HIGH-LEVEL EXPRESSION OF HEPATITIS B SURFACE ANTIGEN IN *PICHIA PASTORIS*, ITS PURIFICATION AND IMMUNOLOGICAL CHARACTERIZATION

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HIGH-LEVEL EXPRESSION OF HEPATITIS B SURFACE ANTIGEN IN *PICHIA PASTORIS*, ITS PURIFICATION AND IMMUNOLOGICAL CHARACTERIZATION

submitted by **HANDE SELAMOĞLU** in partial fulfillment of the requirements for the degree of **Master of Science Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. İnci Eroğlu _____
Head of Department, **Biotechnology**

Prof. Dr. Gülay Özcengiz _____
Supervisor, **Biology Dept., METU**

Prof. Dr. H. Avni Öktem _____
Co-Supervisor, **Biology Dept., METU**

Examining Committee Members:

Prof. Dr. Ufuk Bakır _____
Chemical Eng. Dept., METU

Prof. Dr. Gülay Özcengiz _____
Biology Dept., METU

Assoc. Prof. Dr. Çağdaş Son _____
Biology Dept., METU

Assoc. Prof. Dr. Ayşe Gül Gözen _____
Biology Dept., METU

Assoc. Prof. Dr. Bülent İçgen _____
Biology Dept., Kırıkkale Univ.

Date: 11.12.2009

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Hande Selamoğlu

Signature :

ABSTRACT

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Selamoğlu, Hande

M.Sc., Department of Biotechnology
Supervisor: Prof. Dr. Gülay Özcengiz
Co-Supervisor: Prof. Dr. H. Avni Öktem

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Hepatitis B virus (HBV), which belongs to the family Hepadnaviridae, is responsible for acute and chronic hepatitis. The vaccines presently used to immunize patients against HBV are recombinant subunit vaccines consisting of viral surface antigens (S protein). However, they are expensive and their use is limited in poor countries. For that reason, HBV remains an important worldwide health problem. Of the 2 billion people who have been infected with the HBV, more than 350 million have chronic (lifelong) infections, who face increased risk of developing cirrhosis and hepatocellular carcinoma.

In this study, high-level expression of recombinant Hepatitis B surface Antigen (rHBsAg), PreS2-S was achieved in the methylotrophic yeast, *Pichia pastoris*. For this aim, a single copy of HBV *M* gene (*PreS2-S*) was inserted at the downstream of the alcohol oxidase (*AOX1*) promoter of the pPICZA vector. rHBsAg protein could then be expressed intracellularly by induction with methanol. High cell density

fermentation was followed by chromatographic separation to obtain pure rHBsAg. Humoral response after immunization with the purified protein was observed in mice using commercial Hepatitis B surface antigen kits. It was verified by the atomic force microscopy that rHBsAg has been produced in the desired conformation.

Keywords: Hepatitis B, M protein, *Pichia pastoris*, purification, recombinant protein, protein purification.

ÖZ

HEPATİT B YÜZEY ANTİJENİNİN *PICHIA PASTORIS*'DE YÜKSEK MİKTARDA EKSPRESYONU, SAFLAŞTIRILMASI VE İMMÜNOLOJİK KARAKTERİZASYONU

Selamoğlu, Hande

Yüksek Lisans, Biyoteknoloji Bölümü
Tez Yöneticisi: Prof. Dr. Gülay Özcengiz
Ortak Tez Yöneticisi: Prof. Dr. H. Avni Öktem

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Hepadnaviridae ailesine bağlı olan Hepatit B virüsü, akut ve kronik hepatit hastalığına neden olmaktadır. Bu enfeksiyona karşı geliştirilen aşılar 1982 yılından beri kullanılmasına rağmen, pahalı olması ve fakir ülkelerde yaygın olarak kullanılamaması nedeniyle HBV hala dünya çapında önemli bir sağlık problemi olmayı sürdürmektedir. 2 milyar hepatit B taşıyıcısının 350 milyondan fazlası, yüksek siroz ve karaciğer kanseri riski taşıyan kronik hastalardır. Günümüzde kullanılan hepatit aşıları, viral yüzey antijenlerinden oluşan recombinant alt ünite aşılardır (S proteini).

Bu çalışmada Hepatit B yüzey Antijeninin (rHBsAj) metilotrofik bir maya olan *Pichia pastoris*'de yüksek miktarda ekspresyonu gerçekleştirilmiştir. Bu amaçta HBV *M* geni tek kopya olarak alkol oksidaz (*AOX1*) promotorunun altına yerleştirilerek pPICZA vektörüne klonlanmıştır. Klonlama çalışmalarından sonra rHBsAj protein sentezi hücre içinde metanolle indüklenmiştir. Yüksek hücre konsantrasyonlu fermentasyon çalışmalarının ardından, rHBsAj kromatografik

teknikler kullanılarak saflaştırılmıştır. Rekombinant HBsAj proteini ile bağışıklanan farelerde oluşan immun yanıt ticari Hepatit B yüzey antijen kiti kullanılarak gösterilmiştir. Üretilen rekombinant antijenin istenilen konformasyonda olduğu AFM (Atomic Force Microscopy) mikroskopu ile doğrulanmıştır.

Anahtar kelimeler: Hepatit B, M proteini, *Pichia pastoris*, saflaştırma, rekombinant protein, protein saflaştırılması.

To My Family and Its New Member,

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TABLE OF CONTENTS

LIST OF FIGURES

FIGURES

LIST OF TABLES

TABLES

LIST OF ABBREVIATIONS

AFM	: Atomic Force Microscopy
bp (s)	: Base pair (s)
EDTA	: Ethylenediaminetetraacetic acid
Hour	: h
kDa	: Kilodalton
kb	: Kilobase
P. pastoris	: *Pichia pastoris*
lacZ	: β-galactosidase
ORF	: Open Reading Frame
E. coli	: Es*cherichia coli*
EtBr	: Ethidium bromide
IPTG	: Isopropyl-β D-thiogalactopyranoside
OD	: Optical density

CHAPTER 1

INTRODUCTION

1.1 Hepatitis B

Hepatitis is an inflammation of liver commonly caused by viral infections. There are five main types of Hepatitis viruses infecting liver, namely, Hepatitis A, B, C, D and E. Hepatitis A and E are transferred enterically; whereas hepatitis B, C and D are transferred through parenteral contact with infected body fluids and may progress into chronic stage of inflammation. A form of hepatitis transmissible through blood (later known as hepatitis B) was first reported with the occurrence of jaundice in 15 factory workers inoculated with a small pox vaccine prepared from human sera in Germany (Lurman, 1885). Hepatitis B virus (HBV) was first visualized in an electron microscope by Dane *et al*. in 1970.

1.1.1 Symptoms and Incidence of Disease

Discovery of Australia antigen by Blumberg *et al*. in 1965 (later known as hepatitis B surface antigen) and its relationship with hepatitis B virus (HBV) in early 1970s led to development of diagnosis tests for hepatitis B. Jaundice (yellowing of the skin and eyes), dark urine, extreme fatigue, nausea, vomiting and abdominal pain are among the symptoms of Hepatitis B (World Health Organization, 2009). Worldwide, more than 2 billion people have been infected with the virus and about 400 million of these live with the chronic infection. Chronic hepatitis B patients may be

asymptomatic, but can spread the infection and are at high risk of developing fatal diseases such as cirrhosis and hepatocellular carcinoma. An estimated number of 200,000 and 300,000 chronic Hepatitis B virus carriers die each year from cirrhosis and hepatocellular carcinoma, respectively. Incidence of developing chronic hepatitis B is higher in individuals infected by perinatal route (90%) or during childhood (20-30%) (Perz *et al*., 2006; Lok *et al*., 2007).

1.1.2 Route of Infection

Route of infection of Hepatitis B varies between geographical regions. Hepatitis B is most prevalent in central Asian republics, South-east Asia, sub-Saharan Africa, the Amazon Basin and is least prevalent in the United States, northern Europe, Australia, and parts of South America. The Middle East, some countries of Eastern Europe, and the Mediterranean Basin are considered areas of intermediate endemicity (Zanetti *et al*., 2008). The geographic distribution of the HBV carriers varies significantly ranging from 10% to 20% in Asia and Sub-Saharan Africa to less than 1% in Northern Europe and America (Hilleman *et al*., 2003). 5-8 % of the Turkish population is HBV carriers (Balık S., 1992). In areas of low to intermediate endemicity, sexual contact and intravenous drug use are the most common routes of infection. Besides these, percutaneous routes of infection, such as needle stick injury, mucous membrane splash in health care setting, tattooing, piercing, sharing razors or toothbrushes also plays role in spreading the virus (Lee *et al*., 1997).

1.1.3 Acute and Chronic Stages of Inflammation

Chronic stage of inflammation is described in four phases: immune tolerance, immune clearance, low or non-replication, and reactivation. Not every patient goes through each phase and the length of phases varies from patient to patient. Minority of chronic hepatitis B patients are immunotolerant to the virus and they show no clinical signs of disease. Immunotolerant phase can be characterized by hepatitis B e

antigen (HBeAg) and HBV-DNA being present in the patient's sera due to high viral replication. Serum alanine aminotransferase (ALT) levels and liver functions are normal at this stage. This initial phase of the disease may persist for decades, if perinatally acquired. About 5% of the immunotolerant patients may develop cirrhosis (Rizetto *et al*., 2007). Hepatitis B surface antigen (HBsAg) is the first detectable antigen in the serum during the course of infection and for that reason, it is routinely used to screen for the disease (Blumberg *et al*., 1968). In adult infection, immunotolerant phase marks the incubation period of the acute disease. Depending on the age of the patient and route of Hepatitis B virus acquisition, immune tolerance to the virus is lost and the immune system attacks on the infected hepatocytes. This second phase of the disease starts with the progressive decrease of HBV-DNA and increase of ALT levels in the serum, accompanied with moderate to severe necroinflammation. In acute infections, clinical symptoms and jaundice usually lasts three to four weeks before viral clearance starts. Duration of viral clearance may be years long and correlates with the risk of developing hepatocellular carcinoma and cirrhosis. Seroconversion from HBeAg to anti-HBe, marks the transition to the low or non-replication phase (inactive HBsAg carrier state). Patients at this stage are HBeAg negative and anti-HBe positive. HBV-DNA and ALT levels are undetectably low. Although HBV is cleared from blood, these patients remain occult carriers of the virus in the liver. Roughly 10% to 20% of inactive carriers may have reactivation of HBV replication and exacerbations of hepatitis after years of quiescence. The situation arises because of the emergence of HBeAg mutants rather than reactivation of the original wild-type HBeAg expressing virus (Lok *et al*., 2007; Rizetto *et al*., 2007). Under circumstances where the host remains infected but is successfully clearing the virus, IgM antibodies to the hepatitis B core antigen (anti-HBc IgM) may be the only serological evidence of disease (Bonino *et al*.,1987). It has been suggested that cell-mediated immune (CMI) responses, especially antigen-specific cytotoxic T lymphocytes (CTL), play an important role in controlling HBV infections (Chisari *et al*., 2000). The factors leading to chronic HBV infection are not well understood. Patients with acute infection develop vigorous polyclonal T-cell

responses against HBV antigens, including core, polymerase and envelope proteins. In patients who develop chronic infection, these prime determinants for viral clearances are not present (Rehemann *et al*., 1995) and anti-HBsAb antibodies are barely detectable (Chisari *et al*., 1997). Early immune responses to the viral infection may determine whether a host will become a chronic carrier or not (Rehemann *et al*., 1996). Clinical and virologic profiles of hepatitis B patients are summarized in Table 1.1.

Table 1.1 Clinical and virologic profile of HBsAg carriers (Rizetto *et al*., 2007).

	Immune-tolerant	Chronic hepatitis B (high replication)(HBeAg+/−)	Chronic hepatitis B (low replication) "inactive"
HBsAg	+	+	+
HBeAg	+	+/−	−
anti-HBe	−	−/+	+
ALT	Normal	↑/↑	Normal
HBV-DNA (copies/ml)	>105	>105/>103	<103
Histology	Normal/mild	Active	Normal

1.1.4 Treatment and Prevention

Today, two immunomodulatory agents (conventional interferon α and peginterferon α-2a) and five oral nucleos(t)ide analogues (lamivudine, adefovir dipivoxil, entecavir, telbivudine and tenofovir) have been registered for the treatment of hepatitis B in the USA and Europe. In registration trials, these drugs have been shown to result in hepatitis B virus (HBV) DNA reduction, alanine aminotransferase (ALT) normalization and histological improvements (Vincent *et al*., 2009).

Mechanism of action of oral nucleos(t)ide analogues are based on the idea to inhibit replication of the virus. These drugs have some drawbacks such as immunosuppression and sustained or transient seroconversion (HBeAg+ to anti-HBe+ (Thoma, 2000). Vaccination is the most important measure to prevent and control hepatitis B virus infection and its long term consequences. Plasma derived vaccines for hepatitis B was developed in France and are commercially available since 1981 (Szmuness *et al*., 1980). These vaccines contained highly purified 22 nm HBsAg particles inactivated through a combination of urea, pepsin, formaldehyde, and heat (Zanetti, 2008). Although plasma derived vaccines have been safely and effectively used for a long time, they have several drawbacks: (i) the stringent manufacturing process to inactivate the infectious virus, (ii) risk of acquiring blood transmitted diseases such as acquired immuno-defficieny syndrome, (iii) the high cost of production, which is an obstacle for the implementation of mass immunization programs especially by poor countries where hepatitis B infection is most prevalent , (iv) limited availability of human plasma, (iiv) requirement of a lenghty innocuity test (6 months) performed in chimpanzees (Hardy *et al*., 2000). These risk factors led to development of second generation vaccines produced by recombinant DNA technology to express HBsAg (Szmuness *et al*., 1980). HBsAg peptide vaccines are produced in a variety of cell lines such as *E. coli* (Pumpen *et al*., 1984), *S. cerevisiae* (Emini *et al*., 1986) , *P. pastoris* (Cregg *et al*., 1987), Chinese hamster ovary cells (Belew *et al*., 1991), mouse cells (Denniston *et al*., 1984) and Drosophila Schneider-2 cell (Demi *et al*., 1999). During the 1990s, the so-called third-generation hepatitis B vaccines were developed in HBV transfected mammalian cells which express and secrete the small S and the middle Pre-S2 proteins or all the three envelope proteins; S, Pre-S2 and the large Pre-S1 proteins. The new Pre-S/S HBV vaccines as well as some novel new adjuvants have been shown to enhance the immunogenicity of such vaccines in immunocompromised hosts and non-responders to conventional vaccines (Zanetti *et al*., 2008).

Full course of vaccination involves 3 doses which is administered at 0, 1 and 6 months, respectively. Seroprotection rates to antibodies aganist HBsAg (anti-HBs) is close to 100% in children and 95% in adults which persists over periods of at least 10-15 years. Although antibody concentrations decrease over time, breakthrough infections are rare in succesfully vaccinated people. In some sporadic cases of vaccine failure, both HBsAg and anti-HBsAb can be found in a patient simultaneously. This situation indicates unsuccessfull viral neutralization and virus multiplication in the presence of humoral immunity to the antigen. Vaccinated people show a rapid anamnestic response when boosted with additional dose of vaccine or when exposed to HBV several years after the primary course of vaccination. This means that the immunological memory for HBsAg can outlast the anti-HBs detection, providing long-term protection against acute disease and the development of an HBsAg carrier state. For immunocompromised patients, regular testing and booster administrations, when anti-HBs antibody level falls below 10mIU/ml are also recommended (Jilg *et al*., 1988; West *et al*., 1996).

1.2 Hepatitis B Virus

The human hepatitis B virus (HBV) is a partially double-stranded DNA virus of the Hepadnaviridae family.

1.2.1 Genotypes and Subtypes

Virus isolates are divided into eight genotypes (A to H) according to their phylogenetic origin and different genotypes shows a distinct geographical distribution between and even within regions (Norder *et al*., 2004). Table 1.2 summarizes relation of subtypes and geographic distribution. Structural and functional differences between genotypes can influence the severity, course and likelihood of complications, hepatitis B e antigen (HBeAg) seroconversion and response to treatment of HBV infection and possibly vaccination against the virus

(Chu *et al*., 2002). Genotype D predominates in the Middle East and Medditarian basin, including Turkey (Ozdemir *et al*., 2005).

Table 1.2 Geographic distribution of Hepatitis B genotypes (Rizetto *et al*., 2007).

Subtypes	Genotype	Geographic distribution
ayw 1	A	Northern Europe, United States
adw 2		
ayw 1	B	Eastern Asia, Far East
adw		
ayr	C	Eastern Asia, Far East.
adr		
adrq	D	Worldwide; prevalent in Mediterranean area, near and Middle East, South Asia
ayw2		
ayw3		
ayw4	E	Western sub-Saharan areas
ayw4q-	F	United States, Africa
adw2	G	Recently isolated in France and United States

Major and minor subtypes of Hepatitis B virus have been serologically identified at the hepatitis B surface antigen (HBsAg) level. The common determinant a and two pairs of mutually exclusive determinants, *d/y* (Le ouvier, 1971) and *w/r* (Bancroft *et al*., 1972), enables the distinction of four major subtypes of HBsAg: *adw*, *adr*, *ayw* and *ayr*. Subtypes of the virus are also associated with geographic distribution and different clinical outcomes. Additional determinants designated sub determinants of *w* (*wl* to *w4*) have allowed the definition of four serotypes of *ayw* (*aywl*, *ayw2*, *ayw3*

and *ayw4*) and two serotypes of adw, i.e. *adw2* and *adw4* (Courouc *et al*., 1976). The determinants *wl* to *w4* were once designated subdeterminants of *a*, since they were considered to be variations of the major a determinant (Soulier and Courouc-Pauty, 1973; Courouc-Pauty and Soulier, 1974). Apart from these, the *q* determinant, originally described as present on all subtypes but *adw4* (Magnius *et al*., 1975), was later also found to be absent from adr strains in the Pacific region, thus defining *adr* as either *q*+ or *q*- (Courouc-Pauty *et al*., 1978). Subtype variations are associated with the point mutations at the hydrophilic domains of HBsAg (Gerin *et al*., 1983; Okamoto *et al*., 1987; Ashton-Rickardt & Murray, 1989). The important allelic substitutions associated with d/y and w/r expression are found at residues 122 and 160 respectively. Both of these sites have the subtypic changes mediated by a shift from Lys to Arg (Okamoto *et al*., 1987). Lys160 specificity is found to be responsible for w reactivity (Okamoto *et al*., 1989) and position 127 for *w2*-*w4* reactivity. Phe134 and Ala159 are found to be involved in the expression of *w1* reactivity (Norder *et al*., 1992).

1.2.2 Virus Life Cycle

The virion (Dane particle) is a spherical particle of 42 nm in diameter (Dane *et al*., 1970), consisting of an icosahedral nucleocapsid core (HBcAg) of approximately 30 nm in diameter and an outer lipoproteinaceous envelope (HBsAg) composed of three surface proteins and lipids of host cell origin (Blumberg *et al*., 1968; Almeida *et al*., 1971; Hoofnagle *et al*., 1973). Nucleocapsid core surrounds a DNA genome of 3.2 kb and DNA polymerase with reverse transcriptase activity (Licornini, 2004). The structure of hepatitis B virus is shown in Figure 1.1.

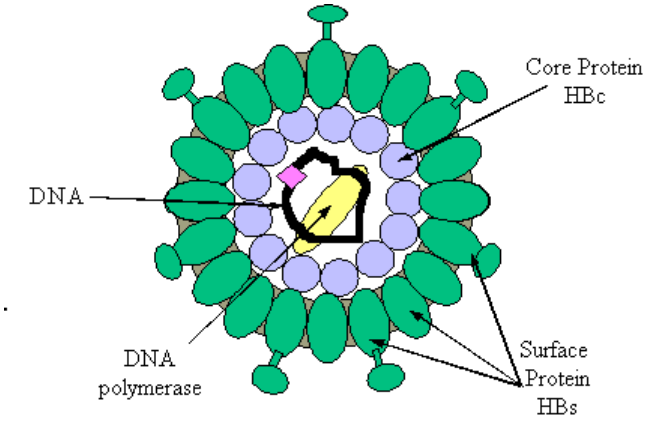


Figure 1.1 The structure of hepatitis B virus (www.hon.ch/Library/Theme/HepB/virology.html).

The HBV life cycle begins with binding of virions to the receptors at the surface of liver hepatocyte cells, which is mediated by a 180 kDa host protein (Kuroki *et al*., 1995). Upon entry of viral nucleocapsids to the cell, viral genome is delivered to the host cell. The first event of replication is the conversion of relaxed circular (rc) DNA genome into covalently closed circular (ccc) DNA in nucleus. Conversion of rcDNA to cccDNA in the liver is detected within the first 24 h following virus inoculation (Mason *et al*., 1983). cccDNA serves as a template for transcription of four viral RNAs that are 3.5, 2.4, 2.1 and 0.7 kb long (Tagava *et al*., 1986). These transcripts are then polyadenylated and transported to the cytoplasm, where they are translated into the viral nucleocapsid and precore antigen (C, pre-C), polymerase (P), envelope L (large), M (medium), S (small), and transcriptional transactivating proteins (X) (Guidotti *et al*., 1994; Chisari *et al*., 1997; Ganem *et al*., 2001). During budding, linear, cytosolic preS domain of envelope proteins interacts with the binding sites on the capsid surface before virions are secreted into the blood (Ueda *et al*., 1991). HBV virions bud into an endoplasmic reticulum (ER)-associated intracellular compartment, but the mechanisms of HBV assembly, budding, and release remains poorly understood.

The genomic structure of hepatitis B virus (HBV) is shown in Figure 1.2. The inner circles represent the full-length minus (-) strand (with the terminal protein attached to its 5' end) and the incomplete plus (+) strand of the HBV genome. The thin black lines represent the 3.5, 2.4, 2.1 and 0.7 kilobase mRNA transcripts, which are all terminated near the poly (A) (polyadenylation) signal. The outermost colored lines indicate the translated HBV proteins: that is, large, middle and small HBV surface proteins, polymerase protein, X protein, and core and pre-core proteins. Hepatitis b e antigen (HBeAg) is a processing product of the core protein (HBcAg). The viral life cycle is shown in Figure 1.3.

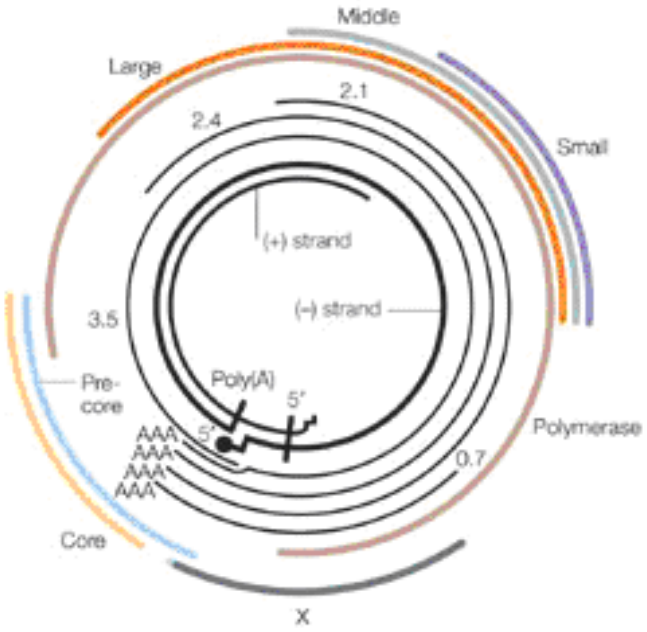


Figure 1.2 The genomic structure of Hepatitis B virus (Rehermann *et al*., 2005).

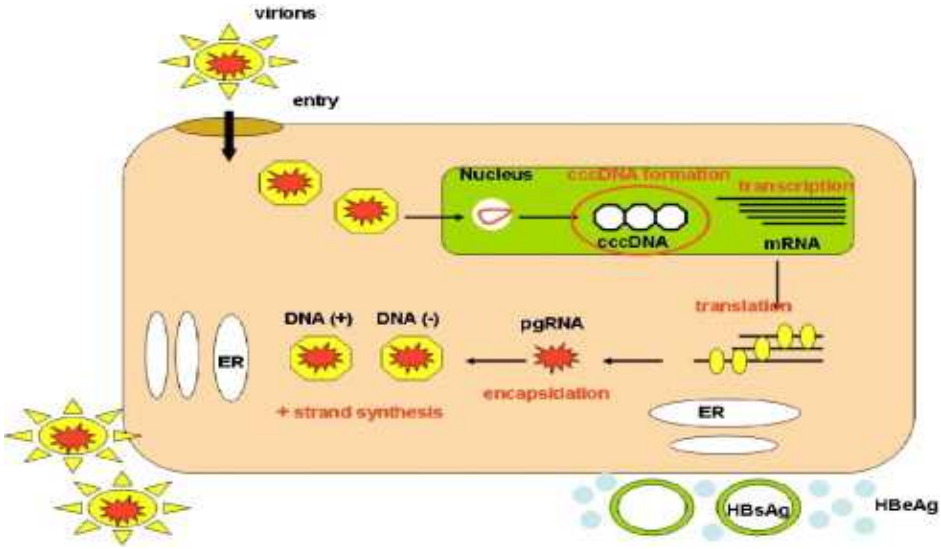


Figure 1.3 Life cycle of HBV (Rizetto *et al*., 2007).

1.2.3 Subviral Particles

Hepatitis B envelope is composed of three related surface proteins; namely small (S), middle (M) and large (L) surface antigens. They are encoded by a single open reading frame (ORF) called E containing 389 to 400 amino acids depending on the genotype (Heermann *et al*., 1984). The large surface protein is the translation product of the entire ORF (389 to 400 a.a.), the middle surface protein lacks 119 a.a. from the N terminal of L protein (the preS1 sequence) and the small surface protein lacks 55 a.a. of M protein (the preS2 sequence) as summarized in Figure 1.4.

Figure 1.4 Structure of the hepatitis B surface proteins (Bo *et al*., 2005).

Six protein products are derived from the open reading frame : a glycosylated and nonglycosylated form of the major protein (gp27 and p24 respectively) translated from the S domain only; middle protein having one or two polysaccharide chains (gp33 and gp36 respectively) translated from preS2 and S region and the glycoslated (gp42) and non-glycosylated (p39) form of the large protein encoded by the entire lenght ORF (Gavilanes *et al*., 1982). All three proteins are glycosylated at N146 residue, except for gp36 (M protein) which is glycosylated at N4 residue (Gavilanes *et al*., 1982). Hepatitis B surface antigens are suitable candidates for vaccine development because of their role in viral clearance (Brechot *et al*., 2000).

The pre-S1 region directly interacts with hepatocytes, the pre-S2 region has been implicated in the polymerized albumin mediated interaction and the S region contains the complete information for self assembly in the absence of capsids to form higly antigenic subviral particles. These particles can be in spherical or filamentous form and they are highly overexpressed relative to virions and reach a 10 000-fold higher concentration in serum. It is assumed that the massive HBsAg overproduction influences the host's immune system in a way that is advantageous for the virus (McAleer *et al*., 1984). The type of particle formed seems to be determined by the ratio of S to L proteins coassembling during morphogenesis. The spherical 22-nm HBsAg particles isolated from the serum of infected patients contain only traces of L protein. The coassembly of a higher proportion of L protein with S results in formation of the filamentous form of HBsAg with variable lenghts (Heermann *et al*., 1984). Chaperons like calnexin (Prange *et al*., 1999) and BIP (Cho *et al*., 2003) supports protein maturation, but how the HBsAg protein escapes the membrane and mobilizes lipid during subviral particle formation is not clear. Lipids are 25% by weight and thus not organized in a bilayer structure in subviral particles (Gavilanes *et al*., 1982).

The high L content inhibits particle secretion (Persing *et al*., 1986) due to specific retention motifs in this protein (Gallina *et al*., 1995) and/or the inefficient export of the filamentous particles (Roingeard, 1998). The significance of secretion inhibition of L protein for the viral life cycle remains unclear. The L protein plays a key role in virus assembly and infectivity and is therefore present in large proportions in the virion envelope. The M protein behaves like S protein with respect to particle formation and it is found in similar proportions with S (Heermann *et al*., 1984; Bruss *et al*., 1991; Bruss *et al*., 1995). Host proteins are efficiently excluded during the morphogenesis of subviral particles. Even the HBV and DHBV S proteins sharing 25% identical amino acids do not form mixed particles during coexpression (Gerhart *et al*., 1995).

Foreign protein domains can only be incorporated into subviral HBsAg particles when they are fused to the S protein (Lambert *et al*., 2004; Bruss *et al*., 1991). Like typical membrane proteins, Hepatitis B envelope proteins are synthesized at endoplasmid reticulum and fold by forming disulfide linked homo and heterodimers as shown in Figure 1.5. HBsAg is found to contain 14 cysteine residues. Reduction and alkylation of the disulfide bonds results in complete loss of antigenicity, however reduction alone doesn't effect serological activity of the protein. Therefore, it is assumed that conformation of antigenic determinant is determined by disulfide bonds (Vyas *et al*., 1972).

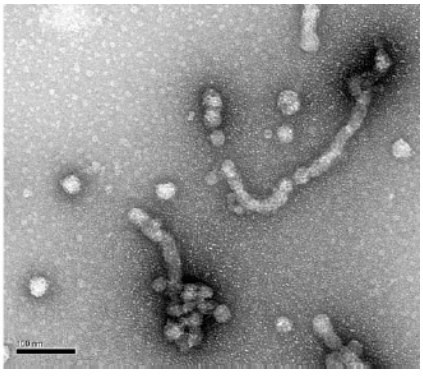


Figure 1.5 Negative staining of of the intracellular subviral envelope filaments purified from the cells producing the HBV S protein (Patient *et al*., 2007).

Insertion of the S protein into the ER membrane is initiated by an N-terminal signal sequence (aa 8 to 22) which is not cleaved by the host's signal peptidase. A second signal (aa 80 to 98) directs the translocation of the peptide chain downstream of this signal through the ER membrane into the ER lumen, whereas the region upstream of the signal remains in the cytosol. The signal itself anchors the protein as a transmembrane domain in the lipid bilayer. The C-terminal hydrophobic 57 amino acids of S are believed to be embedded in the ER membrane. Foreign domains fused to the C terminus of S are oriented towards the ER lumen, suggesting that the C terminus of S is also oriented toward this compartment (Eble *et al*., 1987). This configuration causes the region between residues 23 and 79 to form a loop at the cytosolic side of the ER membrane, whereas the loop between amino acids 99 and approximately 169 is on the luminal side. The luminal loop carries the major conformational epitope (a determinant) of the HBV surface protein antigen (HBsAg) and is N-glycosylated in approximately half of the S molecules at asparagine (asn) residue 146 (Peterson *et al*., 1982). Major conformational epitope (amino acids 124 to 148) is shown in Figure 1.6 and HBsAg membrane topology is presented in Figure 1.7. Neutralizing antibodies aganist HBsAg are specific for the for the group a determinant which appears to be a conserved immunodominant B cell epitope (Vyas *et al*., 1972). The cytosolic loop contains 4 of the 14 cysteine residues.

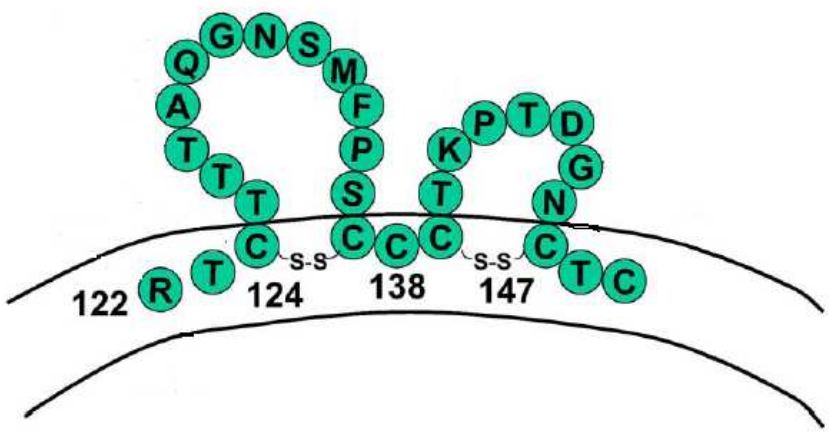


Figure 1.6 Major conformational loop (a determinant) (Weber *et al*., 2005).

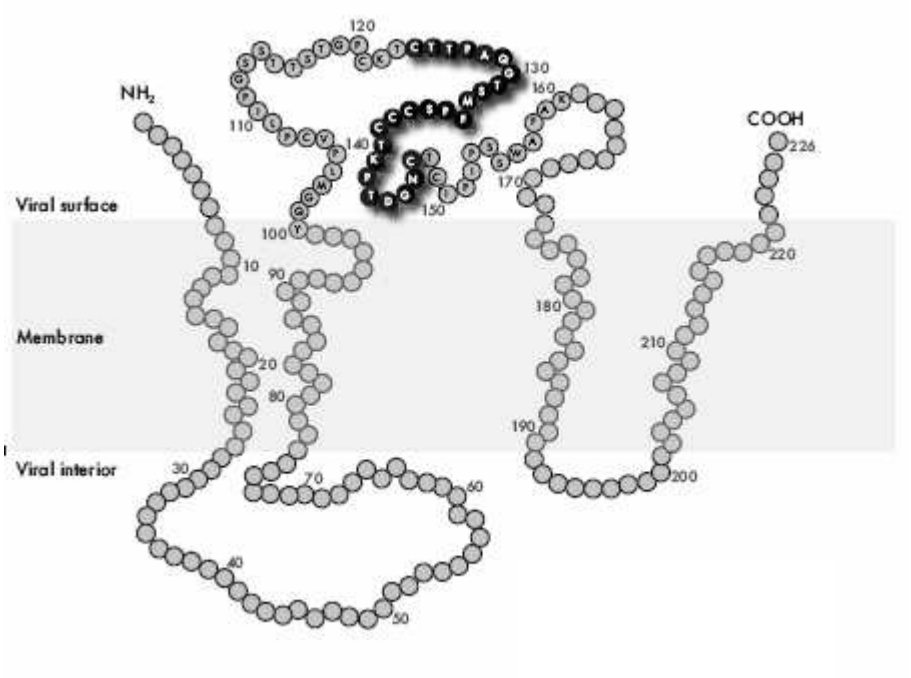


Figure 1.7 Model for the structure of HBsAg. Circles represent amino acids. Major hydrophilic region (MHR) is shown by circles with letters and black circles represent "a" determinant loop (Lei *et al*., 2000).

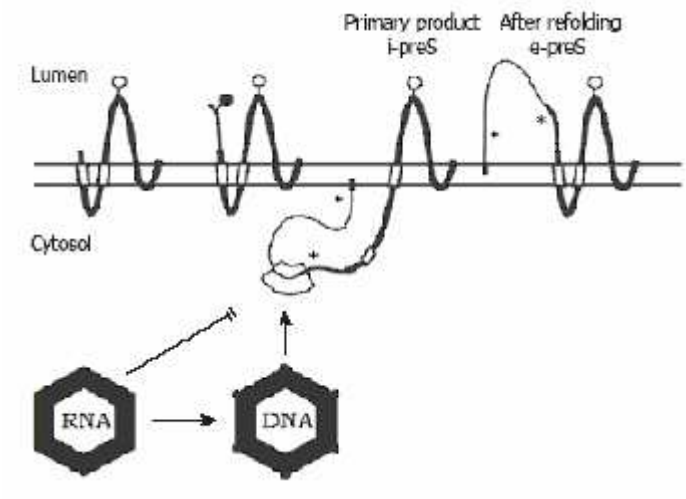


Figure 1.8 Transmembrane topology of the HBV envelope proteins and model for envelope-capsid interaction (Bruss *et al*., 2009).

The transmembrane topology of the M protein is identical to that of S. The M protein is N-glycosylated at aspargine 4 (Stibbe *et al*., 1983) and the preS2 domain is O-glycosylated in some but not all HBV genotypes (Schmitt *et al*., 2004). The N-terminal preS2 domain (55 aa) is translocated into the ER lumen probably by the action of the first signal in the S domain (Eble *et al*., 1990). The preS1 and preS2 domains at the N terminus of L initially remain at the cytosolic side of the ER membrane during L translation (i-preS conformation), as shown in Figure 1.8. The central signal in the S domain of L anchors the protein in the ER membrane and causes the translocation of downstream sequences. Therefore, asn 146 in the S domain of L is partially N-glycosylated, while asn 4 of the preS2 as well as a further potential N-glycosylation site in preS1 remain unmodified. These sites are used when the N terminus of L is forced to cotranslationally translocate by the artificial fusion of a signal sequence to the N terminus of preS1 (Bruss *et al*., 1995). In about half of the L chains, the transmembrane topology changes after translation. The preS domains then appear on the luminal side of the ER membrane (e-preS conformation, Figure 1.8). In spite of the several hypotheses existing, how the preS domains move through the membrane is not known exactly. Probably, the N-terminal signal in the S domain crosses the membrane in this conformation similar to the M and S proteins (Bruss *et al*., 1994; Prage *et al*., 1995; Ostapchuk *et al*., 1994; Swameye *et al*., 1997; Guo *et al*., 1997).

1.3 The Expression System: *Pichia Pastoris*

Pichia pastoris is an efficient system for the expression of heterologues proteins which combines the advantages of both prokaryotic (high expression levels, inexpensive growth media and easy scale up) and eukaryotic systems (post-translational modifications) (Eckard *et al*., 1996). Moreover, the problems encountered with the other widely used recombinant host *Saccharomyces cerevissia* such as mitotic instability of recombinants and overglycosylation are not practiced (Gellissen and Hollenberg, 1997).

1.3.1 Methanol Metabolism

Pichia is one of the four genera capable of metabolizing methanol. The other genera include *Candida*, *Hansenula* and *Torulopsis*. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde, generating hydrogen peroxide in the process, by the enzyme alcohol oxidase (AOX) as explained in Figure 1.9 (Anthony *et al*., 1982). This process takes place in peroxisome to avoid hydrogen peroxide toxicity to the cell.

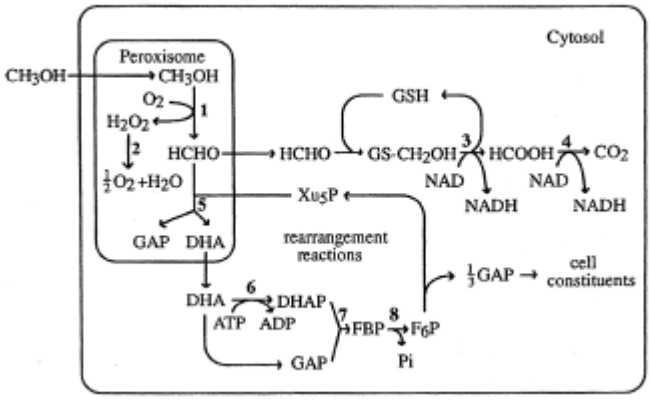


Figure 1.9 Methanol pathway in *Pichia pastoris*. 1: alcohol oxidase, 2: catalase, 3: formaldeyde dehydrogenase, 4: formate dehydrogenase, 5: dihydroxyacetone synthase, 6:dihydroxyacetone kinase, 7: fructose 1,6-biphosphate aldolase, 8: fructose 1,6-biphosphatase (Cereghino and Cregg, 2000).

AOX is a homo-octomer with each subunit containing one non-covalently bound FAD (flavin adenine di-nucleotide) co-factor. AOX has a poor affinity for oxygen and metylotrophic yeasts compensate this deficiency by synthesizing large amounts of this enzyme, which can substitute for up to 30% of protein production in the cell. Expression of the *AOX1* gene is controlled at the level of transcription. In methanol-

grown cells, approximately 5% of polyA+ RNA is from the *AOX1* gene whereas in cells grown on other carbon sources, *AOX1* message is undetectable (Couderc *et al*., 1980; Veenhuis *et al*., 1987; Ellis *et al*., 1987, Tschopp *et al*., 1987). There are two genes in *P. pastoris* that code for AOX which are 97% homologous—*AOX1* and *AOX2*. The *AOX1* product appears to be the major methanol oxidizing activity in methanol-grown cells, since *AOX1*-defective mutants (Mut^s strains) grow slowly on methanol and have low AOX activity levels (Cregg *et al*., 1985). The regulation of the *AOX1* gene (similar to the regulation of the *GAL1* gene of *S.* c*erevisiae*) involves two mechanisms: a repression/de-repression mechanism plus an induction mechanism. However, unlike *GAL1* regulation, de-repressing conditions (the absence of a repressing carbon source such as glucose or glycerol in the medium) do not result in substantial transcription of the *AOX1* gene. The presence of methanol appears to be essential to induce high levels of transcription (Tschopp *et al*., 1987). The strong and tightly regulated AOX promoter is mostly used for protein expression in *Pichia*. The *P. pastoris* expression systems relies on integration of introduced DNA into yeast genome by homologous recombination (Cregg *et al*., 1985). Although there are autonomous replicative plasmids, chromosomal integration is preffered because these plasmids are of low copy number, unstable and invariably integrate at one or more chromosomal loci (Sreekrishna *et al*., 1997).

Type of host strains varies in regard to their ability to utilize methanol due to deletions at one or both of the *AOX* genes. However, the most commonly used expression host is wild-type or GS115 (*his4*), which contains both of the the *AOX1* and *AOX2* genes and grow on methanol at the wild-type rate (methanol utilization plus or Mut^+ phenotype). KM71 (*his4 arg4 aox1*::*ARG4*) is a strain in which the chromosomal AOX1 gene is largely deleted and replaced with the *S. cerevisiae ARG4* (argininosuccinate lyase) gene. As a result, this strain must rely on the much weaker *AOX2* gene for AOX and grows on methanol at a slow rate (methanol utilization slow or Mut^S phenotype). KM71H strain (*arg4 aox1*::*ARG4*) has a mutation in the *arg4* gene that prevets strains from growing in the absense of

arginine. The expression vector series of Invitrogen: pPICZ and pPICZα (containing the α factor signal sequence of *S.cerevisiae*) contains the *Sh ble* gene from *Streptoalloteichus hindustanus* which encodes for 13.665 Da protein conferring resistance to drug Zeocin, which is the selective marker in the system. Gene insertion events at the *AOX1* (GS115) or *aox1*::*ARG4* (KM71H) loci arise from single crossover event between the loci and either one of the two *AOX1* regions on the pPICZ or pPICZα vectors: the AOX1 promoter or AOX1 transcription termination region. This results in insertion of one or more copies of linearized vector upstream or downstream of either *AOX1* (Mut^+) or *aox1*::*ARG4* (Mut^S). Recombination frequency lowers with the use of non-linearized vector. The gene insertion event is further explained in Figure 1.10 (Invitrogen: Easy Select Pichia Expression Kit Manuel, 2005).

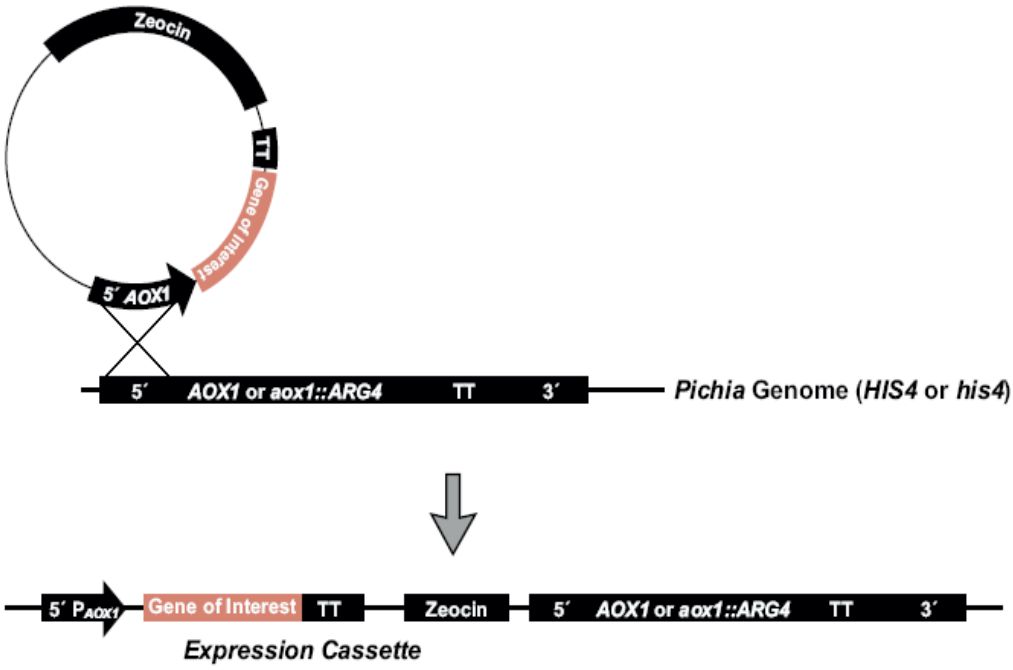


Figure 1.10 Gene insertion at *AOX1* or *aox1*::*ARG4*

CHAPTER 2

MATERIAL AND METHODS

2.1 Bacterial Strains and Plasmids

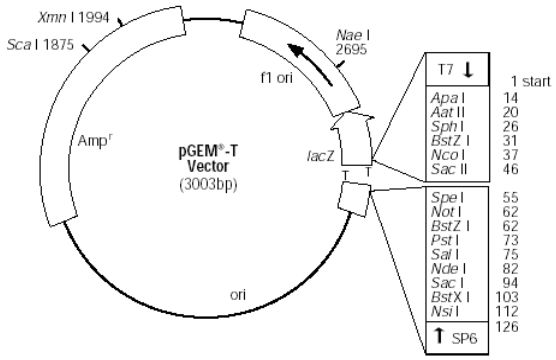
Bacterial and yeast strains as well as the plasmids used in the study are listed in Table 2.1.

Structure of the plasmid vectors are shown in Figure 2.1.

Table 2.1 List of bacterial and yeast strains and plasmids used in the present study.

Strain	**Characteristics**	**Reference**
Escherichia coli DH5α	F' ϕd*lac*ZΔ(*lac*ZY A-argF)U169 *sup*E44λ$^-$ *thi*-1 *gyr*A *rec*A1 *rel*A1 *end*A1 *hsd*R17	American Type Culture Collection; Hanahan (1983).
Pichia pastoris GS115	*his4*, His$^-$, Mut$^+$	Invitrogen
Plasmid	**Characteristics**	**Reference**
pGEM®-T	Amp^r *lac*Z	Promega
pPICZA	*Zeo^r*	Invitrogen
pS2-S/pIL-2	*Amp^r*	Dr. Erkan Özcengiz

(a)



(b)

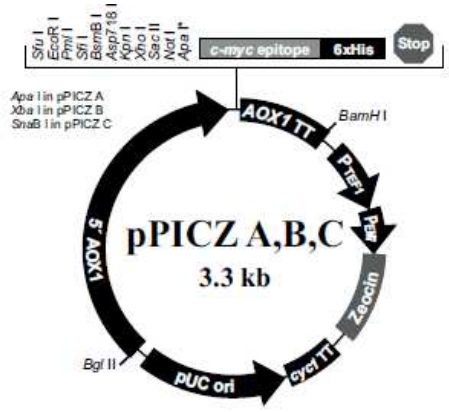


Figure 2.1 Structure of the plasmid vectors: (a) pGEM®-T, (b) pPICZA

2.2 Culture Media

The composition and the preparation of the culture media are given in Appendix A.

2.3 Buffers and Solutions

Buffers, solutions, their composition and preparation are listed in Appendix B.

2.4 Chemicals and Enzymes

The chemicals and the enzymes used and their suppliers are listed in Appendix C.

2.5 Growth Conditions and Maintenance of Bacterial and Yeast Strains

E. coli DH5α strain was routinely grown in Luria Broth (LB) or Low Salt Luria Broth (LSLB) medium at 37°C. Cultures grown on Luria agar (LA) or Low Salt Luria Agar (LSLA) plates were stored at 4°C, for short term (up to one month) preservation. For long term storage, cultures grown until mid-log phase in LB or LSLB were preserved in 50% glycerol, at -80°C. LA and LB were supplemented with 100 μg/mL ampicilin, whenever required. LSLA or LSLB were supplemented with 100 μg/mL zeocin, whenever required.

P. pastoris strains were grown in Yeast Extract Peptone Dextrose (YPD) medium for seed culture preparation and Yeast Extract Peptone Dextrose Sorbitol (YPDS) medium for cloning purposes. YPDS was supplemented with 100μg/ml Zeocin, whenever required. Buffered Minimal Glycerol (BMGH) and Buffered Minimal Methanol (BMMH) medium were used for expression studies. *P. pastoris* were routinely grown at 30°C unless otherwise stated. Cultures grown on YPD or YPDS agar plates were stored at 4°C, for short term (up to one month) preservation. For long term storage, yeast colonies grown on YPD plates were suspended in 50% glycerol, and stored at -80°C.

2.6 Plasmid Isolation

Qiagen Plasmid Purification Mini and Midi Kits (Qiagen Inc., Valencia, CA) were mostly used for isolation of *E. coli* plasmid DNA as specified by the manufacturers.

2.7 Agarose Gel Electrophoresis

A horizontal submarine electrophoresis apparatus was used for electrophoresis. 0.8% agarose gel was prepared in TAE buffer and run at 90 Volts for 45-60 min after the samples are loaded. The gel was stained in TAE buffer containing ethidium bromide at a final concentration of 0.5 μg/mL for 15 minutes. DNA bands were visualized on a shortwave UV transilluminator and photographed by using Vilber Lourmat Gel Imaging System. *Pst*I digested lambda DNA marker (Appendix A) was used to determine the molecular weights of DNA bands.

2.8 Ligation Reactions

Ligation of PCR products to pGEM®-T vector was performed as follows: 5 μL 2X ligase buffer, 0.5 μL (55 ng/μL) pGEM®-T vector, 500 ng insert DNA, 1 μL T4 DNA ligase (3 Units/μL) were mixed and the volume was completed to 10 μL with dH_2O. Ligation mixture was incubated overnight at 4°C. Ligation of restriction digests to expression vector, pPICZA was performed as follows: Before addition of ligase buffer and enzyme, insert DNA were mixed in a 1:7 ratio with the vector and incubated at 45°C for 10 min and then on ice for 5 min. The mixture was then incubated overnight at 4°C after addition of buffer and enzyme.

2.9 Restriction Enzyme Digestion

Restriction enzymes *Kpn*I, *Sac*II and *Sac*I in a suitable buffer (as described by the manufacturer) were used to introduce 2 Units enzyme per μg of DNA. The mixture was incubated at 37°C for 3-4 hs.

2.10 Colony Polymerase Chain Reaction (Colony PCR)

For verification of the transformants colony PCR method was used as described by Amberg *et al*., 2000. PCR reaction mixture contained: 2µl of 10X Colony PCR Buffer (Appendix B), 1,2 µl of 25mM $MgCl_2$, 0,4µl of 10mM dNTPs, 10 pmole of each primer, 5 Units of Taq Polymerase, 0,25 µl of yeast colonies added using a pipette tip and sterile dH_2O to complete the volume to 20µl. PCR cycle profile was as follows: 4 minutes at 94°C for initial denaturation, 10 minutes at 72°C for final extension and 35 cycles of 1 minute at 94°C, 1 minute at 55°C, 2 minutes at 72°C.

2.11 Polymerase Chain Reaction (PCR)

PCR mixture contained 5 µL 10X PCR buffer, 5 µL of 10X $MgCl_2$, 1 µL 10mM dNTP, 10 pmols of each primer, 0.1 µg template DNA, 5 Units of Taq polymerase and sterile dH_2O to complete the volume to 50 µL. PCR conditions are shown in Table 2.2. Two primers were used for PCR amplification: MOF; forward primer: (5'gggtaccag**aacaata**atgcagtgg3') and MOR reverse primer: (5' ttccgcggtgaattgaagctt 3'). Restriction sites, *KpnI* and *SacII* are shown underlined. KOZAK sequence is shown in bold.

Table 2.2 PCR conditions for the genes amplified.

Product	**Template-Primers**	**PCR Conditions**
PreS2S-21 (843 bp)	pS2S-pIL2 plasmid with MOF and MOR primers	Initial denaturation: 1 min at 94 °C Denaturation: 1 min at 94 °C Annealing: 1 min at 55 °C Extension: 1 min at 72 °C Final extension: 10 min at 72 °C 35 cycles

2.12 Preparation of *E. coli* DH5α Competent Cells

For the preparation of chemically competent *E. coli* cells, the protocol described by Sambrook *et al*., 1989 was used with slight modifications. *E. coli* cells were inoculated into 5 ml LB medium (Appendix A) from freshly grown LB Agar plates and incubated overnight at 37°C on a rotary shaker. 300 µl of this seed culture were inoculated into 50 ml of LB broth and incubated at 37°C with vigorous shaking until OD_{600}: 0.6 to obtain an exponentially growing culture. Then the culture was split into two prechilled centrifuge tubes aseptically and spun down at 4000 rpm for 10 min at 4°C. Supernatant was decanted and cells were resuspended in 5 ml cold 10 mM $CaCl_2$ by brief vortexing. After pooling the cell suspension into one tube, cells were spun down at 4000 rpm for 10 min at 4°C. Supernatant was decanted and cells were resuspended in 75 mM $CaCl_2$ solution very gently. Competent cells were stored frozen at -80°C with 50% glycerol in 200 µl aliquots.

2.13 Transformation of *E. coli* DH5α Cells

The chemically competent *E. coli* cells were transformed as described by Sambrook *et al*. (1989). 200 µl aliquots of competent *E. coli* cells were taken from -80 C° and stored 15 min on ice. 100 µl of cells were mixed with appropriate amount of DNA (1- 50 ng in a volume of maximum 10µl) gently. Other 100 µl aliquot was left as the control. The mixture was left on ice for 30 min. After a brief heat shock at 42°C for 90 sec, tubes were immediately put on ice for 5 min. 900 µL of LB broth was added to each tube at the end of the period and the cultures were incubated for 80 min at 37°C. Cells were spun down at 4000 rpm for 10 min and supernatant was decanted. The pellet was resuspended in 100 µl LB and spread onto appropriate selective plates of LB for overnight incubation at 37°C.

2.14 Transformation of *P. pastoris* Cells

For the transformation of *P. pastoris* cells by electroporation the following protocol was used with slight modifications (Thompson *et al.,* 1998). *P. pastoris* cells were inoculated into 5 ml YPD medium (Appendix A) from freshly grown YPD agar plates and incubated at 30C° on a rotary shaker. 0.1-0.5 ml of this seed culture was inoculated into 500 BMGH medium (Appendix A) and incubated at 37C° with vigorous shaking until OD_{600}: 1.3-1.5. The culture was then spun down at 1500g for 5 min at 4°C. The pellet was resuspended in 50 ml of Electroporation buffer (Appendix B) and incubated 1 h at room temperature. The cells were spun down at 5000 rpm, 4°C and washed twice in ice cold dH_2O. Resultant pellet was resuspended in 10 ml of ice cold 1 M sorbitol. The cells were spun down at 5000 rpm, 4C° again and resuspended in 100 µl of ice cold 1 M sorbitol. Cells were kept cold on ice to be used in the same day. 80 µl of the electrocompetent cells were mixed with 5-10 µg linearized DNA (in 5-10 µl sterile water) and transferred to an ice cold 0.2 cm electroporation cuvette. Cuvettes were incubated 5 min on ice. The Bio-Rad MicroPulser^{TM} apparatus was set to Pic program (2 kV) and pulsed according to the manufacturer's instructions for yeast. After electroporation, 1 ml ice cold 1M sorbitol was added immediately to the cuvette. Tube content was transferred to a sterile tube and incubated for 1 h at 30°C without shaking. After 1 h, 1 ml YPD (Appendix A) was added and incubated at 30°C, at 300 rpm for 3 more h. After the incubation period, the cells were spreaded onto YPDS plates (Appendix A) with an appropriate antibiotic and incubated 3-5 days until colonies formed.

2.15 Expression of the *M* gene in *P. pastoris*

Expression studies in recombinant *P. pastoris* were done according to the instructions of Invitrogen. *P. pastoris* glycerol stock was grown on YPD plates (Appendix A) for 3 days at 28-30°C. 50 ml of BMGH medium (Appendix A) was inoculated with a single colony of *P. pastoris* grown on YPD plates and incubated

overnight at 30°C to OD_{600}: 2-6 (with appropriate dilution), which corresponded to the logarithmic phase of growth. Cells were harvested at 1500-3000g for 10 min at room temperature and resuspended in 50 ml BMMH (Appendix A) medium aseptically. Cells were grown at 30°C with constant shaking at 300 rpm for five days. 100% methanol was added to a final concentration of 0.5% every 24 h until the end of the experiment. Cells were centrifuged at 3000 g, 15 min at +4°C and cell pellet was frozen in liquid nitrogen and stored at -80°C.

Scale up studies was performed by increasing the culture volume in shake flasks or fermentation in 5L fermentor (Bioengineering). For shake flask experiments, *P. pastoris* glycerol stock was initially grown on YPD agar plates at 30°C. BMGH medium was inoculated with a single colony from the plate and grown at 30°C, 300 rpm for 24 h. This seed culture was used to inoculate 100 ml of BMGH medium and grown for 16-18 h to OD_{600}: 2-6. ~3 g wet cell weight of *P. pastoris* cells obtained from this culture were resuspended in 200 ml BMMH medium and induced with 100% methanol at a final concentration of 0.5% every 24 h for 5 days.

Protein extraction was performed as described by Vassileva *et al*., 2000 with slight modifications. Aliquot of cells corresponding to 100 absorbance units at 600nm were pelleted by low speed centrifugation. 50U/ml Lyticase (Sigma) dissolved in 0.5 ml, 1M Phosphate buffer (Appendix B) were added to the pellet which was then kept 30 min in a 30°C water bath. 0.5 ml Lysis Buffer (Appendix B) containing 0.5 g of 0.2 mm acid washed glass beads were added to the mixture and lysed by 1 min bursts of vortex for 10 times. Mixture was chilled on ice between the intervals. Cell lysate was centrifuged at 6000 rpm for 10 min at 4°C and supernatant was frozen with liquid nitrogen and stored at -20°C for further analysis.

For batch fermentation, *P. pastoris* cells were grown to OD_{600}: 2-6 in 50 ml BMGH medium overnight and this culture was used to inoculate 3.5 L BMGH medium in the fermentor. Fermentation was performed at 30°C with 750 rpm and constant aeration.

Dissolved oxygen level was monitored to determine the state of culture. Batch culture was first grown in the BMGH medium until glycerol was completely consumed. Consumption of glycerol was followed by the DO spike. This phase of glycerol consumption was named as Glycerol Batch Phase and after this initial phase was over, methanol induction was started at 7.3 ml/hr/liter feed rate for four hs. After four hs, DO level started to decrease and then methanol (1% of culture volume) and casamino acid (0.1% of culture volume) was added every 24 h, for four days to keep induction. Cell pellet was frozen with liquid nitrogen and stored at -80°C. For the extraction of proteins, Dyna-mill Bead Agitator was used at the Central Laboratory of METU. Cell pellet frozen at -80°C was dissolved to OD_{600}:100 with 300 ml lysis buffer (Appendix B) and agitated under the following conditions: 2950 rpm, 10 min, +4°C. Cell lysate was centrifuged at 6000 rpm for 10 min at 4°C, the supernatant was frozen in liquid nitrogen and stored at - 20°C till purification.

2.16 SDS Polyacrylamide Gel Preparation

A vertical electrophoresis apparatus was used for SDS polyacrylamide gel electrophoresis (PAGE). SDS Polyacrylamide gel was prepared as shown in Table 2.3. Proteins to be loaded were diluted 1:1 in 2X Laemmli sample buffer (Appendix B) and put into a boiling water bath for 15 min (Laemmli, 1970). Samples were loaded 10 μg/well to the gel and run in Running Buffer (Appendix B) at 25 mA until loading dye reaches end of the gel.

Table 2.3 SDS Polyacrylamide Gel Preparation

	Stacking Gel 0.125 M Tris, pH 6.8	**Separating Gel** 0.375 M Tris, pH 8.8
Monomer concentration	4.5%	12%
Acrylamide/bis dH2O 1.5 M Tris-HCl, pH 8.8 0.5 M Tris-HCl, pH 6.8 10% (w/v) SDS 10% Ammonium persulphate (fresh) TEMED	1.3 mL 6.1 mL - 2.5 mL 100 μL 50 μL 10 μL	4 mL 3.35 mL 2.5mL - 100 μL 50 μL 5 μL
TOTAL MONOMER	10 mL	10 mL

Proteins were stained by Silver Staining as described by Switzer *et al*., 1979. Gel was soaked into fixation solution (Appendix B) for at least one h and washed in 50% ethanol for 15 minutes. After washing, gel was soaked into pretreatment solution (Appekdix B) for 1 minute and washed with dH_2O for 20 seconds. Gel was then immersed into impregration solution (Appendix B) for 20 minutes and washed twice with dH_2O for 20 seconds. The gel was then washed twice with dH_2O and soaked into development solution (Appendix B). Reaction was stopped with stop solution after protein bands became visible.

2.17 Protein Quantitation

Protein concentration measurements were done by measuring optical density at 280 nm wavelength or by using Bradford method (1976). Bradford assay is based on the observation that certain basic amino acids bind to the acidic solution of Coomassie Brilliant Blue G-250 dye in a spectral shift at 595 nm. 1 mg/ ml Bovine Serum

Albumin (BSA) protein was used to prepare a calibration curve. Bradford stock reagent (Appendix B) was diluted 1:5 and gravity filtered using a Whatman No.1 filter paper. Standards and samples were prepared as shown in Table 2.4 in duplicates. A calibration curve of absorbance at 595 nm versus protein concentration in microgram was drawn at 595 nm wavelength was constructed accordingly.

Table 2.4 Preparation of standards for Bradford Assay.

BSA	**dH_2O**	**Bradford 1X**
2µl	98µl	900µl
4µl	96µl	900µl
6µl	94µl	900µl
8µl	92µl	900µl
10µl	90µl	900µl
Sample	**dH_2O**	**Bradford**
2µl	98µl	900µl
Blank		
	dH_2O	Bradford
	100µl	900µl

2.18 Dot Blot

Nitrocellulose membrane was prewetted in TBS (Appendix B) for 15 min and dried completely under laminar flow. Proteins to be analyzed were spotted onto the nitrocellulose membrane at known concentrations and dried completely. Membrane was blocked in 5% skim milk solution (Appendix B) overnight at 4°C and washed 3 times (5 min) in TBS with 0.5% Tween-20. Membrane was then incubated for 1 h at room temperature in mice anti-HBsAb solution diluted (1:300) in 5% skim milk (Appendix B) with constant shaking. Serum of Balb-C mice, immunized twice in 15

day intervals with Sanofi Pasteur's Hepatitis B vaccine was used as antibody unless otherwise stated. The membrane was then washed 3 times (5 min) in TBS with 0.5% Tween-20 and incubated 1 h at room temperature in alkaline phosphatase conjugated, goat anti-mouse IgG (Sigma) solution prepared in 5% skim milk with constant shaking. Conjugate was diluted according to manufacturer's instructions. After incubation with conjugate, membrane was washed 3 times (5 min) in TBS solution. Biorad AP (Alkaline Phosphatase) Substrate kit was used for visualization of proteins. Membrane was incubated in substrate solution (prepared according to the manufacturer's instructions) for 5-20 min until protein spots became visible. Colour development (purple when positive) was stopped with dH_2O.

2.19 Western Blot

Western Blotting was performed as described by Towbin *et al*., 1979 with slight modifications. Whatman (3D) papers and nitrocellulose sheet (Biorad) cut to the size of SDS-PAGE gel were prewet in transfer buffer (Appendix B) and placed onto semidry gel electrophoresis apparatus (Cleaver Scientific). Proteins were transferred onto nitrocellulose membrane sheet at 1.5 μA / cm^2 for 1.5 h. Nitrocellulose sheet was incubated 2 h at 37°C in 10% skim milk (Appendix B) for blocking purposes to prevent non-specific binding of serum. After blocking, nitrocellulose sheet was washed 3 times in TBS with 0.5% Tween-20. Membrane was then incubated for one h at room temperature in mice anti-HBsAb solution diluted (1:300) in 5% skim milk (Appendix B) with constant shaking. Serum of Balb-C mice, immunized twice in 15 day intervals with Sanofi Pasteur's Hepatitis B vaccine was used as antibody unless stated otherwise. After antibody incubation, membrane was washed 3 times (5 min) in TBS with 0.5% Tween-20 and incubated 1 h at room temperature with alkaline phosphatase conjugated, goat anti-mouse IgG (Sigma) solution prepared in 5% skim milk with constant shaking. After incubation in conjugate, membrane was washed 3 times (5 min) in TBS solution. Biorad AP Substrate kit was used for the detection of proteins colorimetrically. Membrane was incubated in substrate solution prepared

according to the manufacturer's instructions 10-40 min until protein bands were visible. Colour development was stopped with dH_2O.

2.20 Adsorption Chromatography

Batch chromatography was performed in two parts. First part was performed as described by Bardiya, 2005 with slight modifications. 4% Aerosil 380 (Degusa) was prepared in 0.15 M NaCl solution and pH was adjusted to 7.3. Soluble proteins were obtained by centrifugation at 13000 rpm, 10 min, 4°C and dialyzed overnight against Phosphate Buffered Saline (PBS) solution (Appendix B) using cellulose membrane tubing with 12000 MW cut off. Proteins were mixed with 4% Aerosil in 1:1 ratio (one volume total) and adsorption was carried out by gentle stirring on a magnetic stirrer for 16-18 h at +4°C. Upon completion of adsorption, protein adsorbed aerosil was recovered by centrifugation at 7000 rpm for 15 min at +4°C. Resulting slurry was washed twice in PBS (7000 rpm for 15 min at +4°C). Desorption was performed in one volume of 10 mM carbonate buffer, pH: 9.2 (Appendix B) for 6 h at room temperature with gentle stirring. Desorbed HBsAg from aerosol was recovered in the supernatant after centrifugation of the mixture at 26000 rpm, 30 min, +4°C and stored at -20°C.

Second part was performed as described by Wijnendaele *et al*, 1987. Insoluble protein precipitate obtained after clarification was dissolved in one volume of H buffer, containing 4M urea (Appendix B) with gentle stirring on a magnetic stirrer, for 1 h at room temperature. 4% Aerosil was prepared in N buffer (Appendix B) and dissolved protein pellet was gently mixed with the solution on a magnetic stirrer in 1:1 ratio, overnight at +4°C. After adsorption, slurry was washed twice with PBS as described in the first part. Desorption was performed in one volume of 10 mM carbonate buffer, pH: 9.2 for 6 h at room temperature with gentle stirring. Desorbed HBsAg from aerosol was recovered in the supernatant after centrifugation of the

mixture at 26000 rpm, 30 min, +4°C and concentrated using cross flow filtration through a 100.000 Da cut off membrane.

2.21 Size Exclusion Chromatography

Size exclusion chromatography was performed with Hi load 16/60 Superdex200 prep grade column (Pharmacia) at a flow rate of 2ml/min at the Central Laboratory, METU. Separation range of the column varied between 10.000-600.000 Da with 120-124 ml volume. Column was equilibrated with 10 mM carbonate buffer at pH: 9.2 (as running buffer) for 15 h at 0.5 ml/min flow rate at +4°C. Maximum pressure applied to the column was 55 psi. The reaction was performed in 200 min time and the fraction collection was done in 2 ml. Protein measurements were performed spectrophotometrically at 280 nm wavelength.

2.22 Mice Immunization

The recombinant HBsAg protein was adsorbed onto $Al(OH)_3$ gel for one hour, at room temperature with constant stirring. BalbC mice were immunized subcutaneously with 0.5 ml of this preparation. Vaccination was repeated in 15 days intervals for twice. Serum was collected after one month from the initial vaccine administration and pooled in a single tube.

2.23 Enzyme Linked Immunosorbent Assay (ELISA)

Dia.pro HBsAb commercial ELISA plates coated with HBsAg from human origin were used for ELISA. Mouse sera (control negative serum, control positive serum and test serum) was added to plates and incubated for 2 h at room temperature. The plate was washed with saline containing 0.05% Tween 20 (washing solution), for three times. Alkaline phsopatase conjugated, goat anti-mouse IgG (Sigma) was diluted with the PBS, containing 2% fetal calf serum and 0.05% Tween 20 and added to plates at 1/15000 concentration. The plate was incubated for two h at room

temperature and washed with the washing solution for three times. Biorad AP Substrate was prepared according to manufacturer's instructions and added to the plates. Colour development was observed to judge for the positive or negative results. 100 µl working volume was used during the assay.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Cloning of *PreS2+S* gene in *P. pastoris*

HBsAg *PreS2+S* gene (*M* gene) was amplified from pS2-S/pIL2 plasmid by PCR. MOF and MOR primers, designed by Dr. Melek Özkan were used in the reaction as stated in Table 2.2 in Material and Methods. The expected band size of 864 bp (843 bp from *M* gene and 21 bp from primer added restriction digest and KOZAK sequences) was obtained as shown in Figure 3.1.

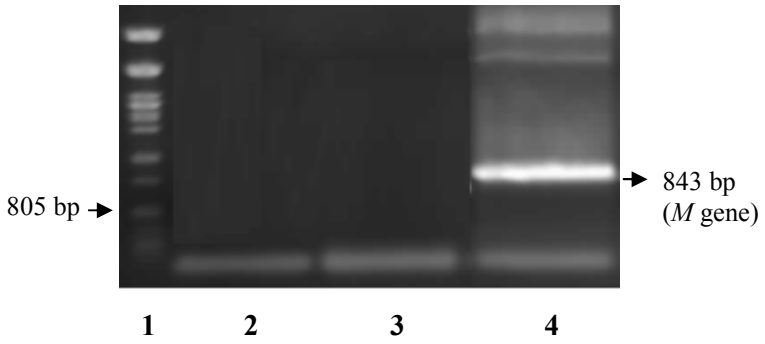


Figure 3.1 PCR amplification of *PreS2-S*. Lane 1: λ Pst Marker, Lane 2-3: Negative control Lane 4: *M* gene (843 bp) amplified by PCR.

Amplified fragments were purified from agarose gel using Qiagen Gel extraction kit and ligated with pGEMT® sub-cloning vector. Ligation product was transformed into competent *Escherichia coli* DH5α cells and recombinants were selected on X-gal + IPTG + ampicillin containing LA plates. Plasmids were isolated from putative

recombinant white colonies and presence of insert was verified by double digestion of restriction enzymes, *Kpn*I and *Sac*II (Figure 3.2).

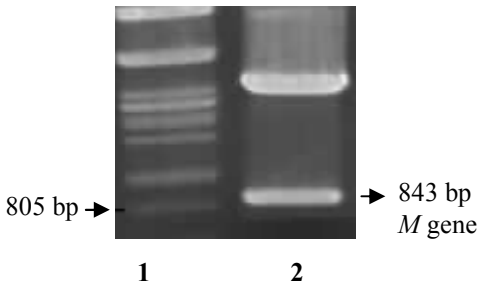


Figure 3.2 Restriction analysis of pGEMT® clones. Lane 1: λ Pst Marker. Lane 2: *Kpn*I and *Sac*II digested pGEMT® vector carrying *M* gene.

For cloning into *P. pastoris* expression vector (pPICZA), both this vector and the pGEMT vector carrying *M* gene were digested with *Kpn*I and *Sac*II enzymes and ligated after purification from agarose gel. Ligation product was cloned into *E. coli* DH5α cells and recombinants were selected on LSLB plates with zeocin. Since high salt concentration inhibits the drug zeocin, LB plates were prepared to contain low salt concentration. Plasmids were purified from the recombinants grown on selective plates and the presence of the gene was verified by PCR. For transformation into *P. pastoris*, the pPICZA plasmid containing the *M* gene was linearized with *Sac*I enzyme, the site of which is in the 5' *AOX1* region. Linearized plasmid was than transferred into *P. pastoris* by. Pretreatment of yeast cells with LiCl (Lithium chloride) and DTT (dithiothreitol) before electroporation had been reported to increase transformation efficiency (Wu *et al*., 2004). Very consistent with this report, our transformation efficiency have increased up to 25 fold when yeast cells were treated with 0.1M LiCl and 10mM DTT before electroporation.

For the strain GS115, gene insertion event was expected to occur at *AOX1* loci and either one of the two *AOX1* regions on the pPICZA vector by homologous recombination. Integrants of such shows stability in the absence of selective pressure. Recombinants were selected on the selective plates containing zeocin.

Transformation of *P. pastoris* with *M* gene was verified by PCR as seen on Figure 3.3. The resultant recombinant strain was named as *P. pastoris* 21/2. The gene inserted into *P. pastoris* 21/2 was amplified with the reverse primer MOR, which does not contain stop codon. Therefore the protein was expected to be expressed with 6xHis tag at its C terminus.

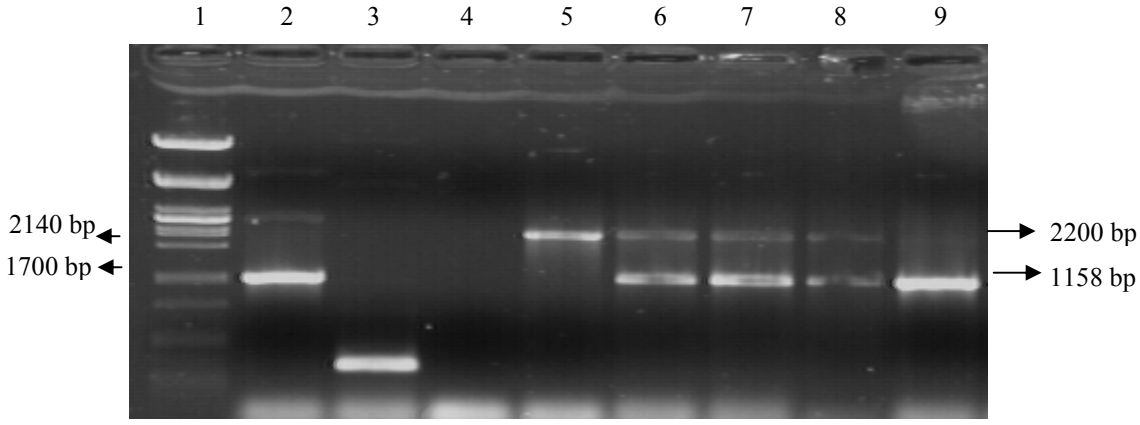


Figure 3.3 Verification of transformation of *P. pastoris* by PCR. *Lane 1*: λ Pst Marker. *Lane 2*: PCR product of pS2-S/pIL2 plasmid using MOF and MOR primers. *Lane 3*: PCR product of pPICZA plasmid using 5'AOX1 and 3' AOX1 primers. *Lane 4*: PCR negative control. *Lane 5*: PCR product of genomic DNA of *P. pastoris* using 5'AOX1 and 3' AOX1 primers. *Lane 6-9*: PCR product of recombinant *P. pastoris* using 5'AOX1 and 3' AOX1 primers.

As seen on Figure 3.3., there were two bands on agarose gel for recombinant colonies. Parent plasmid pPICZA gave rise to the amplification of a 1158 bp PCR product (846 bp gene + 325 bp from the plasmid) and 2.2 kb corresponding to the *AOX1* gene. The presence of two bands on agarose gel showed that the *AOX1* gene was not disrupted so that the resultant colonies were all Mut^+. HBsAg particles can be fold in the desired 22 nm conformation more efficiently in Mut^s strain when compared to Mut^+ strain. This is assumed to be due to slow growth of Mut^s strain which prevents aggregation of HBsAg particles to a larger extent (Cregg *et al*, 1987). However, the time required for the production of optimum HBsAg is much longer in

Mut^{s} (up to 240 h) because of the low metabolic activity of this strain during methanol induction when compared to fast growing Mut^{+} strain. Ottone *et al*., 2007 achieved expression of HBsAg in Mut^{+} strain with similar productivity, when cells were maintained at a limited growth during induction.

3.2 Expression of PreS2+S gene in *P. pastoris*

Expression studies were first performed in shake flask cultures to verify recombinant protein production. Instructions in Invitrogen: Easy select Pichia Expression Kit manuel was followed with slight modifications. Temperature was set up to 30°C throughout the expression experiments. *P. pastoris* grows optimally at temperatures between 28-30°C. Culture volume was 20% of the total flask volume to maintain sufficient aeration throughout the experiment. Although aeration is not critical when obtaining initial biomass, it is very important during induction. Initial growth in the medium with glycerol is important to obtain sufficient biomass before induction. Presence of glycerol (or glucose) in the medium represses *AOX1* gene. For that reason, it is critical to remove glycerol completely from the medium, before methanol induction is started. After sufficient growth (exponential phase) in the BMGH medium, cells were centrifuged and resuspended in 50 ml BMMH medium to start induction with methanol. After cells were transferred to BMMH medium, methanol induction was carried out for five days with 0.5% MeOH.

Growth of recombinant *P. pastoris* 21/2 (under the conditions stated above), was compared to that of the wild type (GS115) *P. pastoris* (Figure 3.4). There was no significant difference in the growth of recombinant and wild type strains when grown in BMGH and BMMH medium.

Gene insertion event occurs at the *AOX1* loci for GS115 strain by homologous recombination. Recombinant strains' growth rate was almost the same to that of the

wild type strain, indicating that gene insertion had no significant effect on growth of *P. pastoris*.

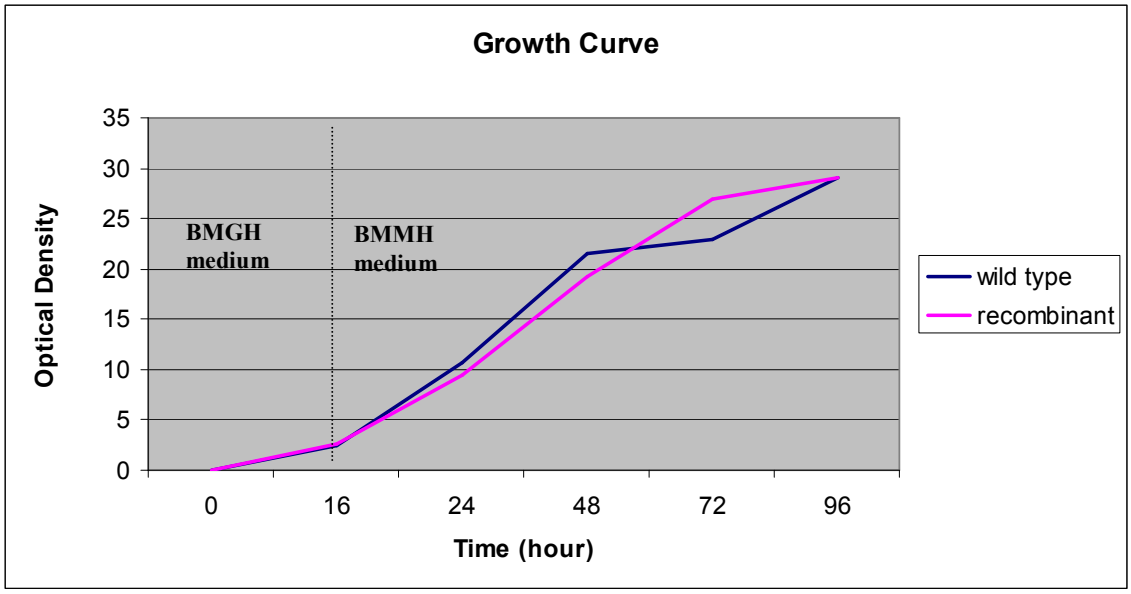


Figure 3.4 Growth curves of the wild type (GS115) and the recombinant *P. pastoris* strains (21/2) in BMGH and BMMH medium.

After 5 days induction with methanol, the yeast lysate was prepared by using the glass beads to analyze the expression of HBsAg. pPICZA vector does not contain secretion signal, so the protein is expected to be expressed intracellularly. Extraction of HBsAg from *P. pastoris* was performed using glass bead-vortex system for shake flask cultures. Efficiency of the cell lysis was examined under microscope by looking at the cellular morphology. It was observed that glass bead vortex system alone resulted in an inefficient cell lysis. A similar observation was reported by Vassileva *et al*., 2000. To increase efficiency of cell lysis, lyticase treatment was used before glass bead extraction as described in Material and Methods, Section 2.15.

HBsAg accumulates in the membranous fraction. For this reason, addition of a detergent to the lysis buffer is required. Triton X-100 was added to the cell disruption buffer as described by Chauhan *et al*., 1998. Protein expression was analyzed by dot blot analysis using the anti-HBsAb serum obtained from mice immunized with Pasteur's hepatitis B vaccine by subcutaneous injection at two weeks intervals. *P. pastoris* 21/2 which was not induced with methanol was used as a negative control. HBsAg protein (especially PreS2+S protein) is known to be very sensitive to proteolytic digestion (Ottone *et al*., 2007). For this reason, all lysing steps were carried out on ice in the presence of PMSF (1mM) added to the breaking buffer to minimize proteolysis. Expression of recombinant HBsAg protein was verified by dot blot analysis (Figure 3.5).

Figure 3.5 Dot blot analysis of shake flask cultures. 1- Negative control. 2- *P. pastoris* 21/2 cell lysate induced with methanol.

Scale up of the expression was first achieved in shake flask cultures as explained in Material and Methods, Section 2.15. Dot blot analysis of these cultures are shown in Figure 3.6.

Figure 3.6 Dot blot analysis of shake flask cultures for scale up of expression.
1- Negative control. 2- *P. pastoris* 21/2 cell lysate induced with methanol.

For further scale up of expression, fermentation was done in 3.5 liter BMGH medium. Inoculum seed was used to grow the culture in 50 ml of BMGH medium (Appendix B) at 30ºC, 300 rpm, 18 h to OD_{600}: 5 (exponential phase). 35 ml of the seed culture (1% of the fermentation volume) was inoculated into a 3.5 liter of BMGH medium. Fermentation was carried out in two phases: Glycerol batch phase and Methanol fed batch phase. Fermentation parameters were set as 30ºC temperature, 600 rpm stirring, constant aeration (50% aeration rate). pH set to 6 initially and left uncontrolled from then on. Cell growth was monitored by the absorbance measurement at 600 nm (Figure 3.7). Dissolved oxygen concentration (relative percent of the oxygen in the O_2 saturated medium) was set to 20%. Dissolved oxygen (DO) concentration is an important parameter during the fermentation of *P. pastoris* because it provides information about the state of the culture. During growth, the culture consumes oxygen, by keeping the DO concentration low. When the carbon source (glycerol) is limiting, metabolic rate of the culture decreases and DO level rises.

At 20^{th} h of growth, there was a sudden decrease in the DO level. Cells were actively growing on glycerol medium at that time and set up parameters for aeration (600 rpm and 50% aeration rate) could not satisfy the required oxygen level. Aeration rate was increased to 100% and rpm was increased to 750 to supply culture with more oxygen. Culture was grown for 44 h in Glycerol batch phase until the glycerol was completely consumed. Consumption of glycerol was evident by an increase in DO level. Methanol was then slowly introduced to the culture for adaptation. Methanol was fed at 3.6 ml/h/l initial 4 hours. *P. pastoris* can assimilate methanol but cannot tolerate high methanol concentrations because of the accumulation of formaldehyde and hydrogen peroxide inside the cells, both of which being the oxidized products of methanol by the alcohol oxidase and are toxic to the cells (Couderc and Baratti, 1980). After the adaptation of the culture on methanol, induction was carried out for 4 days with 1% MeOH and 0.1% Casamino acids. Chauhan *et al*. (1998) reported

that addition of 0.1% Casamino acids to the fermentation medium increases expression levels of HBsAg, possibly by supplying precursors needed for protein synthesis with. pH also being an important parameter for protein expression in *P. pastoris*. *P. pastoris* cell are best grown in pH: 5-6, but it is also resistant to lower pH values of pH: 3. BMGH and BMMH medium was initially buffered with phosphate buffer to pH 6. After an initial 3 h of growth, pH of the medium decreased to pH 5.8. At 20^{th} h of fermentation in glycerol medium, pH of the culture decreased to 5.4 and remained stable until all glycerol was consumed at 44 h. At the beginning and on the first day of methanol induction, pH level remained constant at 5.4. However during the second day of methanol induction, pH decreased to 4.6. This decrease in pH can be attributed to by products of methanol metabolism, hydrogen peroxide and formaldehyde. Formation of by products indicated that, cell metabolism was switched to the methanol use as the carbon source. Slight increase in the optical density of the culture in the second day of methanol induction supported this idea as well. pH was then re-adjusted to 5.4 and stabilized there in order not to decrease growth rate of culture. At 4 days of induction, DO level and the optical density continued to increase. This increase was slower when compared to that in glycerol medium as expected. At 4^{th} day of induction, DO level increased to 16%, although optical density of the culture remained the same. This indicated that, cellular metabolism slowed down and cells were no more using the O_2. Essential nutrients other than the carbon source might have become limited in the fermentation broth, which might have slowed down the growth.

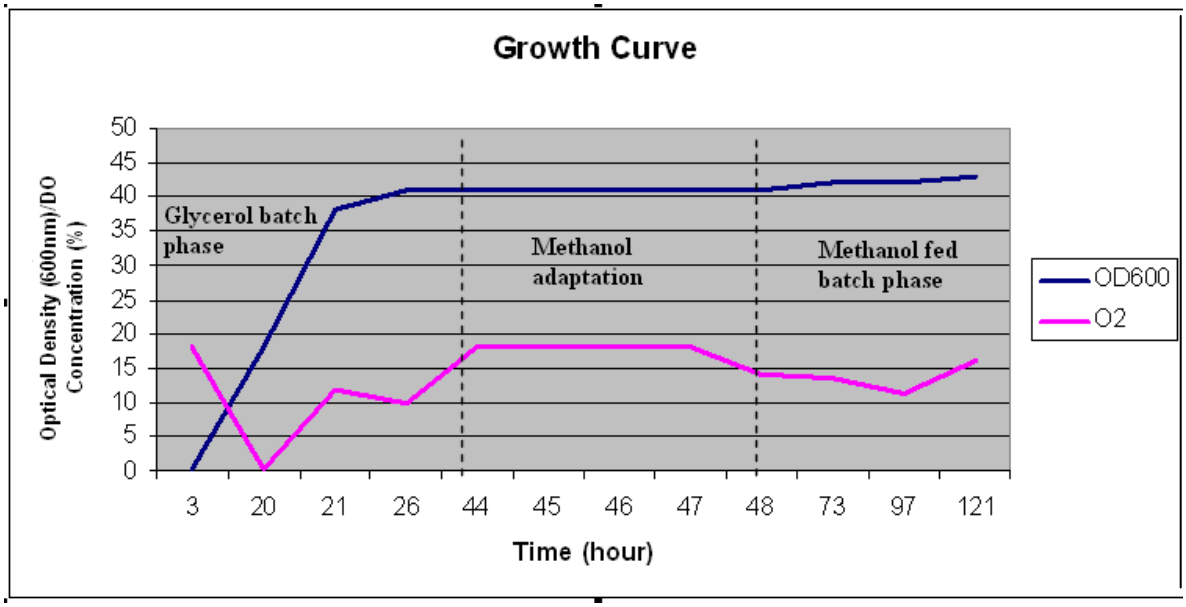
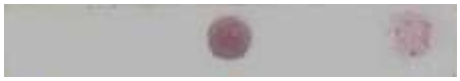


Figure 3.7 Growth curve of *P. pastoris* in a 3.5 L fermentor.

Dyna-mill bead agitator was used for protein extraction from 4 days old fermentor culture. Pellet was frozen in liquid nitrogen and resuspended in lysis buffer to an OD_{600}: 100 and agitated under conditions described in Material and Methods, Section 2.15. Extraction was performed in two cycles, under the same conditions, to increase the protein yield. Extracted proteins from both cycles were subjected to dot-blot analysis and recombinant protein expression was verified. *P. pastoris* 21/2 without methanol induction was used as a negative control (Figure 3.8).



1 2 3

Figure 3.8 Dot Blot analysis of the fermentor culture. 1- Negative control. 2- Proteins obtained from the first cycle of extraction. 3- Proteins obtained from the second cycle of extraction.

HBsAg protein obtained from the fermentor culture, formed an insoluble aggregate upon storage at -80°C. Protein was clarified by centrifugation at 30.000g, 15 min at +4°C. It has been shown by dot blot analysis that both clarified portion (soluble HBsAg) and precipitate (insoluble HBsAg) gave a positive reaction with anti-HBsAb (Figure 3.9). Insoluble fraction of HBsAg could not be dissolved in lysis buffer without presence of at least 2 M Urea.

1 2

Figure 3.9 Dot Blot analysis of HBsAg. (1) Soluble fraction and (2) Insoluble fraction**.**

3.3 Purification of HBsAg protein

3.3.1 Adsorption Chromatography

There are several techniques described in literature for the large scale purification of HBsAg protein such as a combination of ion-exchange chromatography, size exclusion chromatography and ultracentrifugation (Samanta *et al*, 1989), ultracentrifugation (Deml *et al*, 1999), ammonium sulfate precipitation followed by gel permeation chromatography (Kim *et al*, 1996), affinity chromatography of 6xHis-tagged (Bisht *et al*, 2002), or GST-tagged recombinant proteins (Maeng *et al*, 2001) or a single-step purification with monoclonal antibodies (Ibarra *et al*., 1998). Purification with immunoaffinity chromatography using monoclonal antibodies is a fast and efficient method; however, this technique is difficult and expensive.

Adsorption of HBsAg to diatomaceous earth matrix (Agraz *et al*., 1994) or colloidal silica matrix such as Aerosil 380 (Bardiya, 2005) has been an initial step of purification described in the literature. We have used Aerosil 380 (Degusa) for

adsorption chromatography. Soluble and insoluble HBsAg were processed separately to increase the product yield as described in Material and Methods, Section 2.20. 4 M Urea was added to the adsorption buffer to solubilize HBsAg aggregates. Solubilization of HBsAg inclusion bodies at an urea concentration lower than 2 M was unsuccessful. HBsAg is known to be adsorbed onto colloidal silica non-specifically and the protein is known to be less tightly bound onto aerosil than contaminating proteins. For that reason, desorption of rHBsAg was expected to be more specific than the contaminating proteins by use of a basic pH buffer (10 mM carbonate buffer). As noted in the literature, that extended desorption time beyond 2 h does not cause desorption of contaminating proteins (Bardiya, 2005). It was observed in our experiment that the protein desorption was not complete in 2 h. For that reason, the desorption time was extended to 6 h to increase the protein yield. Another important factor was the pH of the desorption buffer. At pH lower than 9.2, desorption efficiency was very low even at 6 h desorption time as seen on Table 3.1.

Table 3.1 Adsorption chromatography of extracted proteins at different pH values.

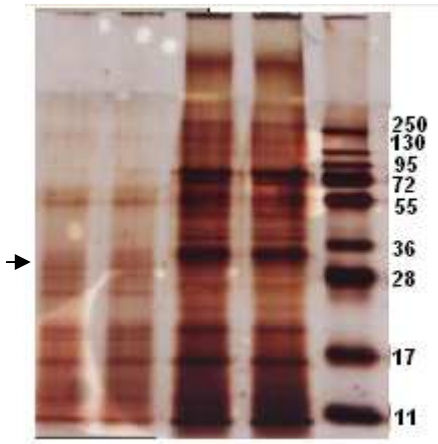
	OD_{280}	
	pH 9.2	pH 8.6
Protein loaded	1.508	0.745
Unbounded proteins	0.735	0.335
First wash	0.272	0.193
Second wash	0.100	0.141
Eluted proteins	0.382	0.007

Dot blot analyses of batch chromatography products were performed as described in Material and Methods, Section 2.18 (Figure 3.10).

1 2 3 4 5

Figure 3.10 Dot blot analysis of batch chromatography. 1-Original protein loaded. 2- Unbound proteins 3- First wash 4- Second wash 5- Eluted proteins.

As seen on Figure 3.10, positive reaction of rHBsAg in the unbound proteins indicated that, capacity of the matrix was exceeded for adsorption of rHBsAg at given concentration (OD_{280}: 1.508). There was no significant amount of rHBsAg desorbed from matrix during washing with PBS at pH 7.5. This result was consistent with the observation that specific desorption of HBsAg only occurs under basic conditions. Likewise it has been approved on dot blot analysis that, HBsAg was specifically desorbed from aerosil matrix at pH 9.2 using carbonate buffer.



1 2 3 4 5

Figure 3.11 SDS PAGE of adsorption chromatography. Lane 1: Original protein loaded to matrix. Lane 2: Desorption product.

SDS page analysis of adsorption chromatography has been performed as described in material and methods (Figure 3.11) and it has been shown that, rHBsAg has been

removed from the bulk extracted proteins and semi-purified using adsorption chromatography.

After desorption from aerosil matrix, cross flow filtration has been chosen to concentrate the protein products. 100.000 Da cut off membrane was used for that aim and the protein has been successfully concentrated almost seven fold. Cross flow filtration was also preferred to remove any possible trace of urea, which may interfere with the antigenic structure of HBsAg. Partially purified proteins appeared as a cloudy precipitate after being concentrated with cross flow filtration.

3.3.2 Size Exclusion Chromatography

Pharmacia Hiload 16/60 Superdex 200 prep grade column was used for the size exclusion chromatography. Separation range of the column varies between 10.000 to 600.000 Da and the column volume is 120 ml. Proteins appeared as a cloudy precipitate after concentrated with cross flow filtration as described above. The insoluble precipitates have been removed by centrifugation and supernatant has been loaded into the column. 10 mM carbonate buffer (pH: 9.2) was used for equilibration of the column. A total volume of 5 ml protein could be loaded and each fraction was collected in 2 ml after a 45 ml of void volume. Protein absorbance readings were done at 280 nm wavelength and the flow rate was set up to 2ml/min. Chromatogram of the size exclusion chromatography was as shown in Figure 3.12.

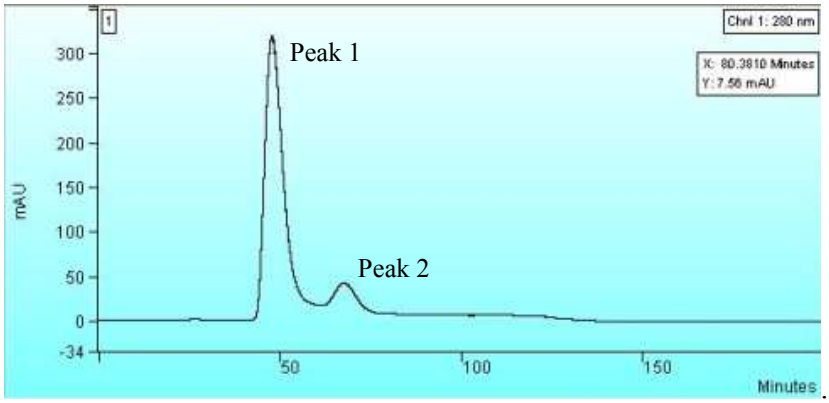


Figure 3.12 Size exclusion chromatography at 2ml/min flow rate.

Two peaks obtained from the size exclusion chromatography were analyzed with dot blot analysis which revealed that HBsAg was eluted in the first peak. Cell lysate of *P. pastoris* 21/2 which was not induced with methanol was used as the negative control (Figure 3.13).

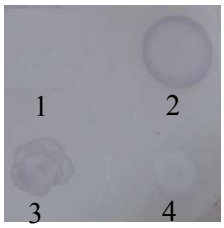


Figure 3.13 Dot blot analysis of size exclusion chromatography. (1) Negative control (2) Protein loaded to size exclusion chromatography (3) Peak 1 (4) Peak 2

SDS PAGE analysis was performed as described in material and methods to check for the purity of the eluted Peak 1 obtained from size exclusion chromatography (Figure 3.14).

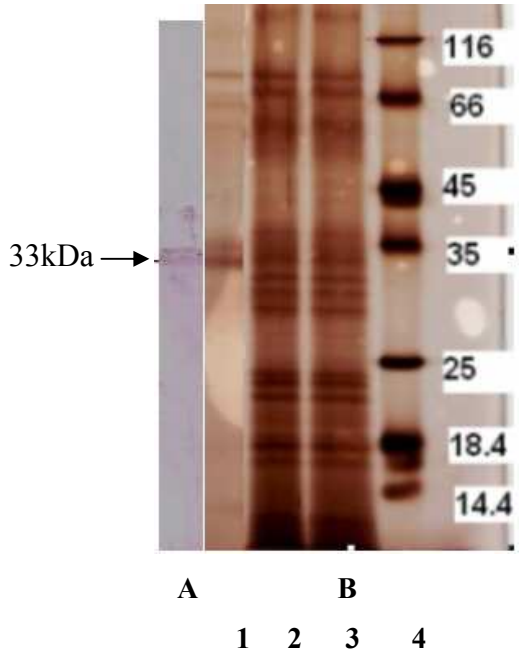


Figure 3.14 SDS Page and Western blot analyses of the eluate from size exclusion chromatography. A) Western Blot analysis of Peak 1 from the size exclusion chromatography. B) Lane1: Peak 1 from the size exclusion chromatography. Lane 2-3: The total protein loaded to size exclusion chromatography. Lane 4: Protein size marker.

It has been reported in the literature that HBsAg protein has been produced in non-glycosylated form in *P. pastoris* expression system (Bo *et al*., 2005). M protein is 32 kDa in unglycosylated form and it is expected to be expressed as a ~33kDa protein along with 0.84 kDa from 6xHis Tag. Expression of rHBsAg protein in non-glycosylated form (33kDa) has been confirmed by SDS PAGE and Western blot analyses (Figure 3.14). Purification of rHBsAg protein by adsorption chromatography and ultrafiltration, followed by size exclusion chromatography was also verified through SDS Page and Western blot analyses as shown in Figure 3.14. During *in vitro* purification of yeast derived antigen, numerous disulfide bonds form between monomers creating mature particles. Formation of high molecular weight aggregates of HBsAg in size exclusion chromatography has also been reported in the literature (Bardiya, 2005). For that reason, HBsAg was expected to be eluted in

earlier fractions of size exclusion chromatography; considering that larger proteins are eluted faster than smaller proteins. Accordingly, rHBsAg was eluted as the first peak of the chromatogram. HBsAg mature particles migrate as a monomer under reducing conditions (Wampler *et al*., 1985). For that reason, we have observed rHBsAg as a 33 kDa protein in SDS PAGE and Western blot analyses.

3.4 Immunogenicity of rHBsAg

Mice immunogenicity test was done to check for the immunogenicity of HBsAg purified from the recombinant *P. pastoris*. 60µg/ml protein obtained from the size exclusion chromatography was adsorbed onto $Al(OH)_3$ gel and this preparation was used to immunize mice to evaluate IgG response elicited by the antigen. Production of anti-HBsAb in test animals was evaluated with Dia.pro HbsAb commercial ELISA Kit. The kit was coated with the heat inactivated HBsAg (ad/ay subtypes) obtained from a human origin (plasma). Unvaccinated BALB-C mice serum was used as a negative control. Mice immunized with Pasteur's Hepatitis B vaccine (as described in Material and Methods, Section 2.22) was used as positive control. The test serum and the control serums were used without dilution. The results of ELISA test is shown in Figure 3.15.

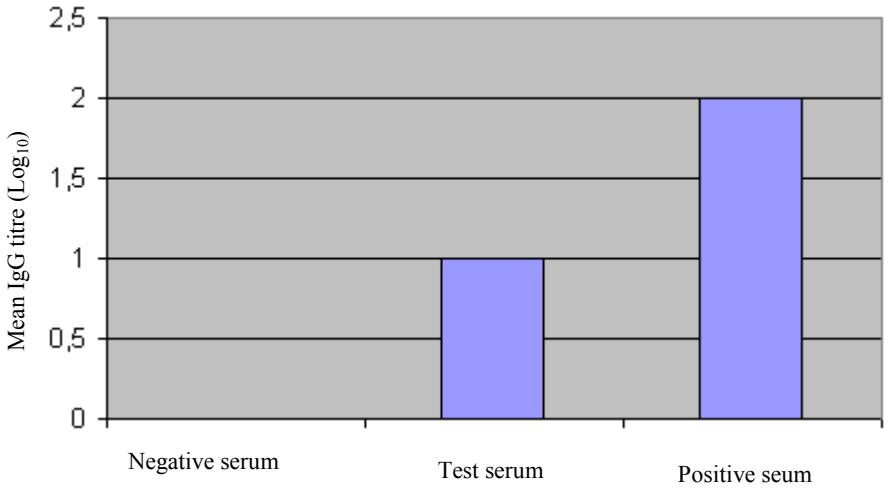


Figure 3.15 Specific IgG levels in mice, immunized with rHBsAg (ELISA test). Column 1: Negative serum (unvaccinated mice). Column 2: Test serum (anti-rHBsAb). Column 3: Positive serum (mice immunized with Pasteur's Hepatitis B vaccine).

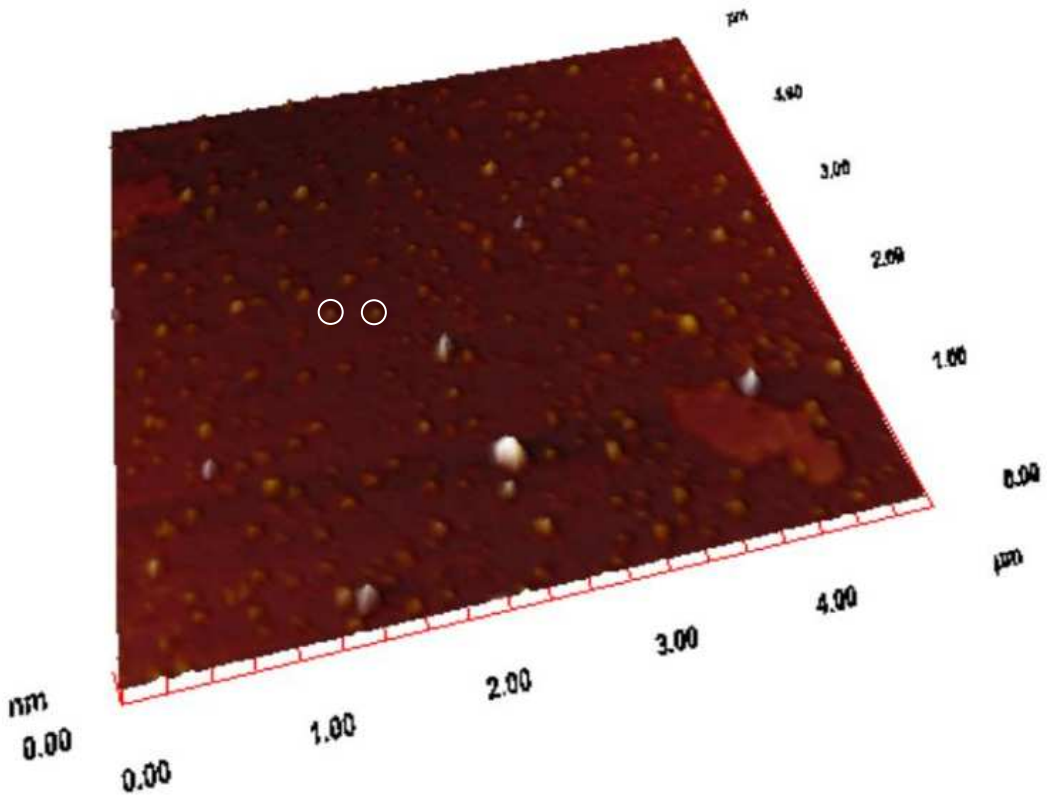
ELISA results have shown that mice immunized with rHBsAg elicited specific anti-HBsAb. This proved that, recombinant protein has been produced in the desired immunogenic conformation.

3.5 Atomic Force Microscopy image of rHBsAg

Atomic Force Microscopy (AFM) images of purified rHBsAg, obtained from size exclusion chromatography were taken by using 'NanoMagnetics Instruments AFM'. Cross Sectional and three dimensional images of HBsAg spherical particles are shown in Figure 3.16 and 3.17. Majority of the particles were between 20-25 nm in size as expected.

HBsAg particles were immunogenic both in monomer and particle forms (~100 monomer units). Still, the 22 nm particle form is used in vaccine formulations, as it is known to be 1000 times more immunogenic than HBsAg monomers. Correct folding of the antigen is very important for its recognition by the immune system (Wampler *et al*., 1985) as verified in the present study.

(a)



(b)

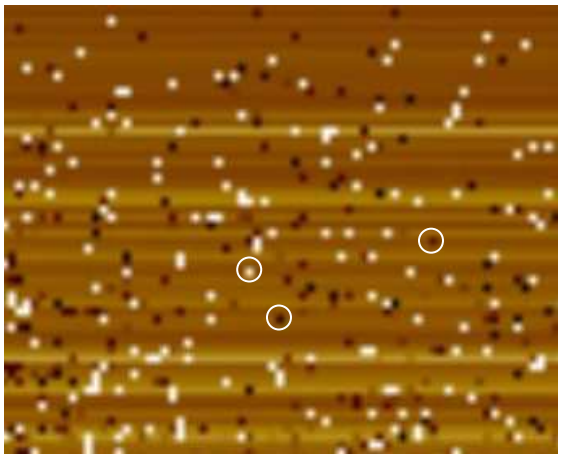
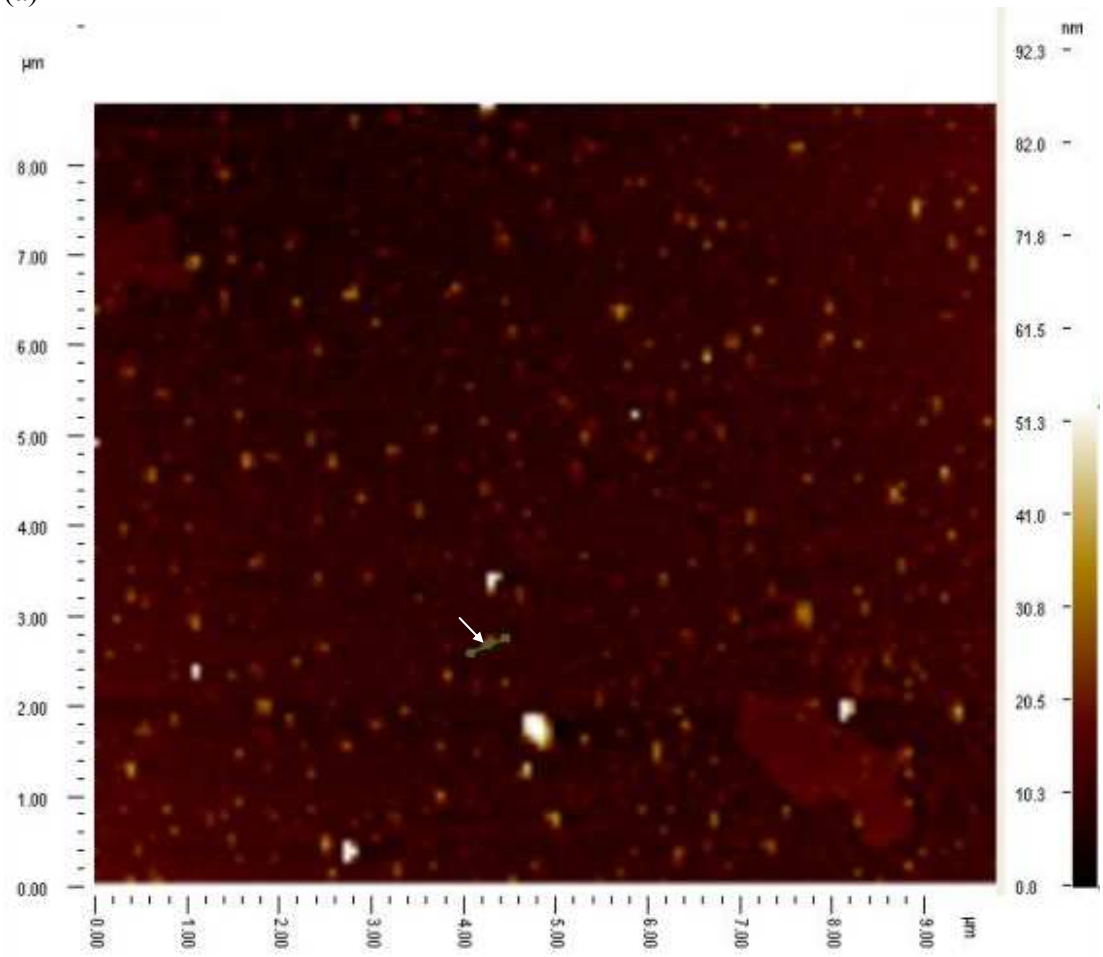


Figure 3.16 AFM image of the rHBsAg particles. (a) Three dimensional image. (b) Phase contrast image. Particles marked with white circles, are standing for rHBsAg particles.

(a)



(b)

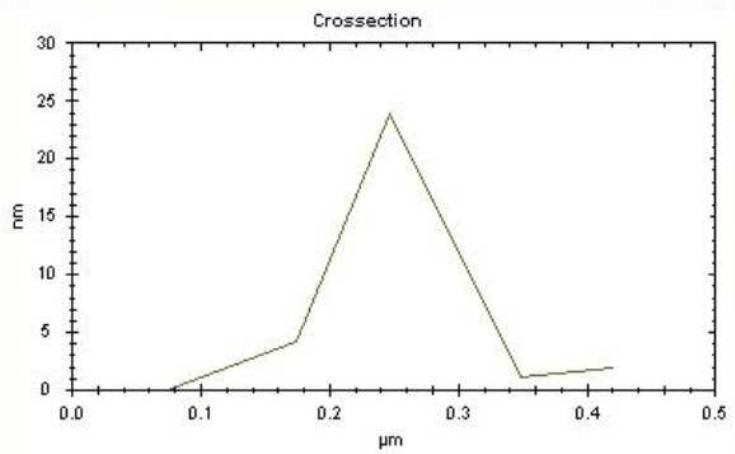


Figure 3.17 AFM image of the rHBsAg particles. (a) Cross Sectional image. (b) Dimension of the rHBsAg particle shown by the white arrow.

HbsAg contains 14 cysteine residues per monomer. It has been noted in the literature that, during *in vitro* purification of yeast derived recombinant antigen, numerous disulfide bonds form between monomers creating mature particles. Besides these, high number of disulfide bonds formed in the antigen is also responsible for the formation of some heterogeneous (larger) particles, due to intermolecular interactions. Formation of high molecular weight aggregates of HBsAg during size exclusion chromatography has also been reported (Bardiya*,* 2005*)*. In this work, majority of the particles obtained were between 20-25 nm in diameter. However, the average diameter of the particles was around 35 nm because of the inhomogeneous particle distribution caused by the existence of some larger particles. Formation of larger heterogeneous particles, besides 22 nm particle formation was something expected and observed as previously mentioned.

To conclude, these results shows us that rHBsAg protein produced in methylotrophic yeast *P*. *pastoris* elicited specific anti-HBsAb production and it was suitable for immunization purpose with it's ~22nm particulate form.

CHAPTER 4

CONCLUSION

- Hepatitis B surface antigen *M* gene (*PreS2-S*) was cloned into pPICZA expression vector which was then transformed to *P. pastoris*. Recombinants were verified by colony PCR.

- Protein product was successfully expressed at a large scale in a batch fermentor, with methanol induction.

- The purification of rHBsAg was accomplished in two steps which involved. Aerosil adsorption-desorption followed by size exclusion chromatography on Superdex 75 column.

- Purified rHBsAg protein was visualized by using Atomic Force Microscopy and determined to be organized in 20-25 nm particles.

- Immunogenicity of purified rHBsAg proteins was demonstrated in BALC-C mice which were shown to produce specific anti-HBsAb response.

REFERENCES

Agraz A., Quiiiones Y., Exposito N., Breiia F., Madruga J., Penton E. (1994). Adsorption-Desorption of Recombinant Hepatitis B Surface Antigen (r-HBsAg) from *P. pastoris* on a Diatomaceous Earth Matrix: Optimization of Parameters for Purification *Biotechnology and Bioengineering* 42 :1238- 1244.

Almeida J.D., Rubenstein D., Stott E.J. (1971). New antigen-antibody system in Australia-antigen-positive hepatitis. *Lancet* 2:1225-1227.

Amberg D.C., Burke D.J., Strathern J.N.**,** Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, 2000.

Anthony C., The biochemistry of methylotrophs, p. 269- 295. Academic Press, Inc., New York, 1982.

Ashton P. G., Murray K. (1989). Mutants of the hepatitis B virus surface antigen that define some antigenically essential residues in the immunodominant a region. *Journal of Medical Virology* **29:**196-203.

Balık S. (1992). Dünyada ve Türkiye'de hepatit B epidemiyolojisi. Ankara, Viral Hepatit Derneği Yayınları, 1992.

Bancroft W. H., Mundon F. K., Russel P.K. (**1972).** Detection of additional antigenic determinants of hepatitis B antigen. *Journal of Immunology* **109:**842-848.

Bardiya N. (2005). Expression in and purification of Hepatitis B surface antigen (S-protein) from methylotrophic yeast Pichia pastoris. Anaerobe **12:** 194–203

Barker L.F., Almeida J.D., Hoofnagle J.H., Gerety R.J., Jackson D.R., McGrath P.P. (1974). Hepatitis B core antigen: immunology and electron microscopy. *J Virol* **14:**1552-8.

Belew M., Mei Y., Li B., Berglo J.H., Janson J.C. (1991). Purification of recombinant Hepatitis B surface antigen (r-HBsAg) produced by transformed CHO cells grown in culture. *Bioseparation* **1:**397-408.

Bisht H., Chugh D.A., Raje M., Swaminathan S., Khanna N. (2002). Recombinant dengue virus type 2 envelope/hepatitis B surface antigen hybrid protein

expressed in *Pichia pastoris* can function as a bivalent immunogen. *J Biotechnol* **99:** 97–110.

Blum H., Bier H. and Gress H.J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8:**93-99.

Blumberg B.S., Sutnick A.I., London W.T. (1968). Hepatitis and leukemia. Their relation to Australia antigen. *Bull NY Acad Med* **44:**1566-1586.

Bo H., Minjan L., Gouqiang L., Zhaoxia L., Zyenhu Z., Lin L. (2005). Expression of Hepatitis B Virus S Gene in Pichia pastoris and Application of the Product for Detection of Anti-HBs Antibody. *Jounal of Biochemistry and Molecular Biology* **38(6):**683-689.

Bonino F., Chiaberge E., Maran E., Piantino P. (1987). Serological markers of HBV infectivity. *Ann Ist Super Sanita* **24 (2):**217–23.

Brechot C., Gozuacik D., Murakami Y., Brechot P.P. (2000). Molecular basis for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). *Semin Cancer Biol* **10:**211-31.

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Bruss V., Ganem D. (1991). The role of envelope proteins in hepatitis B virus assembly. *Proc Natl Acad Sci USA* **88:**1059-1063.

Bruss V., Lu X., Thomssen R., Gerlich W.H. (1994). Post-translational alterations in transmembrane topology of the hepatitis B virus large envelope protein. *EMBO J* **13:**2273-2279.

Bruss V., Vieluf K. (1995). Functions of the internal pre-S domain of the large surface protein in hepatitis B virus particle morphogenesis. *J Virol* **69:**6652-6657.

Bruss V. (1997). A short linear sequence in the pre-S domain of the large hepatitis B virus envelope protein required for virion formation. *J Virol* **71:**9350-9357.

Chauhan K., Arora D., Khanna N. (1998). A novel feeding strategy for enhanced protein production by fed-batch fermentation in recombinant Pichia pastoris. Process Biochemistry **34:**139–145

Chisari F.V., Ferrari C., Viral Hepatitis. In: Nathanson N. *et al*., eds. Viral Pathogenesis, Philadelphia, Lippincott - Raven, 745-778, 1997.

Chisari F.V. (1997). Cytotoxic T cell and viral hepatitis. *J Clin Invest* **99(7):**1472-7.

Chisari F.V. (2000). Rous–Whipple award lecture. Viruses, immunity, and cancer: lessons from hepatitis B. *Am J Pathol* **156:**1117–32.

Cho D.Y., Yang G.H., Ryu C.J., Hong H.J. (2003). Molecular chaperone GRP78/BiP interacts with the large surface protein of hepatitis B virus in vitro and in vivo. *J Virol* **77:**2784-2788.

Chu C.J., Lok A.S.F. (2002). Clinical significance of hepatitis B virus genotypes. *Hepatology* **35:**1274-1276.

Couderc R., Baratti J. (1980). Oxidation of methanol by the yeast *Pichia pastoris*. Purification and properties of alcohol oxidase. *Agric Biol Chem* **44:**2279-2289.

Couroc A.M., Holland P.V., Muller J.Y. Soulier J.P. (1976). HBs antigen subtypes. *Bibliotheca Haematologica* 42, 1

Courouc-Pauty A.M., Soulier J.E. (1974). Further data on HBs antigen subtypes - geographical distribution. *Vox Sanguinis* **27:**533-549.

Courouc-Pauty A.M., Lemaire J.M., Roux J.F. (1978). New hepatitis B surface antigen subtypes inside the ad category. *Vox Sanguinis* **35:**304-308.

Cregg J.M., Barringer K.J., Hessler A.Y., Madden K.R. (1985). *Pichia pastoris* as a host system for transformations. *Mol Cell Biol* **5:**3376-3385.

Cregg J.M., Madden K. R. (1987). Development of yeast transformation systems and construction of methanol-utilization- defective mutants of *Pichia pastoris* by gene disruption. *Biological Research on Industrial Yeasts* **2:**1-18.

Cregg J.M., Tschopp J.F., Stillman C., Siegel R., Akong M., Craig W.S., Buckholz R.G., Madden K.R., Kellaris P.A., Davis G.R., Smiley B.L., Cruze J., Torregrossa R., Velicelebi G., Thill G.P. (1987). High-level expression and efficient assembly of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris*. *Biotechnology* **5:**479-485.

Cereghino J.L., Cregg J.M. (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiology Reviews* **24:**45-66.

Demi L., Schirmbeck R., Reimann J., Wolf H., Wagner R. (1999). Purification and characterization of hepatitis B virus surface antigen particles produced in Drosophila Schneider-2 cells. *J Virol Methods* **79:**205-17.

Dane D.S., Cameron C.H., Briggs M. (1970). Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet* **i:**695–698.

Denniston K.J., Yoneyama T., Hoyer B.H., Gerin J.L. (1984). Expression of hepatitis B virus surface and e antigen genes cloned in bovine papillomavirus vectors. *Gene* **32:**357–68.

Eble B.E., MacRae D.R., Lingappa V.R., Ganem D. (1987). Multiple topogenic sequences determine the transmembrane orientation of the hepatitis B surface antigen. *Mol Cell Biol* **7:**3591-3601.

Eble B.E., Lingappa V.R., Ganem D. (1990). The N-terminal (pre-S2) domain of a hepatitis B virus surface glycoprotein is translocated across membranes by downstream signal sequences. *J Virol* **64:**1414-1419.

Eckart M.R., Bussineau C.M. (1996). Quality and authenticity of heterologous proteins synthesized in yeast. *Curr Opin Biotechnol* **7:**525–530.

Ellis S.B., Brust P.F., Koutz P.J., Waters A.F., Harpold M.M., Gingeras T.R. (1985). Isolation of alcohol oxidaseand two other methanol-regulatable genes from the yeast *Pichia pastoris*. *Mol Cell Biol* **5:**1111-1121.

Emini E.A., Ellis R.W., Miller W.J., McAleer W.J., Scolnick E.M., Gerety R.J. (1986). Production and immunological analysis of ecombinant hepatitis B vaccine. *J Infect* **13:**3-9.

Ganem D., Schneider R.J., Hepadnaviridae: The Viruses and Their Replication. In: Knipe DM *et al*., eds. Fields Virology, 4th ed. Philadelphia, Lippincott Williams & Wilkins:2923-2969, 2001.

Gallina A., Gazina E., and Milanes G. I. (1995). A C-terminal PreS1 sequence is sufficient to retain hepatitis B virus L protein in 293 cells. *Virology* **213:**57-69.

Gavilanes F., Gonzalez-Ros J.M., Peterson D.L. (1982). Structure of hepatitis B surface antigen. Characterization of the lipid components and their association with the viral proteins. *J Biol Chem* **257:**7770-7777.

Gellissen G., Hollenberg C.P. (1997). Application of yeasts in gene expression studies: a comparison of Saccharomyces cerevisiae, Hansenula polymorpha and Kluyveromyces lactis - a review. *Gene* **190:**87-97.

Gerhardt E., Bruss V. (1995). Phenotypic mixing of rodent but not avian hepadnavirus surface proteins into human hepatitis B virus particles. *J Virol* **69:** 1201-1208.

Gerin J.L., Alexander H., Purcell R.H., Dapolito G., Engle R., Green N., Sutcliffe G., Shcnikk, T.M. and Lerner R.A. (1983). Chemically synthesized peptides of hepatitis B surface antigen duplicate the d/y specificities and induce subtype specific antibodies in chipanzees. *Proceedings of the National Academy of Sciences USA* 80:2365-2369.

Guidotti L.G. (1994). Hepatitis B virus nucleocapsid particles do not cross the hepatocyte nuclear membrane in transgenic mice. *Journal of Virology* **68:**5469-5475.

Guo J.T., Pugh J.C. (1997). Topology of the large envelope protein of duck hepatitis B virus suggests a mechanism for membrane translocation during particle morphogenesis. *J Virol* **71:**1107-1114.

Hardy E., Martinez E., Diago D., Diaz R. (2000). Large-scale production of recombinant hepatitis B surface antigen from *Pichia pastoris*. *Journal of Biotechnology* **77:**157-167.

Heermann K.H., Goldmann U., Schwartz W., Seyffarth T., Baumgarten H., Gerlich W.H. (1984). Large surface proteins of hepatitis B virus containing the pre-s sequence. *J Virol* **52:**396-402.

Hoofnagle J.H., Gerety R.J., Barker L.F. (1973). Antibody to hepatitis-B-virus core in man. *Lancet* **2:**869-873.

Hopwood D.A., Bibb M.J., Chater K.F., Keiser T., Bruton C.J., Kieser H.M., Lydiate D.J., Smith C.P., Ward J.M. and Scrempf H., Genetic Manipulation of *Streptomyces*: a Laboratory Manual. Norwich: John Innes Foundation, 1985.

Huovila A.P., Eder A.M., Fuller S.D. (1992). Hepatitis B surface antigen assembles in a post-ER, pre-Golgi compartment. *J Cell Biol* **118:**1305-1320.

Invitrogen:Easy select Pichia Expression Kit, A manuel of Methods for Expression of Recombinant Proteins Using pPICZ and pPICZA in Pichia Pastoris. Version H, 25-0172, 2005.

Ibarra N., Caballero A., Gonzales E., Valdes R. (1998). Comparison of different elution conditions for the immunopurification of recombinant hepatitis B surface antigen. Journal of Chromatography B, 735: 271–277.

Jilg W., Schmidt M., Deinhardt F. (1988). Persistence of specific antibodies after hepatitis B vaccination. *J Hepatol* **6:**201-7.

Kidd-Ljunggren K., Miyakawa Y., Kidd A.H. (2002). Genetic variability in hepatitis B viruses. *J Gen Virol* **83:**1267–80.

Kim H.S., Kim Y.K., Ryu S.E., Hong H.J. (1996). Production of hepatitis B virus preS polypeptide in E. coli by mutation of the 50-end coding sequence and its purification and characterization. *Gene* **177:**173–7.

Kuroki K., Ishikawa T., Harada F., Ganem D. (1995). gp180, a host cell glycoprotein that binds duck hepatitis B virus particles, is encoded by a novel member of the carboxypeptidase gene family. *J Biol Chem* **270:**15022-15028.

Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680-685.

Lambert C., Thome N., Kluck C.J., Prange R. (2004). Functional incorporation of green fluorescent protein into hepatitis B virus envelope particles. *Virology* **330:**158-167.

Le Bouvier G.L. (**1971).** The heterogeneity of Australia antigen. *Journal of Infectious Diseases* **123:**671-675.

Lee W.M. (1997). Hepatitis B virus infection. *N Engl Med* **337:**1733–45.

Le Pogam S., Shih C. (2002). Influence of a putative intermolecular interaction between core and the pre-S1 domain of the large envelope protein on hepatitis B virus secretion. *J Virol* **76:**6510-6517.

Lei J., Bruce A., Cervantes C. (2000). Detection of Hepatitis B Virus Surface Antigen (HBsAg) Mutants by IMMULITE® 2000 HBsAg Assay. *Technical Report of DPC Co*. ZB248-A ©2006.

Locarnini S. (2004). Molecular virology of hepatitis B virus. *Semin Liver Dis* 24 Suppl 1: 3-10. doi:10.1055/s-2004-828672. PMID 15192795.

Lok A.S., McMahon B.J. (2007). Chronic hepatitis B. *Hepatology* 45 (2): 507–39.

Maeng C.Y., Oh M.S., Park I.H., Hong H.J. (2001) Purification and structural analysis of the Hepatitis B virus PreS1 expressed from *E. coli*. *Biochem Biophys* Res Commun **282:**787–92.

Mason W.S., Halpern M., England. J.M., Seal G.J., Egan, L. (1983). Experimental transmission of duckvhepatitis B virus. *Virology* **131:**375-384.

McAleer W.J., Buynak E.B., Maigetter R.Z., Wampler D.E, Miller W.J, Hilleman M.R. (1984). Human hepatitis B vaccine from recombinant yeast. *Nature* **307:**178-180.

Mushahwar I.K., Robertson B.H., Locarnini S., Magnius L.O. (2004). Genetic Diversity of Hepatitis B Virus Strains Derived Worldwide: Genotypes, Subgenotypes, and HBsAg Subtypes. *Intervirology* **47:**289-309.

Norder H., Couroucé A.M., Magnius L.O. (1992). Molecular basis of hepatitis B virus serotype variations within the four major subtypes. *J Gen Virol* **73:**3141-3145.

Norder H., Hammas B., Löfdahl S., Couroucé A.M., Magnius L.O. (1992). Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* **73:**1201-1208

Norder H., Hammas B., Lee S.D., Bile K., Couroucé A.M., Mushahwar I.K., Magnius L.O. (1993). Genetic relatedness of hepatitis B viral strains of diverse geographical origin and natural variations in the primary structure of the surface antigen. *J Gen Virol* 74:1341-1348.

Norder H., Courouce A.M., Coursaget P., Echevarria J.M., Leef S.D., Hilleman M.R. (2003). Critical overview and outlook: pathogenesis, prevention, and treatment of hepatitis and hepatocarcinoma caused by hepatitis B virus. *Vaccine* **21:**4626-49.

Okamoto H., Imai M., Tsdua F., Tanaka T., Miyakawa Y., Mayumi M. (1987). Point mutation in the S-gene of hepatitis B virus for a d/y or w/r subtypic change in two blood donors carrying a surface antigen of compound subtype adyr or adwr. *Journal of Virology* **61:**3030-3034.

Okamoto H., Omi S., Wang Y., Itoh Y., Tsuda F., Tanaka T., Akahane Y., Miyakawa Y., Mayumi M. (1989). The loss of subtypic determinants in alleles, d/y or w/r, on hepatitis B surface antigen. *Mol Immunol* **26:**197-205.

Ostapchuk P., Hearing P., Ganem D. (1994). A dramatic shift in the transmembrane topology of a viral envelope glycoprotein accompanies hepatitis B viral morphogenesis. *EMBO J* **13:**1048-1057.

Ottone S., Nguyen X., Bazin J., Berard C. (2007). Expression of Hepatitis B surface antigen major subtypes in Pichia pastoris and purification for in vitro diagnosis. Protein Expression and Purification **56:**177–188.

Ozdemir F.T., Duman D., Ertem D., Avsar E., Eren F, Özdogan O., Kalayaci C., Aslan N., Bozdayi A.M., Tozun N. (2005). Determination of hepatitis B genotypes in patients with chronic hepatitis B virus infection in Turkey. *The Turkish Journal of Gastroenterology* **16:**183-187.

Persing D.H., Varmus H.E., Ganem D. (1986). Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. *Science* **234:**1388-1391.

Perz J.F., Armstrong G.L., Farrington L.A., Hutin Y.J., Bell B.P. (2006). The contribution of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* **45:**529-538.

Peterson D.L., Nath N., Gavilanes F. (1982). Structure of hepatitis B surface antigen. Correlation of subtype with amino acid sequence and location of the carbohydrate moiety. *J Biol Chem* **257:**10414-10420.

Poisson, F., Severac A., Hioux C., Goudeau A., Roingeard P. (1997). Both pre-S1 and S domains of hepatitis B virus envelope proteins interact with the core particle. *Virology* **228:**115-120.

Prange R., Streeck R.E. (1995). Novel transmembrane topology of the hepatitis B virus envelope proteins. *EMBO J* **14:**247-256.

Prange R., Werr M., Loffler-Mary H. (1999). Chaperones involved in hepatitis B virus morphogenesis. *Biol Chem* **380:**305–314.

Pumpen P., Kozlovskaya T.M., Borisova G.P., Bichko V.V., Dishler A., Kalis J. (1984). Expression of hepatitis B virus surface antigen gene in *Escherichia coli*. *Gene* **30:**201-10.

Rehermann B., Fowler P., Sidney J., Person J., Redeker A., Brown M. (1995). The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. *J Exp Med* **181:**1047-58.

Rehermann B., Lau D., Hoofnagle J.H., Chisari F.V. (1996). Cytotoxic T lymphocyte responsiveness after resolution of chronic hepatitis B virus infection. *J Clin Invest* **97(7):**1655-65.

Rehermann B., Nascimbeni M. (2005). Immunology of hepatitis B virus and hepatitis C virus infection. *Nature Reviews Immunology* **5:**215-229.

Rizzetto M., Ciancio A. (2007). Chronic HBV Related Liver Disease. *Molecular Aspects of Medicine* **29(1-2):**72-84.

Roingeard P., Sureau C. (1998). Ultrastructural analysis of hepatitis B virus in HepG2-transfected cells with special emphasis on subviral filament morphogenesis. *Hepatology* **28:**1128-1133.

Samanta H., Youn B.W. (1989). Expression of hepatitis B virus surface antigen containing the pre-S region in mammalian cell culture system. *Vaccine* **7:**69–76.

Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: a Laboratory Manual, 2nd edn., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.

Shmitt S., Glebe D., Tolle T.K., Lochnit G., Linder D., Geyer R., Gerlich W.H. (2004). Structure of pre-S2 N- and O-linked glycans in surface proteins from different genotypes of hepatitis B virus. *J Gen Virol* **85:**2045-2053.

Soulier J.P., Courouc-Pauty A.M. (1973). New determinants of hepatitis B antigen (Au or Hb antigen). *Vox Sanguinis* 25, 212.

Sreekrishna K., Brankamp R.G., Kropp K.E., Blankenship D.T., Tsay J., Smith P.L., Wierschke J.D., Subramaniam A., Birkenberger L.A. (1997). Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast Pichia pastoris. *Gene* **190:**55-62.

Stephenne J. (1988). Recombinant versus plasma derived hepatitis B vaccines: issues of safety, immunogenecity and cost-effectiveness.*Vaccine* **6:**299-303.

Stibbe W., Gerlich W.H. (1983). Structural relationships between minor and major proteins of hepatitis B surface antigen. *J Virol* **46:**626-628.

Swameye I., Schaller H. (1997). Dual topology of the large envelope protein of duck hepatitis B virus: determinants preventing pre-S translocation and glycosylation. *J Virol* **71:**9434-9441.

Switzer, R.C., Merril, C.R., Shifrin, S(1979). A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal Biochem*. **98:**1-231-7.

Szmuness W., Stevens C.E., Harley E.J., Zang E.A., Oleszko W.R., William D.C. (1980). Hepatitis B vaccine: demonstration of efficacy in a controlled clinical trial in a high-risk population in the United States. *N Eng J Med* **303:**833-41.

Tagawa M., Omata M. Okuda K. (1986). Appearance of viral RNAvtranscripts in the early stage of duck hepatitis B virus infection. *Virology* **152:**477-482.

Thoma H. (1992). US. Pat. Publ. No. US 6,589,530 B1

Thomson J.E., Register E., Curotto J., Kurtz M., Kelly R. (1998). An improved protocol for the preparation of yeast cells for transformation by electroporation. *Yeast* **14:**565-571.

Tschopp J.F., Brust P.F., Cregg J.M., Stillman C.A., Gingeras T.R. (1987). Expression of the *lacZ* gene from two methanol regulated promoters in *Pichia pastoris*. *Nucleic Acids Res* **15:**3859-3876.

Towbin H. , Staehelin T. , Gordon J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Authors Proc Natl Acad Sci USA* **76(9):**4350-4.

Ueda K., Tsurimoto T., Matsubara K(1991). Three envelope proteins of hepatitis B virus: large S, middle S, and major S proteins needed for the formation of Dane particles. *J Virol* **65:**3521-3529.

Vassileva A., Chugh D.A., Swaminathan S., Khanna N. (2000). Effect of copy number on the expression levels of Hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification* **21:**71-80.

Veenhuis M., Harder W. Metabolic significance and biogenesis of microbodies in yeasts, p. 436-458. In H. D. Fahimi and H. Sies (ed.), Peroxisomes in biology and medicine, Springer-Verlag KG, Berlin, 1987.

Vincent W., Joseph J.S. (2009). Antiviral therapy for chronic hepatitis B: are we doing any good to patients. *Journal of Antimicrobial Chemotherapy* **64:**223-226.

Vyas G.N., Rao D.R., Ibrahim A.B. (1972). Australia antigen (hepatitis B antigen). A conformational antigen dependent on disulfide bonds. *Science* **178:**1300.

Wampler D., Lehman D., Boger J., McAleer J., Scolnick M. (1985). Multiple chemical forms of Hepatitis B surface antigen produced in yeast. Proceedings of the National Academy of Sciences of the United States of America. **82:** 6830-6834.

Weber B. (2005). Genetic variability of the S gene of Hepatitis B virus: clinical and diagnostic impact. *Journal of Clinical Virology* **32:**102-112.

West D.J., Calandra G.B. (1996). Vaccine induced immunologic memory for hepatitis B surface antigen: implications for policy on booster vaccination. *Vaccine* **14:**1019-27.

Wu S., Lechworth G. (2004). High efficiency transformation by electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol. *Biotechniques.* **36(1):**152-4.

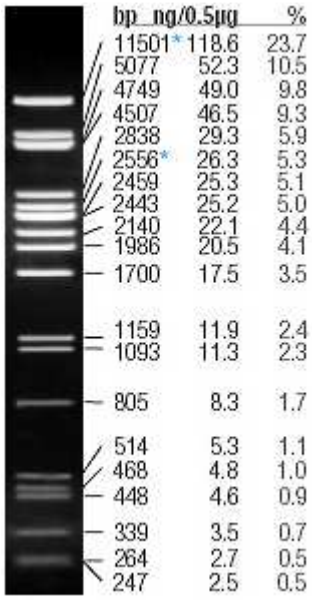
Wijnendaele V., Simonet F. (1987). United States Patent 4649192

World Health Organization ,<http://www.who.int/topics/hepatitis/en/>, 5/10/2009.

Zanetti A.R., Damme P.V., Shouval D. (2008). The global impact of vaccination against hepatitis B: A historical overview. *Vaccine* **26(49):**6266-73.

APPENDIX A

CULTURE MEDIA



Lambda Pst Marker

Luria Broth (LB)

	$g.L^{-1}$
Luria Broth	25

Sterilized at 121 C° for 15 minutes. For solid plates 15g/L agar is added to the medium.

Low Salt Luria Broth (LSLB)

	$g.L^{-1}$
Tryptone	10
Yeast Extract	5
NaCl	5

pH is adjusted to 7.0. Sterilized at 121 C° for 15 minutes. For solid plates 15g/L agar is added to the medium.

Yeast Peptone Dextrose (YPD)

	$g.L^{-1}$
Yeast Extract	10
Peptone	20
Dextrose	20

pH is adjusted to 7.0. Sterilized at 121 C° for 15 minutes. For solid plates 15g/L agar is added to the medium. For Yeast Peptone Dextrose Sorbitol (YPDS) add 182.2 g Sorbitol to YPD medium/liter.

BMGH

	$ml.\ L^{-1}$
Potassium phosphate (1M)	100
YNB (13.4%)	100
Biotin (0.02%)	2
Glycerol (10%)	100
Histidine (0.4%)	10
dH_2O	690

pH is adjusted to 6.0. Sterilized at 121 C° for 20 minutes. Histidine, biotin and YNB are added filter sterilized. For BMMH 100 ml of 5% MeOH added instead of glycerol.

APPENDIX B

BUFFERS AND SOLUTIONS

Colony PCR Buffer

	mM
Tris HCl pH: 8	125
KCl	56

Running Buffer 5X

	$g.L^{-1}$
Tris Base	15
Glycine	72
SDS	5

Laemmli Sample Buffer

	ml
dH2O	3
Tris HCl (0.5M)	1
Glycerol	1.6
SDS (10%)	0.4
β-mercaptoethanol	0.4
Bromophenol blue (0.5%, w/v)	0.4

Colony PCR Buffer

	mM
Tris HCl pH: 8	125
KCl	56

Lysis Buffer

	mM
PMSF	1
EDTA	1
$NaPO_4$	10
NaCl	500

Triton X-100 is added at 0.1% final concentration.

TBS

	$g.L^{-1}$
Tris	2.42
NaCl	29.24

pH is adjusted to 7.5 500 µl Tween-20 is added for TTBS. Skim milk is prepared in TBS at required concentration.

TAE Buffer (50X)

	g.L-^{1}
Tris base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL

Fixation Buffer

MeOH	50%
AcOH	12%
HCOH (37%)	0.5 ml

Pretreatment Buffer

	g.L^{-1}
$Na_2S_2O_3.5H_2O$	0.2

Impregnate Buffer

	g.L^{-1}
$AgNO_3$	2
HCOH (37%)	0.5ml

Development Buffer

	g.L^{-1}
Na_2CO_3	60
HCOH (37%)	0.5ml
$Na_2S_2O_3.5H_2O$	0.004

Stop Solution

MeOH	50%
AcOH	12%

Phosphate Buffered Saline (PBS)

	mM
NaCl	137
KCl	2.7
Na_2HPO_4	10
KH_2PO_4	2

Phosphate Buffer

	L^{-1}
K_2HPO_4 (1M)	132
KH_2PO_4 (1M)	868

Bradford Reagent

	L^{-1}
Coomassie Brilliant Blue G250	50 ml
Phosphoric acid (85%)	100 ml

Transfer Buffer

	L^{-1}
Methanol	200 ml
Tris	3.63 gr
Glycine	14.4 gr
SDS	0.37 gr

Electroporation Buffer

	M
LiCl	0.1
DTT	0.01
Tris (pH.7.5)	0.01
EDTA	0.001

H Buffer

	L^{-1}
Na_2HPO_4	0.6 gr
EDTA	0.12 gr
Tween 20	0.9 ml
Isopropanol	9 ml
PMSF	0.06 gr
Urea	36 gr

N Buffer

	L^{-1}
Na_2HPO_4	0.7 gr
Tween 20	300 µl
Urea	24 gr

APPENDIX C

CHEMICALS AND ENZYMES

Acetic Acid	Merck
Acrylamide	Merck
Agar Bacteriological	Merck
Agarose	Prona
$AgNO_3$	Merck
Ammonium persulfate	AppliChem
Arginine	Sigma
Bovine Serum Albumin	Sigma
Bromophenol Blue	Sigma
$CaCl_2.2H_2O$	Merck
Casamino acid	AppliChem
Chloroform	Merck
Cloramphenicol	Sigma
Coomassie Brillant Blue G-250	Merck
Coomassie Brillant Blue R-250	Sigma
Dimethylformamide	Merck
dNTPs	MBI Fermentas
EDTA	AppliChem
Ethanol	Botafarma
Ethidium bromide	Sigma
Formaldehyde	Merck
Glacial Acetic Acid	Merck
Glucose	Merck
Glycerol	Merck

Glycine	Merck
HCl	Merck
Histidine	Sigma
IPTG	Sigma
Isopropanol	Merck
K_2HPO_4	Merck
KCl	Merck
KH_2PO_4	Merck
Luria Broth	Q-Biogene
Mercaptoethanol	Sigm
Methanol	Merck
MgSO4.7H2O	Merck
MnCl2.2 H2O	Merck
MnSO4.7H2O	Carlo Erba
N, N'-Methylene bis-acrylamide	Sigma
NaCl	Merck
NaOH	Merck
Nutrient Agar	Merck
Nutrient broth	Merck
Peptone	Sigma
SDS	Merck
Sucrose	Merck
TEMED	Sigma
Tris Base	Merck
Tris-HCl	Merck
Yeast extract	Difco