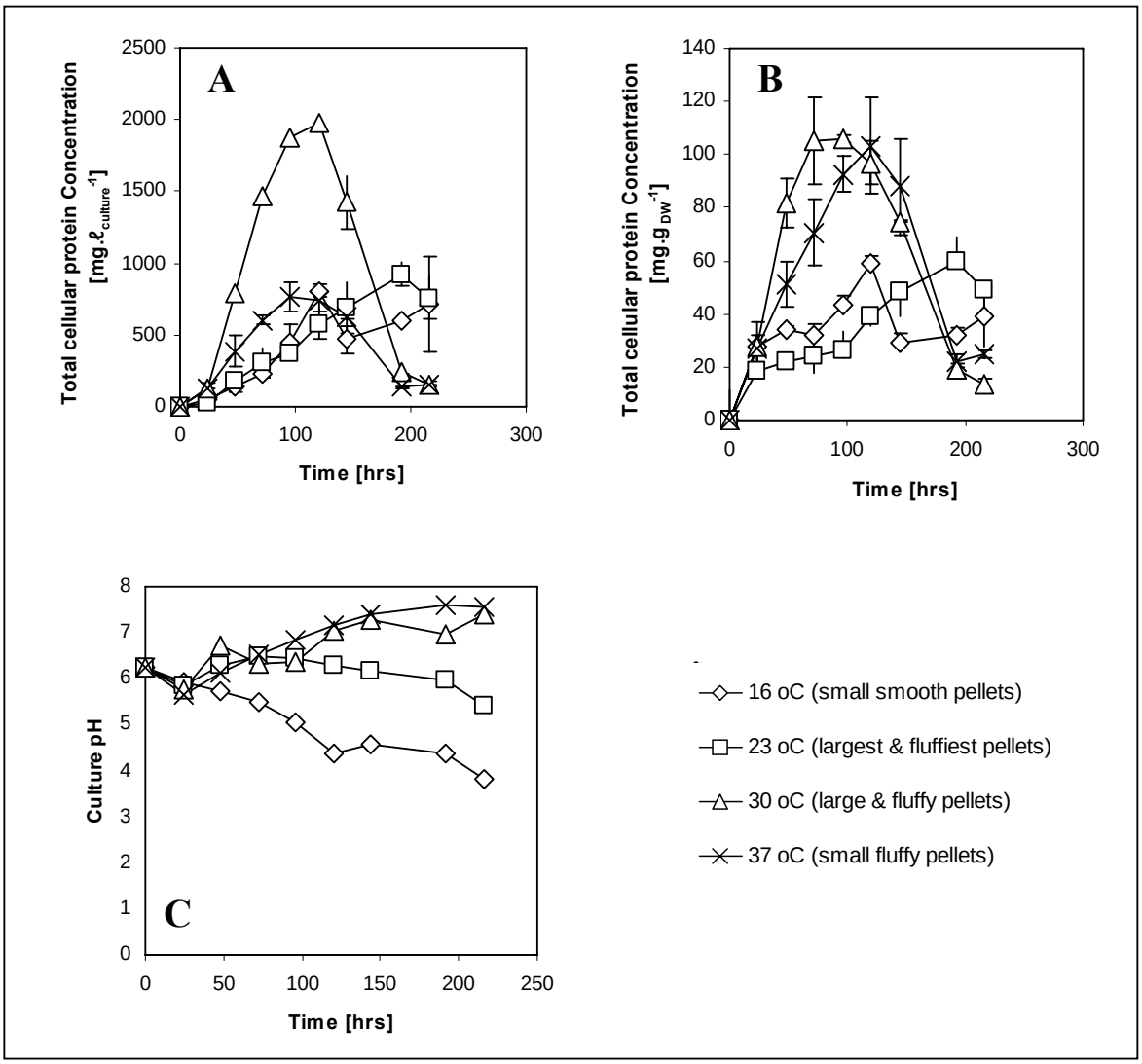


**FIGURE 4.2:** Effect of the bioprocessing parameter temperature ($^{o}$C) in a batch culture (shake flask) on (A) Total cellular protein production. Results are given in milligrams liter$_{culture}^{-1}$ and (B) milligrams per gram$_{dry\ weight}^{-1}$ . (C) Profile of culture pH. Shown are data for (◇) 16 $^{o}$C, (□) 23 $^{o}$C, (△) 30 $^{o}$C and (×) 37 $^{o}$C.

### 4.2.2.4 Effect of temperature on cell morphology

In cultures grown at 16 $^{o}$C small pellets with a black inner core were formed within the first 36 hours. Pellet formation was noted to be small and smooth throughout the culture period, indicating suppressed growth of hyphal structures. Only at 216 hours, pellets were noted to become fluffy and the first signs of cell fragmentation were evident (fluffy bits of hyphae were

observed in the media). Due to a low biomass yield, the culture was observed to have a low viscosity.

At the culture temperature of 23 $^{o}$C, an increase in pellet size was observed, in comparison to 16 $^{o}$C cultures. These pellets were also described as small and white, and increased in size and number during the exponential growth phase. Similar to 16 $^{o}$C cultures, the morphology of the pellets was also smooth, implying that growth of hyphae were restricted. After 120 hours, the media became stained with a dark orange colour, resulting in an orange-like coloured pellets. At 192 hours pellets became fluffy. At 216 hours cell fragmentation was observed.

The pellet formation of cultures at 30 $^{o}$C was noted to be of similar magnitude to that observed in the 23 $^{o}$C cultures, although a larger number of pellets were noted in the 30 $^{o}$C cultures. Pellet morphology was fluffy, indicating the growth of protruding hyphal structures. This phenomenon became increasingly pronounced with culture age. At 96 hours cell fragmentation occurred and progressed until 216 hours. With culture age, media staining increased. At 120 hours, pellet formation could not be identified and after 216 hours the broth just contained cell fragments.

Cultures grown at 37 $^{o}$C, could initially be described as small, fluffy and white. These pellets were smaller than and not as numerous as cultivations grown at 30 $^{o}$C. Fragmentation was noted after just 48 hours and the pellets were noted to be an off-white/cream colour becoming increasingly fluffy with culture age. Media became rapidly darker with culture age. After 216 hours the broth was observed to contain only tiny pellet fragments.

FIG. 4.3 graphically depicts cell morphology on the macro-scale of shake flask cultivations grown under varying temperature conditions. Photographs were taken after just 24 hours.

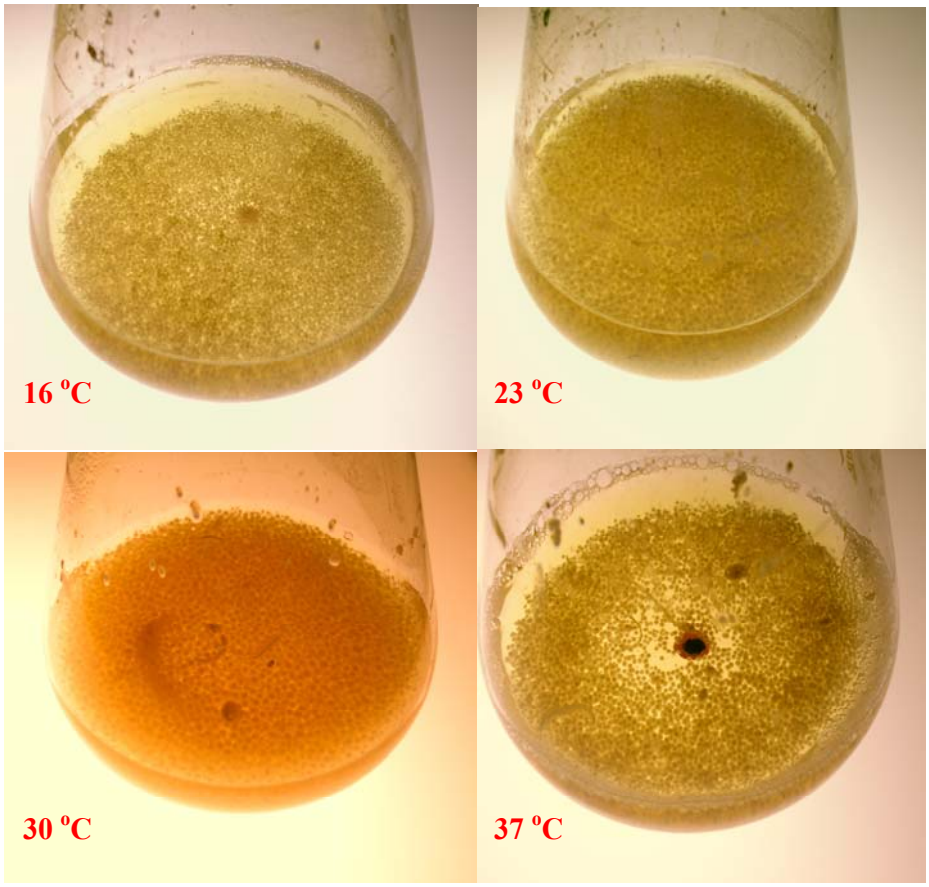


**FIGURE 4.3:** Effect of temperature on cell morphology. Photographs taken after 24 hours of growth at an initial inoculum concentration of $1\times10^{6}$ spores.mℓ$^{-1}$.

### 4.2.3 Inoculum concentration optimisation

#### 4.2.3.1 Effect of inoculum concentration on biomass production

The analysis of biomass production over varying inoculum concentrations, illustrated in FIG. 4.4A, demonstrated an increased lag phase of 24 hours, at low inoculum concentrations of $10^{3}$ and $10^{5}$ spores.mℓ$^{-1}$. As the spore concentration increased to $10^{6}$ and $10^{8}$ spores.mℓ$^{-1}$, the lag phase was reduced to 12 hours and 6 hours, respectively. Similar specific growth rates were observed for all inoculum concentrations and were recorded to be in the range of 0.028 h$^{-1}$. However, biomass production levels varied significantly with the optimum of 20.5 $g_{DW}.\ell_{culture}^{-1}$

at 120 hours in cultures grown at $10^6$ spores.m$\ell^{-1}$. Thereafter, cell death phase was evident in all cultures that progressed for the remainder of the cultivation.

4.2.3.2 Effect of inoculum concentration on protein production yield

- **Total protein production**

The analysis of specific total intracellular protein concentration (FIG. 4.5B) demonstrated a correlation with the change in inoculum concentration. With the increase in inoculum concentration, the specific production rate (0.0346 mg.g$^{-1}_{DW}$.h$^{-1}$ at $10^3$ spores/ml; 0.0416 mg.g$^{-1}_{DW}$.h$^{-1}$ at $10^5$ spores/ml; 0.0627 mg.g$^{-1}_{DW}$.h$^{-1}$ at $10^6$ spores/ml; 0.1021 mg.g$^{-1}_{DW}$.h$^{-1}$ at $10^8$ spores.ml$^{-1}$) as well as the production levels of total intracellular protein. All cultures peaked at varying intervals, which was followed by a decrease in total intracellular protein concentration that corresponded with the cell death phase (FIG. 4.5B). An optimum specific total intracellular protein concentration of 135 mg.$g_{DW}^{-1}$ at 120 hours was observed in cultures grown at $10^8$ spores.m$\ell^{-1}$. The analysis of volumetric (FIG. 4.5A) total intracellular protein concentration indicated a clear resemblance to the biomass growth profile (FIG. 4.4A). Optimum levels of 2000 mg.$\ell_{culture}^{-1}$ at 120 hours at an inoculum concentration of $10^6$ spores.m$\ell^{-1}$ was obtained.

- **HBsAg production**

The specific intracellular HBsAg production (FIG. 4.4D) was significantly low for all cultures until the 72$^{nd}$ hour. This was followed by a rapid increase in HBsAg production, where all cultures had a similar HBsAg production rate (0.0386 $\mu$g.g$^{-1}_{DW}$.h$^{-1}$). Similar to the culture temperature experiments, specific HBsAg production was most efficient during the latter stages of the cultivation. Neither the rate nor the yield of the specific HBsAg production was directly dependent on inoculum concentration. Maximum specific HBsAg production of 59 and 57 $\mu$g.$g_{DW}^{-1}$ at 216 hours was obtained by cultures grown at $10^5$ and $10^6$ spores.m$\ell^{-1}$, respectively. The volumetric HBsAg production followed similar trends to the biomass growth profile (FIG. 4.4C). Due to the high biomass concentrations obtained by cultures grown at $10^6$ spores.m$\ell^{-1}$,

optimum volumetric HBsAg production of 1000 μg.$\ell^{-1}$ at 144 hours was obtained at these inoculum concentrations.

- **Percentage cellular protein that HBsAg represents**

Similar to the culture temperature experiments, the significance of the percentage cellular protein that HBsAg represents (FIG. 4.4B) demonstrated the stability and robustness of the HBsAg particles during the latter stages of the cultivation. A distinct relation between inoculum concentration and the percentage cellular protein that HBsAg represents was also evident at 192 and 216 hours. The ratio increased with the increase in inoculum concentration form $10^3$ and $10^6$ spores.m$\ell^{-1}$. Thereafter, a rapid decrease in the percentage HBsAg was observed at $10^8$ spores.m$\ell^{-1}$. Thus, cultures grown at $10^6$ spores.m$\ell^{-1}$ obtained the highest ratio of percentage cellular protein that HBsAg representsof 0.42% at 216 hours.

4.2.3.3 Effect of inoculum concentration on culture pH

The analysis of the culture pH profile in relation to varying inoculum concentration illustrated in FIG. 4.5C, demonstrated a sudden decrease in pH (initial pH 6.5) within the first 24 hours of culture growth. The largest decrease in pH during this period was observed to be cultures grown at $10^3$ spores.m$\ell^{-1}$ (pH 4.28), followed by $10^5$ spores.m$\ell^{-1}$ (pH 4.5), $10^8$ (pH 5.3) and finally $10^6$ spores.m$\ell^{-1}$ (pH 5.76). Thereafter, all cultures rapidly increased to a pH above 6 at 72 hours, where the culture pH stabilised at approximately 7 for the remainder of the cultivation.

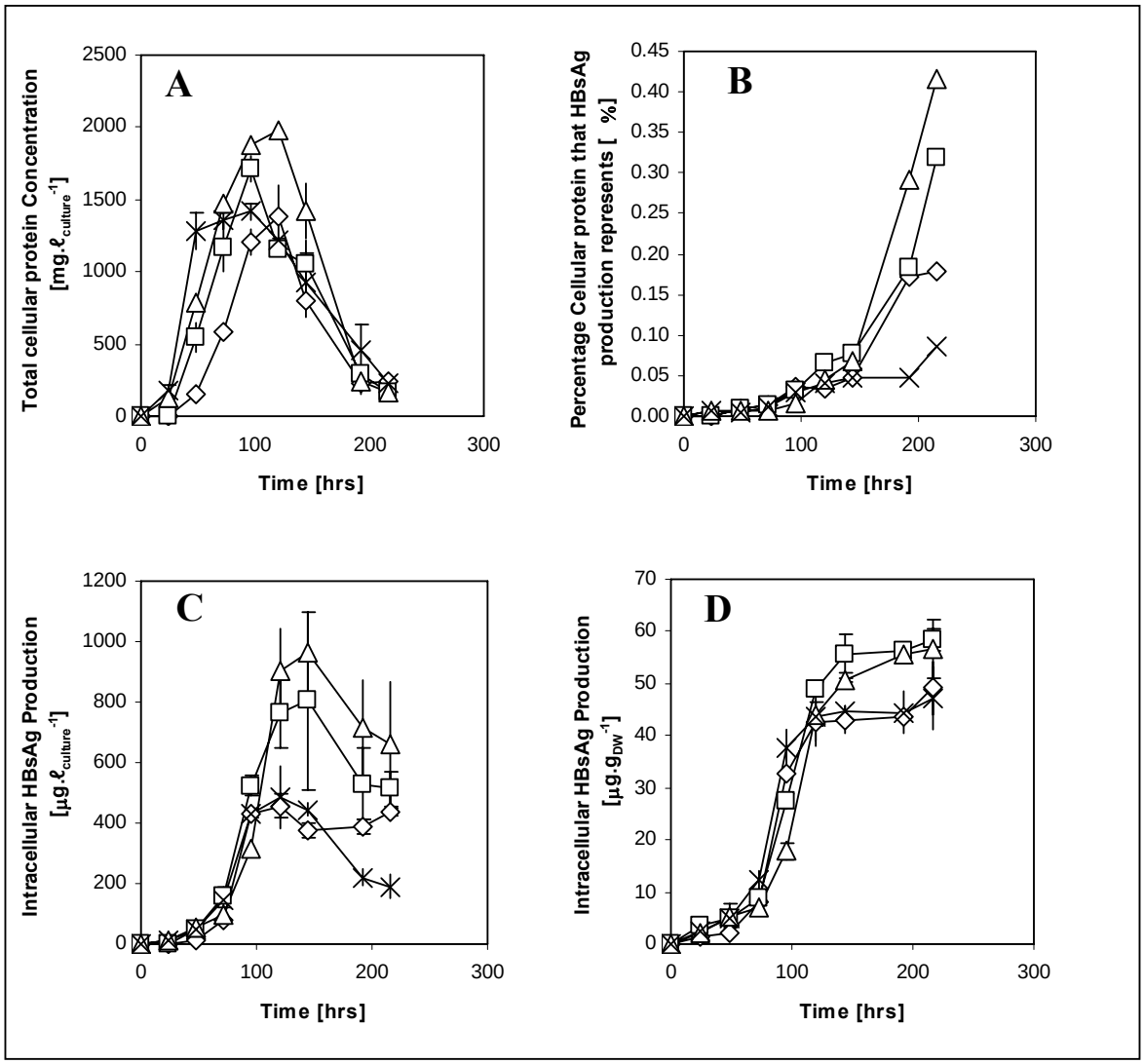


**FIGURE 4.4:** Effect of the bioprocessing parameter inoculum concentration (spores/ml) in a batch culture (shake flask) on (A) Biomass dry mass concentration. Results are given in grams dry weight $liter_{culture}^{-1}$. (B) Percentage cellular protein that HBsAg represents. Results are given in % HBsAg. (C) Intracellular HBsAg production. Results are given in micro grams $liter_{culture}^{-1}$ and (D) micro grams per $gram_{dry\ weight}^{-1}$ .Shown are data for (◇) $1\times10^{3}$ spores/ml, (□)$1\times10^{5}$ spores/ml, (△)$1\times10^{6}$ spores/ml and (×)$1\times10^{8}$ spores/ml. Results are based on three replications over a cultivation time of 220 hours.

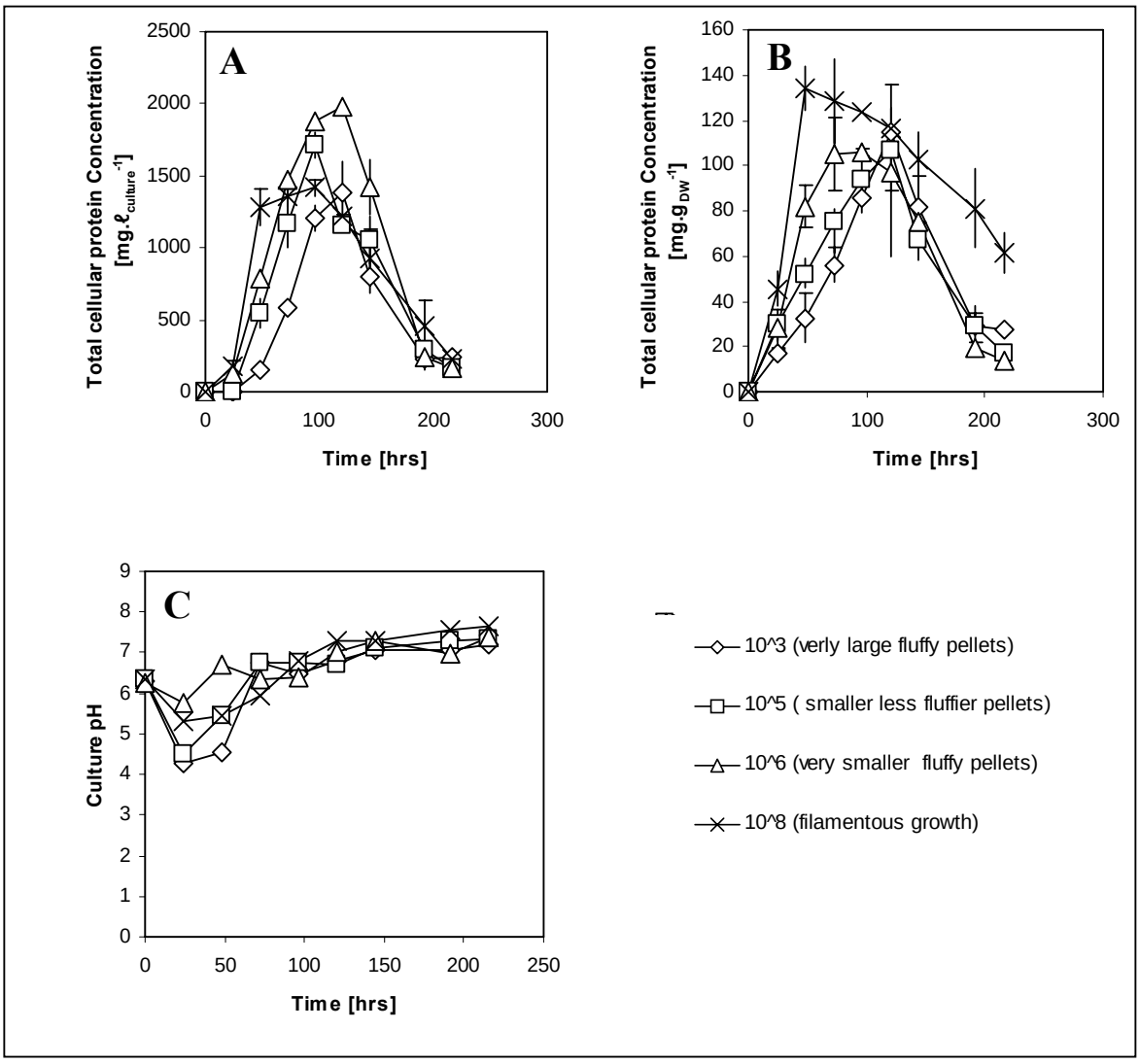


**FIGURE 4.5:** Effect of the bioprocessing parameter inoculum concentration (spores/ml) in a batch culture (shake flask) on (A) Total cellular protein production. Results are given in milligrams liter$_{culture}^{-1}$ and (B) milligrams per gram$_{dry\ weight}^{-1}$ . (C) Profile of culture pH. Shown are data for (◇) $1\times10^{3}$ spores/ml, (□)$1\times10^{5}$ spores/ml, (△)$1\times10^{6}$ spores/ml and (×)$1\times10^{8}$ spores/ml. Results are based on three replications over a cultivation time of 220 hours.

4.2.3.4 Effect of inoculum concentration on broth morphology

Cell morphology was affected by inoculum concentration. In cultures grown at $10^3$ spores.m$\ell^{-1}$, pellet formation was observed. Pellets grew very large in size but did not significantly increase in number after 72 hours. During this period, pellets were observed to be very large, white and fluffy (suggesting long and numerous hyphae structures). After 96 hours, the media started to darken and the first signs of cell fragmentation were evident. After 192 hours, pellets could not be identified, and after 216 hours the broth was observed to contain only bits of cell fragments.

$10^5$ spores.m$\ell^{-1}$ cultures also formed pellets. Pellets were smaller in size but present in larger numbers. After 40 hours, pellets were large, white and fluffy (suggesting long hyphae structures). After 72 hours, the first signs of cell fragmentation were evident, broth became darker and pellet colour changed to a cream colour. Cell fragmentation increased as the culture age increased. After 192 hours pellet formation could not be identified in the culture, and the broth could be described as bits of cell fragments in suspension.

In cultures grown at $10^6$ spores.m$\ell^{-1}$, pellet growth was observed. Pellets were very small in comparison to the above mentioned cultures and were in large numbers. Pellet morphology was noted to be fluffy and became increasingly pronounced with culture age. At 96 hours fragmentation occurred. As the fragmentation increased, media colour darkened. At 120 hours, pellet formation could not be identified in the culture, and after 192 hours the broth could be described as bits of cell fragments in suspension.

$10^8$ spores.m$\ell^{-1}$ cultures formed filamentous clumps. At 24 hours, dispersed mycelia was noted and as the culture age increased these mycelia formed thick filamentous clumps. The biomass production levels in this culture was not high. Cell fragmentation was observed after 72 hours, after which the rapid disintegration of clumps was observed until just fragments of dispersed mycelia was visible in the broth at 216 hours. The colour of the media remained black throughout the culturing period, which indicated that many of the spores did not germinate.

FIG. 4.6 graphically depicts cell morphology on the macro-scale of shake flask cultivations grown under varying inoculum concentrations. Photographs were taken after just 24 hours.

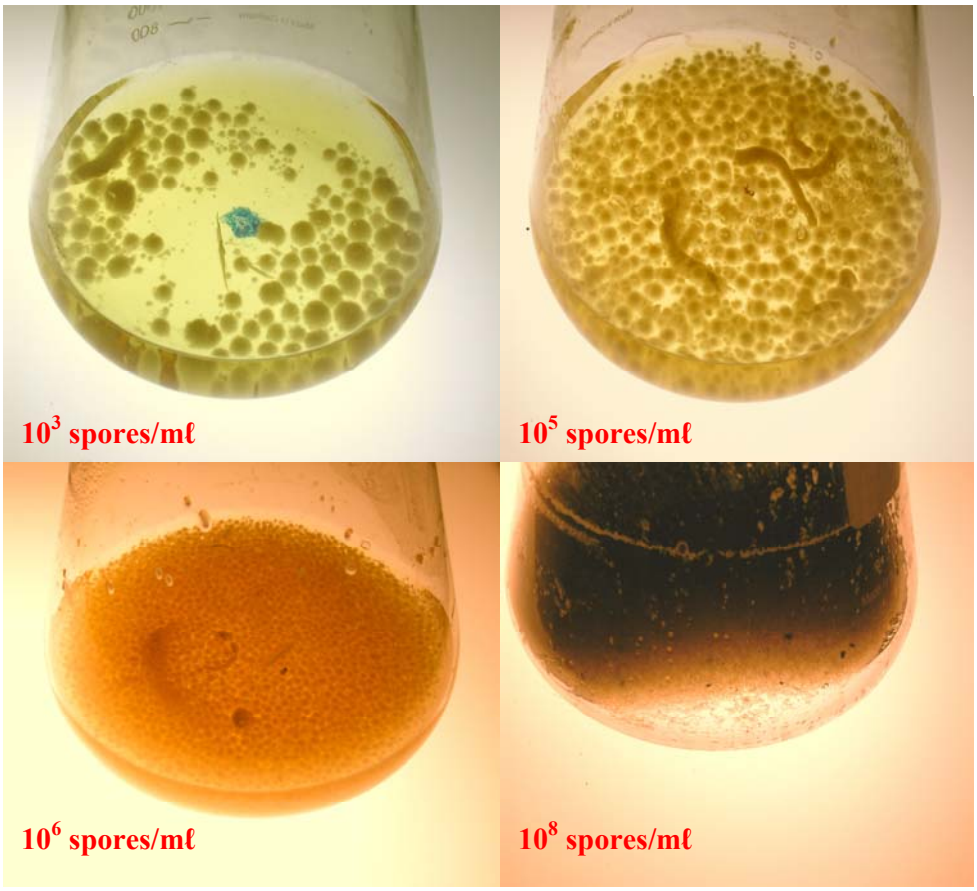


**FIGURE 4.6:** Effect of inoculum concentration on cell morphology. Photographs taken after 24 hours of growth at a cultivation temperature of 30 $^{o}$C.

## 4.3 Bioreactor cultivations

### 4.3.1 Introduction

The aim of the bioreactor cultivations was to determine the effects of agitation, $dO_2$ concentration and culture pH on mycelial morphology, culture growth, and homologous as well as VLP (HBsAg) concentration levels and the glucose utilisation and specific oxygen uptake rates. Special interest was also taken in the potential interdependent relationship existing between agitation and $dO_2$ concentration. In cases where high levels of HBsAg production was measured in the cytoplasmic fraction of the microorganism, further analysis of extracellular HBsAg production was performed on the broth supernatant. All processed data illustrated in these graphs are available in a tabulated form in Appendix C & D.

### 4.3.2 Agitation rate optimisation

#### 4.3.2.1 Effect of agitation rate on biomass yield

High agitation intensity (600 rpm) demonstrated the highest biomass specific growth rate (0.062 $h^{-1}$). Lower biomass specific growth rates of 0.051 $h^{-1}$ and 0.043 $h^{-1}$ were observed at agitation rates of 100 rpm and 350 rpm, respectively (TABLE. 4.1). High maximum biomass concentrations of 12.9 $g_{DW}.\ell_{culture}^{-1}$ (60 hours) and 12.8 $g_{DW}.\ell_{culture}^{-1}$ (42 hours) were recorded at agitation rates of 100 rpm and 600 rpm, respectively, while maximum growth at 350 rpm was significantly less at 7.8 $g_{DW}.\ell_{culture}^{-1}$ (48 hours) (FIG. 4.7A).

#### 4.3.2.2 Effect of agitation rate on protein production yield

- **Total protein production**

No direct correlation between specific total intracellular protein concentration and agitation rate was observed (FIG. 4.8A, B). Maximum concentrations for all cultures were obtained at

approximately 90 hours. This was followed by a decrease in total intracellular protein concentration that corresponded with the cell death phase. An optimum specific total intracellular protein concentration of 150 $mg.g_{DW}^{-1}$ at 90 hours was observed in cultures grown at 100 rpm (FIG. 4.8B). Cultures grown at 600 rpm also obtained high specific yields of 120 $mg.g_{DW}^{-1}$ after 90 hours, while at 350 rpm specific concentration was reduced to 100 $mg.g_{DW}^{-1}$ after 90 hours. The analysis of volumetric total intracellular protein concentration (FIG. 4.8A) indicated a clear resemblance to the biomass growth profile (FIG. 4.7A). Optimum levels of 1250 $mg.\ell_{culture}^{-1}$ at 82 hours with an agitation rate of 100 rpm was obtained.

- **HBsAg production**

HBsAg production was localised intracellularly (intracellular membrane + cytoplasmic fraction) (FIG. 7C, D). No intracellular HBsAg production was observed during the first 37 hours of cultivation, followed by a sudden exponential increase. The intracellular HBsAg specific production rate ($\mu g.g_{DW}^{-1}.h^{-1}$) increased with the increase in agitation intensity (TABLE 4.1). However, maximum volumetric HBsAg production levels increased with the decrease in agitation intensity (FIG. 7C). In cultures grown at 100 rpm, optimum intracellular HBsAg levels of 3600 $\mu g.\ell_{culture}^{-1}$ (82 hours) were obtained. Cultures grown at 350 rpm demonstrated maximum levels of approximately 1000 $\mu g.\ell_{culture}^{-1}$, while volumetric yields were reduced to 600 $\mu g.\ell_{culture}^{-1}$ at 600 rpm.

Although the majority of HBsAg production was associated with the intracellular membrane fraction, production of HBsAg in the intracellular cytoplasmic fraction could be achieved by varying agitation rates. No significant amount of cytoplasmic associated HBsAg (i.e. less than 25 $\mu g.\ell_{culture}^{-1}$) was observed in cultures grown at 350 rpm and 600 rpm (data not shown). However, cultures grown at 100 rpm demonstrated significant amounts of HBsAg production in both the intracellular membrane (3000 $\mu g.\ell_{culture}^{-1}$) and cytoplasmic fraction (584 $\mu g.\ell_{culture}^{-1}$). In these cultures the HBsAg concentration in the membrane and cytoplasmic fractions peaked after 82 hours, followed by a rapid decrease in VLP concentration and the release of significant amounts of HBsAg into the culture supernatant (FIG. 6). The highest level of 970 $\mu g.\ell_{culture}^{-1}$ of HBsAg in

the extracellular medium was achieved at 160 hours, while the volumetric HBsAg levels of the membrane and cytoplasmic fractions were reduced to 557 and 156 $\mu g.\ell_{culture}^{-1}$ respectively, during the corresponding cultivation period (see FIG. 4.18).

- **Percentage cellular protein that HBsAg represents**

In cultures where agitation rates were varied, the percentage of total cellular protein represented by HBsAg (%) increased steadily with cultivation time (FIG. 7B), emphasising the stability, robustness and recalcitrance of the HBsAg VLPs to proteolytic degradation. The stability of the HBsAg VLP also increased with the decrease in agitation intensity. At the highest levels, the percentage HBsAg decreased from 0.45 % to 0.18 % of the total cellular protein with the increase in agitation intensity.

#### 4.3.2.3 Effect of agitation rate on culture glucose concentration

No direct correlation between agitation intensity and the rate of specific glucose consumption ($g.g_{DW}^{-1}.h^{-1}$) of the microorganism was noted (TABLE 4.1). Cultures grown under high agitation intensity of 600 rpm had the highest specific glucose consumption rate of 0.214 $g.g_{DW}^{-1}.h^{-1}$. Cultures grown at low agitation intensities of 100 rpm recorded levels of 0.147 $g.g_{DW}^{-1}.h^{-1}$, while cultures grown at an intermediate level of 350 rpm had the lowest specific glucose consumption rate of 0.127 $g.g_{DW}^{-1}.h^{-1}$. Glucose was completely consumed in all cultures: At 600 rpm glucose was depleted after 42 hours, while cultures at 350 rpm and 100 rpm glucose were depleted after 50 and 48 hours, respectively (data not shown).

#### 4.3.2.4 Effect of agitation rate on culture pH

The analysis of the culture pH profile in relation to varying agitation rates (FIG. 4.19C), demonstrated a sudden decrease in pH (initial pH 6.5) within the first 28 hours of culture growth. The largest decrease in pH during this period was observed to be cultures grown at 600 rpm (pH 3.6 at 17 hours), followed by 350 rpm (pH 4.3 at 28 Hours), and finally 100 rpm (pH 5.1 at 22

hours). Thereafter, the culture pH of all cultures rapidly increased and stabilised between the hours of 58 and 82. Cultures grown at 100 rpm stabilised at a pH value of approximately 8, while cultures grown at 350 rpm and 600 rpm stabilised at a pH value around 7.4 and 6.7, respectively.

#### 4.3.2.5 Effect of agitation rate on broth morphology

From FIG. 4.9, pellet formation was observed in cultures grown at 100 rpm and grew greater in size compared to cultures grown at higher agitation rates. The fluffiness of the pellets (indicating the growth of hyphal structures) became increasingly pronounced with culture age. Pellet colour turned from a white to a brown colour after 48 hours. Cell fragmentation occurred at 103 hours. As cell fragmentation increased in these cultures, biomass as well as broth viscosity decreased.

In cultures grown at 350 rpm, pellet formation was also observed, but slightly smaller and less fluffy compared to cultures grown at 100 rpm. Pellet discoloration was observed and cell fragmentation was evident after a culture age of 40 hours, progressively increasing for the remainder of the cultivation.

In cultures grown at high agitation rates of 600 rpm, the broth was observed to consist of brown filamentous clumps. Biomass concentration rapidly increased during the exponential growth phase. With culture age, filamentous clumps became dispersed with the action of cell fragmentation. Cell fragmentation was evident from as early as the $30^{th}$ hour. This phenomenon progressively increased during the latter stages of the cultivation and as a result biomass concentration decreased from the $58^{th}$ hour onwards.

#### 4.3.2.6 Effect of agitation rate on the specific oxygen uptake rate (SOUR)

The maximum specific oxygen uptake rate (max. SOUR; $mmol_{O2}.g_{DW}^{-1}.h^{-1}$) of the microorganism had no distinct relation to the agitation intensity (TABLE 4.1). Cultures grown at 100 rpm and 600 rpm obtained high SOURs (7.13 $mmolO_2.g_{DW}^{-1}.h^{-1}$ and 6.41 $mmolO_2.g_{DW}^{-1}.h^{-1}$, respectively), while 350 rpm cultures obtained the lowest (4.44 $mmolO_2.g_{DW}^{-1}.h^{-1}$).

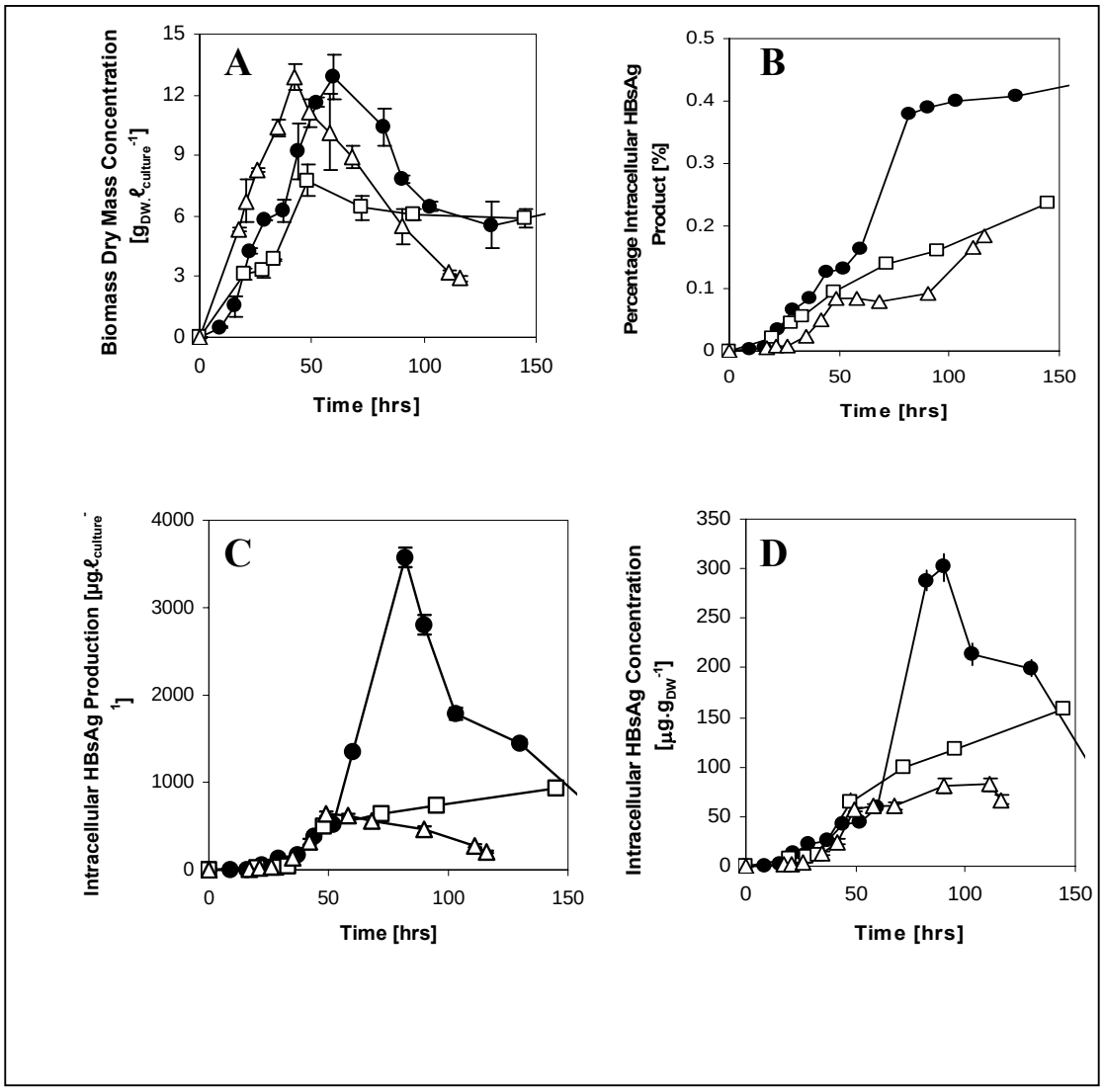


**FIGURE 4.7:** Effect of the bioprocessing parameter agitation (rpm) in a batch culture (bioreactor) on (A) Biomass dry mass concentration. Results are given in grams dry weight $liter_{culture}^{-1}$. (B) Percentage cellular protein that HBsAg represents. Results are given in % HBsAg. (C) Intracellular HBsAg production. Results are given in micro grams $liter_{culture}^{-1}$ and (D) micro grams per $gram_{dry\ weight}^{-1}$. Shown are data for (●) 100 rpm, (□) 350 rpm, (△) 600 rpm. Results are based on two replications over a cultivation time of 150 hours. Points without error bars have an associated error that is < 10 % of the value of the point.

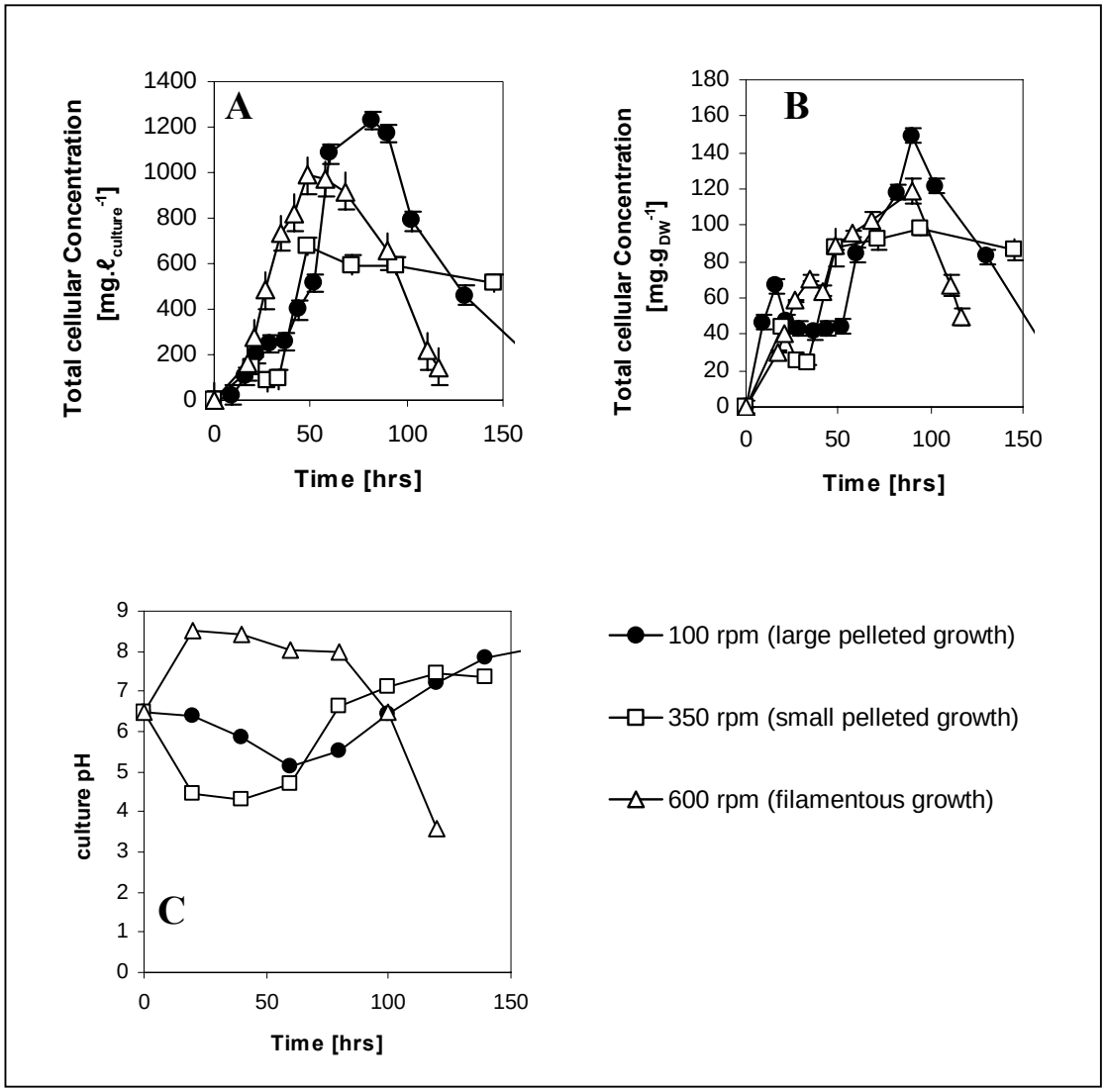


**FIGURE 4.8:** Effect of the bioprocessing parameter agitation (rpm) in a batch culture (bioreactor) on (A) Total cellular protein production. Results are given in milligrams $liter_{culture}^{-1}$ and (B) milligrams per $gram_{dry\ weight}^{-1}$. (C) Profile of culture pH. Shown are data for (●) 100 rpm, (□) 350 rpm, (△) 600 rpm. Results are based on two replications over a cultivation time of 150 hours. Points without error bars have an associated error that is < 10 % of the value of the point.

**TABLE 4.1:** Calculated yields and specific rate parameters from the three different agitation intensities

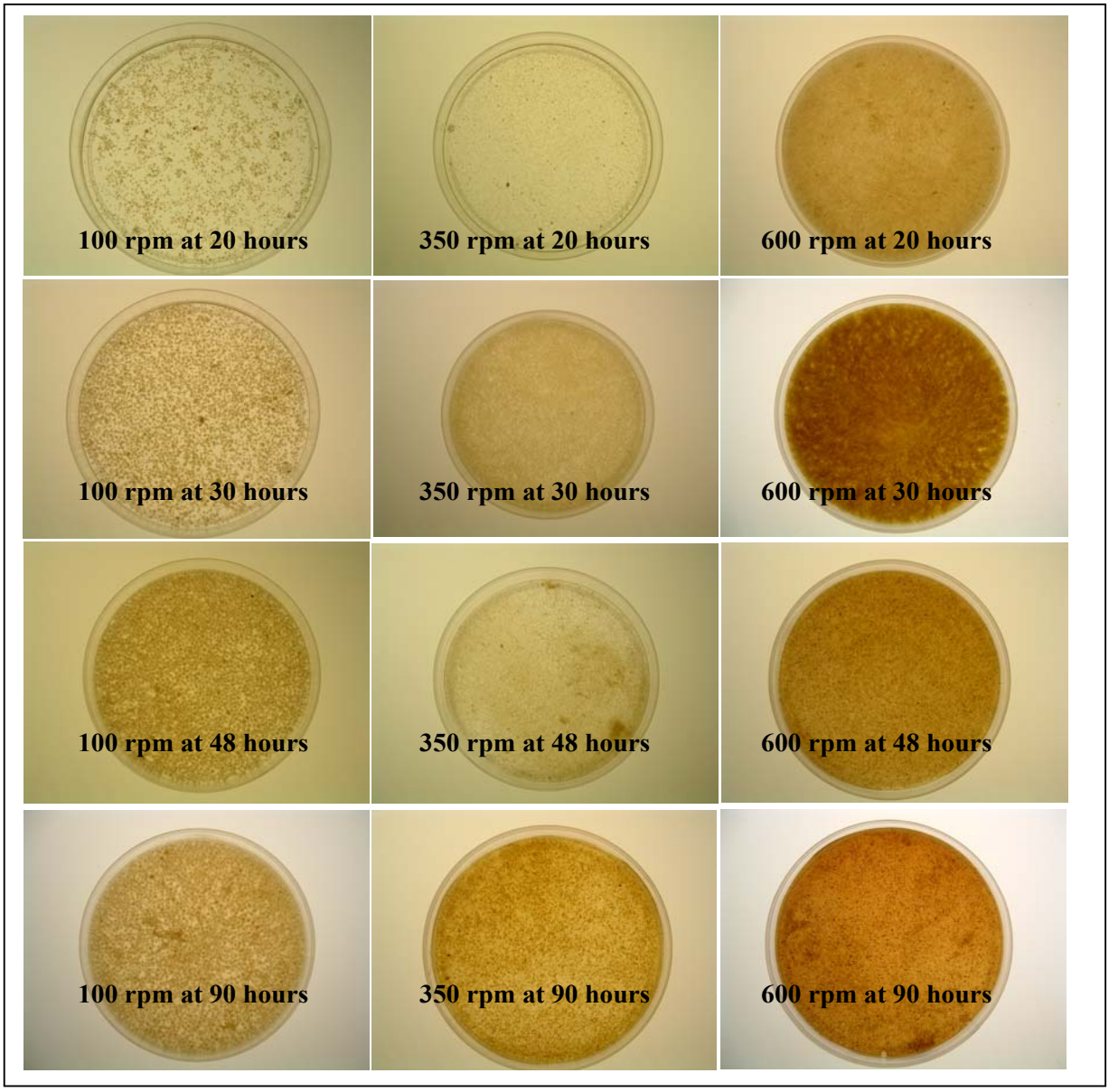


**FIGURE 4.10:** Effect of agitation on cell morphology. All cultivations were controlled at 50% $dO_2$ concentration.

### 4.3.3 $dO_2$ concentration optimisation

#### 4.3.3.1 Effect of $dO_2$ concentration on biomass yield

A distinct relation between $dO_2$ concentration and the biomass specific growth rate was observed (TABLE. 4.2), where the specific growth rate ($h^{-1}$) increased with an increase in culture $dO_2$ concentration. However, the highest biomass production levels were obtained at the lower oxygenation levels under oxygen-limited conditions (2 % $dO_2$ concentration; 11.9 $g_{DW}.\ell_{culture}^{-1}$ after 101 hours) and intermediate oxygen levels (25 % $dO_2$ concentration; 11.1 $g_{DW}.\ell_{culture}^{-1}$ after 72 hours) (FIG. 4.10A). Cultures grown under oxygen-rich conditions of 95 % and 50 % $dO_2$ obtained lower biomass concentrations of 9.6 $g_{DW}.\ell_{culture}^{-1}$ (55 hours) and 7.8 $g_{DW}.\ell_{culture}^{-1}$ (48 hours), respectively.

#### 4.3.3.2 Effect of $dO_2$ concentration on protein production yield

- **Total protein production**

No relation between specific total (membrane associated) intracellular protein concentration and $dO_2$ concentration was observed (FIG. 4.11B). All cultures obtained similar maximum specific yields between 100 and 80 $mg.g_{DW}^{-1}$. These production levels were observed to stabilise at these levels for most of the cultivation period. The analysis of volumetric total intracellular protein concentration (FIG. 4.11A) indicated a clear resemblance to the biomass growth profile (FIG. 4.10A). Optimum levels of 1020 $mg.\ell_{culture}^{-1}$ at 86 hours without $dO_2$ control were recorded and was followed by cultures grown at 25% $dO_2$ concentration, 930 $mg.\ell_{culture}^{-1}$ at 72 hours.

The levels of intracellular cytoplasmic associated total protein production were not affected by $dO_2$ concentration. No significant amount of specific as well as volumetric production was observed in all $dO_2$ concentration varied cultures (data not shown). Recorded values were observed to be below the range of 25 $mg.g_{DW}^{-1}$ and 200 $mg.\ell_{culture}^{-1}$ through out the cultivation.

- **HBsAg production**

Similar to cultures grown at various agitation intensities, no intracellular HBsAg production was observed during the first 35 hours of cultivation, followed by a sudden exponential increase (FIG. 4.10C, D). VLP production was most efficient in cultures grown at intermediate oxygen levels (25 % $dO_2$), with optimum intracellular HBsAg levels of 1710 $\mu g.\ell_{culture}^{-1}$ (72 hours). Under oxygen limited conditions (2 % $dO_2$) and oxygen-rich conditions (50 % and 95 % $dO_2$ concentration), HBsAg production was significantly reduced. The maximum specific rate of intracellular HBsAg production in cultures grown at 25 % $dO_2$ concentration was 0.116 $\mu g.g_{DW}^{-1}.h^{-1}$ (TABLE 4.2). Cultures grown at 50 % $dO_2$ concentration had lower rates (0.104 $\mu g.g_{DW}^{-1}.h^{-1}$), while cultures grown at 2 % and 95 % had the lowest HBsAg specific production rates of 0.072 and 0.090 $\mu g.g_{DW}^{-1}.h^{-1}$, respectively (TABLE 4.2).

- **Percentage cellular protein that HBsAg represents**

The percentage of total cellular protein represented by HBsAg (%) increased steadily with cultivation time (FIG. 4.10B), emphasising the stability, robustness and recalcitrance of the HBsAg VLPs to proteolytic degradation. The highest levels were observed in cultivation of intermediate oxygen levels and low levels were obtained in oxygen rich/limited cultivations. Cultures grown without $dO_2$ control obtained the optimum ratio of 0.31% at 52 hours. This was followed by cultivations grown at 25% $dO_2$ concentration at 0.24% at 145 hours, 50% cultivations of 0.182% at 145 hours, 2% cultivations of 0.172% at 140 hours, and 95% cultivations of 0.16% at 101 hours Insignificant levels of intracellular cytoplasmic associated production ratios were recorded in all cultures. Values in the range of below 0.04% were observed (data not shown).

#### 4.3.3.3 Effect of $dO_2$ concentration on culture glucose concentration

The specific glucose consumption rate ($g.g_{DW}^{-1}.h^{-1}$) increased with an increase in $dO_2$ concentration (TABLE 4.2). Glucose was depleted after 43 hours in cultures grown at 95 % $dO_2$,

while cultures grown at 50 %, 25 % and 2 % $dO_2$ concentration depleted the supply of glucose by the $50^{th}$, $54^{th}$ and $64^{th}$ hour, respectively (data not shown).

#### 4.3.3.4 Effect of $dO_2$ concentration on culture pH

The analysis of the culture pH profile in relation to varying $dO_2$ concentrations illustrated in FIG. 11C, clearly demonstrated a sudden decrease in pH (initial pH 6.5) within the first 40 hours of culture growth at 50 % and 95 % $dO_2$. Thereafter, the culture pH of all cultivations increased and stabilised at various levels. Cultures grown at 2% $dO_2$ concentrations stabilised at a pH value of approximately 6. Similarly, cultures grown at 25% and 50% $dO_2$ concentrations stabilised at approximately pH 7, while at 95% pH stabilisation was measured at approximately 6.5.

#### 4.3.3.5 Effect of $dO_2$ concentrations on broth morphology

From FIG. 4.12, filamentous formation was observed in cultures grown at 2% $dO_2$ concentration, which was noted to form large clumps as the culture age increased. No broth or cell discoloration was evident throughout the cultivation, but cell fragmentation occurred at 52 hours and was seen to progressively increase for the remainder of the cultivation. High biomass levels were associated with filamentous clump formation. With culture age and the action of cell fragmentation, filamentous clumps became increasingly dispersed.

In cultures grown at 25% $dO_2$ concentration, pellet growth was observed. The pellets were very small in size and the pronounced fluffiness suggested long hyphae. Discoloration of broth and cells was noted, as pellets being initially white gradually changed to a brown/orange colour. Cell fragmentation was observed after 48 hours and was seen to progressively increase for the remainder of the cultivation.

In cultures grown at 50% $dO_2$ concentration (not illustrated in fig), pellet formation was observed. The fluffiness of the pellets was considerably less pronounced when compared to cultures grown of lesser $dO_2$ concentration. Similarly, to cultures grown at 25%, pellet coloration

was observed and cell fragmentation was evident after a culture age of 40 hours. This phenomenon increased rapidly during the remainder of the cultivation.

In cultures grown at high $dO_2$ concentrations of 95%, the broth was observed to consist of very small pellets. Pellets were smooth in texture, suggesting a significant reduction in hyphae length and number). After 34 hours the broth was seen to change to a dark brown/orange colour and cell fragmentation was observed. These phenomena were seen to rapidly increase during the remainder of the cultivation.

#### 4.3.3.6 Effect of $dO_2$ concentrations on specific oxygen uptake rate (SOUR)

The maximum SOUR ($mmol_{O2}.g_{DW}^{-1}.h^{-1}$) of the microorganism had no significant relation to the culture $dO_2$ concentration (TABLE 4.2). Cultures grown at intermediate culture $dO_2$ concentration (25 %) obtained the highest maximum SOUR (6.45 $mmolO_2.g_{DW}^{-1}.h^{-1}$). Under oxygen limited conditions (2 % $dO_2$) and oxygen-rich conditions (50 % and 95 % $dO_2$) the SOUR of the microorganism was significantly lower (TABLE 4.2).

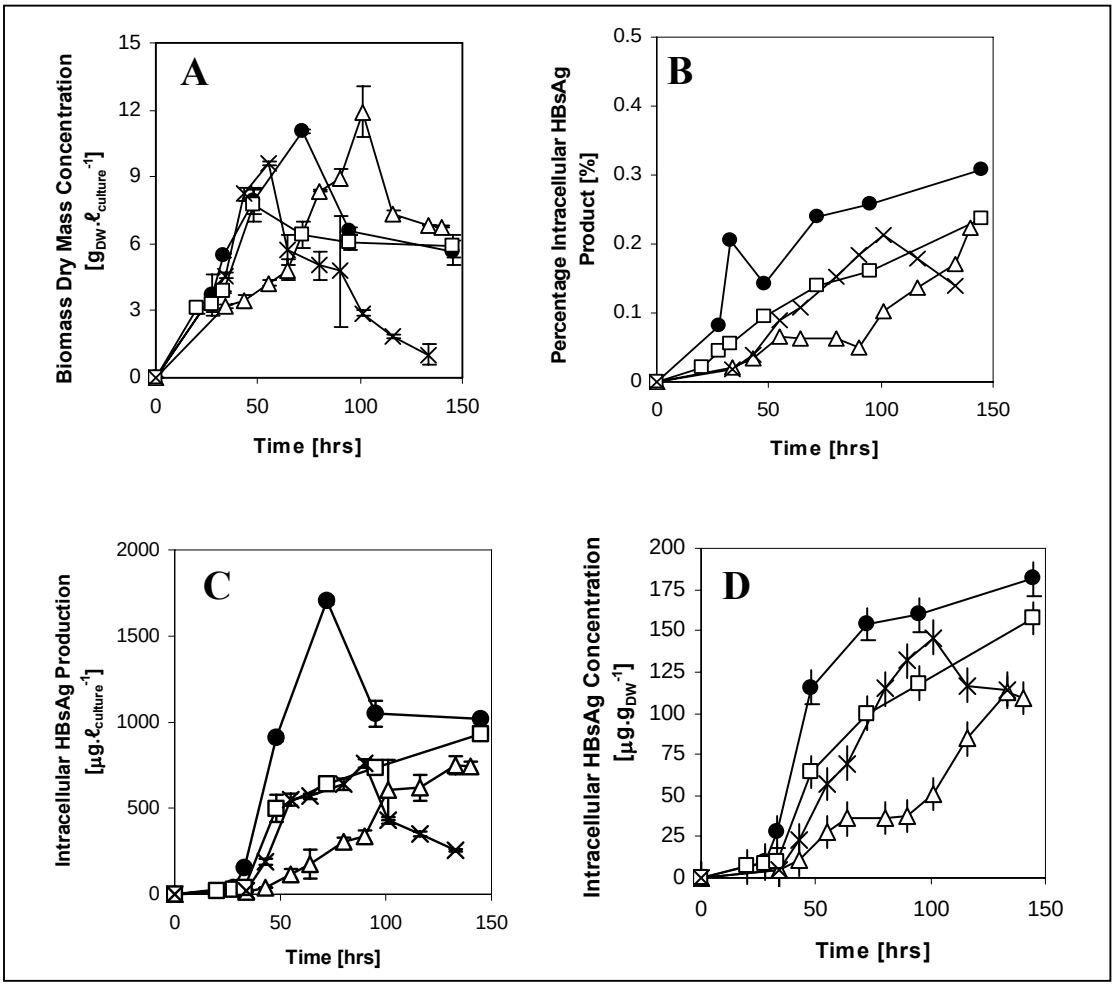


**FIGURE 4.11:** Effect of the bioprocessing parameter dissolved oxygen concentration ($dO_2$ %) in a batch culture (bioreactor) on (A) Biomass dry mass concentration. Results are given in grams dry weight $liter_{culture}^{-1}$. (B) Percentage cellular protein that HBsAg represents. Results are given in % HBsAg. (C) Intracellular HBsAg production. Results are given in micro grams $liter_{culture}^{-1}$ and (D) micro grams per $gram_{dry\ weight}^{-1}$. Shown are data for (△) 2 $dO_2$ %, (●) 25 $dO_2$ %, (□) 50 $dO_2$ %, (×) 95 $dO_2$ %. Points without error bars have an associated error that is < 10 % of the value of the point.

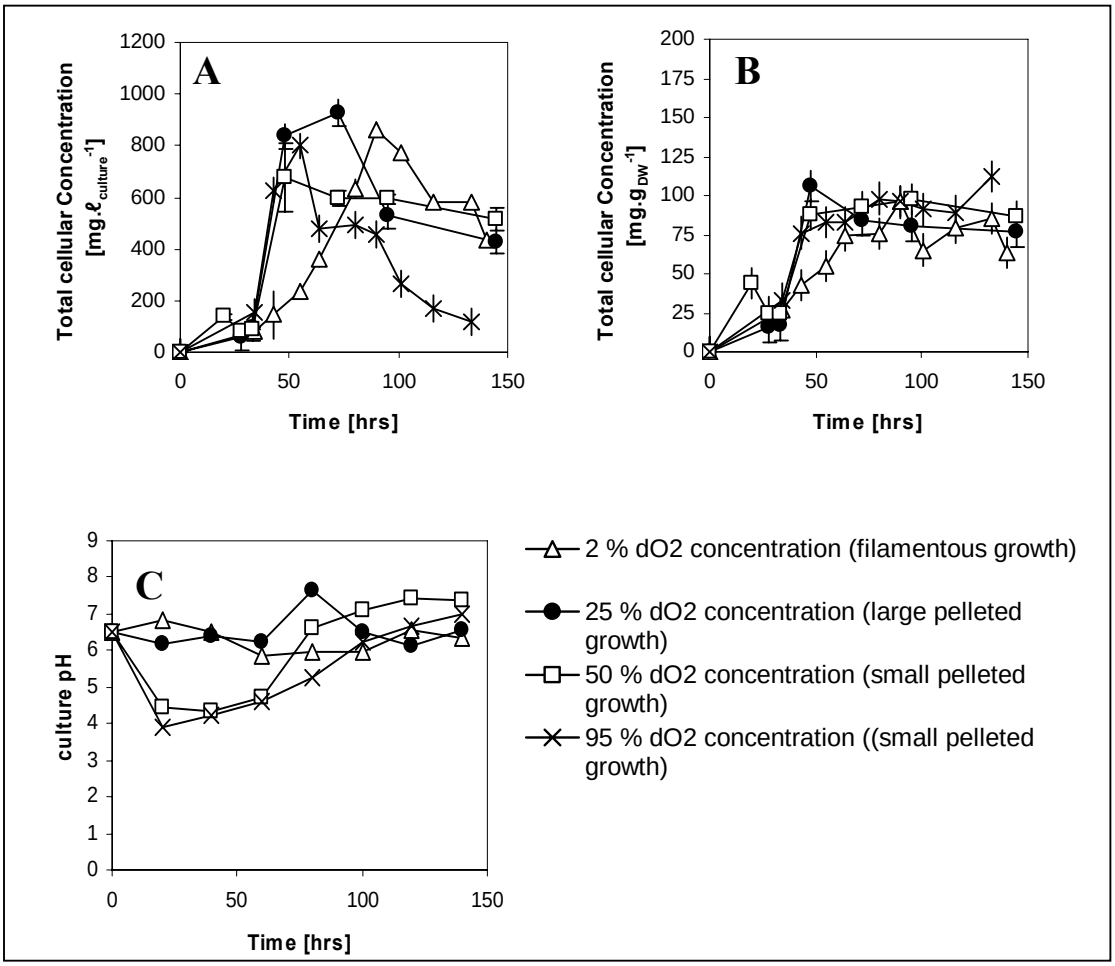


**FIGURE 4.13:** Effect of the bioprocessing parameter dissolved oxygen concentration ($dO_2$ %) in a batch culture (bioreactor) on (A) Total cellular protein production. Results are given in milligrams $liter_{culture}^{-1}$ and (B) milligrams per $gram_{dry\ weight}^{-1}$. (C) Profile of culture pH. Shown are data for (△) 2 $dO_2$ %, (●) 25 $dO_2$ %, (□) 50 $dO_2$ %, (×) 95 $dO_2$ %. Points without error bars have an associated error that is < 10 % of the value of the point.

**TABLE 4.2:** Calculated yields and specific rate parameters from the four different dissolved oxygen concentrations

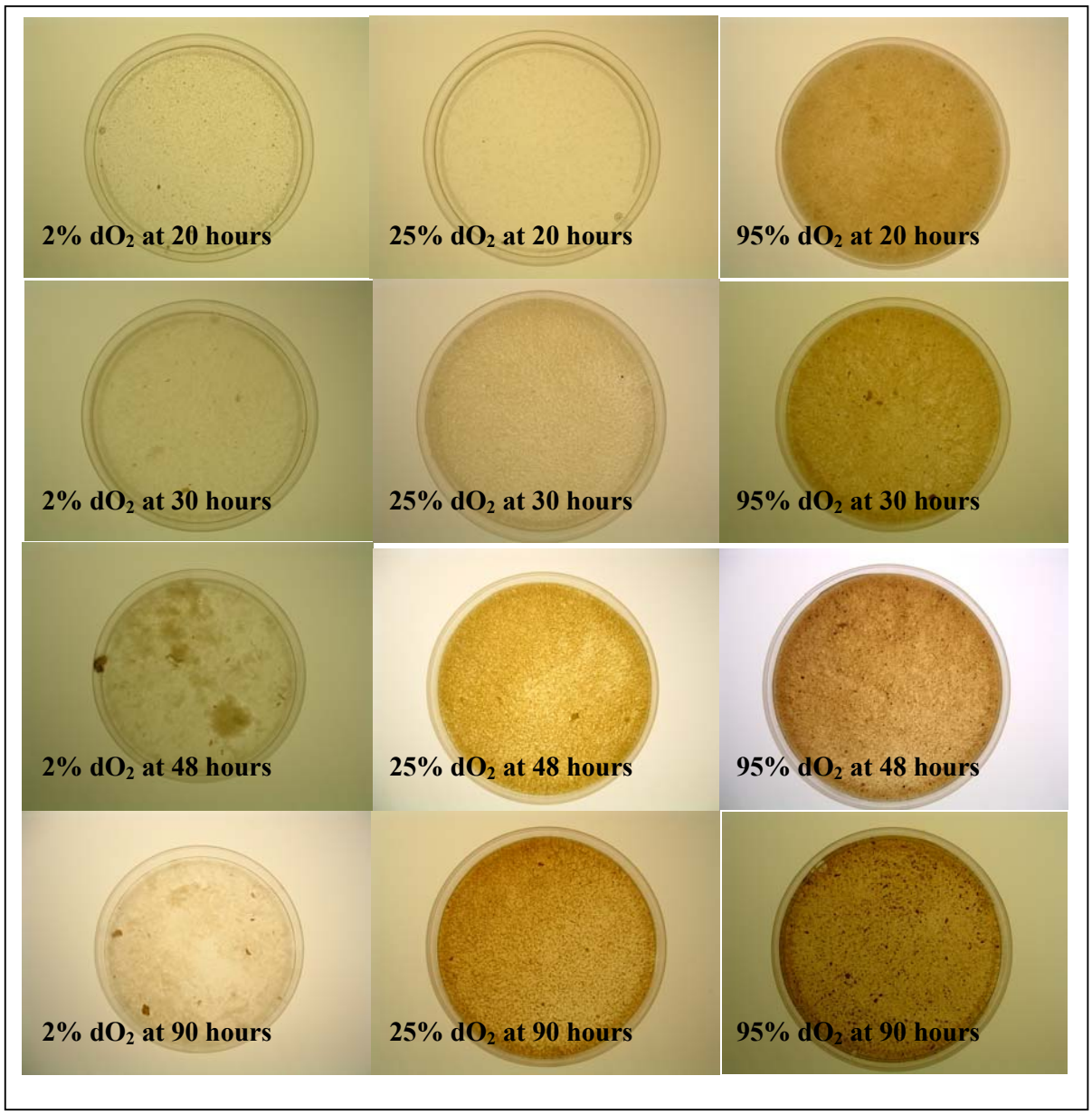


**FIGURE 4.15:** Effect of $dO_2$ concentration on broth rheology and cell morphology. All cultivations were controlled at an agitation of 350 rpm.

### 4.3.4 Interdependent effects of agitation rate and $dO_2$ concentration

#### 4.3.4.1 Interdependent effect of agitation rate and $dO_2$ concentration on biomass yield

The central composite rotary design (CCRD) contour plots indicated that the highest levels of biomass concentration ($g_{DW}.\ell_{culture}^{-1}$) were obtained at low agitation rates of 100 and 250 rpm corresponding to $dO_2$ concentrations ranging between 20 - 50 % (FIG. 4.13). At high agitation rates (450 and 600 rpm) corresponding to $dO_2$ concentrations ranging between 2-50 % $dO_2$, high levels of biomass were also obtained.Biomass concentration was significantly reduced at agitation rates of 350, 450, and 600 rpm under severe oxygen enriched (80-95% $dO_2$) conditions.

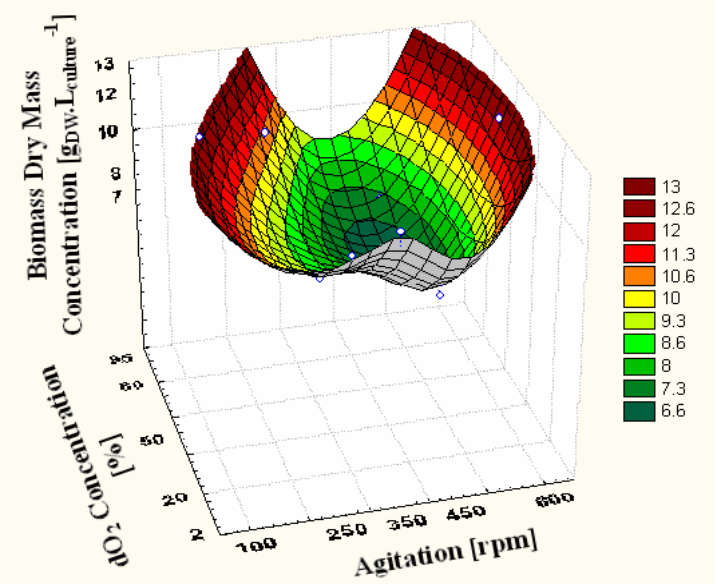


**FIGURE 4.16:** Biomass concentration illustrating interdependent effects of agitation and $dO_2$ concentration (3D Contour Plot)

4.3.4.2 Interdependent effect of agitation rate and $dO_2$ concentration on protein production

- **Total protein production**

A distinct correlation between total (membrane and cytoplasmic associated) intracellular protein concentration and the interdependent effect of $dO_2$ concentration and agitation was observed (FIG. 4.14 and FIG. 4.15). With the increase in agitation rate from 250 rpm to 600 rpm and the increase in $dO_2$ concentration from 2% to 95%, a significant increase in the total (membrane and cytoplasmic associated) intracellular protein concentration was observed. Similarly, the decrease in agitation rate from 250 rpm to 100 rpm and the increase in $dO_2$ concentration from 2% to 95% $dO_2$, also resulted in a significant increase in the specific total intracellular protein concentration. One fact that was highlighted in this data was that the effects of agitation had a more pronounced effect on volumetric total (membrane and cytoplasmic associated) intracellular protein concentration than $dO_2$ concentration. This observation was made by substantial increases in volumetric concentration as a result of varied agitation rates, in comparison to that of $dO_2$ concentration.

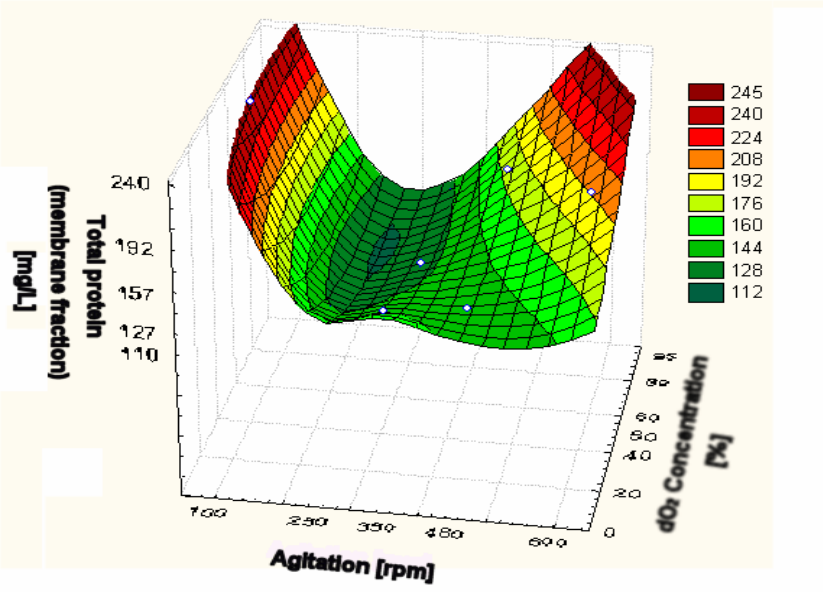


**FIGURE 4.17:** Volumetric total protein production (membrane fraction) [mg.$\ell^{-1}$] illustrating interdependent effects of agitation and $dO_2$ concentration (3D Contour Plot).

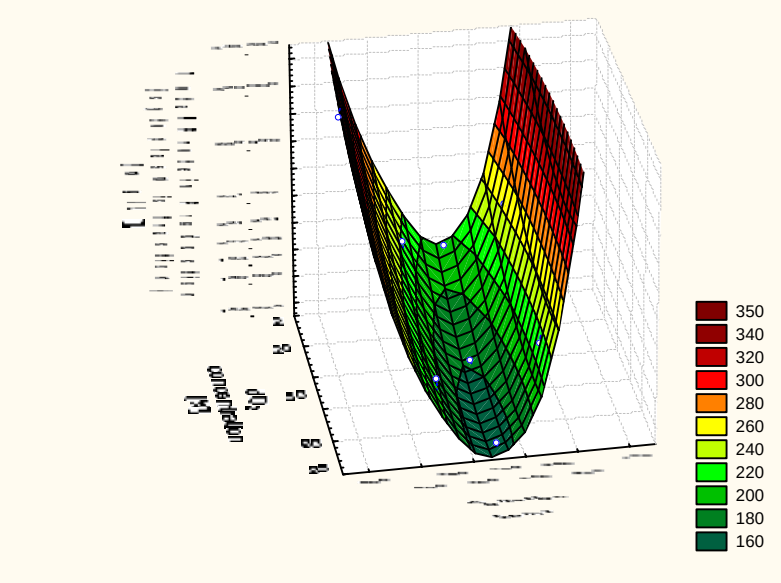


**FIGURE 4.18:** Volumetric total protein production (cytoplasmic fraction) [mg.$\ell^{-1}$] illustrating interdependent effects of agitation and $dO_2$ concentration (3D Contour Plot).

- **HBsAg production**

The maximum intracellular HBsAg production (µg.$\ell_{culture}^{-1}$) was primarily dominated by varying the agitation intensity (FIG. 4.16). With a decrease in agitation from 600 to 100 rpm, maximum HBsAg production levels increased substantially. Variation of the $dO_2$ concentration had no significant effect on intracellular HBsAg production under low agitation intensities. However, under high agitation intensities the intracellular HBsAg production increased with the increase in $dO_2$ concentration.

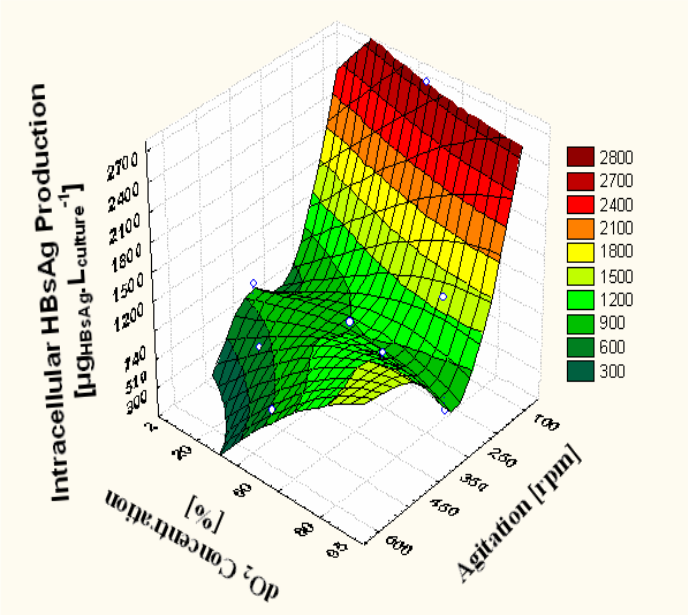


**FIGURE 4.19:** Volumetric HBsAg production [µg.$\ell^{-1}$] illustrating interdependent effects of agitation and $dO_2$ concentration (3D Contour Plot).

- **Percentage cellular protein that HBsAg represents**

The percentage of total cellular protein comprising of HBsAg (%) was primarily dominated by the $dO_2$ concentration (FIG. 4.17). With the increase in $dO_2$ concentration from 2 % to 25 %, the percentage HBsAg increased considerably. No significant effects were observed by further increasing the $dO_2$ concentration to 50 %. However, under extreme oxygen-rich conditions (80 and 95 % $dO_2$), the percentage of cellular protein comprised of HBsAg decreased substantially. No significant changes in the percentage HBsAg were observed at low and intermediate agitation intensities. However under high agitation intensities (450 and 600 rpm) the percentage HBsAg in cellular protein was considerably lower.

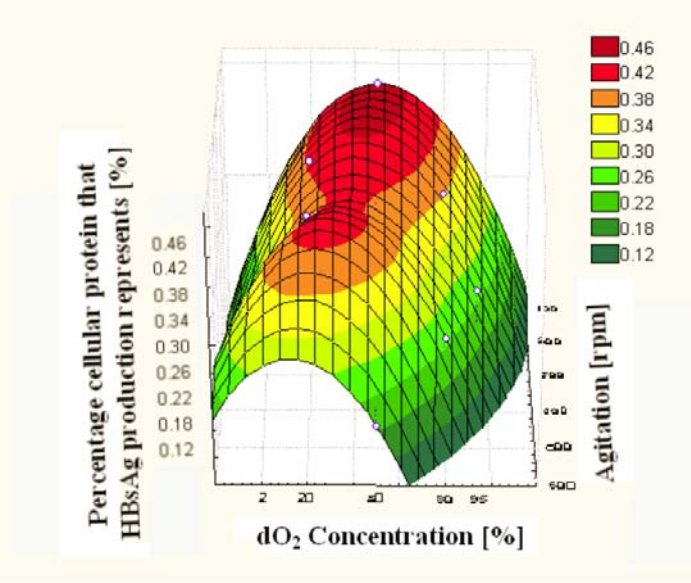


**FIGURE 4.20:** Percentage cellular protein that HBsAg represents [%] illustrating interdependent effects of agitation and $dO_2$ concentration (3D Contour Plot).

4.3.4.3 Interdependent effect of agitation rate and $dO_2$ concentration broth morphology

At low agitation rates, pellet formation was favoured and with the increase of agitation these pellets were observed to become smaller until filamentous formation facilitated growth at very high agitation rates of 600 rpm (data not shown). No significant influence with regards to pellet size was observed as a result of an increase in $dO_2$ concentration but a definite difference in pellet fluffiness (hyphae number and length) was evident. Similar trends were observed with regards to agitation rates, where with the increase in agitation and $dO_2$ concentration, the following variables were influenced;

1) hyphae length and number was reduced,
2) growth rate and product formation increased,
3) fragmentation increased, and
4) media staining increased.

### 4.3.5 Extracellular HBsAg production

Results show that cultures grown at an agitation rate of 100 rpm and a $dO_2$ concentration of 50%, demonstrated significantly high HBsAg production levels in both the intracellular membrane (3 $mg.\ell_{culture}^{-1}$ at 82 hours and 300 $\mu g.g_{DW}^{-1}$ at 90 hours) and cytoplasmic fraction (62 $\mu g.g_{DW}^{-1}$ at 103 hours and 584 $\mu g.\ell_{culture}^{-1}$ after 60 hours). These results are illustrated in FIG. 4.18. Optimum cellular HBsAg production, including the membrane as well as the cytoplasmic cellular fractions, was achieved at 82 hours and a yield of 3.6 $mg.\ell_{culture}^{-1}$ and 350 $\mu g.g_{DW}^{-1}$. In addition, after the optimum peak at 82 hours volumetric HBsAg production in the membrane fraction cytoplasmic fraction rapidly decreased as cell fragmentation increased (FIG. 4.18). Similar volumetric HBsAg concentration trends were observed in the cellular cytoplasm. Consequently, corresponding supernatant samples tested positive for HBsAg at 90 hours, and the volumetric concentration profile increased linearly for the remainder of the cultivation. As a result supernatant samples surpassed the volumetric HBsAg concentration levels of both the membrane and cytoplasmic samples. An optimum of 968.73 $\mu g.\ell_{culture}^{-1}$ HBsAg concentration was achieved at 160 hour in the extracellular media, while the volumetric HBsAg levels of the membrane and cytoplasmic fractions only managed 556.8 $\mu g.\ell_{culture}^{-1}$ and 156 $\mu g.\ell_{culture}^{-1}$ respectively, at the corresponding time period. Cumulatively, a HBsAg concentration total of 1682 $\mu g.\ell_{culture}^{-1}$ was obtained.

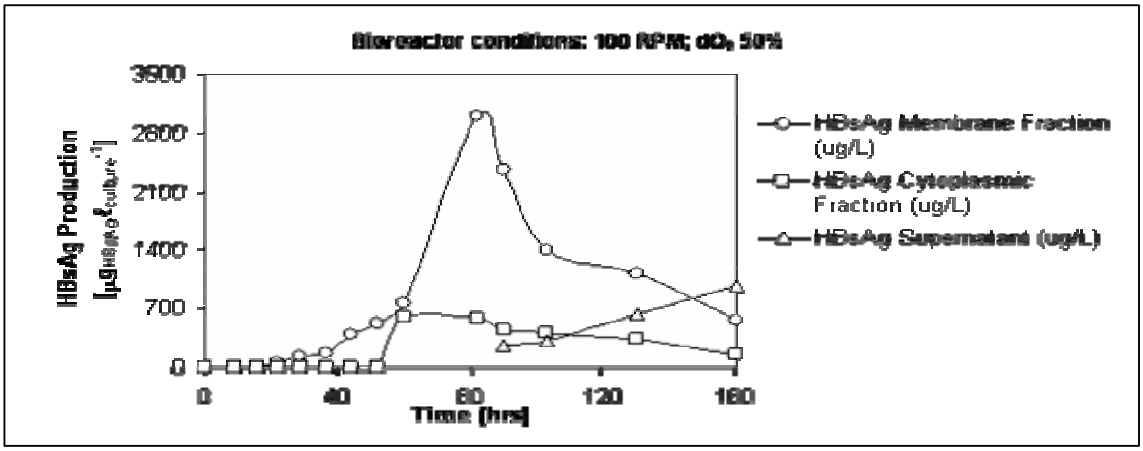


**FIGURE 4.21:** Volumetric HBsAg production in terms of intracellular membrane fraction, intracellular cytoplasmic fraction and extracellular supernatant [$\mu g.\ell^{-1}$].

### 4.3.6 Culture pH optimisation

#### 4.3.6.1 Effect of pH control on biomass yield

Culture pH had no significant impact on biomass production rate as all cultures had similar rates at approximately 0.043 $h^{-1}$. Culture pH affected biomass production levels at an agitation rate of 350 rpm and 50 % $dO_2$ concentration. Cultures grown at pH 6 had similar growth patterns to cultures grown without pH control: An optimum peak of 7.3 $g_{DW}.\ell_{culture}^{-1}$ at 45 hours and 7.74 $g_{DW}.\ell_{culture}^{-1}$ at 48 hours, respectively (FIG. 4.19A). Biomass production levels at cultures grown at pH 7 were significantly reduced. A maximum of 4.4 $g_{DW}.\ell_{culture}^{-1}$ at 83 hours was obtained. Thereafter, a gradual decrease in biomass levels was observed for the remainder of the cultivation (3.73 $g_{DW}.\ell_{culture}^{-1}$ at 140 hours).

#### 4.3.6.2 Effect of pH control on protein production yield

- **Total protein production**

Cellular protein production illustrated similar production profiles for cultures grown at pH 6 and cultures grown without pH control (FIG. 4.20A, B). A maximum biomass peak was obtained in both cultures, where at pH 6 the production stabilised to 103.5 $mg.g_{DW}^{-1}$ (650 $mg.\ell_{culture}^{-1}$) and cultures without pH control stabilised at 98 $mg.g_{DW}^{-1}$ (670 $mg.\ell_{culture}^{-1}$). In comparison, cultures grown at pH 7 obtained significantly lower cellular protein concentrations in the range of 10 $mg.g_{DW}^{-1}$ (40 $mg.\ell_{culture}^{-1}$).

- **HBsAg production**

The intracellular HBsAg production in cultures grown at pH 6 and without pH control followed similar profiles until the 72$^{nd}$ hour (FIG. 4.19C, D). Thereafter, pH 6 cultures obtained a maximum yield of 100 $\mu g.g_{DW}^{-1}$ (630 $\mu g.\ell_{culture}^{-1}$) at 79 hours, while cultures grown without pH control continued to increase in HBsAg production, eventually obtaining an optimum yield of

160 $\mu g.g_{DW}^{-1}$ (930 $\mu g.\ell_{culture}^{-1}$) at 145 hours. HBsAg production was significantly reduced in cultures grown at pH 7. A maximum HBsAg yield of 60 $\mu g.g_{DW}^{-1}$ (220 $\mu g.\ell_{culture}^{-1}$) at 47 hours was observed. In terms of pH controlled cultivations (pH 6 and pH 7), HBsAg production decreased during the latter cultivation periods, while cultivations without pH control (including shake flask and bioreactor experiments) were observed to increase. The analysis of intracellular cytoplasmic associated HBsAg production was not analysed for pH-controlled cultivations.

- **Percentage cellular protein that HBsAg represents**

In cultivations grown without pH control, the percentage cellular protein represented by HBsAg increased with time (FIG. 4.19B). An optimum ratio of 0.18% at 145 hours was obtained. Cultures grown at pH 6 obtained similar levels until the last 50 hours of cultivation where the % HBsAg was reduced substantially to 0.08 %. Conversely, cultures grown at pH 7 did not follow similar trends and % HBs|Ag levels were significantly higher. At its highest level a % HBsAg of 0.85 % was recorded.

#### 4.3.6.3 Effect of pH control on broth morphology

In cultures grown at pH 6, the broth was observed to consist of small pellets (data not shown). Pellet fluffiness was significantly reduced in these cultures. Broth and cell discoloration was emphasized greatly and after just 36 hours the broth changed to a dark brown/orange colour. Cell fragmentation was evident after 102 hours and seen to rapidly increase during the remainder of the cultivation.

In the pH 7 cultures, pellets were noted to be smaller in size compared to pH 6 cultures (data not shown). Pellet fluffiness was also significantly reduced in these cultures and cell fragmentation was evident after a culture age of 59 hours. This phenomenon increased rapidly during the remainder of the cultivation. Broth and cell discoloration was emphasized greatly and after just 30 hours, the broth was not only seen to change to a dark brown/orange colour, but a slimy mucilage layer around individual pellets was noted to develop (indicated by very slow filtration

of the mycelial broth). The development of this mucilage layer was suggested to arise from pH adjustment of the broth and seen to become increasingly pronounced with the addition of KOH.

In cultures grown without pH control (data not shown), pellet formation was observed. The fluffiness of the pellets was considerably more pronounced when compared to cultures grown with pH control. Pellet discoloration was observed in these cultures during the latter stages of the cultivation but with the absence of a slime layer around the outer layer of the pellet. Cell fragmentation was evident after a culture age of 40 hours. This phenomenon increased rapidly during the remainder of the cultivation.

#### 4.3.6.4 Effect of pH control on specific oxygen uptake rate (SOUR)

An apparent link between SOUR and pH control was observed (Appendix D). With the increase in culture pH control SOUR decreased. This was attributed the development of the slimy mucilage layer in pH 7 cultures. Cultures grown without pH control obtained the highest specific OUR of 4.5 $mmolO_2.g_{DW}^{-1}.h^{-1}$ at 20 hours. This was followed by pH 6 cultures (4.4 $mmolO_2.g_{DW}^{-1}.h^{-1}$) and finally cultures grown at pH 7 obtained reduced SOUR levels of 2.1 $mmolO_2.g_{DW}^{-1}.h^{-1}$ at 20 hours Appendix D.

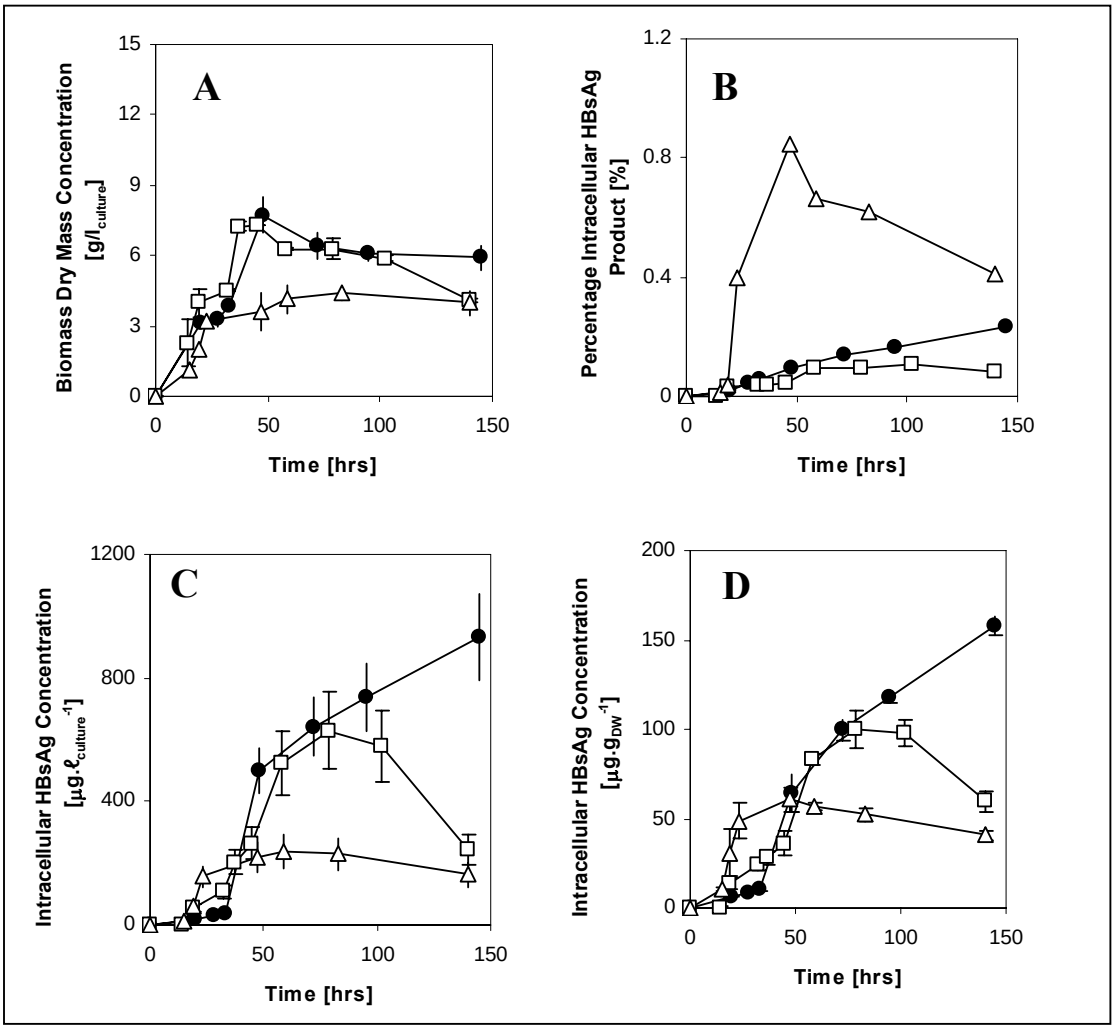


**FIGURE 4.22:** Effect of the bioprocessing parameter culture pH in a batch culture (bioreactor) at 350 rpm and 50 % $dO_2$ concentration on (A) Biomass dry mass concentration. Results are given in grams dry weight $liter_{culture}^{-1}$. (B) Percentage cellular protein that HBsAg represents. Results are given in % HBsAg. (C) Intracellular HBsAg production. Results are given in micro grams $liter_{culture}^{-1}$ and (D) micro grams per $gram_{dry\ weight}^{-1}$. Shown are data for (●) control, ((△) pH 6, (□) pH 7. Points without error bars have an associated error that is < 10 % of the value of the point.

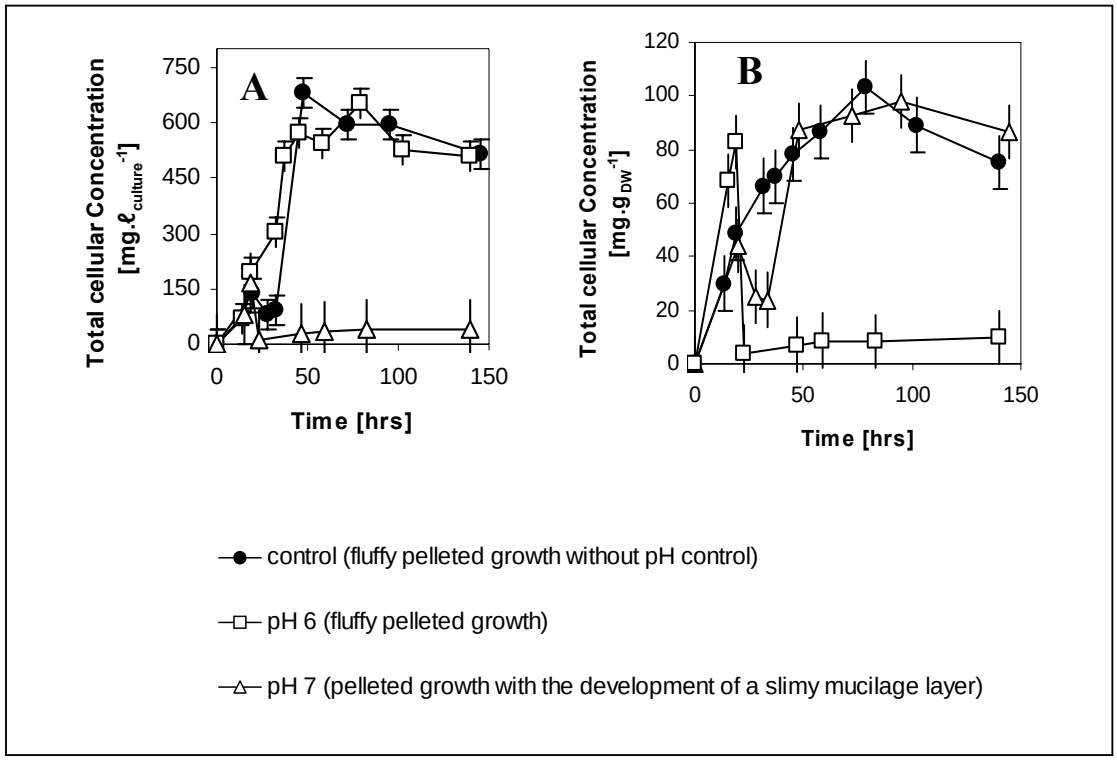


**FIGURE 4.25:** Effect of the bioprocessing parameter culture pH in a batch culture (bioreactor) at 350 rpm and 50 % $dO_2$ concentration on (A) Total cellular protein production. Results are given in milligrams $liter_{culture}^{-1}$ and (B) milligrams per $gram_{dry\ weight}^{-1}$. Shown are data for (●) control, ((△) pH 6, (□) pH 7. Points without error bars have an associated error that is < 10 % of the value of the point.

# 5 DISCUSSION

## 5.1 Introduction

HBsAg VLPs have been successfully produced in *A. niger* (Plüddemann and van Zyl, 2003). However, very little is known regarding the capacity of *A. niger* to produce and assemble complex immunogenic viral proteins into VLPs. The production of HBsAg as a model VLP by recombinant *A. niger* was therefore used to compare production levels with benchmarks set by *S. cerevisiae*, *P. pastoris* and *H. polymorpha* to determine the potential of the current *Aspergillus* system as a feasible VLP production system. Such comparison can only be performed under well-controlled conditions where five interrelated bioprocessing parameters, culture temperature, inoculum concentration, agitation intensity and dissolved oxygen ($dO_2$) concentration and culture pH, are of significant importance. The effects of these parameters on VLP production, biomass formation, the glucose consumption rate, the specific oxygen uptake rate (SOUR) and morphology of the microorganism during micro-pelleted growth in batch cultivation were therefore assessed. The dynamic relationship between the environmental conditions created by these parameters and VLP production was determined, to deduce relationships between the process variables and microbial behaviour.

## 5.2 Temperature effects

The culture temperature effects, in shake flask cultivations, indicated a significant impact on the specific growth rate of the microorganism. The increase in culture temperature resulted in the the increase in the specific growth rate. However, this had no effect on the maximum biomass and HBsAg production levels obtained. Beyond a culture temperature of 30$^{o}$C, biomass concentration, and HBsAg production levels were lowered. (FIG. 4.1A, B, C, D). These findings correlated with the study by Carlsen *et al.* (1995) on the microorganism, *A. oryzae*, who suggested that the optimum growth

temperature, in terms of achieving maximum biomass concentrations and protein production levels for filamentous fungi, lie in the range of 28 to 30$^{o}$C.

Total protein concentration (FIG. 4.2A, B) and HBsAg concentration (FIG. 4.1C, D) were growth associated, following similar trends to the biomass growth profile of the *A. niger* strain. Cellular protein production levels (FIG. 4.2A, B) decreased during the latter stages of the cultivation and were attributed to intracellular protein degradation (Archer and Peberdy, 1997). Conversely, HBsAg concentration (FIG. 4.1C, D) profiles progressed to maximum levels during the latter stages of the cultivation, bringing attention to the post-translational assembly, stability, robustness and recalcitrance of the HBsAg VLPs to proteolytic degradation (FIG. 4.1B).

Changes in the culture temperature affected culture pH during the latter stages of the cultivation (FIG. 4.2C). With the decrease in culture temperature culture pH also decreased, resulting in a decrease in the specific HBsAg concentration.

In relation to cell morphology, major morphological shifts between pelleted and filamentous growth were not enforced by the change in culture temperature, but, on a micro-scale, the distinct fluffiness of the pellets was observed to increase as the cultivation temperature increased (FIG. 4.3). This phenomenon suggested that hyphal growth increased with the increase in culture temperature and correlated with the study by Miles and Trinci (1983) on the microorganism, *Pe. chrysogenum*. These observations were also supported by the fact that dissolved oxygen tension is dependent on the culture temperature (saturated $dO_2$ concentration decreases with the increase in cultivation temperature). Furthermore, cell fragmentation was observed to become increasingly pronounced with the increase in culture age and culture temperature. Specific HBsAg production was influenced by cell fragmentation, increasing with culture age as well as culture temperature to 30$^{o}$C. Beyond a culture temperature of 30$^{o}$C, the rate of cell fragmentation continued to increase but the HBsAg concentration decreased. These results complied with the findings of Schügerl *et al.* (1998), who suggested that with the increase in culture temperature, the oxygen supply to the

microorganism became increasingly inadequate, causing an increase in environmental stress, reducing total cellular protein, and accelerating the rate of cell fragmentation.

Thus, changes in culture temperature produced simultaneous changes to culture variables (metabolic rate, growth rate, cell micro-morphology, culture pH, culture $dO_2$ concentration, and cell fragmentation). The interdependent effects these culture variables have on one another are not fully understood but in terms of intracellular HBsAg production levels, cell micro-morphology and cell fragmentation were observed to be the dominant culture variables affecting production. Therefore, in order to optimise the growth conditions and more importantly optimise HBsAg production in this study, results indicated that cultures grown at 30$^{o}$C achieved the highest biomass as well as volumetric HBsAg yields.

## 5.3 Inoculum concentration effects

In shake flask cultivations, inoculum concentration was observed to have a critical role in the development of cell morphology (FIG. 4.6). At low inoculum concentrations ($10^3$ spores.m$\ell^{-1}$), very large fluffy white pellets with a low broth viscosity were observed, and were noted to decrease in size as the inoculum concentration and broth viscosity increased. This phenomenon became increasingly pronounced as the inoculum concentration increased, until filamentous growth forms were observed at a very high broth viscosity in cultures grown at $10^8$ spores.m$\ell^{-1}$. These results correlated with many studies published in the literature (Hermersodofer *et al.*, 1989; Calam, 1976; Carmichael and Pickard, 1989), and was attributed to the competitive nature of the microorganism to survive. At low inoculum concentrations, the competition for space, oxygen, and nutrition was minimal, and thus the growth of large pellets was encouraged. As the number of spores in the culture increased, competition between the germinating spores increased. Space, oxygen, and nutrition became limiting and as a result, the growth of pellets were suppressed and the microorganism adapted to an alternative growth form (filamentous clumps).

Biomass concentration (FIG. 4.4A) and HBsAg production (FIG. 4.4C, D) were affected by changes in morphology. In cultures grown at $10^3$ spores.m$\ell^{-1}$, low biomass and HBsAg concentrations were obtained. These findings were attributed to the size and compactness of pellets affecting the efficacy of individual fungal cells. Oxygen as well as nutrient gradients were suggested to form with the increased size in pellet growth (Carlsen *et al.*, 1995). As pellet size increased, these gradients were predicted to increase and the inner cells became deprived of oxygen and nutritional resources. Cell growth and protein synthesis were therefore suppressed and cell starvation and consequently cell death (cell autolysis) was inevitable. As a result of cell autolysis, the development of a hollow inner layer was anticipated (Carlsen *et al.*, 1995), justifying the reduced biomass and HBsAg concentration observed in this study. Cultures grown at $10^5$ spores.m$\ell^{-1}$ and $10^6$ spores.m$\ell^{-1}$ were noted to have very similar morphological forms. A significant difference between the two cultures was that the pellets grown at $10^5$ spores.m$\ell^{-1}$ were fewer in number, fluffier and slightly larger than the pellets grown at $10^6$ spores.m$\ell^{-1}$. As a result, biomass concentration wsa slightly higher at $10^6$ spores.m$\ell^{-1}$, while specific HBsAg concentrations achieved higher production levels in cultures grown at $10^5$ spores.m$\ell^{-1}$. In filamentous cultures grown at $10^8$ spores.m$\ell^{-1}$, low biomass and HBsAg concentration were also obtained, and were attributed to the oxygen and nutritional limitations within the culture. It was apparent that these resource limitations hindered the full development and further growth of the microorganism (retarding biomass as well as specific HBsAg concentration). Cultures were noted to remain black during the entire cultivation period (data not shown), which suggested that the limitations of these resources were so severe that the germination of many of the *A. niger* spores were suppressed.

The results of this section showed that changes in inoculum concentration had a significant impact on cell morphology. In turn, an apparent relation between cell morphology and biomass as well as specific HBsAg concentration was evident. In terms of optimising the volumetric HBsAg production yield in this study, results indicated that cultures grown at $10^6$ spores.m$\ell^{-1}$, attaining to numerous, small, fluffy and loosely packed pellets, achieved the highest yields.

## 5.4 Agitation rate effects

Agitation intensity affected a number of variables in the VLP production system. The highest levels in the specific rate parameters; biomass specific growth rate, specific HBsAg production rate, specific glucose consumption rate and maximum SOUR, were obtained under high agitation intensities of 600 rpm (TABLE 4.1). However, maximum cellular protein, intracellular HBsAg production ($\mu g.\ell_{culture}^{-1}$) and the percentage of cellular protein represented by HBsAg (%) increased with a decrease in agitation intensity (FIG. 4.7B, C, D and FIG. 4.8 A, B). Optimum VLP production corresponding to a high biomass concentration and large fluffy pelleted growth was observed at a low agitation intensity of 100 rpm (FIG. 4.7A, B; FIG. 4.9).

These results were attributed to the morphological adaptations by the microorganism to different agitation intensities (FIG. 2). Suppressed VLP production at the higher agitation rates were attributed to the increase in shear intensity, apparently shearing-off of protruding hyphal structures, and causing an increased level of cellular stress (Nielsen *et al.,* 1995). Shear forces apparently reached a critical point above 100 rpm, and inflicted injury to the micro-pelleted fungal cells (van Suijdam and Metz, 1981; Braun and Vecht-Lipshitz, 1991) causing increased levels of cellular stress (environmental stress). This resulted in the decrease in the intracellular HBsAg production and percentage of cellular protein consisting of HBsAg (FIG. 4.7B, C, D). The microorganism apparently adapted its morphology to these environmental changes in order to reduce the level of cellular stress: Similarly to the results obtained by Nielsen *et al.* (1995), the size and roughness of the pellets became less pronounced as the rate of agitation increased (100 to 450 rpm). Higher agitation intensities (600 rpm) demonstrated a morphological shift from pelleted to filamentous growth (FIG. 2). These findings correlated with the studies of Smith *et al.* (1990), Metz *et al.* (1981), and Papagianni *et al.* (1999), who suggested that high agitation intensities would enforce a higher shear on the microorganism that would increase cellular shearing, resulting in shorter, thicker and highly branched filamentous hyphae.

In terms of justifying these results, it should be noted that culture pH was affected by the variation in agitation activity (FIG. 4.8C). With the increase in agitation intensity, culture pH decreased. The study of O' Donnell *et al.* (2001), suggested that protease activity was dependent on the culture pH; as culture pH decreased, intracellularly as well as extracellularly protease activity increased. In the current study, one could speculate that the increase in agitation increased cellular stress and cell damage causing cell autolysis. This resulted in a decrease in culture pH and therefore actuated an increase in the protease activity. Studies by Archer and Peberdy (1997) and Van den Hombergh *et al.* (1997c) have suggested that proteolytic activity is a major factor responsible for protein degradation and retardation, especially concerning heterologous proteins. Therefore, in the current study, the estimated increase in protease activity with the increase in agitation, could have caused the suppressed specific HBsAg concentration during the latter periods of the cultivation. Under these high agitation intensities (600 rpm) and increased levels of environmental (cellular) stress, morphological adaptation to filamentous growth significantly benefited biomass production (FIG. 4.7A) and the specific rate parameters (TABLE 4.1), although HBsAg production was significantly suppressed. The highest HBsAg and biomass production occurred under low levels of cellular stress at a low agitation rate of 100 rpm, where a distinct morphology was favoured; large, loosely packed, fluffy pellets (FIG. 4.9).

## 5.5 $dO_2$ concentration effects

$DO_2$ concentration significantly affected the mycelial morphology and thereby VLP (HBsAg) production. Under oxygen-limited conditions (2 % $dO_2$), the microorganism adopted a filamentous morphology that apparently increased the surface area per volume exposed to the environment in an effort to increase the efficiency of oxygen uptake. These oxygen-limited conditions demonstrated the highest maximum biomass concentration (FIG. 4.10A). However, intracellular HBsAg production and the percentage of cellular protein represented by HBsAg as well as the SOUR was considerably reduced, which suggests that limited $O_2$ availability within the submerged

culture may become a limiting factor negatively affecting VLP production (FIG. 4.10B, C, D; TABLE 4.2). In addition, other specific rate parameters; biomass specific growth rate, specific HBsAg production rate and specific glucose consumption rate, demonstrated the lowest rates under these conditions (TABLE 4.2).

Intermediate oxygenation (25% $dO_2$) resulted in a morphology shift, which was characterised by the adhesion of mycelia and formation of large fluffy pellets (FIG. 4.12). These conditions resulted in the highest HBsAg yields (FIG. 4.10B, C, D; TABLE 4.2), which coincided with a high biomass concentration and SOUR (FIG. 4.10A; TABLE 4.2). An increase in the $dO_2$ concentration (50-95 %) resulted in the formation of smaller and smoother pellets (FIG. 4.12). This morphological adaptation was considered as a 'protective strategy' against the increased levels of intracellular reactive oxygen species (ROS) availability causing increased levels of oxidative stress within the microorganism (Bai *et al.,* 2003). Under high culture $dO_2$ concentrations, the studies of Bai *et al.* (2003) proposed that fungal cells generally adopt morphological forms (adhesion of mycelia, (Wongwicharn *et al.*, 1999); formation of smaller and smoother pellets (Higashiyama *et al.*, 1999; FIG. 4.12); and increased pellet density, (Cui *et al.*, 1998)) which have the net effect of protecting the inner cells of the pellet from the potential ROS-mediated damage, and thus reducing levels of intracellular oxidative stress.

Despite the efforts of reducing the intracellular oxidative stress of the inner cells of the pellet caused by elevated $dO_2$ concentrations in the culturing environment, lower biomass concentration, depressed HBsAg production levels and reduced maximum SOURs were observed at high $dO_2$ levels (FIG. 4.10A, B, C, D; TABLE 4.2). However, this had not have a detrimental effect on the microorganisms specific growth rate and glucose consumption rate, which was at its highest levels under oxygen rich conditions (95 % $dO_2$) (TABLE 4.2).

## 5.6 Interdependent effects between agitation and $dO_2$ concentration

The sufficient transfer of $O_2$ to active cells is critical for aerobic cultivation. The regulation of agitation intensity and $dO_2$ concentration within the bioreactor are obvious responses to overcome any $O_2$ limitations. However, the interdependent effects of these two process parameters have drastically affected biomass and VLP production by *A. niger* in the present study, due to morphological shifts caused by these changes in the environmental conditons. Cell morphology has therefore become a critical bioprocessing parameter for filamentous fungi (Znidarsic and Pavko, *2001*). In the present study, the CCRD (FIG. 3.4) contour plots indicated that agitation had a more dominant impact on biomass, total cellular protein and intracellular HBsAg production, compared to $dO_2$ concentration (FIG. 4.13, 4.14, 4.15, 4.16, and 4.17). On the other hand, the $dO_2$ concentration had a greater impact on the percentage of cellular protein comprised of HBsAg (FIG. 4.14 and 4.15).

Morphological shifts were influenced by both process parameters. Filamentous growth was induced at high agitation intensity (600 rpm) and under oxygen-limited (2 %) conditions. Under these growth conditions VLP (HBsAg) production was suppressed and was attributed to the increased levels of environmental (cellular) stress enforced on the microorganism. Low agitation rates and intermediate oxygenated cultivations were optimal cultivation parameters resulting in the least environmental stress and stimulating the growth and formation of large, loosely packed fluffy pellets and enhancing biomass production as well as optimum HBsAg concentration (FIG. 4.16 and 4.17).

## 5.7 pH effects

The effect of pH had an impact on pellet morphology, biomass concentration (FIG. 4.19A) as well as HBsAg production (FIG. 4.19B, C, D). Similar fluffy pellets were observed in cultures grown without pH control and at pH 6. At an elevated culture pH of 7, reduced pellet size and fluffiness was observed. Biomass concentration obtained maximum yields in a culture pH of 6. Similar biomass yields were achieved in cultures grown without pH control, while a significant reduction in biomass concentration was observed in pH 7 cultures. These findings were attributed to a thin slimy mucilage layer that was observed to develop around the pellet surface (as indicated by very slow filtration of the mycelial broth), as a result of the pH adjustment (KOH addition). The formation of the slimy mucilage layer, whose presence in *A. niger* has already been reported (Kisser *et al.*, 1980 and Mischak *et al.*, 1985), became increasingly pronounced with culture age and appeared to lower SOUR of the microorganism. The slimy mucilage layer was considered to reduce the nutrient transfer capabilities and extracellular secretion efficiency of the microorganism and therefore retarded cell growth.

Similar total intracellular protein concentrations were obtained in cultures grown without pH control and pH 6, in the absence of the mucilage layer. The specific total intracellular protein concentrations increased significantly in pH 7 cultures and were apparently caused by the trapping of secreted proteins in the mucilage layer (Mischak *et al.*, 1985).

HBsAg concentration became increasingly suppressed as the culture pH increased to pH 7. These results suggested that the apparent development of the mucilage layer inhibited the secretion efficiency of the extracellular proteins, causing an accumulation of proteins intracellularly as well as within the mucilage layer. Consequently, the accumulation of intracellular proteins may have either blocked or suppressed the secretion pathway of the HBsAg. The study by Punt *et al.* (2002) suggested

contradictory evidence to the above mentioned results. By using a similar *A. niger* host strain, it was shown that the biomass concentration decreased and the total protein as well as the heterologous protein concentration increased as the culture pH was increased from pH 6 to pH 7.5. These findings were attributed to a less active proteolytic system at increased pH levels. In the current study, even without pH control, the protease-deficient *A. niger* strain contributed to a stable culture pH (culture pH typically remained above 6 for most of the cultivations) that suggested insignificant levels of proteolytic activity in the supernatant.

Therefore, pH control affected the development of a slimy mucilage layer that not only enforced a change in pellet morphology but also reduced biomass levels as well as inhibited the production of the HBsAg. In order to optimise the HBsAg production system in this study, results indicated that the protease-deficient *A. niger* VLP producing strain was negatively affected by pH control of the media. The impact of culture pH control on HBsAg production was significantly greater compared to the potential threat of protease activity. Thus, cultivation without pH control is recommended.

## 5.8 Parameter effects on HBsAg assembly and extracellular release

In the *A. niger* host strain, VLP production of the S-protein containing pseudoviral particles was achieved intracellularly and was seen to be associated with the membrane organelles of the cell (Plüddemann and van Zyl, 2003). These findings were confirmed in the current study. Specific intracellular HBsAg concentration accumulated slowly over time and maximum concentrations were observed during the latter stages of the cultivation. This brought attention to the HBsAg assembly, stability, robustness and recalcitrance of the HBsAg VLPs to proteolytic degradation..

The HBsAg synthesis and assembly has been extensively discussed in the literature. The HBsAg is synthesized co-translationally and incorporated into the membrane of the ER.

Rapid dimmer formation by intermolecular disulfide bonding follows and the particles bud into the ER, provoking dilation and proliferation of the ER network (Patzer *et al.*, 1986). These particles accumulate in a dilated ER network (dilated ribosomes-studded vesicles), which has been observed in human hepatocytes (Tanaka *et al.*, 1977), mammalian (Patzer *et al.*, 1986), yeasts (Biemans *et al.*, 1992; Kitano *et al.*, 1987) and plant cell expression systems (Smith *et al.*, 2002). A similar HBsAg synthesis is considered for the *A. niger* expression system. Insignificant levels of HBsAg were observed in the cytoplasmic fraction of the cell, suggesting that the HBsAg particles did not progress further than the ER/Golgi apparatus.

Much has been said concerning protease accumulation during the latter periods of cultivation. This accumulation of proteases has caused the degradation of heterologous as well as native protein in many biological systems, and was therefore an issue of concern (Broekhuijsen *et al.*, 1993; van Hartingsveld *et al.*, 1990; Roberts *et al.*, 1992). The first signs of possible protein degradation in the current study were evident, when typically, specific total protein concentration levels were noted to peak, followed by a sudden decrease in concentration. This was not considered a result of protease accumulation and was substantiated by the high levels and stability of the culture pH during the latter stages of the cultivation. Degradation of cellular protein was apparent due to severe nutrient limitations within the cultivation broth, causing the cell to turn to alternative resources; incorrectly folded proteins within the cell (Schröder and Kaufman, 2004). In terms of stability and robustness of the HBsAg particles, the particle's association with the membranous ER/Golgi network was considered to be protected from intracellular degradation (Schröder and Kaufman, 2004).

The extracellular production of heterologous protein is frequently desirable as this simplifies purification procedures. In the current study, during the latter stages of the cultivation, at very low shear conditions (100 rpm) and intermediate $dO_2$ concentration levels (50%), significant yields of cytoplasmic associated HBsAg concentration were observed (FIG. 4.18). Furthermore, when corresponding supernatant samples were assayed, significant levels of HBsAg concentration were also noted. The intracellular

cytoplasmic and supernatant HBsAg concentration seemed to correlate with the ever-increasing effect of cell fragmentation, as the culture age increased. The mechanisms of cell fragmentation have been well documented by Papagianni (2004), and results suggest that during the latter stages of a cultivation cells fragment by the method of cell autolysis. The main cause of cell lysis is the material imbalance in hyphae caused either by internal or external factors. Of the internal factors, it could be a disturbance of the organelles or an accumulation of toxic metabolites. The external factors could be physical or chemical factors, the lack of nutrients, as well as enzyme influences, which disturb the cell wall structures (Fencl, 1978; Nombela *et al.*, 1993). Cell autolysis will also occur as a result of the weakening of structural elements caused by the natural aging process of vacuolation (Papagianni, 2004). In terms of the current study, cell autolysis appeared to result from a combination of events; cell age and the weakening of structural elements, accumulation of toxic metabolites as well as nutrient limitations, especially taking into account that glucose levels were exhausted within the $50^{th}$ hour of the cultivation (Appendix D). The combination of these factors would increase stress levels and disturb the organelles within the cell. The rate of cell autolysis would be enhanced, disintegrating the cell membrane and releasing all the membrane associated HBsAgs into the cytoplasmic fraction of the cell as well as into the broth supernatant. These results correlate with the study by Shen *et al.* (1989) on *H. Polymorpha*, where the partial digestion of the cell wall yielded HBsAg particle secretion.

Thus, results indicated that the optimum harvesting period of the HBsAg under the optimum growth conditions was during the latter stages of the cultivation (at 96 hours). The high yields of specific HBsAg concentration during the latter stages of the cultivation emphasised the stability and robustness of the heterologous protein. Under the optimum growth conditions ($30^{o}$C, $10^{6}$ spores.m$\ell^{-1}$, 100 rpm, without $dO_2$ control and no culture pH control), significant yields of specific HBsAg concentration were observed in the cell cytoplasm as well as in the broth supernatant. This concentration of HBsAg was noted to increase with cell age due to the apparent action of cell fragmentation and autolysis. Therefore, the stability and robustness as well as the extracellular release of the HBsAg makes the current production system commercially

attractive by simplifying purification procedures and therefore significantly reducing purification costs.

## 5.9 Benchmark comparison with alternative recombinant systems

In the present study, a 9-fold increase in VLP production in a batch culture was achieved in comparison to the yields obtained by Plüddemann and van Zyl, (2003). HBsAg production reached 3.6 mg.$\ell_{culture}^{-1}$ (350 µg.$g_{DW}^{-1}$) at a biomass concentration of 10.5 $g_{DW}.\ell_{culture}^{-1}$ in comparison to 0.4 mg.$\ell_{culture}^{-1}$ (200 µg.$g_{DW}^{-1}$) at a biomass concentration of 2 $g_{DW}.\ell_{culture}^{-1}$ in shakeflask cultivation (Plüddemann and van Zyl, 2003). These results emphasize that HBsAg VLP production is predominantly growth-associated.

The *A. niger* production system achieved production levels comparable to the three commercially competing recombinant microorganisms. In batch cultivations, *S. cerevisiae* has achieved HBsAg yields of 2 mg.$\ell_{culture}^{-1}$ (280 µg.$g_{DW}^{-1}$) (Gu *et al.,* 1991). This production system has been significantly improved by using a fed-batch process, resulting in HBsAg levels of 10 - 20 mg.$\ell_{culture}^{-1}$ (670 µg.$g_{DW}^{-1}$) (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 mg.$\ell_{culture}^{-1}$ (46 µg.$g_{DW}^{-1}$). The implementation of fed-batch cultivation increased HBsAg production levels substantially to 8-9 mg.$\ell_{culture}^{-1}$ (225 µg.$g_{DW}^{-1}$) (de Roubin *et al.*, 1991). In shakeflask cultivation recombinant *P. pastoris* obtained low HBsAg production levels of 0.14 mg.$\ell_{culture}^{-1}$ (270 µg.$g_{DW}^{-1}$), however large-scale fed-batch cultivation resulted in a significant increase in HBsAg production of 0.38 g.$\ell_{culture}^{-1}$ (6.4 mg.$g_{DW}^{-1}$) (Cregg *et al.,* 1987).

In all of the previously reported studies, VLP production was quantified by means of polyclonal immunoassays, as opposed to the method of monoclonal immunoassays exclusively used in the current study. Comparing the HBsAg production levels of the present study to polyclonal-determined levels may not give a fair indication of the

potential of the Aspergillus VLP production system. Although polyclonal immunoassays have long been used as a means in detecting antigens (Harlow and Lane, 1988), this method has one major disadvantage: The antibody response to a given antigen tends to be broad, covering both specific and cross-reactive epitopes. As a result immunoassays based solely on polyclonal antibodies may not be very specific to correctly folded and assembled VLPs (Andreotti *et al.,* 2003), and result in the over-estimation of VLP production levels. Monoclonal immunoassays have improved the specificity of the assay by simplifying the selection of antibodies directed against chosen molecular targets, and has thus been deemed as a more reliable and accurate means to quantify the production of correctly folded and assembled VLPs (Andreotti *et al.,* 2003).

# 6 CONCLUSIONS

Bioprocessing strategies proved to be a successful tool for the optimisation of the HBsAg VLPs production in a batch culture by the *Aspergillus* expression system. In terms of highlighting the significant findings of this study, the following points are concluded:

1) The bioprocessing parameters (temperature, inoculum concentration, agitation, $dO_2$ concentration, and pH) had a significant impact on the environmental stress levels within the bioreactor. The morphological type and physiology of *A. niger* were strongly dependent on the environmental conditions created by these parameters and significantly affected biomass formation and VLP production. In an attempt to relieve stress, the recombinant *A. niger* was able to adapt into various morphological forms that not only affected biomass concentration but specific HBsAg production levels as well.
   a) HBsAg production in the *A. niger* expression system was extremely sensitive to shear intensity, elevated $O_2$ levels and pH control within the bioreactor.
      i) High shear intensities and low $O_2$ levels within the cultivation induced filamentous growth. Suppressed HBsAg production levels were observed under these conditions, indicating that filamentous growth is not favourable in terms of maximising HBsAg production.
      ii) At high $dO_2$ concentrations an apparent toxic build up of ROS affected mycelial morphology and the metabolic OUR and specific HBsAg concentration decreased. This phenomenon indicated a shift in the respiratory pathway and was attributed to a protective strategy adopted by the microorganism in an attempt to alleviate excess (toxic) intracellular ROS. Maximum HBsAg yields were obtained under intermediate oxygen levels and were attributed to the relative energy-efficient conventional pathway.

iii) A slimy mucilage layer developed, with the controlled adjustment of culture pH. The development of this layer negatively affected the transport properties in and out of the cell and therefore reduced pellet size and volumetric HBsAg production. Maximum HBsAg yields were obtained in cultivations without pH control.

2) Thus, the control of bioprocessing parameters, amounting to the least amount of environmental stress resulted in optimum fungal morphology (large, loosely packed fluffy pellets) and optimum volumetric HBsAg production yields:
   a) Optimum control of bioprocessing parameters;
      i) culture temperature of $30^{o}C$,
      ii) inoculum concentration of $10^{6}$ spores.m$\ell^{-1}$,
      iii) low agitation rate of 100 rpm,
      iv) intermediate oxygen enrichment (20%-30%) without $dO_2$ control, and
      v) no culture pH control.

3) HBsAg production was primarily associated with the membrane protein fraction and correlated with the study of Plüddemann and van Zyl (2003).
   a) HBsAg production was most pronounced during the latter stages of the cultivation and was attributed to the stability and robustness of the HBsAg particles. Results indicate that the optimum harvesting period of the HBsAg particles, under optimum production conditions, is between 96 and 100 hours.
   b) During optimum production conditions, significant yields of specific HBsAg concentration were observed in the cell cytoplasm as well as in the broth supernatant. This concentration of HBsAg was noted to increase with cell age due to the apparent action of cell fragmentation and autolysis. Cell fragmentation was suggested to disrupt intracellular membrane organelles, releasing HBsAg particles into the cytoplasm, as well as disintegrating the cell wall and releasing the cytoplasmic-associated HBsAg particles into the culture broth.

c) Therefore, the stability and robustness as well as the extracellular release of the HBsAg makes the current *A. niger* production system commercially attractive, by simplifying purification procedures and therefore significantly reducing purification costs.

4) The optimum HBsAg production levels and benchmark comparisons:
   a) Initial volumetric HBsAg production yields of 0.4 $mg.\ell_{culture}^{-1}$ (Plüddemann and van Zyl, 2003), were exceeded 9 fold. Optimum HBsAg production levels were in the range of 3.6 $mg.\ell_{culture}^{-1}$ and 350 $\mu g.g_{DW}^{-1}$, with a corresponding biomass concentration of 10.5 $g_{DW}.\ell_{culture}^{-1}$ .
   b) In terms of a batch mode optimisation, the current *A. niger* production process demonstrated competitive HBsAg production levels compared to two commercially competing recombinant microorganisms, *S. cerevisiae* and *P. pastoris.*
   c) With the implementation of an alternative fed-batch process, a realistic estimate of HBsAg production places the current *Aspergillus* expression system above the recombinant *S. cerevisiae*, *P. pastoris,* and *H. polymorpha* processes.

5) The competitive VLP production levels, stability and robustness of the *A. niger* expression system, along with the extracellular release of HBsAg, makes the system attractive for further development, including the use of a fed-batch cultivation strategy for growth-associated HBsAg VLP production.

# 7 FUTURE PROSPECTS

The results obtained from the current research project have proven that a cheaper generic Hepatitis B vaccine is indeed possible through the cultivation of *A. niger*. This current production system has by no means reached its full potential, and the already firmly established base demands for continued research and development.

Further bioprocessing strategies regarding the development of the production process will be of enormous importance for the further exploitation of the current *Aspergillus* production system. Various types of cultivation and modes of operation warrant investigation. Although most current processes rely on submerged fermentation, the use of traditional solid-state fermentation processes should be considered. Several results have indicated that the solid-state fermentation process result in improved levels of various secreted fungal hydrolases (Pandey *et al.,* 2000). Moreover, the processes involved are extremely competitive, particularly when considering process sustainability. Until now, most of the research in the field of solid-state fermentation has been focused on process and fermenter design (Weber *et al.,* 1999) treating the microorganism involved as a black box. Already, the first results of molecular research, in this type of cultivation, show distinct differences in the level of gene expression and regulation compared with submerged fermentation (Ishida *et al.,* 1998). In terms of modes of operation, fed batch process strategies are expected to have the greatest impact on biomass yields and specifice HBsAg production. Statistical evidence suggests that biomass levels can potentially increase 10 to 20 fold in fed batch operations compared to batch cultivations (Cregg *et al.*, 1987; Gu *et al.*, 1991; Hsieh *et al.*, 1988). The implications of such a biomass increase implies that the volumetric HBsAg production system will also increase and a realistic estimate places the current *Aspergillus* system in a league of its own, superseding any competitive processes.

The present study confirms that the morphological type and the related physiology are the key variables, necessary to explain and predict the bioreactor performance and to

design a bioprocess. They strongly depend on the environmental conditions and affect the rheological behaviour of the broth, which in turn affect transport phenomena in a bioreactor. Image analysis is now a well-established method for the characterization of complex mycelial morphology, while in physiological studies it has provided better understanding and determination of microbial activities. Therefore, it is an important instrument for the research and design of the current filamentous production process.

Mathematical modelling has become a powerful engineering tool in a complex system such as growth of filamentous microorganisms. Besides simple unstructured models, also numerous morphologically structured models have been proposed, considering different morphological forms of mycelial culture as a consequence of metamorphosis reactions. However, they may contain a large number of parameters, which makes these models too complex and not usable for process automation. Therefore, the main goal of mathematical modelling, if it can be applied to the current filamentous system, should be to develop a reliable mathematical model of the bioprocess with minimal complexity and necessary data to control the mycelial cultivations.

Clearly, the past decade has provided an abundance of new data regarding the use of filamentous fungi as cell factories for heterologous protein production. Strain optimization is a critical step in enhancing the production levels of the current production process. The basis for strain improvement strategies lies in the development of molecular tools, which have initiated a detailed analysis of the fungal secretion pathway (Conesa *et al.,* 2001). Current research in several research groups (including the EUROFUNG consortium; www.eurofung.net) are focused on unraveling the key features of this pathway. With this information at our finger tips, the words of Peberdy, refering to protein secretion in filamentous fungi as “a highly productive black box” (Peberdy, 1994), will be something of the past and strain optimization will become a realistic approach in process design.

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. Obviously, these technologies rely heavily on sophisticated laboratory techniques, development of data analysis and pattern-recognition tools (bioinformatics). Currently, the focus of the fungal research community is still primarily on the acquisition of DNA sequence data coupled with functional analysis of identified genes (Yoder and Turgeon, 2001; Sweigard and Ebbole, 2001; Hamer *et al.,* 2001). Also the first reports on fungal proteomic approaches have appeared (Lim *et al.,* 2001). In the near future, requirements for '-omics' research in filamentous fungi will undoubtedly be met, allowing the fungal research community to address the various biological questions that it still faces.

Once strain, nutrient, and bioprocessing conditions have been optimised, the next step is to design a large-scale commercial production process. This involves a comprehensive scale-up analysis that is not easy and involves several compromises. In general, it is not possible to provide the same environment in large-scale and small-scale vessels because impeller tip speed, superficial air velocity and the corresponding flooding tendency, the quality of mixing and heat transfer surface to volume ratio change with size. Therefore, scale-up is still an art and not exact science, especially for the mycelial submerged cultures.

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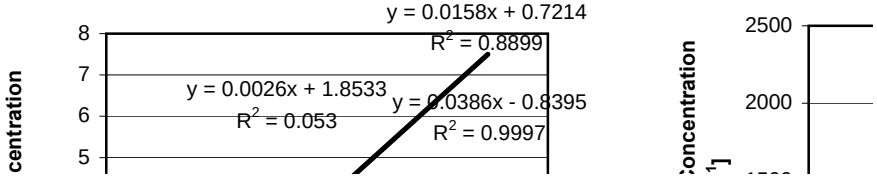
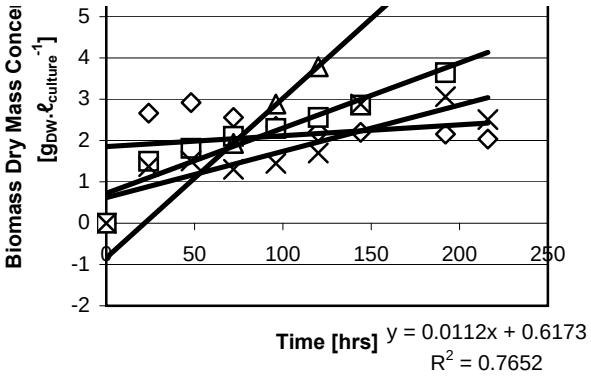
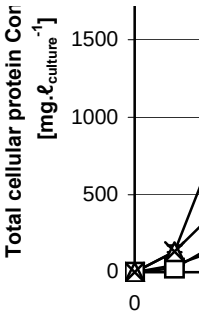
# 9 APPENDICES

Appendix A, B, C and D are in excel format and are wriiten on CD, placed at the back of the manuscript. Appendix A contains all the processed data concening shake flask experiments of temperature optimisation. Appendix B contains all the processed data concening shake flask experiments of inoculum concentration and Appendix C contains all bioreactor related processed data. This includes agitation, $dO_2$ concentration and pH optimisation. Appendix D contains all the processed data realating to the specific OUR of the microorganism.

| Temperature | Time | Biomass Dry Weight (DW) | | Bradford Production | | HBsAg P |
|---|---|---|---|---|---|---|
| [°C] | [Hrs] | Average [gDW/l] | Deviation [gDW/l] | Average [mg/L] | Deviation [mg/L] | Average [ug/L] |
| (small smooth p | 0 | #NUM! | 0.000 | 0.000 | 0.000 | 0.000 |
| 16 | 24 | 2.664 | 0.545 | 45.403 | 15.055 | 23.599 |
| 16 | 48 | 2.919 | 0.883 | 135.655 | 35.078 | 73.720 |
| 16 | 72 | 2.559 | 0.299 | 230.493 | 22.280 | 92.991 |
| 16 | 96 | 2.341 | 1.976 | 447.053 | 120.716 | 106.934 |
| 16 | 120 | 2.207 | 0.160 | 809.688 | 46.860 | 124.385 |
| 16 | 144 | 2.200 | 1.472 | 466.221 | 91.316 | 143.142 |
| 16 | 192 | 2.155 | 1.215 | 602.911 | 2.437 | 161.061 |
| 16 | 216 | 2.036 | 3.202 | 710.179 | 329.791 | 140.116 |
| argest & fluffiest | 0 | #NUM! | 0.000 | 0.000 | 0.000 | 0.000 |
| 23 | 24 | 1.499 | 0.481 | 26.276 | 8.839 | 6.344 |
| 23 | 48 | 1.812 | 0.762 | 172.438 | 21.651 | 47.381 |
| 23 | 72 | 2.103 | 0.624 | 308.717 | 100.121 | 103.348 |
| 23 | 96 | 2.293 | 0.553 | 371.219 | 29.465 | 139.256 |
| 23 | 120 | 2.560 | 1.300 | 572.111 | 100.531 | 189.535 |
| 23 | 144 | 2.856 | 1.061 | 692.126 | 180.274 | 249.738 |
| 23 | 192 | 3.643 | 0.624 | 923.910 | 80.059 | 587.783 |
| 23 | 216 | | 0.624 | 754.253 | 147.758 | 615.256 |
| (large & fluffy p | 0 | | 0.000 | 0.000 | 0.000 | 0.000 |
| 30 | 24 | | 1.348 | 132.229 | 3.307 | 10.176 |
| 30 | 48 | | 1.750 | 786.879 | 53.192 | 51.844 |
| 30 | 72 | 1.932 | 1.788 | 1470.424 | 38.551 | 98.287 |
| 30 | 96 | 2.885 | 0.240 | 1873.171 | 49.932 | 316.975 |
| 30 | 120 | 3.785 | 2.107 | 1980.963 | 37.419 | 902.029 |
| 30 | 144 | | 2.446 | 1423.514 | 189.622 | 965.955 |
| 30 | 192 | | 2.666 | 244.846 | 19.604 | 715.322 |
| 30 | 216 | | 2.423 | 159.369 | 10.186 | 663.558 |
| C (small fluffy pe | 0 | #NUM! | 0.000 | 0.000 | 0.000 | 0.000 |
| 37 | 24 | 1.369 | 0.484 | 133.161 | 38.950 | 19.383 |
| 37 | 48 | 1.505 | 0.811 | 385.035 | 107.593 | 34.563 |
| 37 | 72 | 1.304 | 1.962 | 603.372 | 31.863 | 31.726 |
| 37 | 96 | 1.448 | 0.470 | 771.040 | 101.558 | 35.636 |
| 37 | 120 | 1.698 | 0.534 | 735.878 | 71.912 | 39.284 |
| 37 | 144 | 2.885 | 1.682 | 630.348 | 18.229 | 128.644 |
| 37 | 192 | 3.063 | 0.999 | 139.515 | 6.759 | 133.919 |
| 37 | 216 | 2.507 | 0.997 | 156.666 | 16.288 | 75.789 |





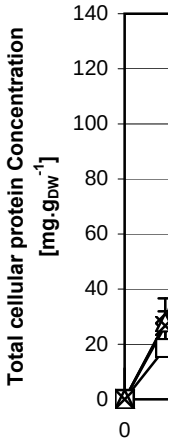



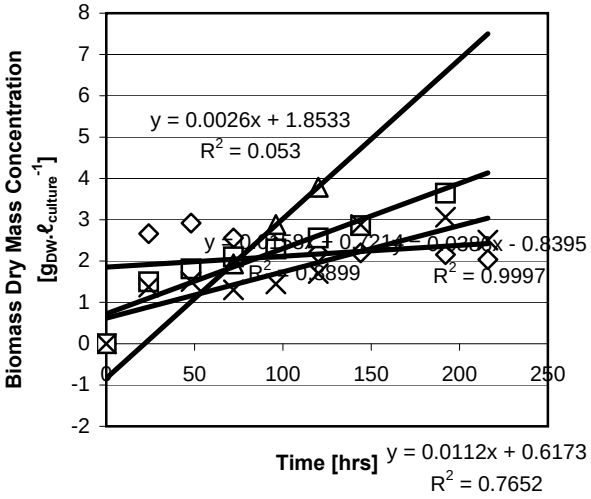

16 oC (small smooth pellets)

23 oC (largest & fluffiest pellets)

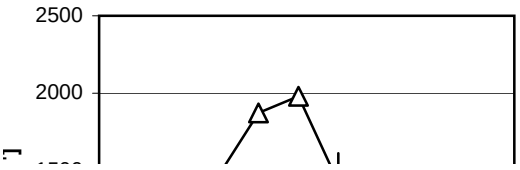
30 oC (large & fluffy pellets)

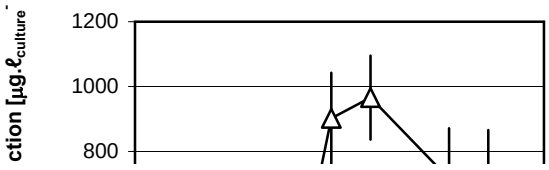
37 oC (small fluffy pellets)

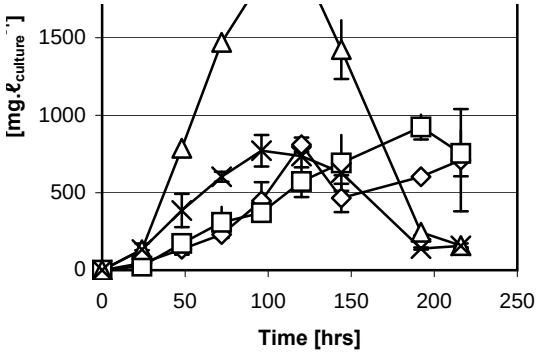


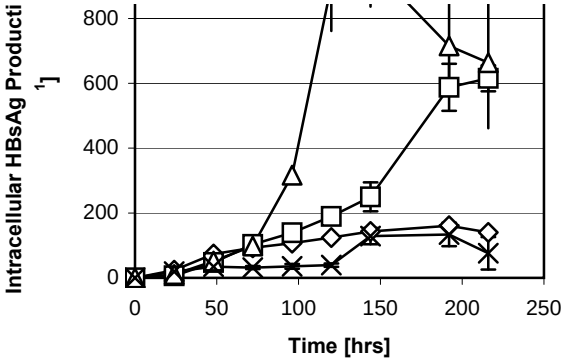



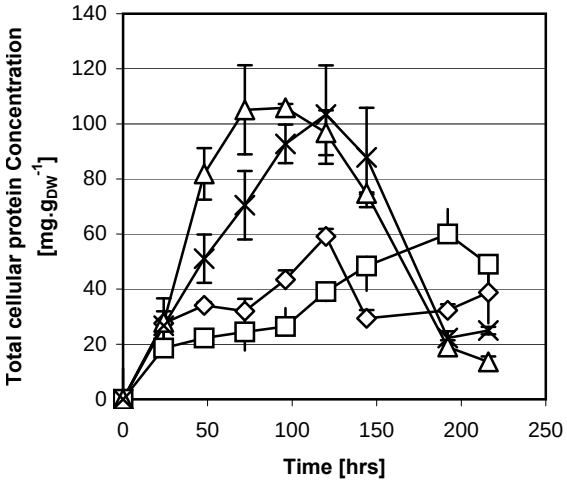
| roduction | HbsAg Production / Bradford Production | Bradford Production | | HBsAg Production | |
|---|---|---|---|---|---|
| Deviation [ug/L] | [%] | Average [mg/gDW] | Deviation [mg/gDW] | Average [ug/gDW] | Deviation [ug/gDW] |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 7.092 | 0.052 | 27.621 | 0.000 | 14.357 | 0.446 |
| 11.105 | 0.054 | 34.094 | 1.246 | 18.528 | 1.323 |
| 14.356 | 0.040 | 32.037 | 4.427 | 12.925 | 2.532 |
| 7.809 | 0.024 | 43.438 | 3.389 | 10.390 | 1.236 |
| 14.084 | 0.015 | 59.159 | 2.731 | 9.088 | 0.923 |
| 8.719 | 0.031 | 29.398 | 3.029 | 9.026 | 1.388 |
| 11.620 | 0.027 | 32.292 | 2.233 | 8.626 | 0.061 |
| 24.184 | 0.020 | 38.820 | 11.233 | 7.659 | 0.018 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 3.102 | 0.024 | 18.594 | 0.069 | 4.476 | 0.668 |
| 2.245 | 0.027 | 22.260 | 0.607 | 6.124 | 0.311 |
| 2.773 | 0.033 | 24.446 | 6.720 | 8.188 | 0.185 |
| 10.269 | 0.038 | 26.411 | 3.136 | 9.904 | 1.121 |
| 9.374 | 0.033 | 39.093 | 3.397 | 12.932 | 0.510 |
| 44.677 | 0.036 | 48.379 | 9.014 | 17.397 | 1.829 |
| 72.449 | 0.064 | 60.030 | 2.770 | 38.203 | 6.254 |
| 39.775 | 0.082 | 49.006 | 11.586 | 39.899 | 4.204 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 2.955 | 0.008 | 27.966 | 8.672 | 2.155 | 0.011 |
| 11.933 | 0.007 | 81.860 | 9.370 | 5.085 | 2.223 |
| 5.473 | 0.007 | 105.097 | 16.188 | 6.901 | 1.289 |
| 23.695 | 0.017 | 105.829 | 1.384 | 17.908 | 1.582 |
| 140.477 | 0.046 | 96.781 | 8.134 | 44.039 | 2.327 |
| 129.666 | 0.068 | 74.782 | 0.352 | 50.748 | 0.291 |
| 155.969 | 0.292 | 19.017 | 2.415 | 55.564 | 0.610 |
| 202.283 | 0.416 | 13.616 | 1.949 | 56.753 | 5.544 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 2.597 | 0.015 | 26.735 | 5.222 | 3.931 | 0.900 |
| 1.661 | 0.009 | 51.066 | 8.778 | 4.505 | 0.713 |
| 4.681 | 0.005 | 70.467 | 12.427 | 3.685 | 0.302 |
| 7.512 | 0.005 | 92.740 | 6.975 | 4.254 | 0.661 |
| 5.219 | 0.005 | 103.392 | 17.856 | 5.462 | 1.147 |
| 25.176 | 0.020 | 87.823 | 18.039 | 17.910 | 0.692 |
| 36.357 | 0.096 | 22.239 | 2.465 | 21.398 | 2.395 |
| 49.994 | 0.048 | 24.968 | 1.373 | 12.265 | 9.887 |

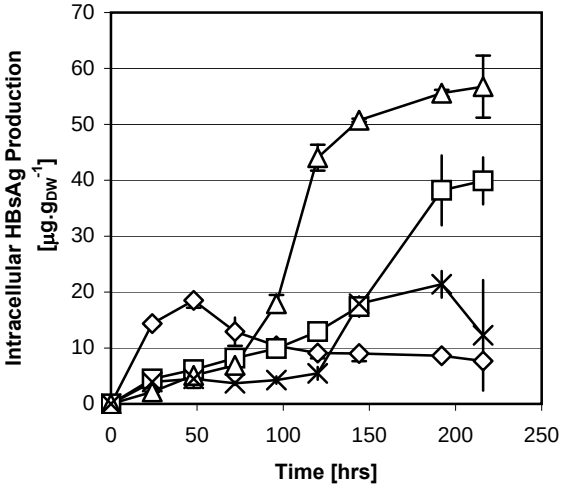


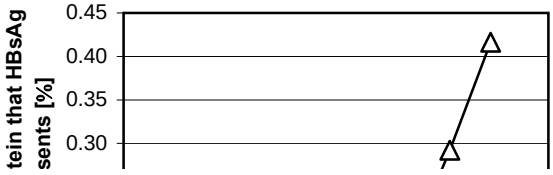

[mg.ℓculture-1]
1500
1000
500
0
0
50
100
150
200
250
Time [hrs]

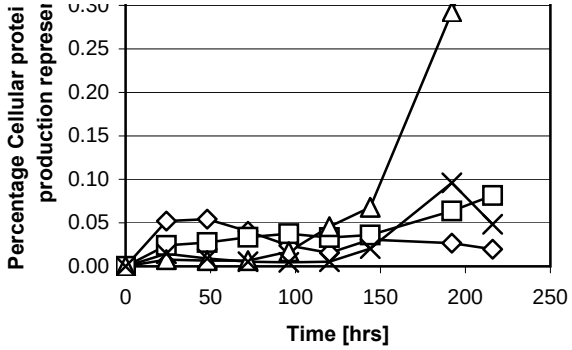

Intracellular HBsAg Producti
-1]
800
600
400
200
0
0
50
100
150
200
250
Time [hrs]

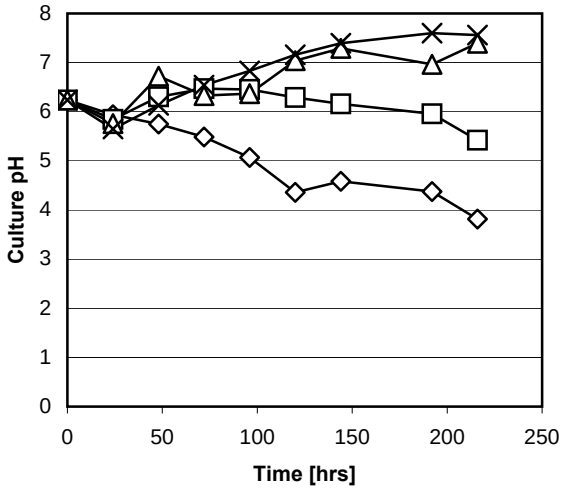

Total cellular protein Concentration
[mg·gDW-1]
140
120
100
80
60
40
20
0
0
50
100
150
200
250
Time [hrs]


Intracellular HBsAg Production
[µg·gDW-1]
70
60
50
40
30
20
10
0
0
50
100
150
200
250
Time [hrs]

| HbsAg Production / Bradford Production | pH | |
|---|---|---|
| [%] | Average | Deviation |
| 0.000 | 6.240 | 0.000 |
| 0.052 | 5.940 | 0.000 |
| 0.054 | 5.750 | 0.198 |
| 0.040 | 5.485 | 0.007 |
| 0.024 | 5.070 | 0.085 |
| 0.015 | 4.360 | 0.212 |
| 0.031 | 4.585 | 0.205 |
| 0.027 | 4.375 | 0.078 |
| 0.020 | 3.815 | 0.262 |
| 0.000 | 6.240 | 0.000 |
| 0.024 | 5.845 | 0.007 |
| 0.028 | 6.305 | 0.021 |
| 0.033 | 6.470 | 0.057 |
| 0.038 | 6.455 | 0.078 |
| 0.033 | 6.290 | 0.014 |
| 0.036 | 6.160 | 0.127 |
| 0.064 | 5.960 | 0.085 |
| 0.081 | 5.420 | 0.467 |
| 0.000 | 6.240 | 0.000 |
| 0.008 | 5.760 | 0.000 |
| 0.006 | 6.725 | 0.021 |
| 0.007 | 6.330 | 0.184 |
| 0.017 | 6.370 | 0.170 |
| 0.046 | 7.040 | 0.113 |
| 0.068 | 7.290 | 0.028 |
| 0.292 | 6.965 | 0.049 |
| 0.417 | 7.395 | 0.021 |
| 0.000 | 6.240 | 0.000 |
| 0.015 | 5.650 | 0.141 |
| 0.009 | 6.130 | 0.099 |
| 0.005 | 6.540 | 0.184 |
| 0.005 | 6.830 | 0.042 |
| 0.005 | 7.160 | 0.042 |
| 0.020 | 7.395 | 0.191 |
| 0.096 | 7.600 | 0.453 |
| 0.049 | 7.560 | 0.509 |




Percentage Cellular protei
production represe
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
250
Time [hrs]

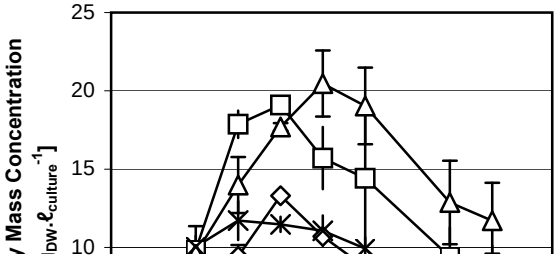

Culture pH
8
7
6
5
4
3
2
1
0
0
50
100
150
200
250
Time [hrs]

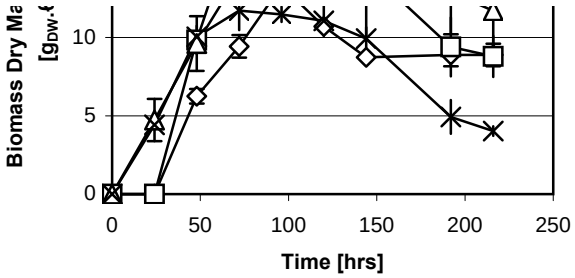
| Temperature | Time | Biomass Dry Weight (DW) | | Bradford Production | | HBsAg P |
|---|---|---|---|---|---|---|
| [°C] | [Hrs] | Average [gDW/l] | Deviation [gDW/l] | Average [mg/L] | Deviation [mg/L] | Average [ug/L] |
| (small smooth p | 0 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 16 | 24 | 1.644 | 0.545 | 45.403 | 15.055 | 23.599 |
| 16 | 48 | 3.979 | 0.883 | 135.655 | 35.078 | 73.720 |
| 16 | 72 | 7.195 | 0.299 | 230.493 | 22.280 | 92.991 |
| 16 | 96 | 10.292 | 1.976 | 447.053 | 120.716 | 106.934 |
| 16 | 120 | 13.687 | 0.160 | 809.688 | 46.860 | 124.385 |
| 16 | 144 | 15.859 | 1.472 | 466.221 | 91.316 | 143.142 |
| 16 | 192 | 18.671 | 1.215 | 602.911 | 2.437 | 161.061 |
| 16 | 216 | 18.294 | 3.202 | 710.179 | 329.791 | 140.116 |
| argest & fluffiest | 0 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 23 | 24 | 1.413 | 0.481 | 26.276 | 8.839 | 6.344 |
| 23 | 48 | 7.747 | 0.762 | 172.438 | 21.651 | 47.381 |
| 23 | 72 | 12.629 | 0.624 | 308.717 | 100.121 | 103.348 |
| 23 | 96 | 14.055 | 0.553 | 371.219 | 29.465 | 139.256 |
| 23 | 120 | 14.635 | 1.300 | 572.111 | 100.531 | 189.535 |
| 23 | 144 | 14.306 | 1.061 | 692.126 | 180.274 | 249.738 |
| 23 | 192 | 15.391 | 0.624 | 923.910 | 80.059 | 587.783 |
| 23 | 216 | 15.391 | 0.624 | 754.253 | 147.758 | 615.256 |
| (large & fluffy p | 0 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 30 | 24 | 4.728 | 1.348 | 132.229 | 3.307 | 10.176 |
| 30 | 48 | 9.613 | 1.750 | 786.879 | 53.192 | 51.844 |
| 30 | 72 | 13.991 | 1.788 | 1470.424 | 38.551 | 98.287 |
| 30 | 96 | 17.700 | 0.240 | 1873.171 | 49.932 | 316.975 |
| 30 | 120 | 20.468 | 2.107 | 1980.963 | 37.419 | 902.029 |
| 30 | 144 | 19.035 | 2.446 | 1423.514 | 189.622 | 965.955 |
| 30 | 192 | 12.875 | 2.666 | 244.846 | 19.604 | 715.322 |
| 30 | 216 | 11.705 | 2.423 | 159.369 | 10.186 | 663.558 |
| C (small fluffy pe | 0 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 37 | 24 | 4.981 | 0.484 | 133.161 | 38.950 | 19.383 |
| 37 | 48 | 7.540 | 0.811 | 385.035 | 107.593 | 34.563 |
| 37 | 72 | 8.563 | 1.962 | 603.372 | 31.863 | 31.726 |
| 37 | 96 | 8.314 | 0.470 | 771.040 | 101.558 | 35.636 |
| 37 | 120 | 7.117 | 0.534 | 735.878 | 71.912 | 39.284 |
| 37 | 144 | 7.177 | 1.682 | 630.348 | 18.229 | 128.644 |
| 37 | 192 | 6.273 | 0.999 | 139.515 | 6.759 | 133.919 |
| 37 | 216 | 6.275 | 0.997 | 156.666 | 16.288 | 75.789 |

| roduction | HbsAg Production / Bradford Production | Bradford Production | | HBsAg Production | |
|---|---|---|---|---|---|
| Deviation [ug/L] | [%] | Average [mg/gDW] | Deviation [mg/gDW] | Average [ug/gDW] | Deviation [ug/gDW] |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 7.092 | 0.052 | 27.621 | 0.000 | 14.357 | 0.446 |
| 11.105 | 0.054 | 34.094 | 1.246 | 18.528 | 1.323 |
| 14.356 | 0.040 | 32.037 | 4.427 | 12.925 | 2.532 |
| 7.809 | 0.024 | 43.438 | 3.389 | 10.390 | 1.236 |
| 14.084 | 0.015 | 59.159 | 2.731 | 9.088 | 0.923 |
| 8.719 | 0.031 | 29.398 | 3.029 | 9.026 | 1.388 |
| 11.620 | 0.027 | 32.292 | 2.233 | 8.626 | 0.061 |
| 24.184 | 0.020 | 38.820 | 11.233 | 7.659 | 0.018 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 3.102 | 0.024 | 18.594 | 0.069 | 4.476 | 0.668 |
| 2.245 | 0.027 | 22.260 | 0.607 | 6.124 | 0.311 |
| 2.773 | 0.033 | 24.446 | 6.720 | 8.188 | 0.185 |
| 10.269 | 0.038 | 26.411 | 3.136 | 9.904 | 1.121 |
| 9.374 | 0.033 | 39.093 | 3.397 | 12.932 | 0.510 |
| 44.677 | 0.036 | 48.379 | 9.014 | 17.397 | 1.829 |
| 72.449 | 0.064 | 60.030 | 2.770 | 38.203 | 6.254 |
| 39.775 | 0.082 | 49.006 | 11.586 | 39.899 | 4.204 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 2.955 | 0.008 | 27.966 | 8.672 | 2.155 | 0.011 |
| 11.933 | 0.007 | 81.860 | 9.370 | 5.085 | 2.223 |
| 5.473 | 0.007 | 105.097 | 16.188 | 6.901 | 1.289 |
| 23.695 | 0.017 | 105.829 | 1.384 | 17.908 | 1.582 |
| 140.477 | 0.046 | 96.781 | 8.134 | 44.039 | 2.327 |
| 129.666 | 0.068 | 74.782 | 0.352 | 50.748 | 0.291 |
| 155.969 | 0.292 | 19.017 | 2.415 | 55.564 | 0.610 |
| 202.283 | 0.416 | 13.616 | 1.949 | 56.753 | 5.544 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 2.597 | 0.015 | 26.735 | 5.222 | 3.931 | 0.900 |
| 1.661 | 0.009 | 51.066 | 8.778 | 4.505 | 0.713 |
| 4.681 | 0.005 | 70.467 | 12.427 | 3.685 | 0.302 |
| 7.512 | 0.005 | 92.740 | 6.975 | 4.254 | 0.661 |
| 5.219 | 0.005 | 103.392 | 17.856 | 5.462 | 1.147 |
| 25.176 | 0.020 | 87.823 | 18.039 | 17.910 | 0.692 |
| 36.357 | 0.096 | 22.239 | 2.465 | 21.398 | 2.395 |
| 49.994 | 0.048 | 24.968 | 1.373 | 12.265 | 9.887 |

| HbsAg Production / Bradford Production | pH | |
|---|---|---|
| [%] | Average | Deviation |
| 0.000 | 6.240 | 0.000 |
| 0.052 | 5.940 | 0.000 |
| 0.054 | 5.750 | 0.198 |
| 0.040 | 5.485 | 0.007 |
| 0.024 | 5.070 | 0.085 |
| 0.015 | 4.360 | 0.212 |
| 0.031 | 4.585 | 0.205 |
| 0.027 | 4.375 | 0.078 |
| 0.020 | 3.815 | 0.262 |
| 0.000 | 6.240 | 0.000 |
| 0.024 | 5.845 | 0.007 |
| 0.028 | 6.305 | 0.021 |
| 0.033 | 6.470 | 0.057 |
| 0.038 | 6.455 | 0.078 |
| 0.033 | 6.290 | 0.014 |
| 0.036 | 6.160 | 0.127 |
| 0.064 | 5.960 | 0.085 |
| 0.081 | 5.420 | 0.467 |
| 0.000 | 6.240 | 0.000 |
| 0.008 | 5.760 | 0.000 |
| 0.006 | 6.725 | 0.021 |
| 0.007 | 6.330 | 0.184 |
| 0.017 | 6.370 | 0.170 |
| 0.046 | 7.040 | 0.113 |
| 0.068 | 7.290 | 0.028 |
| 0.292 | 6.965 | 0.049 |
| 0.417 | 7.395 | 0.021 |
| 0.000 | 6.240 | 0.000 |
| 0.015 | 5.650 | 0.141 |
| 0.009 | 6.130 | 0.099 |
| 0.005 | 6.540 | 0.184 |
| 0.005 | 6.830 | 0.042 |
| 0.005 | 7.160 | 0.042 |
| 0.020 | 7.395 | 0.191 |
| 0.096 | 7.600 | 0.453 |
| 0.049 | 7.560 | 0.509 |

| Inoculum concentration | Time | Biomass Dry Weight (DW) | | Bradford Production | | HBsAg Production | |
|---|---|---|---|---|---|---|---|
| [spores/ml] | [Hrs] | Average [gDW/l] | Deviation [gDW/l] | Average [mg/L] | Deviation [mg/L] | Average [ug/L] | Deviation [ug/L] |
| (verly large fluffy p | 0 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| (verly large fluffy p | 24 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| (verly large fluffy p | 48 | 6.249 | 0.468 | 156.698 | 51.319 | 13.057 | 0.977 |
| (verly large fluffy p | 72 | 9.432 | 0.721 | 579.407 | 31.194 | 75.852 | 5.799 |
| (verly large fluffy p | 96 | 13.306 | 0.000 | 1204.923 | 89.613 | 433.250 | 12.289 |
| (verly large fluffy p | 120 | 10.710 | 0.000 | 1386.238 | 218.496 | 456.066 | 37.951 |
| (verly large fluffy p | 144 | 8.733 | 0.000 | 795.928 | 115.575 | 375.782 | 22.608 |
| (verly large fluffy p | 192 | 8.885 | 0.721 | 224.871 | 71.131 | 386.138 | 24.084 |
| (verly large fluffy p | 216 | 8.885 | 0.721 | 243.420 | 19.901 | 436.835 | 9.925 |
| smaller less fluffier | 0 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| smaller less fluffier | 24 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| smaller less fluffier | 48 | 9.842 | 0.908 | 548.157 | 101.253 | 49.876 | 18.365 |
| smaller less fluffier | 72 | 17.866 | 0.871 | 1172.979 | 173.986 | 155.950 | 32.265 |
| smaller less fluffier | 96 | 19.092 | 0.271 | 1712.187 | 85.578 | 523.709 | 35.187 |
| smaller less fluffier | 120 | 15.706 | 1.989 | 1156.032 | 40.263 | 766.362 | 120.141 |
| smaller less fluffier | 144 | 14.415 | 4.320 | 1050.847 | 164.754 | 803.457 | 293.624 |
| smaller less fluffier | 192 | 9.395 | 1.880 | 288.499 | 34.322 | 527.680 | 118.224 |
| smaller less fluffier | 216 | 8.792 | 1.314 | 161.227 | 10.293 | 512.432 | 58.377 |
| very smaller fluffy | 0 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| very smaller fluffy | 24 | 4.728 | 1.348 | 132.229 | 3.307 | 10.176 | 2.955 |
| very smaller fluffy | 48 | 9.613 | 1.750 | 786.879 | 53.192 | 51.844 | 11.933 |
| very smaller fluffy | 72 | 13.991 | 1.788 | 1470.424 | 38.551 | 98.287 | 5.473 |
| very smaller fluffy | 96 | 17.700 | 0.240 | 1873.171 | 49.932 | 316.975 | 23.695 |
| very smaller fluffy | 120 | 20.468 | 2.107 | 1980.963 | 37.419 | 902.029 | 140.477 |
| very smaller fluffy | 144 | 19.035 | 2.446 | 1423.514 | 189.622 | 965.955 | 129.666 |
| very smaller fluffy | 192 | 12.875 | 2.666 | 244.846 | 19.604 | 715.322 | 155.969 |
| very smaller fluffy | 216 | 11.705 | 2.423 | 159.369 | 10.186 | 663.558 | 202.283 |
| 8 (filamentous grov | 0 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 8 (filamentous grov | 24 | 4.424 | 0.058 | 176.958 | 36.939 | 11.237 | 2.305 |
| 8 (filamentous grov | 48 | 10.047 | 0.178 | 1279.254 | 123.105 | 49.948 | 11.562 |
| 8 (filamentous grov | 72 | 11.718 | 1.247 | 1352.031 | 58.178 | 145.162 | 5.929 |
| 8 (filamentous grov | 96 | 11.477 | 0.482 | 1417.506 | 58.172 | 430.101 | 24.682 |
| 8 (filamentous grov | 120 | 11.068 | 0.975 | 1216.927 | 16.397 | 483.129 | 103.481 |
| 8 (filamentous grov | 144 | 9.931 | 0.725 | 929.172 | 198.873 | 444.043 | 20.928 |
| 8 (filamentous grov | 192 | 4.926 | 1.079 | 460.604 | 173.450 | 219.194 | 27.489 |
| 8 (filamentous grov | 216 | 4.027 | 0.308 | 222.712 | 15.676 | 190.432 | 38.646 |






10^3 (verly large fluffy pellets)

10^5 ( smaller less fluffier pellets)

10^6 (very smaller  fluffy pellets)

10^8 (filamentous growth)

| HbsAg Production / Bradford Production | Bradford Production | | HBsAg Production | | HbsAg Production / Bradford Production |
|---|---|---|---|---|---|
| [%] | Average [mg/gDW] | Deviation [mg/gDW] | Average [ug/gDW] | Deviation [ug/gDW] | [%] |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.000 | 17.111 | 1.123 | 1.428 | 0.163 | 0.008 |
| 0.008 | 32.606 | 10.651 | 2.089 | 0.000 | 0.006 |
| 0.013 | 56.060 | 7.593 | 8.042 | 0.000 | 0.014 |
| 0.036 | 85.791 | 6.735 | 32.560 | 0.924 | 0.038 |
| 0.033 | 115.008 | 20.401 | 42.583 | 3.543 | 0.037 |
| 0.047 | 81.779 | 13.234 | 43.028 | 2.589 | 0.053 |
| 0.172 | 29.289 | 5.628 | 43.460 | 0.817 | 0.148 |
| 0.179 | 27.407 | 0.015 | 49.165 | 5.108 | 0.179 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.000 | 30.197 | 7.515 | 3.654 | 0.605 | 0.012 |
| 0.009 | 51.800 | 5.507 | 5.067 | 1.398 | 0.010 |
| 0.013 | 75.131 | 13.401 | 8.729 | 1.380 | 0.012 |
| 0.031 | 93.794 | 5.814 | 27.431 | 1.454 | 0.029 |
| 0.066 | 106.407 | 46.392 | 48.793 | 1.471 | 0.046 |
| 0.076 | 66.820 | 8.598 | 55.738 | 3.664 | 0.083 |
| 0.183 | 29.166 | 2.183 | 56.169 | 1.345 | 0.193 |
| 0.318 | 17.334 | 1.419 | 58.284 | 2.069 | 0.336 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.008 | 27.966 | 8.672 | 2.152 | 0.011 | 0.008 |
| 0.007 | 81.860 | 9.370 | 5.393 | 2.223 | 0.007 |
| 0.007 | 105.097 | 16.188 | 7.025 | 1.289 | 0.007 |
| 0.017 | 105.829 | 1.384 | 17.908 | 1.582 | 0.017 |
| 0.046 | 96.781 | 8.134 | 44.069 | 2.327 | 0.046 |
| 0.068 | 74.782 | 0.352 | 50.745 | 0.291 | 0.068 |
| 0.292 | 19.017 | 2.415 | 55.559 | 0.610 | 0.292 |
| 0.416 | 13.616 | 1.949 | 56.692 | 5.544 | 0.416 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.006 | 45.480 | 7.749 | 2.540 | 0.554 | 0.006 |
| 0.004 | 134.317 | 9.879 | 4.972 | 1.239 | 0.004 |
| 0.011 | 128.564 | 18.647 | 12.388 | 1.824 | 0.010 |
| 0.030 | 123.427 | 0.117 | 37.475 | 3.725 | 0.030 |
| 0.040 | 116.133 | 8.744 | 43.651 | 5.506 | 0.038 |
| 0.048 | 102.438 | 12.551 | 44.713 | 1.155 | 0.044 |
| 0.048 | 81.169 | 17.434 | 44.493 | 4.163 | 0.055 |
| 0.086 | 61.372 | 8.589 | 47.283 | 5.977 | 0.077 |

| pH | |
|---|---|
| **Average** | **Deviation** |
| 6.320 | 0.014 |
| 4.280 | 0.014 |
| 4.565 | 0.014 |
| 6.730 | 0.290 |
| 6.480 | 0.028 |
| 6.780 | 0.000 |
| 7.050 | 0.099 |
| 7.060 | 0.000 |
| 7.195 | 0.297 |
| 6.340 | 0.014 |
| 4.495 | 0.049 |
| 5.430 | 0.127 |
| 6.760 | 0.014 |
| 6.770 | 0.000 |
| 6.700 | 0.184 |
| 7.100 | 0.198 |
| 7.290 | 0.042 |
| 7.345 | 0.092 |
| 6.240 | 0.000 |
| 5.760 | 0.000 |
| 6.725 | 0.021 |
| 6.330 | 0.184 |
| 6.370 | 0.170 |
| 7.040 | 0.113 |
| 7.290 | 0.028 |
| 6.965 | 0.049 |
| 7.395 | 0.021 |
| 6.350 | 0.000 |
| 5.300 | 0.226 |
| 5.430 | 0.127 |
| 5.945 | 0.445 |
| 6.775 | 0.163 |
| 7.290 | 0.042 |
| 7.300 | 0.028 |
| 7.575 | 0.233 |
| 7.655 | 0.191 |

| Agitation | $dO_2$ | Time | Biomass Dry Weight (DW) | | | | Assay Dry w |
|---|---|---|---|---|---|---|---|
| [rpm] | [%] | Hours | Sample A [gDW/L] | Sample B [gDW/L] | Average [gDW/L] | Deviation [gDW/L] | HBsAg Average [gDW/ml] |
| 350 | 50 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 350 | 50 | **20** | 3.323 | 2.948 | 3.135 | 0.265 | 0.012 |
| 350 | 50 | **28** | 3.493 | 3.063 | 3.278 | 0.304 | 0.043 |
| 350 | 50 | **33** | 3.800 | 3.877 | 3.838 | 0.054 | 0.057 |
| 350 | 50 | **48** | 7.183 | 8.297 | 7.740 | 0.787 | 0.018 |
| 350 | 50 | **72** | 6.840 | 5.996 | 6.418 | 0.597 | 0.046 |
| 350 | 50 | **95** | 5.857 | 6.277 | 6.067 | 0.297 | 0.045 |
| 350 | 50 | **145** | 6.250 | 5.553 | 5.902 | 0.493 | 0.050 |
| 100 | 50 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 100 | 50 | **9** | 0.500 | 0.453 | 0.477 | 0.033 | 0.031 |
| 100 | 50 | **16** | 1.917 | 1.177 | 1.547 | 0.523 | 0.028 |
| 100 | 50 | **22** | 4.343 | 4.163 | 4.253 | 0.127 | 0.041 |
| 100 | 50 | **29** | 5.833 | 5.830 | 5.832 | 0.002 | 0.050 |
| 100 | 50 | **37** | 5.907 | 6.687 | 6.297 | 0.552 | 0.053 |
| 100 | 50 | **44** | 8.252 | 10.151 | 9.202 | 1.343 | 0.047 |
| 100 | 50 | **52** | 11.475 | 11.800 | 11.638 | 0.230 | 0.051 |
| 100 | 50 | **60** | 13.680 | 12.100 | 12.890 | 1.117 | 0.054 |
| 100 | 50 | **82** | 9.752 | 11.075 | 10.413 | 0.936 | 0.046 |
| 100 | 50 | **90** | 7.708 | 7.950 | 7.829 | 0.171 | 0.047 |
| 100 | 50 | **103** | 6.653 | 6.290 | 6.472 | 0.257 | 0.032 |
| 100 | 50 | **130** | 6.368 | 4.765 | 5.567 | 1.133 | 0.031 |
| 100 | 50 | **160** | 6.334 | 6.248 | 6.291 | 0.061 | 0.055 |
| 600 | 50 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 600 | 50 | **17** | 5.370 | 5.273 | 5.322 | 0.068 | 0.053 |
| 600 | 50 | **21** | 5.977 | 7.507 | 6.742 | 1.082 | 0.045 |
| 600 | 50 | **26** | 8.203 | 8.363 | 8.283 | 0.113 | 0.047 |
| 600 | 50 | **35** | 10.677 | 10.053 | 10.365 | 0.441 | 0.051 |
| 600 | 50 | **42** | 12.410 | 13.306 | 12.858 | 0.633 | 0.056 |
| 600 | 50 | **49** | 10.630 | 11.583 | 11.107 | 0.674 | 0.043 |
| 600 | 50 | **58** | 11.489 | 8.840 | 10.164 | 1.873 | 0.051 |
| 600 | 50 | **68** | 9.332 | 8.515 | 8.924 | 0.577 | 0.045 |
| 600 | 50 | **90** | 4.885 | 6.106 | 5.496 | 0.863 | 0.043 |
| 600 | 50 | **111** | 3.293 | 3.127 | 3.210 | 0.118 | 0.043 |
| 600 | 50 | **116** | 2.837 | 3.013 | 2.925 | 0.125 | 0.050 |
| 350 | 95 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 350 | 95 | **34** | 4.513 | 4.653 | 4.583 | 0.099 | 0.057 |
| 350 | 95 | **43** | 8.040 | 8.448 | 8.244 | 0.288 | 0.049 |
| 350 | 95 | **55** | 9.690 | 9.520 | 9.605 | 0.120 | 0.038 |
| 350 | 95 | **64** | 6.235 | 5.257 | 5.746 | 0.692 | 0.045 |
| 350 | 95 | **80** | 4.592 | 5.480 | 5.036 | 0.628 | 0.038 |
| 350 | 95 | **90** | 6.496 | 3.028 | 4.762 | 2.452 | 0.046 |
| 350 | 95 | **101** | 2.971 | 2.804 | 2.888 | 0.118 | 0.036 |
| 350 | 95 | **116** | 1.943 | 1.818 | 1.881 | 0.089 | 0.035 |
| 350 | 95 | **133** | 0.710 | 1.377 | 1.043 | 0.471 | 0.008 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 350 | 2 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 350 | 2 | **34** | 3.137 | 3.247 | 3.192 | 0.078 | 0.036 |
| 350 | 2 | **43** | 3.190 | 3.648 | 3.419 | 0.324 | 0.008 |
| 350 | 2 | **55** | 4.140 | 4.320 | 4.230 | 0.127 | 0.030 |
| 350 | 2 | **64** | 4.497 | 5.160 | 4.828 | 0.469 | 0.030 |
| 350 | 2 | **80** | 8.320 | 8.393 | 8.356 | 0.052 | 0.029 |
| 350 | 2 | **90** | 8.690 | 9.229 | 8.959 | 0.381 | 0.045 |
| 350 | 2 | **101** | 11.104 | 12.696 | 11.900 | 1.125 | 0.040 |
| 350 | 2 | **116** | 7.436 | 7.227 | 7.331 | 0.148 | 0.042 |
| 350 | 2 | **133** | 6.833 | 6.808 | 6.821 | 0.018 | 0.041 |
| 350 | 2 | **140** | 6.800 | 6.754 | 6.777 | 0.033 | 0.049 |
| 250 | 20 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 250 | 20 | **9** | 0.637 | 0.573 | 0.605 | 0.045 | 0.000 |
| 250 | 20 | **16** | 2.733 | 2.823 | 2.778 | 0.064 | 0.000 |
| 250 | 20 | **22** | 5.592 | 4.340 | 4.966 | 0.885 | 0.048 |
| 250 | 20 | **29** | 5.907 | 5.960 | 5.933 | 0.038 | 0.053 |
| 250 | 20 | **37** | 6.890 | 6.607 | 6.748 | 0.200 | 0.055 |
| 250 | 20 | **44** | 6.973 | 6.714 | 6.844 | 0.183 | 0.053 |
| 250 | 20 | **52** | 8.687 | 8.087 | 8.387 | 0.424 | 0.050 |
| 250 | 20 | **60** | 8.463 | 8.615 | 8.539 | 0.107 | 0.050 |
| 250 | 20 | **82** | 8.786 | 8.743 | 8.765 | 0.030 | 0.045 |
| 250 | 20 | **90** | 8.048 | 8.030 | 8.039 | 0.013 | 0.041 |
| 250 | 20 | **103** | 7.763 | 7.972 | 7.867 | 0.148 | 0.039 |
| 250 | 20 | **130** | 6.147 | 7.121 | 6.634 | 0.689 | 0.049 |
| 250 | 20 | **160** | 6.084 | 5.967 | 6.025 | 0.083 | 0.047 |
| 250 | 80 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 250 | 80 | **12** | 3.900 | 4.197 | 4.048 | 0.210 | 0.048 |
| 250 | 80 | **21** | 6.237 | 6.060 | 6.148 | 0.125 | 0.059 |
| 250 | 80 | **26** | 6.980 | 6.313 | 6.647 | 0.471 | 0.047 |
| 250 | 80 | **35** | 9.313 | 9.930 | 9.622 | 0.436 | 0.056 |
| 250 | 80 | **42** | 10.380 | 10.017 | 10.198 | 0.257 | 0.047 |
| 250 | 80 | **49** | 11.400 | 10.483 | 10.942 | 0.648 | 0.043 |
| 250 | 80 | **58** | 11.454 | 10.714 | 11.084 | 0.523 | 0.052 |
| 250 | 80 | **68** | 8.660 | 8.797 | 8.728 | 0.097 | 0.042 |
| 250 | 80 | **90** | 8.507 | 8.233 | 8.370 | 0.193 | 0.052 |
| 250 | 80 | **111** | 7.127 | 7.157 | 7.142 | 0.022 | 0.047 |
| 250 | 80 | **116** | 6.300 | 6.400 | 6.350 | 0.071 | 0.049 |
| 450 | 20 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 450 | 20 | **14** | 0.780 | 0.827 | 0.803 | 0.033 | 0.473 |
| 450 | 20 | **19** | 4.097 | 3.823 | 3.960 | 0.193 | 0.039 |
| 450 | 20 | **32** | 7.927 | 8.110 | 8.018 | 0.130 | 0.052 |
| 450 | 20 | **37** | 7.833 | 7.277 | 7.555 | 0.394 | 0.044 |
| 450 | 20 | **45** | 6.818 | 6.682 | 6.750 | 0.096 | 0.046 |
| 450 | 20 | **58** | 6.510 | 6.740 | 6.625 | 0.163 | 0.048 |
| 450 | 20 | **79** | 6.391 | 6.363 | 6.377 | 0.019 | 0.048 |
| 450 | 20 | **102** | 6.297 | 6.118 | 6.207 | 0.127 | 0.050 |
| 450 | 20 | **140** | 5.287 | 5.000 | 5.143 | 0.203 | 0.049 |
| 450 | 80 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 450 | 80 | **16** | 5.503 | 4.277 | 4.890 | 0.867 | 0.033 |
| 450 | 80 | **38** | 6.913 | 6.104 | 6.508 | 0.572 | 0.041 |
| 450 | 80 | **43** | 8.093 | 7.823 | 7.958 | 0.191 | 0.042 |
| 450 | 80 | **50** | 7.793 | 6.787 | 7.290 | 0.712 | 0.045 |
| 450 | 80 | **68** | 6.967 | 7.064 | 7.016 | 0.069 | 0.039 |
| 450 | 80 | **74** | 6.990 | 6.938 | 6.964 | 0.037 | 0.036 |
| 450 | 80 | **94** | 5.956 | 6.077 | 6.016 | 0.085 | 0.042 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 350 | 25 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 350 | 25 | **28** | 4.333 | 3.047 | 3.690 | 0.910 | 0.026 |
| 350 | 25 | **33** | 5.430 | 5.550 | 5.490 | 0.085 | 0.051 |
| 350 | 25 | **48** | 8.263 | 7.517 | 7.890 | 0.528 | 0.037 |
| 350 | 25 | **72** | 11.017 | 11.113 | 11.065 | 0.068 | 0.047 |
| 350 | 25 | **95** | 6.696 | 6.470 | 6.583 | 0.160 | 0.049 |
| 350 | 25 | **145** | 5.992 | 5.237 | 5.614 | 0.534 | 0.047 |
| 350 | <2 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.040 |
| 350 | <2 | **15** | 0.440 | 0.480 | 0.460 | 0.028 | 0.040 |
| 350 | <2 | **19** | 1.110 | 1.253 | 1.182 | 0.101 | 0.040 |
| 350 | <2 | **23** | 2.047 | 1.180 | 1.613 | 0.613 | 0.040 |
| 350 | <2 | **47** | 5.010 | 4.540 | 4.775 | 0.332 | 0.042 |
| 350 | <2 | **59** | 5.750 | 5.440 | 5.595 | 0.219 | 0.046 |
| 350 | <2 | **83** | 5.980 | 6.100 | 6.040 | 0.085 | 0.047 |
| 350 | <2 | **140** | 7.160 | 6.847 | 7.003 | 0.222 | 0.048 |
| 350 | control | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 350 | control | **30** | 1.810 | 1.850 | 1.830 | 0.028 | 0.043 |
| 350 | control | **50** | 9.550 | 8.253 | 8.902 | 0.917 | 0.043 |
| 350 | control | **57** | 8.953 | 10.220 | 9.587 | 0.896 | 0.031 |
| 350 | control | **68** | 9.547 | 10.273 | 9.910 | 0.514 | 0.032 |
| 350 | control | **74** | 11.100 | 10.220 | 10.660 | 0.622 | 0.034 |
| 350 | control | **80** | 12.220 | 11.373 | 11.797 | 0.599 | 0.036 |
| 350 | control | **86** | 12.083 | 11.576 | 11.830 | 0.359 | 0.035 |
| 350 | control | **94** | 12.070 | 11.747 | 11.908 | 0.229 | 0.034 |
| 350 | control | **127** | 12.400 | 14.657 | 13.528 | 1.596 | 0.049 |
| 350 | control | **152** | 10.783 | 18.203 | 14.493 | 5.247 | 0.040 |
| 300 | pH6 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.040 |
| 300 | pH6 | **14** | 1.553 | 2.977 | 2.265 | 1.006 | 0.040 |
| 300 | pH6 | **19** | 3.533 | 4.420 | 3.977 | 0.627 | 0.026 |
| 300 | pH6 | **32** | 4.496 | 4.555 | 4.525 | 0.041 | 0.041 |
| 300 | pH6 | **37** | 7.391 | 7.110 | 7.250 | 0.199 | 0.038 |
| 300 | pH6 | **45** | 7.297 | 7.321 | 7.309 | 0.017 | 0.039 |
| 300 | pH6 | **58** | 6.262 | 6.292 | 6.277 | 0.021 | 0.045 |
| 300 | pH6 | **79** | 5.960 | 6.592 | 6.276 | 0.447 | 0.040 |
| 300 | pH6 | **102** | 5.813 | 5.953 | 5.883 | 0.099 | 0.046 |
| 300 | pH6 | **140** | 4.053 | 4.145 | 4.099 | 0.065 | 0.000 |
| 300 | pH7 | **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 300 | pH7 | **15** | 1.250 | 1.070 | 1.160 | 0.127 | 0.000 |
| 300 | pH7 | **19** | 2.077 | 1.880 | 1.978 | 0.139 | 0.000 |
| 300 | pH7 | **23** | 2.945 | 3.445 | 3.195 | 0.354 | 0.000 |
| 300 | pH7 | **47** | 3.718 | 3.509 | 3.614 | 0.148 | 0.000 |
| 300 | pH7 | **59** | 3.925 | 4.394 | 4.160 | 0.332 | 0.000 |
| 300 | pH7 | **83** | 4.470 | 4.292 | 4.381 | 0.126 | 0.000 |
| 300 | pH7 | **140** | 8.590 | 10.037 | 9.313 | 1.023 | 0.047 |

**NM** **Not measured**

| veight (DW) | HBsAg Concentration Assay (membrane fraction) | | Specific HBsAg Concentration (membrane fraction) | |
|---|---|---|---|---|
| Total protein Average [gDW/ml] | Average [IU/ml] | Deviation [IU/ml] | Average [ug/gDW] | Deviation [ug/gDW] |
| 0.040 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.040 | 3.470 | 0.416 | 6.723 | 0.805 |
| 0.033 | 3.635 | 0.294 | 8.668 | 0.702 |
| 0.047 | 6.205 | 0.699 | 10.134 | 1.141 |
| 0.008 | 6.263 | 0.994 | 64.282 | 10.201 |
| 0.036 | 46.815 | 2.691 | 99.947 | 5.746 |
| 0.035 | 53.453 | 1.634 | 118.019 | 3.608 |
| 0.040 | 81.653 | 2.882 | 157.803 | 5.570 |
| 0.040 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.021 | 0.245 | 0.075 | 0.895 | 0.274 |
| 0.018 | 0.480 | 0.127 | 2.059 | 0.545 |
| 0.031 | 5.015 | 1.481 | 12.377 | 3.656 |
| 0.040 | 11.430 | 1.434 | 21.953 | 2.755 |
| 0.043 | 14.695 | 0.354 | 26.577 | 0.640 |
| 0.032 | 17.180 | 1.495 | 41.737 | 3.631 |
| 0.031 | 17.798 | 1.028 | 44.421 | 2.566 |
| 0.024 | 18.615 | 0.541 | 59.124 | 1.718 |
| 0.021 | 79.223 | 2.882 | 288.243 | 10.487 |
| 0.022 | 84.220 | 3.954 | 301.473 | 14.153 |
| 0.020 | 56.365 | 2.771 | 213.603 | 10.501 |
| 0.021 | 54.550 | 2.279 | 199.411 | 8.330 |
| 0.045 | 51.788 | 3.176 | 88.499 | 5.428 |
| 0.040 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.043 | 0.583 | 0.205 | 1.049 | 0.369 |
| 0.035 | 1.173 | 0.091 | 2.634 | 0.204 |
| 0.037 | 1.890 | 0.156 | 3.922 | 0.325 |
| 0.041 | 7.030 | 0.798 | 13.176 | 1.495 |
| 0.046 | 14.745 | 1.495 | 24.841 | 2.519 |
| 0.033 | 24.213 | 1.075 | 57.121 | 2.537 |
| 0.031 | 24.415 | 0.691 | 60.163 | 1.703 |
| 0.035 | 27.385 | 2.004 | 60.898 | 4.457 |
| 0.033 | 34.330 | 2.953 | 80.621 | 6.934 |
| 0.033 | 34.965 | 2.627 | 82.237 | 6.178 |
| 0.040 | 34.400 | 2.540 | 66.648 | 4.922 |
| 0.040 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.040 | 2.345 | 0.922 | 4.543 | 1.787 |
| 0.040 | 11.788 | 1.146 | 22.838 | 2.221 |
| 0.040 | 29.498 | 1.877 | 57.150 | 3.636 |
| 0.040 | 35.885 | 1.365 | 69.525 | 2.644 |
| 0.040 | 59.165 | 3.344 | 114.629 | 6.479 |
| 0.036 | 61.115 | 2.304 | 131.564 | 4.959 |
| 0.040 | 75.300 | 3.526 | 145.890 | 6.832 |
| 0.040 | 60.275 | 3.759 | 116.780 | 7.282 |
| 0.040 | 59.115 | 3.297 | 114.532 | 6.387 |

| | | | | |
|---|---|---|---|---|
| 0.030 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.026 | 1.435 | 0.387 | 4.253 | 1.146 |
| 0.040 | 5.600 | 0.046 | 10.850 | 0.089 |
| 0.020 | 7.115 | 1.931 | 27.639 | 7.499 |
| 0.020 | 9.298 | 4.505 | 36.027 | 17.456 |
| 0.020 | 9.333 | 0.802 | 36.436 | 3.130 |
| 0.035 | 17.060 | 1.799 | 37.560 | 3.960 |
| 0.030 | 19.928 | 5.758 | 50.884 | 14.702 |
| 0.032 | 34.763 | 4.245 | 84.320 | 10.296 |
| 0.031 | 44.880 | 3.080 | 112.926 | 7.749 |
| 0.039 | 54.418 | 2.009 | 109.255 | 4.033 |
| 0.040 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.040 | 0.328 | 0.095 | 0.635 | 0.184 |
| 0.040 | 0.565 | 0.210 | 1.095 | 0.407 |
| 0.038 | 2.378 | 1.335 | 4.823 | 2.708 |
| 0.043 | 12.713 | 3.993 | 22.753 | 7.146 |
| 0.045 | 14.888 | 3.354 | 25.525 | 5.751 |
| 0.043 | 15.268 | 2.571 | 27.548 | 4.639 |
| 0.040 | 25.058 | 5.522 | 48.487 | 10.684 |
| 0.040 | 27.595 | 6.297 | 53.000 | 12.094 |
| 0.035 | 27.853 | 0.312 | 62.205 | 0.697 |
| 0.031 | 28.390 | 1.167 | 70.518 | 2.900 |
| 0.029 | 30.053 | 4.447 | 79.761 | 11.803 |
| 0.039 | 46.533 | 8.703 | 93.183 | 17.427 |
| 0.037 | 47.313 | 5.637 | 98.433 | 11.727 |
| 0.040 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.038 | 2.435 | 0.434 | 4.921 | 0.878 |
| 0.049 | 2.458 | 0.952 | 3.883 | 1.505 |
| 0.037 | 3.088 | 0.343 | 6.476 | 0.719 |
| 0.046 | 5.058 | 1.885 | 8.548 | 3.186 |
| 0.037 | 9.415 | 0.327 | 19.509 | 0.678 |
| 0.033 | 17.888 | 12.941 | 42.588 | 30.810 |
| 0.022 | 24.080 | 21.484 | 85.212 | 76.026 |
| 0.032 | 48.338 | 19.073 | 115.441 | 45.550 |
| 0.042 | 85.378 | 5.681 | 156.236 | 10.395 |
| 0.037 | 86.015 | 3.690 | 181.882 | 7.804 |
| 0.039 | 90.703 | 5.157 | 180.469 | 10.262 |
| 0.000 | 0.000 | 0.000 | #DIV/0! | #DIV/0! |
| 0.463 | 0.280 | 0.050 | 0.047 | 0.008 |
| 0.029 | 3.263 | 0.962 | 8.779 | 2.588 |
| 0.042 | 9.798 | 1.801 | 17.950 | 3.300 |
| 0.034 | 10.955 | 2.848 | 24.824 | 6.454 |
| 0.036 | 20.260 | 1.489 | 44.042 | 3.237 |
| 0.038 | 26.440 | 0.247 | 54.065 | 0.506 |
| 0.038 | 33.765 | 2.836 | 69.594 | 5.846 |
| 0.040 | 42.573 | 3.061 | 83.210 | 5.983 |
| 0.039 | 36.443 | 3.280 | 72.323 | 6.510 |
| -0.010 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.023 | 3.603 | 0.671 | 12.192 | 2.270 |
| 0.031 | 13.180 | 1.149 | 33.380 | 2.910 |
| 0.032 | 28.610 | 5.484 | 69.072 | 13.240 |
| 0.035 | 50.643 | 6.576 | 113.104 | 14.686 |
| 0.029 | 64.433 | 6.537 | 171.300 | 17.380 |
| 0.026 | 64.728 | 3.531 | 190.010 | 10.366 |
| 0.032 | 67.783 | 6.531 | 161.880 | 15.599 |

| | | | | |
|---|---|---|---|---|
| 0.040 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.040 | 5.165 | 2.131 | 10.007 | 4.128 |
| 0.041 | 14.873 | 1.510 | 27.840 | 2.826 |
| 0.027 | 40.078 | 0.968 | 115.462 | 2.790 |
| 0.037 | 73.085 | 0.916 | 154.121 | 1.932 |
| 0.039 | 80.118 | 5.832 | 159.408 | 11.604 |
| 0.037 | 85.900 | 1.325 | 181.392 | 2.798 |
| 0.040 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.040 | 0.390 | 0.112 | 0.756 | 0.216 |
| 0.040 | 0.418 | 0.136 | 0.809 | 0.263 |
| 0.040 | 0.423 | 0.190 | 0.819 | 0.368 |
| 0.032 | 4.570 | 2.756 | 10.914 | 6.583 |
| 0.036 | 5.675 | 0.348 | 12.082 | 0.742 |
| 0.037 | 13.363 | 3.436 | 28.102 | 7.227 |
| 0.038 | 6.268 | 2.420 | 12.935 | 4.994 |
| -0.010 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.033 | 25.580 | 3.078 | 89.707 | 0.769 |
| 0.033 | 28.968 | 5.174 | 91.334 | 3.564 |
| 0.021 | 32.565 | 6.657 | 93.953 | 11.304 |
| 0.022 | 36.020 | 4.442 | 100.895 | 18.022 |
| 0.024 | 37.983 | 1.533 | 105.816 | 21.631 |
| 0.026 | 38.025 | 0.326 | 109.470 | 13.499 |
| 0.025 | 38.715 | 1.511 | 118.453 | 4.782 |
| 0.024 | 40.870 | 2.534 | 130.343 | 8.081 |
| 0.049 | 92.613 | 3.661 | 146.775 | 5.803 |
| 0.040 | 98.808 | 4.975 | 191.435 | 9.640 |
| 0.040 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.040 | 0.268 | 0.017 | 0.518 | 0.033 |
| 0.016 | 2.843 | 0.770 | 14.121 | 3.823 |
| 0.031 | 9.485 | 1.094 | 24.101 | 2.779 |
| 0.028 | 10.023 | 1.180 | 27.940 | 3.290 |
| 0.029 | 13.740 | 2.516 | 36.218 | 6.632 |
| 0.035 | 37.720 | 1.419 | 83.164 | 3.129 |
| 0.030 | 38.275 | 4.141 | 100.211 | 10.842 |
| 0.036 | 45.355 | 3.396 | 98.182 | 7.351 |
| 0.040 | 30.840 | 3.017 | 59.751 | 5.846 |
| 0.030 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.030 | 4.128 | 0.401 | 10.662 | 1.037 |
| 0.030 | 11.755 | 5.266 | 30.366 | 13.604 |
| 0.030 | 18.863 | 3.863 | 48.727 | 9.979 |
| 0.030 | 23.453 | 2.474 | 60.584 | 6.390 |
| 0.028 | 20.685 | 0.744 | 57.252 | 2.060 |
| 0.030 | 20.265 | 1.381 | 52.350 | 3.569 |
| 0.037 | 19.375 | 1.190 | 40.747 | 2.503 |

| Volumetric HBsAg Concentration (membrane fraction) | | Specific HBsAg Concentration (cytoplasmic fraction) | |
|---|---|---|---|
| Average [ug/L] | Deviation [ug/L] | Average [ug/gDW] | Deviation [ug/gDW] |
| 0.000 | 0.000 | 0.000 | 0.000 |
| 21.079 | 2.525 | 0.000 | 0.000 |
| 28.416 | 2.302 | 0.000 | 0.000 |
| 38.899 | 4.379 | 0.000 | 0.000 |
| 497.545 | 78.955 | 0.103 | 0.000 |
| 641.473 | 36.877 | 0.181 | 0.141 |
| 715.981 | 21.888 | 3.317 | 0.476 |
| 931.301 | 32.874 | 0.019 | 0.000 |
| 0.000 | 0.000 | 0.000 | 0.000 |
| 0.427 | 0.131 | 0.037 | 0.000 |
| 3.184 | 0.843 | 0.086 | 0.000 |
| 52.645 | 15.550 | 0.037 | 0.014 |
| 128.022 | 16.064 | 0.029 | 0.011 |
| 167.348 | 4.032 | 0.018 | 0.000 |
| 384.048 | 33.411 | 0.024 | 0.000 |
| 516.949 | 29.866 | 0.050 | 0.029 |
| 762.108 | 22.147 | 45.347 | 10.172 |
| 3001.602 | 109.209 | 54.967 | 1.988 |
| 2360.229 | 110.801 | 56.531 | 17.695 |
| 1382.365 | 67.956 | 61.894 | 14.766 |
| 1110.079 | 46.372 | 60.509 | 13.751 |
| 556.781 | 34.147 | 24.800 | 2.770 |
| 0.000 | 0.000 | 0.000 | 0.000 |
| 5.580 | 1.965 | 0.000 | 0.000 |
| 17.756 | 1.373 | 0.000 | 0.000 |
| 32.484 | 2.688 | 0.021 | 0.000 |
| 136.565 | 15.496 | 0.103 | 0.050 |
| 319.406 | 32.384 | 0.198 | 0.050 |
| 634.435 | 28.179 | 0.720 | 0.260 |
| 611.519 | 17.310 | 0.850 | 0.051 |
| 543.432 | 39.776 | 1.946 | 0.203 |
| 443.055 | 38.108 | 3.235 | 0.475 |
| 263.982 | 19.831 | 3.610 | 1.130 |
| 194.946 | 14.396 | 3.904 | 0.190 |
| 0.000 | 0.000 | 0.000 | 0.000 |
| 20.824 | 8.189 | 0.000 | 0.000 |
| 188.272 | 18.310 | 0.000 | 0.000 |
| 548.925 | 34.925 | 0.019 | 0.000 |
| 399.468 | 15.190 | 0.029 | 0.011 |
| 577.273 | 32.627 | 0.993 | 0.176 |
| 626.506 | 23.615 | 3.681 | 0.870 |
| 421.289 | 19.728 | 2.955 | 0.257 |
| 219.616 | 13.695 | 6.403 | 1.421 |
| 119.495 | 6.664 | 5.919 | 1.040 |

| | | | |
|---|---|---|---|
| 0.000 | 0.000 | 0.000 | 0.000 |
| 13.573 | 3.659 | 0.000 | 0.000 |
| 37.095 | 0.306 | 0.000 | 0.000 |
| 116.913 | 31.722 | 0.000 | 0.000 |
| 173.950 | 84.282 | 0.000 | 0.000 |
| 304.473 | 26.159 | 0.000 | 0.000 |
| 336.512 | 35.476 | 0.000 | 0.000 |
| 605.533 | 174.960 | 0.000 | 0.000 |
| 618.166 | 75.479 | 0.061 | 0.042 |
| 770.222 | 52.852 | 0.164 | 0.060 |
| 740.414 | 27.331 | 0.291 | 0.136 |
| 0.000 | 0.000 | NM | NM |
| 0.384 | 0.111 | NM | NM |
| 3.041 | 1.130 | NM | NM |
| 23.953 | 13.450 | NM | NM |
| 135.002 | 42.403 | NM | NM |
| 172.254 | 38.810 | NM | NM |
| 188.532 | 31.750 | NM | NM |
| 406.645 | 89.606 | NM | NM |
| 452.572 | 103.274 | NM | NM |
| 545.197 | 6.110 | NM | NM |
| 566.901 | 23.310 | NM | NM |
| 627.491 | 92.860 | NM | NM |
| 618.145 | 115.607 | NM | NM |
| 593.084 | 70.659 | NM | NM |
| 0.000 | 0.000 | NM | NM |
| 19.921 | 3.554 | NM | NM |
| 23.873 | 9.251 | NM | NM |
| 43.041 | 4.776 | NM | NM |
| 82.250 | 30.655 | NM | NM |
| 198.961 | 6.916 | NM | NM |
| 465.986 | 337.115 | NM | NM |
| 944.467 | 842.643 | NM | NM |
| 1007.613 | 397.582 | NM | NM |
| 1307.694 | 87.007 | NM | NM |
| 1298.987 | 55.732 | NM | NM |
| 1145.977 | 65.162 | NM | NM |
| #DIV/0! | #DIV/0! | NM | NM |
| 0.038 | 0.007 | NM | NM |
| 34.765 | 10.249 | NM | NM |
| 143.929 | 26.462 | NM | NM |
| 187.547 | 48.761 | NM | NM |
| 297.286 | 21.853 | NM | NM |
| 358.178 | 3.351 | NM | NM |
| 443.807 | 37.280 | NM | NM |
| 516.507 | 37.138 | NM | NM |
| 371.982 | 33.482 | NM | NM |
| 0.000 | 0.000 | NM | NM |
| 59.617 | 11.099 | NM | NM |
| 217.237 | 18.940 | NM | NM |
| 549.700 | 105.367 | NM | NM |
| 824.525 | 107.059 | NM | NM |
| 1201.807 | 121.935 | NM | NM |
| 1323.220 | 72.190 | NM | NM |
| 973.924 | 93.846 | NM | NM |

| | | | |
|---|---|---|---|
| 0.000 | 0.000 | NM | NM |
| 36.926 | 15.232 | NM | NM |
| 152.843 | 15.515 | NM | NM |
| 910.995 | 22.012 | NM | NM |
| 1705.347 | 21.374 | NM | NM |
| 1049.396 | 76.390 | NM | NM |
| 1018.394 | 15.706 | NM | NM |
| 0.000 | 0.000 | NM | NM |
| 0.348 | 0.100 | NM | NM |
| 0.956 | 0.311 | NM | NM |
| 1.321 | 0.594 | NM | NM |
| 52.115 | 31.434 | NM | NM |
| 67.601 | 4.150 | NM | NM |
| 169.737 | 43.649 | NM | NM |
| 90.590 | 34.978 | NM | NM |
| 0.000 | 0.000 | NM | NM |
| 164.163 | 1.407 | NM | NM |
| 813.028 | 31.725 | NM | NM |
| 900.692 | 108.368 | NM | NM |
| 999.874 | 178.602 | NM | NM |
| 1128.003 | 230.590 | NM | NM |
| 1291.378 | 159.241 | NM | NM |
| 1401.256 | 56.567 | NM | NM |
| 1552.172 | 96.235 | NM | NM |
| 1985.617 | 78.498 | NM | NM |
| 2774.525 | 139.712 | NM | NM |
| 0.000 | 0.000 | NM | NM |
| 1.174 | 0.075 | NM | NM |
| 56.155 | 15.203 | NM | NM |
| 109.062 | 12.578 | NM | NM |
| 202.576 | 23.855 | NM | NM |
| 264.709 | 48.473 | NM | NM |
| 522.038 | 19.644 | NM | NM |
| 628.905 | 68.040 | NM | NM |
| 577.628 | 43.246 | NM | NM |
| 244.929 | 23.965 | NM | NM |
| 0.000 | 0.000 | NM | NM |
| 12.368 | 1.202 | NM | NM |
| 60.075 | 26.912 | NM | NM |
| 155.682 | 31.884 | NM | NM |
| 218.929 | 23.092 | NM | NM |
| 238.146 | 8.567 | NM | NM |
| 229.336 | 15.633 | NM | NM |
| 379.490 | 23.312 | NM | NM |

| Volumetric HBsAg Concentration (cytoplasmic fraction) | | Volumetric Total Protein Concentration (membrane fraction) | | Specific Total Protein Concentration (membrane fraction) | |
|---|---|---|---|---|---|
| Average [ug/L] | Deviation [ug/L] | Average [mg/L] | Deviation [mg/L] | Total Protein (membrane fraction) Culture Average [mg/gDW] | Total Protein (membrane fraction) Culture St dev [mg/gDW] |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.000 | 0.000 | 27.649 | 1.975 | 8.818 | 0.630 |
| 0.000 | 0.000 | 16.323 | 1.377 | 4.979 | 0.420 |
| 0.000 | 0.000 | 18.363 | 0.655 | 4.784 | 0.171 |
| 0.794 | 0.000 | 135.450 | 25.915 | 17.500 | 3.348 |
| 1.165 | 0.905 | 118.852 | 5.825 | 18.518 | 0.908 |
| 20.126 | 2.886 | 118.591 | 3.856 | 19.548 | 0.636 |
| 0.114 | 0.000 | 102.456 | 8.713 | 17.361 | 1.476 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.017 | 0.000 | 4.434 | 0.150 | 9.303 | 0.315 |
| 0.133 | 0.000 | 20.542 | 4.672 | 13.282 | 3.021 |
| 0.157 | 0.061 | 40.183 | 2.681 | 9.447 | 0.630 |
| 0.168 | 0.065 | 50.059 | 9.109 | 8.584 | 1.562 |
| 0.114 | 0.000 | 51.884 | 7.714 | 8.240 | 1.225 |
| 0.224 | 0.000 | 79.324 | 6.360 | 8.621 | 0.691 |
| 0.581 | 0.335 | 102.559 | 12.968 | 8.813 | 1.114 |
| 584.529 | 131.113 | 216.234 | 6.919 | 16.775 | 0.537 |
| 572.397 | 20.698 | 245.558 | 13.473 | 23.581 | 1.294 |
| 442.578 | 138.536 | 233.900 | 17.448 | 29.876 | 2.229 |
| 400.559 | 95.564 | 157.296 | 17.455 | 24.305 | 2.697 |
| 336.839 | 76.550 | 92.024 | 7.615 | 16.531 | 1.368 |
| 156.028 | 17.430 | 43.258 | 13.667 | 6.876 | 2.172 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.000 | 0.000 | 32.309 | 0.803 | 6.071 | 0.151 |
| 0.000 | 0.000 | 55.026 | 8.170 | 8.162 | 1.212 |
| 0.172 | 0.000 | 96.573 | 13.814 | 11.659 | 1.668 |
| 1.068 | 0.514 | 146.591 | 12.021 | 14.143 | 1.160 |
| 2.545 | 0.647 | 164.661 | 17.218 | 12.806 | 1.339 |
| 7.992 | 2.890 | 197.256 | 3.426 | 17.760 | 0.308 |
| 8.641 | 0.521 | 194.430 | 8.671 | 19.128 | 0.853 |
| 17.364 | 1.808 | 183.607 | 15.004 | 20.575 | 1.681 |
| 17.778 | 2.610 | 130.726 | 8.667 | 23.788 | 1.577 |
| 11.589 | 3.628 | 42.931 | 7.771 | 13.374 | 2.421 |
| 11.419 | 0.556 | 29.167 | 3.712 | 9.972 | 1.269 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.000 | 0.000 | 30.651 | 3.431 | 6.688 | 0.749 |
| 0.000 | 0.000 | 125.626 | 18.176 | 15.239 | 2.205 |
| 0.186 | 0.000 | 159.792 | 6.052 | 16.636 | 0.630 |
| 0.167 | 0.064 | 95.489 | 5.079 | 16.619 | 0.884 |
| 5.000 | 0.888 | 98.693 | 7.628 | 19.597 | 1.515 |
| 17.530 | 4.143 | 91.332 | 4.731 | 19.179 | 0.993 |
| 8.532 | 0.743 | 52.608 | 1.129 | 18.218 | 0.391 |
| 12.042 | 2.672 | 33.701 | 2.838 | 17.920 | 1.509 |
| 6.175 | 1.085 | 23.428 | 4.846 | 22.455 | 4.645 |

| | | | | | |
|---|---|---|---|---|---|
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.000 | 0.000 | 16.926 | 3.350 | 5.303 | 1.050 |
| 0.000 | 0.000 | 28.984 | 7.246 | 8.477 | 2.119 |
| 0.000 | 0.000 | 46.647 | 5.981 | 11.028 | 1.414 |
| 0.000 | 0.000 | 72.059 | 16.470 | 14.924 | 3.411 |
| 0.000 | 0.000 | 126.678 | 13.479 | 15.159 | 1.613 |
| 0.000 | 0.000 | 172.752 | 44.037 | 19.282 | 4.915 |
| 0.000 | 0.000 | 155.057 | 7.640 | 13.030 | 0.642 |
| 0.445 | 0.308 | 116.502 | 8.228 | 15.891 | 1.122 |
| 1.116 | 0.409 | 116.462 | 5.328 | 17.075 | 0.781 |
| 1.973 | 0.919 | 86.228 | 6.685 | 12.724 | 0.986 |
| NM | NM | 0.000 | 0.000 | 0.000 | 0.000 |
| NM | NM | 5.851 | 1.199 | 9.670 | 1.981 |
| NM | NM | 12.945 | 0.365 | 4.659 | 0.131 |
| NM | NM | 16.723 | 2.798 | 3.367 | 0.563 |
| NM | NM | 25.755 | 4.176 | 4.341 | 0.704 |
| NM | NM | 37.019 | 2.133 | 5.486 | 0.316 |
| NM | NM | 44.484 | 3.925 | 6.500 | 0.573 |
| NM | NM | 57.834 | 9.895 | 6.896 | 1.180 |
| NM | NM | 88.817 | 37.061 | 10.401 | 4.340 |
| NM | NM | 86.107 | 19.528 | 9.824 | 2.228 |
| NM | NM | 110.342 | 2.489 | 13.726 | 0.310 |
| NM | NM | 125.282 | 20.031 | 15.925 | 2.546 |
| NM | NM | 64.075 | 14.977 | 9.659 | 2.258 |
| NM | NM | 45.732 | 9.031 | 7.590 | 1.499 |
| NM | NM | 0.000 | 0.000 | 0.000 | 0.000 |
| NM | NM | 15.738 | 6.321 | 3.888 | 1.561 |
| NM | NM | 7.265 | 3.198 | 1.182 | 0.520 |
| NM | NM | 9.114 | 0.876 | 1.371 | 0.132 |
| NM | NM | 27.710 | 3.826 | 2.880 | 0.398 |
| NM | NM | 39.663 | 4.785 | 3.889 | 0.469 |
| NM | NM | 67.765 | 18.283 | 6.193 | 1.671 |
| NM | NM | 105.017 | 10.819 | 9.475 | 0.976 |
| NM | NM | 124.403 | 21.180 | 14.253 | 2.427 |
| NM | NM | 98.221 | 13.059 | 11.735 | 1.560 |
| NM | NM | 73.506 | 9.949 | 10.292 | 1.393 |
| NM | NM | 66.556 | 3.407 | 10.481 | 0.537 |
| NM | NM | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| NM | NM | 0.532 | 0.094 | 0.663 | 0.117 |
| NM | NM | 29.719 | 3.103 | 7.505 | 0.784 |
| NM | NM | 116.363 | 11.620 | 14.512 | 1.449 |
| NM | NM | 143.338 | 11.208 | 18.973 | 1.484 |
| NM | NM | 127.030 | 4.302 | 18.819 | 0.637 |
| NM | NM | 120.414 | 6.302 | 18.176 | 0.951 |
| NM | NM | 115.061 | 4.373 | 18.043 | 0.686 |
| NM | NM | 108.234 | 3.306 | 17.437 | 0.533 |
| NM | NM | 95.934 | 14.689 | 18.652 | 2.856 |
| NM | NM | 0.000 | 0.000 | 0.000 | 0.000 |
| NM | NM | 72.312 | 12.441 | 14.788 | 2.544 |
| NM | NM | 107.114 | 5.677 | 16.459 | 0.872 |
| NM | NM | 151.909 | 7.603 | 19.088 | 0.955 |
| NM | NM | 134.807 | 7.681 | 18.492 | 1.054 |
| NM | NM | 161.365 | 4.681 | 23.000 | 0.667 |
| NM | NM | 180.994 | 7.198 | 25.990 | 1.034 |
| NM | NM | 130.077 | 2.565 | 21.621 | 0.426 |

| | | | | | |
|---|---|---|---|---|---|
| NM | NM | 0.000 | 0.000 | 0.000 | 0.000 |
| NM | NM | 11.804 | 0.598 | 3.199 | 0.162 |
| NM | NM | 19.349 | 3.022 | 3.524 | 0.550 |
| NM | NM | 167.253 | 15.369 | 21.198 | 1.948 |
| NM | NM | 185.717 | 10.954 | 16.784 | 0.990 |
| NM | NM | 105.864 | 5.467 | 16.081 | 0.830 |
| NM | NM | 85.912 | 1.971 | 15.302 | 0.351 |
| NM | NM | 0.000 | 0.000 | 0.000 | 0.000 |
| NM | NM | 2.467 | 1.449 | 5.364 | 3.149 |
| NM | NM | 10.662 | 0.000 | 9.023 | 0.000 |
| NM | NM | 8.103 | 0.212 | 5.023 | 0.131 |
| NM | NM | 27.557 | 8.912 | 5.771 | 1.866 |
| NM | NM | 53.938 | 10.011 | 9.640 | 1.789 |
| NM | NM | 48.167 | 15.086 | 7.975 | 2.498 |
| NM | NM | 22.805 | 4.110 | 3.256 | 0.587 |
| NM | NM | 0.000 | 0.000 | 0.000 | 0.000 |
| NM | NM | 17.826 | 3.993 | 9.741 | 2.182 |
| NM | NM | 69.531 | 5.597 | 7.811 | 0.629 |
| NM | NM | 133.995 | 17.011 | 13.977 | 1.774 |
| NM | NM | 156.007 | 11.291 | 15.742 | 1.139 |
| NM | NM | 156.912 | 27.140 | 14.720 | 2.546 |
| NM | NM | 171.945 | 9.416 | 14.576 | 0.798 |
| NM | NM | 203.254 | 16.421 | 17.182 | 1.388 |
| NM | NM | 192.903 | 30.029 | 16.199 | 2.522 |
| NM | NM | 138.012 | 18.714 | 10.202 | 1.383 |
| NM | NM | 179.602 | 24.731 | 12.392 | 1.706 |
| NM | NM | 0.000 | 0.000 | 0.000 | 0.000 |
| NM | NM | 13.564 | 1.880 | 5.989 | 0.830 |
| NM | NM | 38.562 | 8.125 | 9.697 | 2.043 |
| NM | NM | 60.114 | 3.345 | 13.284 | 0.739 |
| NM | NM | 101.300 | 7.296 | 13.972 | 1.006 |
| NM | NM | 114.579 | 7.346 | 15.677 | 1.005 |
| NM | NM | 108.449 | 7.518 | 17.277 | 1.198 |
| NM | NM | 129.863 | 5.379 | 20.693 | 0.857 |
| NM | NM | 104.988 | 5.728 | 17.845 | 0.974 |
| NM | NM | 63.933 | 0.699 | 15.597 | 0.170 |
| NM | NM | 0.000 | 0.000 | 0.000 | 0.000 |
| NM | NM | 15.845 | 4.323 | 13.659 | 3.727 |
| NM | NM | 32.744 | 2.847 | 16.551 | 1.439 |
| NM | NM | 39.211 | 0.000 | 12.273 | 0.000 |
| NM | NM | 77.611 | 15.255 | 21.477 | 4.221 |
| NM | NM | 107.773 | 29.454 | 25.909 | 7.081 |
| NM | NM | 111.512 | 36.645 | 25.455 | 8.365 |
| NM | NM | 158.937 | 21.830 | 17.065 | 2.344 |

| Total Protein Bradford Optical Density (membrane fraction) | | | | | | Total Protein Bradford | | |
|---|---|---|---|---|---|---|---|---|
| A [OD] | B [OD] | C [OD] | D [OD] | Ave | Deviation | Buffer A [ug/ml] | Buffer B [ug/ml] | Buffer C [ug/ml] |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.035 | 0.035 | 0.039 | 0.039 | 0.002 | 0.037 | 31.818 | 31.818 | 35.455 |
| 0.057 | 0.057 | 0.026 | 0.026 | 0.018 | 0.042 | 51.818 | 51.818 | 23.636 |
| 0.025 | 0.025 | 0.014 | 0.014 | 0.006 | 0.020 | 22.727 | 22.727 | 12.727 |
| 0.079 | 0.081 | 0.044 | 0.049 | 0.019 | 0.063 | 71.818 | 73.636 | 40.000 |
| 0.147 | 0.164 | 0.162 | 0.155 | 0.008 | 0.157 | 133.636 | 149.091 | 147.273 |
| 0.186 | 0.152 | 0.193 | 0.113 | 0.037 | 0.161 | 169.091 | 138.182 | 175.455 |
| 0.146 | 0.116 | 0.145 | 0.186 | 0.029 | 0.148 | 132.727 | 105.455 | 131.818 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.110 | 0.110 | 0.077 | 0.077 | 0.019 | 0.094 | 100.000 | 100.000 | 70.000 |
| 0.016 | 0.016 | 0.034 | 0.034 | 0.010 | 0.025 | 14.545 | 14.545 | 30.909 |
| 0.032 | 0.032 | 0.051 | 0.051 | 0.011 | 0.042 | 29.091 | 29.091 | 46.364 |
| 0.056 | 0.055 | 0.067 | 0.045 | 0.009 | 0.056 | 50.909 | 50.000 | 60.909 |
| 0.118 | 0.082 | 0.151 | 0.076 | 0.035 | 0.107 | 107.273 | 74.545 | 137.273 |
| 0.008 | 0.009 | 0.018 | 0.033 | 0.012 | 0.017 | 7.273 | 8.182 | 16.364 |
| 0.071 | 0.043 | 0.036 | 0.032 | 0.018 | 0.046 | 64.545 | 39.091 | 32.727 |
| 0.085 | 0.076 | 0.224 | 0.202 | 0.077 | 0.147 | 77.273 | 69.091 | 203.636 |
| 0.283 | 0.434 | 0.249 | 0.391 | 0.087 | 0.339 | 257.273 | 394.545 | 226.364 |
| 0.310 | 0.328 | 0.199 | 0.209 | 0.067 | 0.262 | 281.818 | 298.182 | 180.909 |
| 0.141 | 0.154 | 0.069 | 0.055 | 0.050 | 0.105 | 128.182 | 140.000 | 62.727 |
| 0.215 | 0.195 | 0.123 | 0.155 | 0.041 | 0.172 | 195.455 | 177.273 | 111.818 |
| 0.215 | 0.195 | 0.123 | 0.155 | 0.041 | 0.172 | 195.455 | 177.273 | 111.818 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.019 | 0.026 | 0.039 | 0.035 | 0.009 | 0.030 | 17.273 | 23.636 | 35.455 |
| 0.015 | 0.015 | 0.024 | 0.013 | 0.005 | 0.017 | 13.636 | 13.636 | 21.818 |
| 0.017 | 0.041 | 0.027 | 0.066 | 0.021 | 0.038 | 15.455 | 37.273 | 24.545 |
| 0.230 | 0.312 | 0.254 | 0.236 | 0.037 | 0.258 | 209.091 | 283.636 | 230.909 |
| 0.253 | 0.309 | 0.278 | 0.259 | 0.025 | 0.275 | 230.000 | 280.909 | 252.727 |
| 0.267 | 0.262 | 0.267 | 0.260 | 0.004 | 0.264 | 242.727 | 238.182 | 242.727 |
| 0.246 | 0.241 | 0.243 | 0.235 | 0.005 | 0.241 | 223.636 | 219.091 | 220.909 |
| 0.306 | 0.261 | 0.212 | 0.181 | 0.055 | 0.240 | 278.182 | 237.273 | 192.727 |
| 0.220 | 0.157 | 0.145 | 0.194 | 0.034 | 0.179 | 200.000 | 142.727 | 131.818 |
| 0.178 | 0.169 | 0.142 | 0.166 | 0.015 | 0.164 | 161.818 | 153.636 | 129.091 |
| 0.182 | 0.182 | 0.140 | 0.140 | 0.024 | 0.161 | 165.455 | 165.455 | 127.273 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.052 | 0.047 | 0.050 | 0.035 | 0.008 | 0.046 | 47.273 | 42.727 | 45.455 |
| 0.135 | 0.180 | 0.165 | 0.162 | 0.019 | 0.161 | 122.727 | 163.636 | 150.000 |
| 0.182 | 0.162 | 0.181 | 0.170 | 0.010 | 0.174 | 165.455 | 147.273 | 164.545 |
| 0.212 | 0.202 | 0.193 | 0.183 | 0.012 | 0.198 | 192.727 | 183.636 | 175.455 |
| 0.249 | 0.234 | 0.203 | 0.193 | 0.026 | 0.220 | 226.364 | 212.727 | 184.545 |
| 0.144 | 0.144 | 0.187 | 0.187 | 0.025 | 0.166 | 130.909 | 130.909 | 170.000 |
| 0.167 | 0.167 | 0.145 | 0.145 | 0.013 | 0.156 | 151.818 | 151.818 | 131.818 |
| 0.144 | 0.144 | 0.147 | 0.147 | 0.002 | 0.146 | 130.909 | 130.909 | 133.636 |
| 0.142 | 0.142 | 0.140 | 0.140 | 0.001 | 0.141 | 129.091 | 129.091 | 127.273 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.022 | 0.022 | 0.022 | 0.022 | 0.000 | 0.022 | 20.000 | 20.000 | 20.000 |
| 0.004 | 0.004 | 0.004 | 0.004 | 0.000 | 0.004 | 3.636 | 3.636 | 3.636 |
| 0.038 | 0.038 | 0.038 | 0.018 | 0.010 | 0.033 | 34.545 | 34.545 | 34.545 |
| 0.076 | 0.052 | 0.052 | 0.037 | 0.016 | 0.054 | 69.091 | 47.273 | 47.273 |
| 0.039 | 0.113 | 0.066 | 0.075 | 0.031 | 0.073 | 35.455 | 102.727 | 60.000 |
| 0.081 | 0.038 | 0.041 | 0.091 | 0.027 | 0.063 | 73.636 | 34.545 | 37.273 |
| 0.108 | 0.111 | 0.126 | 0.104 | 0.010 | 0.112 | 98.182 | 100.909 | 114.545 |
| 0.110 | 0.111 | 0.161 | 0.182 | 0.036 | 0.141 | 100.000 | 100.909 | 146.364 |
| 0.150 | 0.257 | 0.155 | 0.264 | 0.062 | 0.207 | 136.364 | 233.636 | 140.909 |
| 0.136 | 0.244 | 0.266 | 0.127 | 0.072 | 0.193 | 123.636 | 221.818 | 241.818 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.151 | 0.151 | 0.155 | 0.155 | 0.002 | 0.153 | 137.273 | 137.273 | 140.909 |
| 0.055 | 0.055 | 0.119 | 0.119 | 0.037 | 0.087 | 50.000 | 50.000 | 108.182 |
| 0.046 | 0.046 | 0.046 | 0.046 | 0.000 | 0.046 | 41.818 | 41.818 | 41.818 |
| 0.047 | 0.031 | 0.035 | 0.026 | 0.009 | 0.035 | 42.727 | 28.182 | 31.818 |
| 0.139 | 0.142 | 0.134 | 0.122 | 0.009 | 0.134 | 126.364 | 129.091 | 121.818 |
| 0.071 | 0.091 | 0.048 | 0.076 | 0.018 | 0.072 | 64.545 | 82.727 | 43.636 |
| 0.149 | 0.090 | 0.183 | 0.145 | 0.038 | 0.142 | 135.455 | 81.818 | 166.364 |
| 0.141 | 0.165 | 0.163 | 0.150 | 0.011 | 0.155 | 128.182 | 150.000 | 148.182 |
| 0.169 | 0.137 | 0.273 | 0.218 | 0.059 | 0.199 | 153.636 | 124.545 | 248.182 |
| 0.171 | 0.223 | 0.231 | 0.185 | 0.029 | 0.203 | 155.455 | 202.727 | 210.000 |
| 0.127 | 0.172 | 0.258 | 0.209 | 0.056 | 0.192 | 115.455 | 156.364 | 234.545 |
| 0.106 | 0.097 | 0.166 | 0.217 | 0.056 | 0.147 | 96.364 | 88.182 | 150.909 |
| 0.035 | 0.084 | 0.068 | 0.146 | 0.047 | 0.083 | 31.818 | 76.364 | 61.818 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.101 | 0.101 | 0.067 | 0.067 | 0.020 | 0.084 | 91.818 | 91.818 | 60.909 |
| 0.053 | 0.020 | 0.013 | 0.021 | 0.018 | 0.027 | 48.182 | 18.182 | 11.818 |
| 0.044 | 0.025 | 0.020 | 0.020 | 0.011 | 0.027 | 40.000 | 22.727 | 18.182 |
| 0.092 | 0.051 | 0.075 | 0.108 | 0.024 | 0.082 | 83.636 | 46.364 | 68.182 |
| 0.080 | 0.094 | 0.086 | 0.073 | 0.009 | 0.083 | 72.727 | 85.455 | 78.182 |
| 0.183 | 0.180 | 0.127 | 0.118 | 0.034 | 0.152 | 166.364 | 163.636 | 115.455 |
| 0.086 | 0.120 | 0.098 | 0.074 | 0.020 | 0.095 | 78.182 | 109.091 | 89.091 |
| 0.148 | 0.134 | 0.141 | 0.122 | 0.011 | 0.136 | 134.545 | 121.818 | 128.182 |
| 0.113 | 0.118 | 0.153 | 0.163 | 0.025 | 0.137 | 102.727 | 107.273 | 139.091 |
| 0.169 | 0.133 | 0.143 | 0.136 | 0.016 | 0.145 | 153.636 | 120.909 | 130.000 |
| 0.140 | 0.160 | 0.083 | 0.091 | 0.037 | 0.119 | 127.273 | 145.455 | 75.455 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.025 | 0.025 | 0.028 | 0.035 | 0.005 | 0.028 | 22.727 | 22.727 | 25.455 |
| 0.270 | 0.270 | 0.121 | 0.146 | 0.079 | 0.202 | 245.455 | 245.455 | 110.000 |
| 0.218 | 0.218 | 0.198 | 0.221 | 0.011 | 0.214 | 198.182 | 198.182 | 180.000 |
| 0.273 | 0.196 | 0.232 | 0.256 | 0.033 | 0.239 | 248.182 | 178.182 | 210.909 |
| 0.235 | 0.212 | 0.216 | 0.218 | 0.010 | 0.220 | 213.636 | 192.727 | 196.364 |
| 0.116 | 0.138 | 0.129 | 0.129 | 0.009 | 0.128 | 105.455 | 125.455 | 117.273 |
| 0.125 | 0.121 | 0.148 | 0.150 | 0.015 | 0.136 | 113.636 | 110.000 | 134.545 |
| 0.100 | 0.115 | 0.101 | 0.096 | 0.008 | 0.103 | 90.909 | 104.545 | 91.818 |
| 0.043 | 0.094 | 0.076 | 0.084 | 0.022 | 0.074 | 39.091 | 85.455 | 69.091 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.092 | 0.092 | 0.054 | 0.054 | 0.022 | 0.073 | 83.636 | 83.636 | 49.091 |
| 0.097 | 0.140 | 0.080 | 0.123 | 0.027 | 0.110 | 88.182 | 127.273 | 72.727 |
| 0.129 | 0.211 | 0.158 | 0.157 | 0.034 | 0.164 | 117.273 | 191.818 | 143.636 |
| 0.207 | 0.198 | 0.270 | 0.230 | 0.032 | 0.226 | 188.182 | 180.000 | 245.455 |
| 0.139 | 0.149 | 0.145 | 0.158 | 0.008 | 0.148 | 126.364 | 135.455 | 131.818 |
| 0.155 | 0.178 | 0.165 | 0.209 | 0.023 | 0.177 | 140.909 | 161.818 | 150.000 |
| 0.112 | 0.132 | 0.156 | 0.163 | 0.023 | 0.141 | 101.818 | 120.000 | 141.818 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.027 | 0.027 | 0.026 | 0.026 | 0.001 | 0.027 | 24.545 | 24.545 | 23.636 |
| 0.008 | 0.008 | 0.023 | 0.007 | 0.008 | 0.012 | 7.273 | 7.273 | 20.909 |
| 0.127 | 0.097 | 0.097 | 0.110 | 0.014 | 0.108 | 115.455 | 88.182 | 88.182 |
| 0.084 | 0.084 | 0.116 | 0.074 | 0.018 | 0.090 | 76.364 | 76.364 | 105.455 |
| 0.149 | 0.129 | 0.130 | 0.161 | 0.016 | 0.142 | 135.455 | 117.273 | 118.182 |
| 0.126 | 0.145 | 0.117 | 0.108 | 0.016 | 0.124 | 114.545 | 131.818 | 106.364 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.136 | 0.136 | 0.110 | 0.110 | 0.015 | 0.123 | 123.636 | 123.636 | 100.000 |
| 0.113 | 0.113 | 0.082 | 0.082 | 0.018 | 0.098 | 102.727 | 102.727 | 74.545 |
| 0.035 | 0.010 | 0.019 | 0.016 | 0.011 | 0.020 | 31.818 | 9.091 | 17.273 |
| 0.025 | 0.014 | 0.013 | 0.029 | 0.008 | 0.020 | 22.727 | 12.727 | 11.818 |
| 0.009 | 0.007 | 0.070 | 0.040 | 0.030 | 0.032 | 8.182 | 6.364 | 63.636 |
| 0.008 | 0.005 | 0.005 | 0.005 | 0.002 | 0.006 | 7.273 | 4.545 | 4.545 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.261 | 0.246 | 0.246 | 0.244 | 0.008 | 0.249 | 237.273 | 223.636 | 223.636 |
| 0.214 | 0.174 | 0.100 | 0.094 | 0.058 | 0.146 | 194.545 | 158.182 | 90.909 |
| 0.094 | 0.064 | 0.123 | 0.102 | 0.024 | 0.096 | 85.455 | 58.182 | 111.818 |
| 0.048 | 0.061 | 0.035 | 0.059 | 0.012 | 0.051 | 43.636 | 55.455 | 31.818 |
| 0.067 | 0.091 | 0.025 | 0.027 | 0.032 | 0.053 | 60.909 | 82.727 | 22.727 |
| 0.085 | 0.055 | 0.052 | 0.040 | 0.019 | 0.058 | 77.273 | 50.000 | 47.273 |
| 0.055 | 0.070 | 0.084 | 0.059 | 0.013 | 0.067 | 50.000 | 63.636 | 76.364 |
| 0.010 | 0.036 | 0.011 | 0.024 | 0.012 | 0.020 | 9.091 | 32.727 | 10.000 |
| 0.065 | 0.078 | 0.089 | 0.082 | 0.010 | 0.079 | 59.091 | 70.909 | 80.909 |
| 0.093 | 0.128 | 0.130 | 0.152 | 0.024 | 0.126 | 84.545 | 116.364 | 118.182 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.045 | 0.045 | 0.031 | 0.031 | 0.008 | 0.038 | 40.909 | 40.909 | 28.182 |
| 0.022 | 0.022 | 0.016 | 0.019 | 0.003 | 0.020 | 20.000 | 20.000 | 14.545 |
| 0.256 | 0.239 | 0.272 | 0.282 | 0.019 | 0.262 | 232.727 | 217.273 | 247.273 |
| 0.249 | 0.203 | 0.198 | 0.298 | 0.047 | 0.237 | 226.364 | 184.545 | 180.000 |
| 0.198 | 0.183 | 0.217 | 0.214 | 0.016 | 0.203 | 180.000 | 166.364 | 197.273 |
| 0.137 | 0.099 | 0.135 | 0.158 | 0.024 | 0.132 | 124.545 | 90.000 | 122.727 |
| 0.122 | 0.136 | 0.144 | 0.175 | 0.022 | 0.144 | 110.909 | 123.636 | 130.909 |
| 0.161 | 0.133 | 0.135 | 0.185 | 0.025 | 0.154 | 146.364 | 120.909 | 122.727 |
| 0.136 | 0.134 | 0.282 | 0.316 | 0.096 | 0.217 | 123.636 | 121.818 | 256.364 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.074 | 0.074 | 0.131 | 0.131 | 0.033 | 0.103 | 67.273 | 67.273 | 119.091 |
| 0.403 | 0.403 | 0.520 | 0.490 | 0.060 | 0.454 | 366.364 | 366.364 | 472.727 |
| 0.399 | 0.170 | 0.494 | 0.067 | 0.198 | 0.283 | 362.727 | 154.545 | 449.091 |
| 0.500 | 0.413 | 0.392 | 0.392 | 0.051 | 0.424 | 454.545 | 375.455 | 356.364 |
| 0.415 | 0.500 | 0.396 | 0.476 | 0.049 | 0.447 | 377.273 | 454.545 | 360.000 |
| 0.324 | 0.329 | 0.348 | 0.362 | 0.018 | 0.341 | 294.545 | 299.091 | 316.364 |
| 0.319 | 0.282 | 0.332 | 0.339 | 0.025 | 0.318 | 290.000 | 256.364 | 301.818 |

| Concentration (cytoplasm fraction) | | | Volumetric Total Protein Concentration (cytoplasmi | | | | |
|---|---|---|---|---|---|---|---|
| Buffer D [ug/ml] | Buffer Average [ug/ml] | Buffer Deviation [ug/ml] | Culture Ass A [mg/L] | Culture Ass B [mg/L] | Culture Ass C [mg/L] | Culture Ass D [mg/L] | Average[ mg/L] |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 35.455 | 33.636 | 2.099 | 2.494 | 2.494 | 2.779 | 2.779 | 2.637 |
| 23.636 | 37.727 | 16.271 | 5.227 | 5.227 | 2.384 | 2.384 | 3.806 |
| 12.727 | 17.727 | 5.774 | 2.181 | 2.181 | 1.221 | 1.221 | 1.701 |
| 44.545 | 57.500 | 17.696 | 27.794 | 28.497 | 15.480 | 17.239 | 22.253 |
| 140.909 | 142.727 | 7.003 | 34.308 | 38.276 | 37.809 | 36.175 | 36.642 |
| 102.727 | 146.364 | 33.336 | 29.226 | 23.883 | 30.325 | 17.755 | 25.297 |
| 169.091 | 134.773 | 26.142 | 19.534 | 15.520 | 19.400 | 24.886 | 19.835 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 70.000 | 85.000 | 17.321 | 1.589 | 1.589 | 1.112 | 1.112 | 1.351 |
| 30.909 | 22.727 | 9.448 | 1.245 | 1.245 | 2.646 | 2.646 | 1.945 |
| 46.364 | 37.727 | 9.972 | 4.852 | 4.852 | 7.733 | 7.733 | 6.293 |
| 40.909 | 50.682 | 8.178 | 7.358 | 7.226 | 8.803 | 5.912 | 7.325 |
| 69.091 | 97.045 | 31.678 | 20.562 | 14.289 | 26.312 | 13.243 | 18.602 |
| 30.000 | 15.455 | 10.523 | 2.098 | 2.360 | 4.720 | 8.654 | 4.458 |
| 29.091 | 41.364 | 15.998 | 24.192 | 14.651 | 12.266 | 10.903 | 15.503 |
| 183.636 | 133.409 | 70.102 | 40.822 | 36.499 | 107.577 | 97.011 | 70.477 |
| 355.455 | 308.409 | 79.539 | 125.779 | 192.891 | 110.668 | 173.779 | 150.779 |
| 190.000 | 237.727 | 60.841 | 101.910 | 107.828 | 65.420 | 68.707 | 85.966 |
| 50.000 | 95.227 | 45.433 | 40.565 | 44.305 | 19.851 | 15.823 | 30.136 |
| 140.909 | 156.364 | 37.365 | 51.323 | 46.549 | 29.362 | 37.000 | 41.059 |
| 140.909 | 156.364 | 37.365 | 27.115 | 24.593 | 15.512 | 19.548 | 21.692 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 31.818 | 27.045 | 8.178 | 2.135 | 2.922 | 4.383 | 3.933 | 3.343 |
| 11.818 | 15.227 | 4.477 | 2.665 | 2.665 | 4.264 | 2.309 | 2.976 |
| 60.000 | 34.318 | 19.319 | 3.427 | 8.266 | 5.444 | 13.307 | 7.611 |
| 214.545 | 234.545 | 34.015 | 61.921 | 83.997 | 68.382 | 63.536 | 69.459 |
| 235.455 | 249.773 | 22.907 | 64.289 | 78.519 | 70.642 | 65.814 | 69.816 |
| 236.364 | 240.000 | 3.235 | 82.068 | 80.531 | 82.068 | 79.917 | 81.146 |
| 213.636 | 219.318 | 4.223 | 72.278 | 70.809 | 71.396 | 69.046 | 70.882 |
| 164.545 | 218.182 | 49.964 | 71.231 | 60.756 | 49.350 | 42.134 | 55.868 |
| 176.364 | 162.727 | 31.255 | 33.306 | 23.768 | 21.952 | 29.370 | 27.099 |
| 150.909 | 148.864 | 13.973 | 15.764 | 14.967 | 12.576 | 14.702 | 14.502 |
| 127.273 | 146.364 | 22.044 | 12.099 | 12.099 | 9.307 | 9.307 | 10.703 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 31.818 | 41.818 | 6.923 | 5.417 | 4.896 | 5.208 | 3.646 | 4.792 |
| 147.273 | 145.909 | 17.032 | 25.294 | 33.725 | 30.915 | 30.353 | 30.072 |
| 154.545 | 157.955 | 8.668 | 39.730 | 35.364 | 39.511 | 37.110 | 37.929 |
| 166.364 | 179.545 | 11.269 | 27.684 | 26.378 | 25.202 | 23.897 | 25.790 |
| 175.455 | 199.773 | 23.792 | 32.570 | 30.608 | 26.553 | 25.245 | 28.744 |
| 170.000 | 150.455 | 22.569 | 17.316 | 17.316 | 22.487 | 22.487 | 19.902 |
| 131.818 | 141.818 | 11.547 | 11.537 | 11.537 | 10.017 | 10.017 | 10.777 |
| 133.636 | 132.273 | 1.575 | 6.155 | 6.155 | 6.283 | 6.283 | 6.219 |
| 127.273 | 128.182 | 1.050 | 3.367 | 3.367 | 3.320 | 3.320 | 3.343 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 20.000 | 20.000 | 0.000 | 2.128 | 2.128 | 2.128 | 2.128 | 2.128 |
| 3.636 | 3.636 | 0.000 | 0.622 | 0.622 | 0.622 | 0.622 | 0.622 |
| 16.364 | 30.000 | 9.091 | 7.325 | 7.325 | 7.325 | 3.470 | 6.361 |
| 33.636 | 49.318 | 14.666 | 18.533 | 12.680 | 12.680 | 9.023 | 13.229 |
| 68.182 | 66.591 | 27.817 | 15.391 | 44.594 | 26.046 | 29.598 | 28.907 |
| 82.727 | 57.045 | 24.712 | 35.092 | 16.463 | 17.763 | 39.424 | 27.185 |
| 94.545 | 102.045 | 8.732 | 38.497 | 39.566 | 44.913 | 37.071 | 40.012 |
| 165.455 | 128.182 | 32.954 | 22.946 | 23.154 | 33.584 | 37.965 | 29.412 |
| 240.000 | 187.727 | 56.775 | 30.198 | 51.738 | 31.204 | 53.148 | 41.572 |
| 115.455 | 175.682 | 65.418 | 21.707 | 38.944 | 42.456 | 20.270 | 30.844 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 140.909 | 139.091 | 2.099 | 2.076 | 2.076 | 2.131 | 2.131 | 2.104 |
| 108.182 | 79.091 | 33.591 | 3.473 | 3.473 | 7.514 | 7.514 | 5.494 |
| 41.818 | 41.818 | 0.000 | 5.436 | 5.436 | 5.436 | 5.436 | 5.436 |
| 23.636 | 31.591 | 8.144 | 7.613 | 5.021 | 5.669 | 4.212 | 5.629 |
| 110.909 | 122.045 | 8.007 | 18.866 | 19.273 | 18.187 | 16.559 | 18.221 |
| 69.091 | 65.000 | 16.203 | 11.043 | 14.154 | 7.466 | 11.821 | 11.121 |
| 131.818 | 128.864 | 34.984 | 29.895 | 18.057 | 36.717 | 29.093 | 28.440 |
| 136.364 | 140.682 | 10.295 | 35.653 | 41.722 | 41.216 | 37.929 | 39.130 |
| 198.182 | 181.136 | 53.989 | 38.805 | 31.458 | 62.686 | 50.057 | 45.751 |
| 168.182 | 184.091 | 26.405 | 44.633 | 58.205 | 60.293 | 48.287 | 52.854 |
| 190.000 | 174.091 | 50.532 | 31.106 | 42.128 | 63.192 | 51.191 | 46.904 |
| 197.273 | 133.182 | 50.998 | 22.830 | 20.892 | 35.753 | 46.737 | 31.553 |
| 132.727 | 75.682 | 42.312 | 5.147 | 12.352 | 9.999 | 21.469 | 12.242 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 60.909 | 76.364 | 17.845 | 9.693 | 9.693 | 6.430 | 6.430 | 8.061 |
| 19.091 | 24.318 | 16.235 | 6.040 | 2.279 | 1.481 | 2.393 | 3.048 |
| 18.182 | 24.773 | 10.375 | 8.862 | 5.035 | 4.028 | 4.028 | 5.489 |
| 98.182 | 74.091 | 22.175 | 17.551 | 9.729 | 14.308 | 20.604 | 15.548 |
| 66.364 | 75.682 | 8.110 | 19.831 | 23.302 | 21.319 | 18.096 | 20.637 |
| 107.273 | 138.182 | 31.166 | 55.923 | 55.006 | 38.810 | 36.060 | 46.450 |
| 67.273 | 85.909 | 17.838 | 39.568 | 55.211 | 45.089 | 34.047 | 43.479 |
| 110.909 | 123.864 | 10.079 | 36.190 | 32.767 | 34.478 | 29.832 | 33.317 |
| 148.182 | 124.318 | 22.689 | 28.661 | 29.929 | 38.806 | 41.343 | 34.685 |
| 123.636 | 132.045 | 14.889 | 31.350 | 24.672 | 26.527 | 25.229 | 26.944 |
| 82.727 | 107.727 | 34.019 | 23.770 | 27.166 | 14.092 | 15.451 | 20.120 |
| 0.000 | 0.000 | 0.000 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| 31.818 | 25.682 | 4.288 | 0.039 | 0.039 | 0.044 | 0.055 | 0.045 |
| 132.727 | 183.409 | 72.242 | 33.750 | 33.750 | 15.125 | 18.250 | 25.219 |
| 200.909 | 194.318 | 9.632 | 37.567 | 37.567 | 34.121 | 38.084 | 36.835 |
| 232.727 | 217.500 | 30.346 | 54.825 | 39.362 | 46.591 | 51.411 | 48.047 |
| 198.182 | 200.227 | 9.223 | 40.450 | 36.491 | 37.180 | 37.524 | 37.911 |
| 117.273 | 116.364 | 8.232 | 18.882 | 22.463 | 20.998 | 20.998 | 20.835 |
| 136.364 | 123.636 | 13.747 | 19.273 | 18.656 | 22.819 | 23.128 | 20.969 |
| 87.273 | 93.636 | 7.533 | 14.232 | 16.367 | 14.374 | 13.663 | 14.659 |
| 76.364 | 67.500 | 20.088 | 4.622 | 10.104 | 8.169 | 9.029 | 7.981 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 49.091 | 66.364 | 19.945 | 17.860 | 17.860 | 10.483 | 10.483 | 14.171 |
| 111.818 | 100.000 | 24.269 | 18.755 | 27.068 | 15.468 | 23.782 | 21.268 |
| 142.727 | 148.864 | 31.134 | 29.075 | 47.556 | 35.611 | 35.385 | 36.907 |
| 209.091 | 205.682 | 29.208 | 40.708 | 38.938 | 53.097 | 45.231 | 44.493 |
| 143.636 | 134.318 | 7.249 | 30.413 | 32.601 | 31.726 | 34.570 | 32.328 |
| 190.000 | 160.682 | 21.338 | 37.170 | 42.685 | 39.568 | 50.119 | 42.386 |
| 148.182 | 127.955 | 21.195 | 18.877 | 22.248 | 26.294 | 27.473 | 23.723 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 23.636 | 24.091 | 0.525 | 2.264 | 2.264 | 2.180 | 2.180 | 2.222 |
| 6.364 | 10.455 | 6.983 | 0.964 | 0.964 | 2.773 | 0.844 | 1.386 |
| 100.000 | 97.955 | 12.929 | 33.864 | 25.864 | 25.864 | 29.331 | 28.731 |
| 67.273 | 81.364 | 16.623 | 22.992 | 22.992 | 31.751 | 20.255 | 24.498 |
| 146.364 | 129.318 | 14.110 | 22.894 | 19.821 | 19.974 | 24.737 | 21.857 |
| 98.182 | 112.727 | 14.374 | 17.523 | 20.165 | 16.271 | 15.020 | 17.245 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 100.000 | 111.818 | 13.646 | 3.652 | 3.652 | 2.954 | 2.954 | 3.303 |
| 74.545 | 88.636 | 16.271 | 4.143 | 4.143 | 3.007 | 3.007 | 3.575 |
| 14.545 | 18.182 | 9.706 | 4.682 | 1.338 | 2.542 | 2.140 | 2.675 |
| 26.364 | 18.409 | 7.249 | 3.493 | 1.956 | 1.817 | 4.052 | 2.830 |
| 36.364 | 28.636 | 27.075 | 1.341 | 1.043 | 10.430 | 5.960 | 4.694 |
| 4.545 | 5.227 | 1.364 | 1.356 | 0.848 | 0.848 | 0.848 | 0.975 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 221.818 | 226.591 | 7.173 | 13.218 | 12.458 | 12.458 | 12.357 | 12.623 |
| 85.455 | 132.273 | 53.079 | 52.718 | 42.864 | 24.634 | 23.156 | 35.843 |
| 92.727 | 87.045 | 22.223 | 27.307 | 18.592 | 35.732 | 29.632 | 27.816 |
| 53.636 | 46.136 | 10.868 | 14.415 | 18.318 | 10.511 | 17.718 | 15.240 |
| 24.545 | 47.727 | 29.218 | 21.643 | 29.396 | 8.076 | 8.722 | 16.959 |
| 36.364 | 52.727 | 17.392 | 40.514 | 26.215 | 24.785 | 19.065 | 27.645 |
| 53.636 | 60.909 | 11.807 | 29.574 | 37.640 | 45.168 | 31.725 | 36.027 |
| 21.818 | 18.409 | 11.168 | 4.455 | 16.038 | 4.901 | 10.692 | 9.021 |
| 74.545 | 71.364 | 9.166 | 16.348 | 19.617 | 22.384 | 20.623 | 19.743 |
| 138.182 | 114.318 | 22.174 | 30.634 | 42.162 | 42.821 | 50.068 | 41.421 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 28.182 | 34.545 | 7.348 | 2.316 | 2.316 | 1.596 | 1.596 | 1.956 |
| 17.273 | 17.955 | 2.611 | 2.651 | 2.651 | 1.928 | 2.290 | 2.380 |
| 256.364 | 238.409 | 17.127 | 26.329 | 24.580 | 27.974 | 29.003 | 26.972 |
| 270.909 | 215.455 | 42.452 | 59.037 | 48.131 | 46.945 | 70.655 | 56.192 |
| 194.545 | 184.545 | 14.297 | 44.747 | 41.357 | 49.041 | 48.363 | 45.877 |
| 143.636 | 120.227 | 22.260 | 22.242 | 16.072 | 21.917 | 25.651 | 21.471 |
| 159.091 | 131.136 | 20.387 | 23.515 | 26.214 | 27.756 | 33.731 | 27.804 |
| 168.182 | 139.545 | 22.336 | 24.053 | 19.870 | 20.169 | 27.638 | 22.932 |
| 287.273 | 197.273 | 87.001 | 12.670 | 12.484 | 26.272 | 29.439 | 20.216 |
| 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 119.091 | 93.182 | 29.917 | 1.951 | 1.951 | 3.454 | 3.454 | 2.702 |
| 445.455 | 412.727 | 54.682 | 18.120 | 18.120 | 23.380 | 22.031 | 20.413 |
| 60.909 | 256.818 | 179.838 | 57.946 | 24.689 | 71.742 | 9.730 | 41.027 |
| 356.364 | 385.682 | 46.783 | 54.752 | 45.225 | 42.926 | 42.926 | 46.457 |
| 432.727 | 406.136 | 44.767 | 52.311 | 63.025 | 49.916 | 60.000 | 56.313 |
| 329.091 | 309.773 | 15.944 | 43.012 | 43.676 | 46.198 | 48.056 | 45.235 |
| 308.182 | 289.091 | 23.082 | 73.294 | 64.792 | 76.280 | 77.889 | 73.064 |

| ic fraction) | Specific Total Protein Concentration (cytoplasmic fraction) | | Glucose consumption | Volumetric HBsAg in Supernatant |
|---|---|---|---|---|
| Deviation [mg/L] | Average [mg/gDW] | Deviation [mg/gDW] | [g/l] | [ug/L] |
| 0.000 | 0.000 | 0.000 | **35.056** | NM |
| 0.165 | 0.841 | 0.052 | **28.508** | NM |
| 1.641 | 1.161 | 0.501 | **22.512** | NM |
| 0.554 | 0.443 | 0.144 | **20.289** | NM |
| 6.848 | 2.875 | 0.885 | **2.115** | NM |
| 1.798 | 5.709 | 0.280 | **0.168** | NM |
| 5.762 | 4.170 | 0.950 | **0.164** | NM |
| 3.847 | 3.361 | 0.652 | **0.058** | NM |
| 0.000 | 0.000 | 0.000 | **35.056** | NM |
| 0.275 | 2.833 | 0.577 | **30.833** | NM |
| 0.809 | 1.258 | 0.523 | **28.162** | NM |
| 1.663 | 1.480 | 0.391 | **21.397** | NM |
| 1.182 | 1.256 | 0.203 | **9.929** | NM |
| 6.072 | 2.954 | 0.964 | **3.708** | NM |
| 3.036 | 0.484 | 0.330 | **2.597** | NM |
| 5.996 | 1.332 | 0.515 | **2.313** | NM |
| 37.033 | 5.468 | 2.873 | **0.211** | NM |
| 38.886 | 14.479 | 3.734 | **0.073** | NM |
| 22.001 | 10.980 | 2.810 | **0.064** | **253.419** |
| 14.378 | 4.657 | 2.222 | **0.060** | **324.000** |
| 9.811 | 7.376 | 1.763 | **0.058** | **627.230** |
| 5.184 | 3.448 | 0.824 | **0.049** | **968.725** |
| 0.000 | 0.000 | 0.000 | **35.056** | NM |
| 1.011 | 0.628 | 0.190 | **26.947** | NM |
| 0.875 | 0.441 | 0.130 | **14.695** | NM |
| 4.284 | 0.919 | 0.517 | **7.264** | NM |
| 10.073 | 6.701 | 0.972 | **0.951** | NM |
| 6.403 | 5.430 | 0.498 | **0.347** | NM |
| 1.094 | 7.306 | 0.098 | **0.313** | NM |
| 1.365 | 6.974 | 0.134 | **0.068** | NM |
| 12.794 | 6.261 | 1.434 | **0.050** | NM |
| 5.205 | 4.931 | 0.947 | **0.038** | NM |
| 1.361 | 4.518 | 0.424 | **0.036** | NM |
| 1.612 | 3.659 | 0.551 | **0.018** | NM |
| 0.000 | 0.000 | 0.000 | **35.056** | NM |
| 0.793 | 1.045 | 0.173 | **20.165** | NM |
| 3.510 | 3.648 | 0.426 | **0.602** | NM |
| 2.081 | 3.949 | 0.217 | **0.217** | NM |
| 1.619 | 4.489 | 0.282 | **0.142** | NM |
| 3.423 | 5.708 | 0.680 | **0.130** | NM |
| 2.985 | 4.179 | 0.627 | **0.132** | NM |
| 0.877 | 3.732 | 0.304 | **0.126** | NM |
| 0.074 | 3.307 | 0.039 | **0.083** | NM |
| 0.027 | 3.205 | 0.026 | **0.059** | NM |

| | | | | |
|---|---|---|---|---|
| 0.000 | 0.000 | 0.000 | **35.056** | **NM** |
| 0.000 | 0.667 | 0.000 | **20.984** | **NM** |
| 0.000 | 0.182 | 0.000 | **13.090** | **NM** |
| 1.928 | 1.504 | 0.456 | **7.085** | **NM** |
| 3.934 | 2.740 | 0.815 | **1.744** | **NM** |
| 12.075 | 3.459 | 1.445 | **0.621** | **NM** |
| 11.777 | 3.034 | 1.314 | **0.030** | **NM** |
| 3.424 | 3.362 | 0.288 | **0.027** | **NM** |
| 7.562 | 4.012 | 1.031 | **0.022** | **NM** |
| 12.573 | 6.095 | 1.843 | **0.019** | **NM** |
| 11.485 | 4.551 | 1.695 | **0.014** | **NM** |
| 0.000 | 0.000 | 0.000 | **NM** | **NM** |
| 0.032 | 3.477 | 0.052 | **NM** | **NM** |
| 2.333 | 1.977 | 0.840 | **NM** | **NM** |
| 0.000 | 1.095 | 0.000 | **NM** | **NM** |
| 1.451 | 0.949 | 0.245 | **NM** | **NM** |
| 1.196 | 2.700 | 0.177 | **NM** | **NM** |
| 2.772 | 1.625 | 0.405 | **NM** | **NM** |
| 7.721 | 3.391 | 0.921 | **NM** | **NM** |
| 2.864 | 4.582 | 0.335 | **NM** | **NM** |
| 13.637 | 5.220 | 1.556 | **NM** | **NM** |
| 7.581 | 6.575 | 0.943 | **NM** | **NM** |
| 13.614 | 5.962 | 1.731 | **NM** | **NM** |
| 12.082 | 4.756 | 1.821 | **NM** | **NM** |
| 6.844 | 2.032 | 1.136 | **NM** | **NM** |
| 0.000 | 0.000 | 0.000 | **NM** | **NM** |
| 1.884 | 1.991 | 0.465 | **NM** | **NM** |
| 2.035 | 0.496 | 0.331 | **NM** | **NM** |
| 2.299 | 0.826 | 0.346 | **NM** | **NM** |
| 4.653 | 1.616 | 0.484 | **NM** | **NM** |
| 2.211 | 2.024 | 0.217 | **NM** | **NM** |
| 10.477 | 4.245 | 0.957 | **NM** | **NM** |
| 9.028 | 3.923 | 0.815 | **NM** | **NM** |
| 2.711 | 3.817 | 0.311 | **NM** | **NM** |
| 6.330 | 4.144 | 0.756 | **NM** | **NM** |
| 3.038 | 3.773 | 0.425 | **NM** | **NM** |
| 6.354 | 3.168 | 1.001 | **NM** | **NM** |
| #DIV/0! | #DIV/0! | #DIV/0! | **NM** | **NM** |
| 0.007 | 0.055 | 0.009 | **NM** | **NM** |
| 9.933 | 6.368 | 2.508 | **NM** | **NM** |
| 1.826 | 4.594 | 0.228 | **NM** | **NM** |
| 6.704 | 6.360 | 0.887 | **NM** | **NM** |
| 1.746 | 5.616 | 0.259 | **NM** | **NM** |
| 1.474 | 3.145 | 0.222 | **NM** | **NM** |
| 2.332 | 3.288 | 0.366 | **NM** | **NM** |
| 1.179 | 2.362 | 0.190 | **NM** | **NM** |
| 2.375 | 1.552 | 0.462 | **NM** | **NM** |
| 0.000 | 0.000 | 0.000 | **NM** | **NM** |
| 4.259 | 2.898 | 0.871 | **NM** | **NM** |
| 5.162 | 3.268 | 0.793 | **NM** | **NM** |
| 7.719 | 4.637 | 0.970 | **NM** | **NM** |
| 6.318 | 6.103 | 0.867 | **NM** | **NM** |
| 1.745 | 4.608 | 0.249 | **NM** | **NM** |
| 5.629 | 6.086 | 0.808 | **NM** | **NM** |
| 3.930 | 3.943 | 0.653 | **NM** | **NM** |

| | | | | |
|---|---|---|---|---|
| 0.000 | 0.000 | 0.000 | NM | NM |
| 0.048 | 0.602 | 0.013 | NM | NM |
| 0.926 | 0.253 | 0.169 | NM | NM |
| 3.792 | 3.641 | 0.481 | NM | NM |
| 5.005 | 2.214 | 0.452 | NM | NM |
| 2.385 | 3.320 | 0.362 | NM | NM |
| 2.199 | 3.072 | 0.392 | NM | NM |
| 0.000 | 0.000 | 0.000 | NM | NM |
| 0.000 | 0.000 | 0.000 | NM | NM |
| 0.403 | 2.795 | 0.341 | NM | NM |
| 0.656 | 2.216 | 0.407 | NM | NM |
| 1.428 | 0.560 | 0.299 | NM | NM |
| 1.114 | 0.506 | 0.199 | NM | NM |
| 4.438 | 0.777 | 0.735 | NM | NM |
| 0.254 | 0.139 | 0.036 | NM | NM |
| 0.000 | 0.000 | 0.000 | **35.056** | NM |
| 0.400 | 6.898 | 0.218 | **23.200** | NM |
| 14.383 | 4.027 | 1.616 | **6.784** | NM |
| 7.102 | 2.902 | 0.741 | **2.092** | NM |
| 3.590 | 1.538 | 0.362 | **0.940** | NM |
| 10.382 | 1.591 | 0.974 | **0.404** | NM |
| 9.119 | 2.343 | 0.773 | **0.215** | NM |
| 6.983 | 3.045 | 0.590 | **0.107** | NM |
| 5.473 | 0.758 | 0.460 | **0.056** | NM |
| 2.536 | 1.459 | 0.187 | **0.014** | NM |
| 8.034 | 2.858 | 0.554 | **0.005** | NM |
| 0.000 | 0.000 | 0.000 | NM | NM |
| 0.416 | 0.864 | 0.184 | NM | NM |
| 0.346 | 0.598 | 0.087 | NM | NM |
| 1.938 | 5.960 | 0.428 | NM | NM |
| 11.072 | 7.750 | 1.527 | NM | NM |
| 3.554 | 6.277 | 0.486 | NM | NM |
| 3.975 | 3.420 | 0.633 | NM | NM |
| 4.323 | 4.430 | 0.689 | NM | NM |
| 3.671 | 3.898 | 0.624 | NM | NM |
| 8.916 | 4.932 | 2.175 | NM | NM |
| 0.000 | 0.000 | 0.000 | NM | NM |
| 0.868 | 2.330 | 0.748 | NM | NM |
| 2.704 | 10.318 | 1.367 | NM | NM |
| 28.729 | 12.841 | 8.992 | NM | NM |
| 5.635 | 12.856 | 1.559 | NM | NM |
| 6.207 | 13.538 | 1.492 | NM | NM |
| 2.328 | 10.326 | 0.531 | NM | NM |
| 5.834 | 7.845 | 0.626 | NM | NM |

| Agitation | $dO_2$ | Time | Dry Weight | $O_2$ in | $O_2$ out | $dO_2$ | pH |
|---|---|---|---|---|---|---|---|
| rpm | [%] | [hrs] | Average [$g_{DW}/L$] | [%] | [%] | [%] | pH |
| 350 | 50 | 0 | 0.000 | 8.229 | 8.229 | 19.311 | 6.500 |
| 350 | 50 | 20 | 5.609 | 12.996 | 4.658 | 50.310 | 4.470 |
| 350 | 50 | 40 | 7.334 | 23.242 | 17.050 | 56.437 | 4.330 |
| 350 | 50 | 60 | 7.012 | 55.525 | 53.449 | 50.275 | 4.690 |
| 350 | 50 | 80 | 6.498 | 57.066 | 56.215 | 38.142 | 6.615 |
| 350 | 50 | 100 | 5.896 | 19.428 | 18.764 | 50.988 | 7.110 |
| 350 | 50 | 120 | 6.053 | 14.931 | 14.641 | 49.632 | 7.453 |
| 350 | 50 | 140 | 6.211 | 12.475 | 12.203 | 50.028 | 7.365 |
| 100 | 50 | 0 | 0.000 | 17.289 | 17.261 | 47.895 | 6.500 |
| 100 | 50 | 20 | 2.868 | 58.737 | 51.889 | 52.340 | 6.394 |
| 100 | 50 | 40 | 6.912 | 102.881 | 96.913 | 50.500 | 5.848 |
| 100 | 50 | 60 | 13.680 | 98.420 | 94.960 | 50.265 | 5.138 |
| 100 | 50 | 80 | 10.109 | 97.310 | 95.399 | 49.118 | 5.492 |
| 100 | 50 | 100 | 6.897 | 41.785 | 40.988 | 48.059 | 6.444 |
| 100 | 50 | 120 | 6.474 | 38.775 | 38.649 | 50.059 | 7.215 |
| 100 | 50 | 140 | 6.357 | 66.982 | 66.953 | 48.059 | 7.845 |
| 100 | 50 | 160 | 6.334 | 44.824 | 44.367 | 52.029 | 8.060 |
| 600 | 50 | 0 | 0.000 | 33.675 | 33.625 | 50.029 | 8.459 |
| 600 | 50 | 20 | 5.825 | 43.308 | 30.799 | 45.059 | 8.496 |
| 600 | 50 | 40 | 11.915 | 100.259 | 97.570 | 49.059 | 8.439 |
| 600 | 50 | 60 | 11.058 | 23.198 | 22.750 | 47.059 | 8.056 |
| 600 | 50 | 80 | 6.906 | 15.149 | 14.740 | 51.162 | 7.992 |
| 600 | 50 | 100 | 4.127 | 13.219 | 12.976 | 50.868 | 6.500 |
| 600 | 50 | 120 | 2.837 | 21.365 | 21.344 | 45.981 | 3.559 |
| 350 | 95 | 0 | 0.000 | 44.000 | 43.978 | 46.953 | 3.550 |
| 350 | 95 | 20 | 2.655 | 49.878 | 46.351 | 52.870 | 3.920 |
| 350 | 95 | 40 | 6.864 | 21.478 | 17.978 | 49.131 | 4.250 |
| 350 | 95 | 60 | 7.770 | 19.488 | 18.064 | 49.249 | 4.594 |
| 350 | 95 | 80 | 4.592 | 16.059 | 15.525 | 49.632 | 5.270 |
| 350 | 95 | 100 | 3.292 | 15.146 | 14.714 | 49.926 | 6.217 |
| 350 | 95 | 120 | 1.653 | 14.136 | 13.992 | 49.896 | 6.674 |
| 350 | 95 | 140 | 0.710 | 8.428 | 8.147 | 48.837 | 6.981 |
| 350 | 2 | 0 | 0.000 | 7.696 | 7.688 | 55.607 | 6.700 |
| 350 | 2 | 20 | 1.845 | 19.341 | 16.860 | 55.519 | 6.808 |
| 350 | 2 | 40 | 3.172 | 82.851 | 79.513 | 31.388 | 6.500 |
| 350 | 2 | 60 | 4.338 | 97.227 | 94.687 | 100.384 | 5.848 |
| 350 | 2 | 80 | 8.320 | 88.807 | 87.076 | 92.277 | 5.981 |
| 350 | 2 | 100 | 9.304 | 88.999 | 88.465 | 90.245 | 5.970 |
| 350 | 2 | 120 | 7.294 | 22.287 | 22.111 | 95.963 | 6.562 |
| 350 | 2 | 140 | 6.800 | 16.090 | 15.980 | 96.364 | 6.360 |
| 250 | 20 | 0 | 0.000 | 22.647 | 22.624 | 89.140 | 6.173 |
| 250 | 20 | 20 | 4.639 | 42.779 | 42.144 | 90.197 | 6.299 |
| 250 | 20 | 40 | 6.926 | 61.496 | 60.971 | 95.160 | 6.523 |
| 250 | 20 | 60 | 8.463 | 15.130 | 14.780 | 94.749 | 6.731 |
| 250 | 20 | 80 | 8.756 | 3.773 | 3.498 | 1.825 | 6.500 |
| 250 | 20 | 100 | 7.828 | 28.515 | 28.371 | 1.500 | 5.180 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **250** | **20** | **120** | 6.745 | 55.640 | 55.345 | 1.119 | 4.240 |
| **250** | **20** | **140** | 6.126 | 9.774 | 9.742 | 2.824 | 4.960 |
| **250** | **20** | **160** | 6.084 | 22.194 | 22.030 | 2.088 | 6.150 |
| **250** | **80** | **0** | 0.000 | 21.751 | 21.688 | 1.531 | 6.855 |
| **250** | **80** | **20** | 5.977 | 27.375 | 22.349 | 1.707 | 7.247 |
| **250** | **80** | **40** | 10.075 | 25.054 | 23.553 | 1.354 | 6.656 |
| **250** | **80** | **60** | 10.895 | 17.393 | 16.631 | 2.118 | 6.900 |
| **250** | **80** | **80** | 8.576 | 8.134 | 8.037 | 1.500 | 7.438 |
| **250** | **80** | **100** | 7.850 | 19.937 | 19.858 | 2.059 | 6.971 |
| **250** | **80** | **120** | 6.300 | 13.909 | 13.878 | 18.039 | 6.500 |
| **450** | **20** | **0** | 0.000 | 41.628 | 41.614 | 16.991 | 6.376 |
| **450** | **20** | **20** | 4.391 | 29.392 | 24.657 | 20.804 | 3.830 |
| **450** | **20** | **40** | 7.453 | 24.392 | 23.633 | 20.017 | 3.747 |
| **450** | **20** | **60** | 6.499 | 24.283 | 24.007 | 20.026 | 3.879 |
| **450** | **20** | **80** | 6.387 | 22.720 | 22.547 | 20.043 | 4.010 |
| **450** | **20** | **100** | 6.305 | 22.443 | 22.349 | 18.034 | 4.076 |
| **450** | **20** | **120** | 5.818 | 22.923 | 22.804 | 25.043 | 4.141 |
| **450** | **20** | **140** | 5.287 | 23.371 | 23.335 | 20.034 | 4.194 |
| **450** | **20** | **0** | 0.000 | 22.583 | 22.571 | 15.026 | 4.484 |
| **450** | **20** | **20** | 1.807 | 23.989 | 22.621 | 13.026 | 4.696 |
| **450** | **20** | **40** | 4.813 | 50.885 | 50.801 | 24.034 | 5.369 |
| **450** | **20** | **60** | 5.219 | 22.947 | 22.906 | 19.043 | 7.043 |
| **450** | **20** | **80** | 4.259 | 20.673 | 20.277 | 10.718 | 7.694 |
| **450** | **20** | **100** | 4.178 | 50.764 | 50.744 | 81.270 | 6.500 |
| **450** | **80** | **0** | 0.000 | 83.491 | 83.442 | 82.026 | 6.090 |
| **450** | **80** | **20** | 5.759 | 89.958 | 84.755 | 79.034 | 4.397 |
| **450** | **80** | **40** | 7.385 | 96.112 | 93.350 | 75.043 | 4.910 |
| **450** | **80** | **60** | 7.334 | 96.616 | 94.730 | 85.034 | 6.775 |
| **450** | **80** | **80** | 6.680 | 96.041 | 94.942 | 80.043 | 7.002 |
| **450** | **80** | **100** | 5.956 | 94.054 | 94.024 | 74.043 | 5.344 |
| **350** | **25** | **0** | 0.000 | 95.495 | 95.430 | 25.500 | 4.593 |
| **350** | **25** | **20** | 3.095 | 94.586 | 86.179 | 81.206 | 6.193 |
| **350** | **25** | **40** | 6.752 | 78.861 | 73.123 | 80.966 | 6.386 |
| **350** | **25** | **60** | 9.640 | 21.461 | 18.906 | 91.043 | 6.222 |
| **350** | **25** | **80** | 9.514 | 22.032 | 21.275 | 66.311 | 7.631 |
| **350** | **25** | **100** | 6.626 | 7.938 | 7.646 | 16.690 | 6.500 |
| **350** | **25** | **120** | 6.344 | 8.989 | 8.725 | 20.000 | 6.149 |
| **350** | **25** | **140** | 6.062 | 37.233 | 36.970 | 20.000 | 6.543 |
| **350** | **sur $O_2$** | **0** | 0.000 | 61.262 | 61.219 | 23.020 | 6.709 |
| **350** | **sur $O_3$** | **20** | 1.344 | 26.556 | 25.610 | 20.000 | 6.740 |
| **350** | **sur $O_4$** | **40** | 4.146 | 23.623 | 22.733 | 19.811 | 5.631 |
| **350** | **sur $O_5$** | **60** | 5.760 | 19.788 | 18.665 | 19.664 | 5.491 |
| **350** | **sur $O_6$** | **80** | 5.951 | 15.991 | 15.418 | 20.000 | 7.310 |
| **350** | **sur $O_7$** | **100** | 6.332 | 16.009 | 15.294 | 19.841 | 7.440 |
| **350** | **sur $O_8$** | **120** | 6.746 | 23.705 | 22.990 | 20.000 | 7.363 |
| **350** | **sur $O_9$** | **140** | 7.160 | 5.124 | 4.409 | 19.841 | 6.500 |
| **350** | **Normal** | **0** | 0.000 | 5.670 | 5.541 | 19.605 | 6.149 |
| **350** | **Normal** | **20** | 2.096 | 48.065 | 40.337 | 21.783 | 7.550 |
| **350** | **Normal** | **40** | 6.347 | 20.351 | 13.446 | 54.989 | 7.075 |
| **350** | **Normal** | **60** | 9.115 | 19.105 | 15.310 | 20.500 | 6.939 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **350** | **Normal** | **80** | 12.220 | 18.282 | 15.377 | 20.118 | 6.916 |
| **350** | **Normal** | **100** | 8.797 | 17.351 | 15.536 | 43.526 | 8.366 |
| **350** | **Normal** | **120** | 8.997 | 15.233 | 14.315 | 20.059 | 7.987 |
| **350** | **Normal** | **140** | 8.226 | 33.607 | 32.532 | 80.055 | 6.500 |
| **350** | **pH6** | **0** | 0.000 | 69.453 | 69.396 | 65.659 | 3.584 |
| **350** | **pH6** | **20** | 3.607 | 94.259 | 88.790 | 75.578 | 6.490 |
| **350** | **pH6** | **40** | 7.356 | 85.509 | 80.054 | 81.383 | 5.714 |
| **350** | **pH6** | **60** | 6.233 | 70.089 | 68.449 | 79.846 | 6.665 |
| **350** | **pH6** | **80** | 5.954 | 59.661 | 59.048 | 79.977 | 7.325 |
| **350** | **pH6** | **100** | 5.826 | 56.161 | 56.018 | 85.394 | 7.236 |
| **350** | **pH6** | **120** | 4.980 | 47.786 | 47.730 | 99.021 | 7.081 |
| **350** | **pH6** | **140** | 4.053 | 11.066 | 11.030 | 30.837 | 6.500 |
| **350** | **pH7** | **0** | 0.000 | 27.656 | 27.606 | 29.506 | 6.290 |
| **350** | **pH7** | **20** | 2.294 | 32.715 | 31.083 | 20.774 | 4.290 |
| **350** | **pH7** | **40** | 3.493 | 63.829 | 62.365 | 21.864 | 5.491 |
| **350** | **pH7** | **60** | 3.948 | 23.881 | 22.466 | 25.133 | 7.310 |
| **350** | **pH7** | **80** | 4.402 | 16.558 | 15.241 | 25.133 | 7.440 |
| **350** | **pH7** | **100** | 5.699 | 13.153 | 12.487 | 25.365 | 7.363 |
| **350** | **pH7** | **120** | 7.144 | 98.384 | 97.758 | 94.671 | 6.500 |
| **350** | **pH7** | **140** | 8.590 | 13.339 | 12.803 | 98.000 | 5.331 |

| OUR | SOUR |
|---|---|
| [mmol$O_2$/L.hr] | [mmol$O_2$/$g_{DW}$.hr] |
| 0.000 | #DIV/0! |
| 25.139 | 4.482 |
| 18.668 | 2.545 |
| 6.260 | 0.893 |
| 2.566 | 0.395 |
| 2.001 | 0.339 |
| 0.874 | 0.144 |
| 0.819 | 0.132 |
| 0.085 | #DIV/0! |
| 20.648 | 7.200 |
| 17.994 | 2.603 |
| 10.431 | 0.763 |
| 5.761 | 0.570 |
| 2.403 | 0.348 |
| 0.381 | 0.059 |
| 0.088 | 0.014 |
| 1.378 | 0.218 |
| 0.151 | #DIV/0! |
| 37.717 | 6.475 |
| 8.107 | 0.680 |
| 1.351 | 0.122 |
| 1.233 | 0.179 |
| 0.731 | 0.177 |
| 0.063 | 0.022 |
| 0.066 | #DIV/0! |
| 10.636 | 4.006 |
| 10.552 | 1.537 |
| 4.292 | 0.552 |
| 1.608 | 0.350 |
| 1.303 | 0.396 |
| 0.432 | 0.261 |
| 0.846 | 1.192 |
| 0.024 | #DIV/0! |
| 7.482 | 4.055 |
| 10.066 | 3.173 |
| 7.660 | 1.766 |
| 5.220 | 0.627 |
| 1.608 | 0.173 |
| 0.532 | 0.073 |
| 0.332 | 0.049 |
| 0.070 | #DIV/0! |
| 1.916 | 0.413 |
| 1.584 | 0.229 |
| 1.055 | 0.125 |
| 0.828 | 0.095 |
| 0.432 | 0.055 |

| | |
|---|---|
| 0.889 | 0.132 |
| 0.097 | 0.016 |
| 0.493 | 0.081 |
| 0.190 | #DIV/0! |
| 15.156 | 2.536 |
| 4.525 | 0.449 |
| 2.297 | 0.211 |
| 0.293 | 0.034 |
| 0.239 | 0.030 |
| 0.094 | 0.015 |
| 0.042 | #DIV/0! |
| 14.276 | 3.251 |
| 2.288 | 0.307 |
| 0.834 | 0.128 |
| 0.520 | 0.081 |
| 0.281 | 0.045 |
| 0.360 | 0.062 |
| 0.109 | 0.021 |
| 0.036 | #DIV/0! |
| 4.126 | 2.284 |
| 0.251 | 0.052 |
| 0.124 | 0.024 |
| 1.194 | 0.280 |
| 0.060 | 0.014 |
| 0.148 | #DIV/0! |
| 15.688 | 2.724 |
| 8.328 | 1.128 |
| 5.689 | 0.776 |
| 3.313 | 0.496 |
| 0.091 | 0.015 |
| 0.193 | #DIV/0! |
| 25.348 | 8.190 |
| 17.302 | 2.562 |
| 7.705 | 0.799 |
| 2.282 | 0.240 |
| 0.880 | 0.133 |
| 0.795 | 0.125 |
| 0.792 | 0.131 |
| 0.130 | #DIV/0! |
| 2.853 | 2.123 |
| 2.684 | 0.647 |
| 3.385 | 0.588 |
| 1.726 | 0.290 |
| 2.155 | 0.340 |
| 2.155 | 0.319 |
| 2.155 | 0.301 |
| 0.390 | #DIV/0! |
| 23.302 | 11.120 |
| 20.820 | 3.281 |
| 11.444 | 1.255 |

| | |
|---|---|
| 8.757 | 0.717 |
| 5.471 | 0.622 |
| 2.769 | 0.308 |
| 3.240 | 0.394 |
| 0.172 | #DIV/0! |
| 16.489 | 4.571 |
| 16.450 | 2.236 |
| 4.945 | 0.793 |
| 1.847 | 0.310 |
| 0.429 | 0.074 |
| 0.169 | 0.034 |
| 0.109 | 0.027 |
| 0.151 | #DIV/0! |
| 4.921 | 2.145 |
| 4.413 | 1.264 |
| 4.265 | 1.080 |
| 3.972 | 0.902 |
| 2.010 | 0.353 |
| 1.889 | 0.264 |
| 1.617 | 0.188 |