Study 1: Constitutive expression and characterization of Hepatitis B Surface Antigen (HBsAg) purified by metal affinity precipitation
INTRODUCTION &
REVIEW of LITERATURE
4.1 Introduction

Human Hepatitis B virus [HBV] is the prototype for a family of viruses referred to as *Hepadnaviridae* [Table 13]. The most closely related to HBV have been found in woodchucks and ground squirrels. These viruses have 70% homology to HBV but are not known to infect humans or other primates, in contrast HBV is infectious to great apes. The similarities in DNA sequence and genome organization, the viruses infecting mammals are grouped under the genus of *Orthohepadnaviridae*. HBV surface protein was accidentally discovered in the blood of an Australian aborigine by B.S.Blumberg and was referred to as Australia antigen. The association of this antigen with acute hepatitis was discovered.

Table 13. Hepatitis Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term</th>
<th>Definition and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
<td>Etiologic agent of serum hepatitis; also known as Dane particle</td>
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<tr>
<td>HBsAg</td>
<td>Hepatitis B surface antigen</td>
<td>Surface antigen [s] of HBV detectable in large quantity in serum; several serotypes identified</td>
</tr>
<tr>
<td>HBeAg</td>
<td>Hepatitis B core antigen</td>
<td>Core antigen of HBV</td>
</tr>
<tr>
<td>Anti-HBs</td>
<td>Antibodies to HBsAg</td>
<td>Indicates past infection with and immunity to HBV, passive antibody from HBIG, or immune response to HBsAg</td>
</tr>
<tr>
<td>Anti-HBe</td>
<td>Antibodies to HBeAg</td>
<td>Presence in serum of individuals with chronic infection indicates low titer of HBV</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
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<td></td>
</tr>
<tr>
<td>Anti-HBc</td>
<td>Antibodies to HBcAg indicates prior infection at some undefined time in the past</td>
<td></td>
</tr>
<tr>
<td>Pre-S1, Pre-S2</td>
<td>Envelope protein containing pre-SHBs epitopes</td>
<td></td>
</tr>
<tr>
<td>SHBs</td>
<td>Major surface antigen protein</td>
<td></td>
</tr>
<tr>
<td>MHBs</td>
<td>Middle HBs protein</td>
<td></td>
</tr>
<tr>
<td>LHBs</td>
<td>Large HBs protein</td>
<td></td>
</tr>
<tr>
<td>HBIG</td>
<td>Hepatitis B globulin contains high titers of antibody to HBsAg</td>
<td></td>
</tr>
</tbody>
</table>

Electron microscopy of hepatitis B virus-positive serum reveals 3 morphologically distinct forms of particles [Fig.8] [Kann et al., 1997].
**Figure 8.** Schematic representation of hepatitis B virus particles. Individual subunits containing SHBs protein only, HBs protein plus pre-S2 [MHBs], and HBs protein plus pre-S1 and pre-S2 [LHBs] are shown in intact virus, among filaments and spheres. The virus particles contain an internal nucleocapsid [HBc] and viral genome. pol, polymerase.

[1] Small 22nm spherical or tubular forms comprise of virus surface proteins which are synthesized in excess of the 42 nm complete virions.

[2] Complete 42 nm virion [Dane particle]. The HBsAg differs from the HBsAg found in the 22 nm particles in that pre-S1 epitopes are present.

[3] The 27 nm nucleocapsid comprises of the DNA genome surrounded by a second protein, the HBcAg. A third antigen, the HBeAg is found in the soluble forms in virus-positive sera and is related to the core antigen.

**Liver: Target for HEPADNAVIRUS Infection**

The liver plays an essential role in energy storage and conversion, blood homeostasis, chemical detoxification, and immunity to microbial infections. The functional activity of liver resides on hepatocytes that constitute 70%, bile ductile epithelium and Kupffer macrophages. Hepatocytes are the only confirmed site of replication for all members of hepadnaviridae. Extrahepatic sites for replication are controversial and incomplete and so is yet to be determined. Hepatocytes have the capacity for extensive proliferation in response to liver injury. Under normal conditions, they have life times exceeding 6 to 12 months. Following partial
hepatectomy of 70% of liver, virtually every hepatocyte passes through the cell cycle at least once and the liver mass is restored in few days. If the entire hepatocyte population is infected, virus clearance requires either a mechanism for eliminating virus from hepatocytes or complete replacement of infected hepatocytes by proliferation or hypothetically in infected population of progenitor cells. These theories are still under investigation to understand the portal of entry of the virus into hepatocyte.

4.1.2 Genome Replication

Introduction

The life cycle of hepadnaviruses is characterized by the synthesis of a ~3.2 kb partially double stranded, relaxed circular DNA [rcDNA] genome by reverse transcription of an RNA intermediate, the pregenome. The mechanism of RNA directed DNA synthesis has been characterized however, early events of viral life cycle, including entry, uncoating and delivery of viral genome in cell nucleus are not well understood.

Attachment to Hepatocytes

The first step in replication of HBV is entry into hepatocyte after recognition of the specific cell type by receptor mediated interaction. Studies on this subject were hampered by the non-susceptibility of established hepatocyte cell lines to HBV infection. Therefore a variety of techniques were used, such as infection of primary hepatocyte cultures and binding to liver plasma membranes, have been used. The lack of pHSA [polymerized Human Serum Albumin] receptors on hepatoma cell line HepG2 may be one reason for being refractory to HBV infection. In theory,
attachment of HBV should include a ligand, at least one of the three surface proteins of HBV, termed L [Large] HBs, M [Middle] HBs, S [Small] HBs protein. These proteins have identical amino acid sequence at the carboxy terminus, but differ by their amino terminal domains, called preS1 and preS2. The S and preS2 domains can be glycosylated, resulting in at least six different surface proteins SHBs [p24, gp27], MHBs [gp33, gp36], and LHBs [p39, gp42]. The preS1 domain present only in LHBs, is myristylated at its amino terminus. The relevance of different surface proteins for infectivity was demonstrated in vivo by natural HBV variants. In certain patients with chronic hepatitis B, variants devoid of MHBs were observed, whereas variants devoid of LHBs were never identified.

Long term cultivated hepatocytes are not susceptible to HBV, although they are permissive for production of infectious virions. Most of the research on viral uptake is being done using primary hepatocyte cultures. De novo synthesis of secreted HBV occurs 2 days after infection of hepatocytes.

Nuclear Entry of Viral Genome

Release of viral genome from the nucleocapsid and transport into the nucleus of the hepatocyte is an essential step in the hepatitis B replication cycle. The exact mechanism of transportation of viral DNA to karyoplasts is not known. Using immunofluorescence, HBV core particles were found in the cytoplasm of infected hepatocytes. This data was confirmed by the observation of nuclear localization signal on the carboxy terminal portion of the core molecule. A potential candidate that mediates the transport of viral genome was found in viral polymerase that exhibited nuclear localization signal when expressed in eukaryotic cell line.
Formation of covalently closed circular [ccc] molecule

The first event in virus DNA replication accessible to experimental investigation is the conversion of viral relaxed circular DNA [rc DNA] genome in to ccc DNA molecule. Since ccc DNA is the template for transcription of viral mRNAs, its formation indicates a successful initiation of infection. Hepadnaviruses contain a partially double stranded DNA genome with a covalently bound protein at the 5' end of the minus DNA strand and an RNA at the 5' end of virus plus strand. After infection, the formation of cccDNA occurs first [6-16 hrs after infection in embryonic ducks], followed by replication intermediates after 24 hrs. Therefore it was assumed that only the cccDNA serves as a template for transcription of viral RNAs. This assumption was confirmed by the infectivity of cloned, circular HBV DNA in chimpanzees, after intrahepatic injection. The following steps prior to RNA synthesis are implied:

1. Removal of covalently bound polymerase
2. Removal of RNA primer
3. Completion of plus DNA strand
4. Repair and ligation of the gaps between 5' and 3' ends of each strand. cccDNA then acts as a template for transcription of all viral mRNAs.

Viral Gene Expression

Hepadnaviruses multiply their genomes by reverse transcription of an RNA intermediate in a manner similar to retroviruses. Four promoters and two enhancers have been identified as regulatory elements for transcription of HBV mRNAs. The viral mRNAs include pregenomic RNA [pg RNA] that serves as a template for reverse transcription, three sub-genomic mRNAs necessary for translation of envelope proteins and the mRNA for X protein. Like eukaryotic mRNAs, the viral transcripts
include a cap structure at their 5' terminus and are polyadenylated TATAAA at 3' end. Therefore all viral transcripts share a common 3' end. The shortest RNA approximately 700 nucleotides encodes the viral X gene, that plays a important role in infection of hepatocytes and in replication after transfection. 2.1 kb RNA encodes the preS2 and S region that encodes MHBs and SHBs. Therefore the promoter has been termed as preS2/S. 2.4 kb RNA encodes the preS1 region that encodes the LHBs. Two RNAs of ~3.3 kb are controlled by core/e promoter i.e preS1 promoter and is tissue dependant. The viral polymerase consists of four domains: 1. the priming domain that presents the first hydroxyl group for the addition of first nucleotide of minus strand 2. spacer or tether 3. polymerase domain that catalyzes strand elongation 4. RNase H domain that degrades the RNA from the resulting RNA-DNA hybrid [Fig.9].

Regulation of viral gene expression also occurs at the level of translation. The pregenome serves as mRNA not only for the core protein but also for the viral polymerase.

Assembly of the Nucleocapsid and Reverse Transcription

Inferring from genetic experiments, it has been suggested that translation of the polymerase and pgRNA packaging into viral nucleocapsids are tightly coupled events. These experiments revealed that reverse transcriptase is required for RNA packaging and also packaging in cis conformation. It is now well established that the polymerase binds to an RNA stem-loop structure at the 59 end of pgRNA, termed epsilon, and that this event triggers the sequestration of viral RNA and polymerase into core particles. RNA packaging also depends on host factors, in particular polypeptides belonging to the molecular chaperone complex of hsp90.
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Figure 9. Schematic diagram of HBV genome and its organization. The inner circle represents the viral DNA as found in virions. The arrow represent the four different ORFs, polymerase [P], X, Core and pre core [C & preC], S-preS1-preS2 domains. The 5' end of [-I strand DNA is linked with the priming domain, the 3' end of the [+DNA is associated with the reverse transcriptase domain.

Annealing of the primer with complementary sequences at the acceptor is possible due to an 11- to 12-nucleotide sequence homology referred to as direct repeats 1 and 2 [DR1 and DR2]. The 39 end of the RNA primer most probably represents the final RNase H cleavage site on pgRNA. Plus-strand DNA synthesis involves a template switch from the 59 end to the 39 end of minus-strand DNA, which is facilitated by short sequence repetitions at the ends of the DNA template. The final result of the reverse transcription reaction is an rcDNA genome with two modified
ends and, in mammalian viruses, with a less-than-genome-length plus-strand DNA, leaving up to 50% of the rcDNA single stranded.

4.1.3 The Surface Protein HBsAg

Expression of the surface open reading frame of HBV leads to synthesis of the major [S], middle [preS2+S] and large [preS1+preS2+S] surface proteins. These proteins are present in glycosylated and non glycosylated forms in the surface of the 42 nm virions. L and M are present in roughly equal eamounts in Dane particle and together constitute approximately 30% of the envelope protein content. Infected hepatocytes secrete a vast excess of 22 nm spherical particles that lack HBV DNA and the nucleocapsid protein and also are devoid of the large surface protein. The 22 nm subviral particles may be secreted in the absence of HBV replication, by hepatocytes with chromosomal integration of HBV DNA. Tubular structures of 22 nm diameter that contain the large surface protein also may be present in the circulation of viraemic individuals. These sphere and filaments can accumulate to concentrations of several hundred micrograms per milliliter in the blood of HBV infected patients.

The principal component of the virion surface and the subviral particles is the small or major surface protein. This 226 amino acid protein may be glycosylated at the asparagine at the position 146 and glycosylated [gp27] and non glycosylated [p24] forms are present roughly the same molarity. The amino terminus of the major surface protein is exposed on the surface of the particles and preS domains of the larger proteins also are exposed. The middle proteins have a 55 residue amino terminal extension [pre S2 domain] that is glycosylated. The large proteins have a further
extension of 108 residues and are myristylated near the amino terminus. The preS2 domain is not glycosylated in the large surface proteins [Fig. 8].

Subtypes of HBsAg

HBsAg contains a common antigenic determinant, ‘a’, and several major subdeterminants that are specified by the viral genome. The subdeterminants can be detected by the presence of the spurs in the immunodiffusion tests with various antisera. Eight distinct categories and two of the mixed subtype have been recognized. In addition several minor antigenic subtypes have been described. The major subtypes consist of various combinations of the subdeterminants d/y and w/r which appear to constitute two groups, composed of d/y and w1, w2, w3, w4 and r [Table 14]. The two mixed subtypes [adwr and adyr] are extremely rare and may be due to phenotypic or genotypic mixing of immunologic markers during simultaneous infection associated with more than one subtype of HBsAg. HBsAg of adw and ayw subtypes appear to differ in both biophysical and biochemical characteristics.

Table 14.

<table>
<thead>
<tr>
<th>Major subtypes</th>
<th>Minor subtypes</th>
</tr>
</thead>
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<tr>
<td>ayw1</td>
<td>q</td>
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<td>ayw4</td>
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</tr>
<tr>
<td>ayr</td>
<td>j</td>
</tr>
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<td>g</td>
</tr>
<tr>
<td>adr</td>
<td></td>
</tr>
<tr>
<td>adwy</td>
<td></td>
</tr>
<tr>
<td>adyr</td>
<td></td>
</tr>
</tbody>
</table>
Antibodies to HBsAg

Antibodies to HBcAg [anti-HBc] appear [often at the same time that enzyme elevations are first seen] subsequent to infection with HBV. Antibodies to the surface antigen [anti-HBs] usually appear later, sometimes being delayed by 6 to 12 months after the acute episode and often coinciding with the disappearance of circulating HBsAg.

Both animal and human studies indicate that the anti-HBc tends to decrease gradually and may become undetectable after 1 or 2 years, although high titers are found in carriers. During the recovery phase of acute hepatitis B, anti HBc may be present in the absence of HBsAg and anti-HBs, and donations taken during this time can cause post transfusion hepatitis. Anti-HBs last much longer and may persist throughout life. This antibody is responsible for the immunity to further infection with HBV. As mentioned HBeAg is present during the incubation period of acute hepatitis B, and the anti HBe develops either during recovery or with the onset of overt liver disease. The presence of the antibody is considered to be a good prognostic sign [Fig. 10].

![Figure 10. Characteristics of acute hepatitis B with recovery and progression to chronic hepatitis](image-url)
4.1.4 EPIDEMIOLOGY [Andre 2000, Koff 2002]

HBV is transmitted by percutaneous or permucosal exposure to infectious body fluids, by sexual contact with an infected person, and perinatally from an infected mother to her infant. The frequency of HBV infection and patterns of HBV transmission vary markedly in different parts of the world. Approximately 45% of the world’s population live in areas where the prevalence of chronic HBV infection is high [~8% of the population is HBsAg positive], 43% live in areas where the prevalence is moderate [2 to 7% of the population is HBsAg positive], and 12% live in areas of low endemicity [,2% of the population is HBsAg positive]. In areas of high endemicity, the lifetime risk of HBV infection is 60% and most infections occur at birth or during early childhood, when the risk of chronic infection is greatest. Because most early childhood HBV infections are asymptomatic, there is little recognition of acute disease, but rates of chronic liver disease and liver cancer are high. Areas of high endemicity include most of Asia [except Japan and India], most of the Middle East, the Amazon Basin of South America, most Pacific Island Groups, Africa, and other special populations such as Native Alaskans, Australian Aborigines, and Maoris in New Zealand. The mechanisms of early childhood transmission in areas of high endemicity are variable. Generally, infections cluster in households of persons with chronic infection. The major determinants of infection include exposure to a HBsAg-positive mother or sibling. The contribution of perinatal transmission to the overall burden of disease is related to the prevalence of HBeAg among pregnant women. If a mother is HBsAg positive and HBeAg positive, 70 to 90% of her infants will become
infected if not given immuno- prophylaxis. In East and Southeast Asian countries, as well as the Pacific, 35 to 50% of HBsAg-positive women are HBeAg positive. It is estimated that 3 to 5% of all infants in these countries may develop chronic HBV infection at birth and that up to 30 to 50% of all chronic infections among children may result from perinatal transmission.

In India the average carrier rates of HBV in the general population are considered to be approximately 4%. Among these, professional blood donors constitute a major risk factor group with a prevalence rate of 14%. Thallassemic and renal dialysis patients also have a high risk of acquiring HBV infection [Kottili et al., 2005]. These studies have indicated that HBV infection is established in early childhood, probably associated with crowded living conditions and poor hygiene. However, HBV is also associated with acute and sub acute liver failure in adults as well as with a significant proportion of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Community based epidemiological studies have indicated a slightly higher rates of chronic HBV infection in a south Indian tribal population. Several tribal customs including scarification, tribal treatment practices like blood-letting and other practices like tattooing, ear and body-piercing are all suggested to be contributing to the high prevalence rates of HBV. However, it should be noted that many of these studies have very small sample sizes and may have selection biases and may not accurately reflect the real prevalence of HBV infection in India. Use of molecular diagnostics have indicated a higher prevalence rate of HBV infection among the general population in India, surprisingly slightly higher among rural than urban population. In summary HBV infection remains a significant public health hazard in
India and will probably remain so until nationwide vaccination programs and other control measures are fully implemented. Vaccination of all individuals is probably the most cost-effective way to control HBV infection in India.

Worldwide, the consequences of acute and chronic HBV infection are major public health problems. Approximately 5% of the world’s population [300 million persons] have chronic HBV infection, which is the leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma worldwide. It is estimated that 500,000 to 1,000,000 persons die annually of HBV-related liver disease.

Transmission of Hepatitis B

Parenteral infection is considered to be the most important mode of transmission of hepatitis B, infections can be acquired by casual contact with infected blood or serum. The minimum infective dose of plasma from a carrier was estimated to be $1 \times 10^6$ ml. Apart from the transfusion of the blood and its products, that are the common source of infection other recognized modes of transmission of the virus include common needles and syringes among drug addicts, tattooing needles, sharing razors and toothbrushes. Susceptibility to the disease is not confined to any age group. Maternal transmission to the fetus or newborn has been reported and often leads to chronic infection of the infant.

DIAGNOSIS

Because the clinical symptoms of HBV infection are indistinguishable from other forms of viral hepatitis, definitive diagnosis is dependent on serologic testing for HBV infection. A variety of tests are available to make the diagnosis of HBV infection. Acute HBV infection is characterized by the presence of HBsAg in serum and the
development of IgM class antibody [IgM anti-HBc]. Detection of HBsAg has evolved from immunodiffusion methods to reversed passive hemagglutination assays and to the more sensitive enzyme immunoassays and radioimmunoassays, which can detect HBsAg at concentrations of 0.1 ng/ml. HBeAg is also detectable during acute infection. During convalescence, HBsAg and HBeAg are cleared, and anti-HBs, anti-HBc, and anti-HBe develop. Anti-HBs is a protective antibody that neutralizes the virus. The presence of anti-HBs following acute infection indicates recovery and immunity from re-infection. Anti-HBs is also detected among persons who have received hepatitis B vaccine. The detection of IgM anti-HBc is diagnostic of acute HBV infection. In persons with chronic HBV infection, HBsAg remains persistently detectable, generally for life. Most slot or dot blot hybridization assays can detect HBV DNA levels as low as 5 pg/ml, which corresponds to $1.5 \times 10^6$ genomes per ml. A commercial liquid hybridization assay [Abbott] detects 1.5 pg of HBV DNA per ml [$4.0 \times 10^5$ genomes per ml], and the branched-DNA hybridization assay detects 2.5 pg of HBV DNA per ml. PCR is much more sensitive than direct hybridization and detects HBV DNA levels of $10^{23}$ pg/ml [approximately 100 to 1,000 genomes]; however, PCR assays are prone to false-positive results. The clinical significance of detecting HBV DNA by hybridization and by PCR is quite different. Generally, detection by PCR has the same significance as detection of HBsAg and indicates current HBV infection. In contrast, detection by hybridization indicates significant viral replication and a high probability of active liver disease [similar to HBeAg]. Monitoring HBV DNA levels is useful in determining the response of chronic HBV infection to treatment. Nucleic acid sequence analysis has been used to identify
genetic variants of the virus and to investigate common-source outbreaks of HBV infection.

4.1.5 PREVENTION of Hepatitis

4.1.5.1 Passive Immunization [Heijtink et al., 2000]

The discovery that passively acquired anti-HBs could protect individuals from acute clinical hepatitis B and chronic HBV infection if given soon after exposure led to the development of a specific immunoglobulin containing high titer of anti-HBs [HBIG]. HBIG is prepared by the Cohn Oncly fractionation procedure from serum containing high titers of anti-HBs and is standardized to 100,000 IU of anti-HBs/ml. HBIG is effective, often in combination with hepatitis B vaccine, as post exposure prophylaxis following [i] perinatal exposure for an infant born to an HBsAg-positive mother, [ii] percutaneous or mucous membrane exposure to HBsAg-positive blood, or [iii] sexual exposure to an HBsAg-positive person. HBIG is also used to protect patients from severe recurrent HBV infection following liver transplantation.

4.1.5.2 Active Immunization

Safe, immunogenic, and effective hepatitis B vaccines have been commercially available in the United States since 1981. Hepatitis B vaccines are composed of highly purified preparations of HBsAg. The vaccines are prepared either by harvesting HBsAg from the plasma of persons with chronic infection [plasma-derived vaccine] or by inserting plasmids containing the HBsAg gene, and in some cases the pre-S1 and/or pre-S2 gene, into yeast or mammalian cells. The vaccines undergo various inactivation steps, are highly purified, and then are adjuvanted with aluminum phosphate or
aluminum hydroxide and preserved with thimerosal. Vaccines are licensed for all age
groups as a three-dose series consisting of two priming doses given 1 month apart and
a third dose given 5 months after the second dose. The recommended dose varies with
the product, the recipient's age, and, for infants, the mother's HBsAg serologic status.
In general, the vaccine dose for infants and adolescents is 50 to 75% lower than that
for adults.

Recombinant HBsAg Vaccines

4.1.5.2.1 E.coli derived

_E. coli_ derived Hepatitis B virus vaccines did not elicit Immunogenicity due to their
inability to aggregate the HBsAg polypeptides in to 22 nm particles. However novel
strategies were developed that formed 22nm, 24kDa particles [Deml _et al._,1998].

4.1.5.2.2 Yeast derived

Yeast-derived recombinant DNA hepatitis B vaccines were developed in the
mid-1980s. Initial clinical trials were performed in 1984, and by 1986 the first human
vaccines manufactured by recombinant DNA technology were licensed for general
use. The vaccine was shown to be safe, immunogenic and protective against hepatitis
B virus [HBV] infection and clinical HBV disease. It was initially recommended for
individuals at high risk for acquiring infection. However, the use of yeast-derived
vaccine became widely accepted because of its safety and immunogenicity profile,
as well as the fact that it was inexpensive to produce, thus allowing for a near
unlimited supply of vaccine. Vaccines that used yeast as the expression vector were
licensed and gradually replaced the plasma-derived vaccine. Recombivax-HB,
manufactured by Merck Sharp and Dohme [MSD], was licensed in 1986; Engerix-B,
manufactured by SmithKline Biologicals [SKB] was licensed in 1989. To date globally there are many manufacturers of recombinant HBsAg with 5 manufacturers from India [Table 15].

Table 15. United Nations-UN Prequalified Hepatitis B vaccines [As on December 2005]

<table>
<thead>
<tr>
<th>S.no</th>
<th>Trade Name</th>
<th>Recombinant Yeast</th>
<th>Company</th>
<th>Molecule used</th>
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<tbody>
<tr>
<td>1</td>
<td>Recombivax-HB</td>
<td><em>S. cerevisiae</em></td>
<td>Merck &amp; Co., Inc.</td>
<td>S antigen</td>
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<tr>
<td>2</td>
<td>Engerix-B</td>
<td><em>S. cerevisiae</em></td>
<td>GlaxoSmithKline</td>
<td>S antigen</td>
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<tr>
<td>3</td>
<td>Hepavax-Gene</td>
<td><em>P. angusta</em></td>
<td>Berna Biotech</td>
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<td></td>
<td></td>
<td>Korea Green Cross</td>
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<tr>
<td>4</td>
<td>Heberbiovac HB</td>
<td><em>P. pastoris</em></td>
<td>Centre for Genetic Engineering and Biotechnology, Cuba</td>
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<tr>
<td>7</td>
<td>Uni-Ject</td>
<td></td>
<td>P.T Biopharma, Indonesia</td>
<td>S antigen</td>
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</tbody>
</table>

The use of non conventional yeasts like *P. pastoris* for expression of HBsAg has revolutionized the economics of hepatitis B vaccine production. A variety of promoters were successfully employed in various yeast expression systems for successful expression of HBsAg [Table 16].
<table>
<thead>
<tr>
<th>S.no</th>
<th>Recombinant Host</th>
<th>Promoter</th>
<th>Expression strategy</th>
</tr>
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<tbody>
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<td>PGK</td>
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<td>ADH1</td>
<td>Constitutive</td>
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<td>Inducible/Fed batch/</td>
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<td>4</td>
<td><em>Y. lipolytica</em></td>
<td>XPR2</td>
<td>Inducible</td>
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</tbody>
</table>

Table 16. Yeast expression hosts employed for HBsAg protein production in inducible/constitutive mode.

A gene encoding the 226 amino acid hepatitis B surface antigen [HBsAg], subtype adw, was cloned in to a *S. cerevisiae*. Promoters employed initially were of the glycolytic genes alcohol dehydrogenase 1, 3-phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase [Valenzuela *et al.*, 1982, Bitter *et al.*, 1984]. Other promoters employed were galactose 10 [Shen *et al.*, 1989], acid phosphotase [Miyanahora *et al.*, 1983]. High-cell-density fermentations of laboratory strains of Saccharomyces cerevisiae have been developed in which HBsAg production increases linearly with respect to cell mass. The HBsAg is present as a lipoprotein particle in cell lysates and has been purified to homogeneity. The evidence presented
indicates that the HBsAg particles may be formed during lysis of the yeast cells. The purified HBsAg particles have morphology similar to that of the 22 nm particles present in the serum of human chronic carriers of hepatitis B and a buoyant density if 1.16-1.2 g cm\(^{-3}\). The reactivity of the yeast-derived HBsAg particles with a series of monoclonal antibodies is essentially identical to that of human plasma HBsAg. By this analysis, therefore, the structure of the HBsAg protein is similar in yeast and in human particles. The purified yeast HBsAg particles were formulated with alum adjuvant and subsequently were shown to confer immunity in chimpanzees to challenge with two heterologous serotypes \((adr, ayw)\) of hepatitis B virus.

HBsAg 'S' gene (681 bp) cloned in pBSAG151 [Cregg et al., 1987] expression vector containing AOX1 promoter was successfully expressed in \(P.\) pastoris GS115 host by HIS4 integration. pBSAG151 vector components are \(P.\) pastoris AOX1 promoter, AOX1 terminator, HIS4 (histidinol dehydrogenase) gene, Autonomous replication sequence and linker sequences of pBR322. Integration was achieved by linearization of the recombinant expression vector at 3'AOX1 region before transformation. Integration to host chromosomal DNA was established by a one step gene replacement technique of the linearized vector with the host AOX1 gene. Transformation was done by spheroplast generation using zymolyase. Selection of transformants was done by histidine auxotrophy on histidine deficient medium. Continuous production of HBsAg in \(P.\) pastoris was achieved by cloning the HBsAg gene under control of \(P.\) pastoris glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter [Vassileva et al., 2001]. Integration by homologous recombination at GAP
locus of host and selection for transformants integrated with multiple copies by zeocin was established. Transformation was done by electroporation.

The production of HBsAg at high cell density batch fermentation with repression/derepression and induction was successfully employed. The HBsAg expressed intracellularly was released from host cells by lysis and purified by cesium chloride density gradient centrifugation. The HBsAg polypeptide assembled into a 22 nm particle that was confirmed by electron microscopy. Assembly of HBsAg polypeptide to 22 nm particle is essential for the immunogenicity. The stability of the HBsAg expression cassette remained stable after 100 generations indicating that over-expressed HBsAg is not toxic to host cell.

An expression system has been developed for the methylotrophic yeast *P. angusta* and used to co-express both the L [preS1-S2-S] and S hepatitis B surface antigens [HBsAg] under the control of strong methanol-inducible promoters derived from the methanol oxidase [MOX] and from the formate dehydrogenase genes [FMD] [Janowicz et al., 1991]. A unique feature of this *P. angusta* expression system is the possibility of integrating up to 100 copies of an expression cassette via a multimeric integration mechanism. Several multimeric integrants containing various numbers of L and S expression cassettes were constructed to give a spectrum of strains characterized by different L to S ratios. The expression level of S antigen was 5-8% of the total soluble cell protein. Analysis by sucrose and CsCl density gradient centrifugation and by particle-specific immunoassays demonstrated that the synthesized HBsAg spontaneously assembled into composite subviral particles containing both S and L proteins. Only a minor portion of the L protein was found to be glycosylated. These *P.
angusta derived composite particles can be used for the production of a hepatitis B virus vaccine with the potential for improved immunogenicity due to the presence of a wider spectrum of epitopes and negligible glycosylation.

Expression of the gene encoding the hepatitis B virus middle surface antigen [pre-HBsAg] in the yeast Yarrowia lipolytica has been studied [Hamsa et al., 1994]. The preS2-HBsAg gene was expressed from the alkaline extracellular protease-encoding gene [XPR2] promoter. In the fusion construct, the membrane-spanning 'a' domain of preS2-HBsAg has been replaced by the leader peptide and the prol region of the alkaline protease, thus eliminating the epitope responsible for the immune escape mechanism. Expression has been found to be growth-stage dependent with the highest protein accumulation during the stationary phase, accounting for around 2.35% of the total soluble intracellular proteins. The produced protein was assembled into Dane particles and was immunogenic in mice. The expression vector was found to be stable for at least 100 generations under non-selective conditions.

The unicellular eukaryotic marine green alga Dunaliella salina was used for cloning and expression of HBsAg expression. HBsAg expression maintained stably for 60 generations. [Geng et al., 2003]

4.1.5.2.3 Cell line derived

Various cell lines were successfully used for expression of hepatitis B surface antigen. Chinese Hamster Ovary [Diminsky et al., 1999], SP2/0 cells [Qin et al., 2003], HepG2, NIH3T3 [Zhou et al., 2002], Drosophila melanogaster Schneider-2 [DS-2] [Deml et al., 1999], human embryo diploid fibroblast LEP and MRC-5 cells,
rabbit embryo fibroblast REF cells, TK-rat RAT-2 cells, green monkey CV-1 cells [Kutinova et al., 1990], PLC/PRF/5 human hepatoma cell line [Felling et al., 2004] were used to characterize the physical, chemical and immunological stability of HBsAg. Vector systems like pRc/CMV, pSG5UTPL/Flag [Qin et al., 2003], pLXSN retroviral vector [Zhou et al., 2003], inducible drosophila metallothionein promoter [Deml et al., 1999], poliovirus vectors [Yim et al., 1996], vaccinia virus vectors [Kunke et al., 1993], baculovirus expression system [Lanford et al., 1989], BK virus [BKV] episomal plasmid vector [Cputo et al., 1988], were successfully used to clone and express stable immunogenic HBsAg that can be used for vaccine approach. Third generation HBV vaccine with improved immunogenecity and antigenecity are being developed in CHO cell lines system.

Recombinant HBsAg coded by preS1-preS2-S regions of hepatitis B virus was expressed in Bombyx mori silkworm larvae. Recombinant HBsAg was expressed [30-40 mg/mL, 0.1% of the total amount of extracted protein] by larvae infected with recombinant baculovirus rBmNPV-Hep-preS1-S containing cDNA of HBsAg strictly by the polyhedrin gene promoter. Recombinant HBsAg consisting of a polypeptide of molecular weight ~36 kDa [p36] was purified by gel filtration and affinity chromatography to 92% purity [Bachurina et al., 2005]
transformed with the gene encoding hepatitis B surface antigen [HBsAg] linked to a nominally constitutive promoter. Recombinant HBsAg was purified from transgenic plants by immuno-affinity chromatography and examined by electron microscopy. Spherical particles with an average diameter of 22 nm were observed in negatively stained preparations. Sedimentation of transgenic plant extracts in sucrose and cesium chloride density gradients showed that the recombinant HBsAg and human serum-derived HBsAg had similar physical properties. The anti-hepatitis B response to the tobacco-derived rHBsAg was qualitatively similar to that obtained by immunizing mice with yeast-derived rHBsAg [commercial vaccine]. Additionally, T cells obtained from mice primed with the tobacco-derived rHBsAg could be stimulated in vitro by the tobacco-derived rHBsAg and yeast-derived rHBsAg. [Mason et al., 1992, Thanavala et al., 2005]

The immunogenicity of hepatitis B surface antigen [HBsAg] expressed in potatoes and transgenic lupin, lettuce and delivered orally was evaluated. Mice were fed transgenic tubers and leaves expressing HBsAg, both primary and booster serum antibody responses were elicited. These combined experiments demonstrated that plants can express, fold, assemble, and process foreign antigens and can provide both a simple vaccine-manufacturing process as well as a matrix suitable for oral immunization. Transgenic plant material containing HBsAg was the superior means of both inducing a primary immune response and priming the mice to respond to a subsequent parenteral injection of HBsAg. Electron microscopy of transgenic plant samples revealed evidence that the HBsAg accumulated intracellularly; natural bio-encapsulation of the antigen may provide protection from degradation in the digestive
tract until plant cell degradation occurs near an immune effector site in the gut. Human volunteers, fed with transgenic lettuce plants expressing hepatitis B virus surface antigen, developed specific serum-IgG response. [Thanavala *et al.*, 2005].

**DNA vaccines**

Intramuscular injection of plasmids encoding HBV antigens is another novel approach to vaccination, which enables the expression of encoded proteins *in vivo*, in their native conformation and with the appropriate post-translational modifications. Moreover, such proteins are processed intracellularly and the correct epitopes are thus presented to the immune system. Vaccination of two chimpanzees against hepatitis B virus [HBV] by intramuscular injection of plasmid DNA encoding the major and middle HBV envelope proteins induced group-, subtype- and preS2-specific antibodies. These were initially of IgM isotype, and then they were of IgG [predominantly IgG1] isotype. A DNA vaccine against HBV has also been evaluated in healthy human volunteers using the PowderJect system to deliver gold particles coated with plasmid DNA directly to skin cells. The vaccine proved safe, was well tolerated and produced preferentially Th1 helper cell responses.

4.1.5.2.5 Alternative modes of therapy [Karriyanis 2003]

Interferons have immunomodulatory, but also antiproliferative and antiviral effects. Lymphoblastoid and recombinant IFN-γ, have been used in turn since the early 1980s in attempts to achieve sustained suppression of HBV replication, and remission of HBV-related chronic liver disease. The drug is administered by subcutaneous injection and the recommended dosage for adults is 5 MU [million units] daily or 10 MU thrice weekly for a period of 16 weeks in HBeAg positive patients, or 12 months
for those who are HBeAg negative. The recommended dose for children is 6 MU/m² thrice weekly with a maximum of 10 MU. Interleukin-12 [IL-12] is also being used as therapy.

Nucleoside analogues are chemically synthesized drugs that are able to mimic natural nucleosides. As such, they are incorporated into newly synthesized HBV-DNA causing chain termination, and thus inhibiting viral replication. In addition, some of them competitively inhibit the DNA dependent and reverse transcriptase activity of the viral polymerase. For this to occur, the analogues need to be phosphorylated within cells to their triphosphate counterparts. Nucleoside analogues can be produced in their natural D- or unnatural L-configuration, and these are often referred to as enantiomers. Lamivudine, adefovir dipivoxil, enticavir, famciclovir, ganciclovir, lobucavir, are some of the nucleoside analogues that have shown therapeutic value.

Antisense oligodeoxynucleotides [ODN] are synthetic DNA molecules that can inhibit gene expression within cells by binding to complementary mRNA sequences, thus preventing translation. Phosphorothioate ODNs are nuclease resistant, so that they are still biologically active when they reach their intended site of action. Early experiments in cells transiently or stably transfected with plasmids encoding HBV proteins or the whole genome indicated that ODNs were effective in inhibiting viral protein expression and viral replication.

Ribozymes [ribonucleic acid enzymes] are naturally occurring RNA molecules that catalyse RNA sequence-specific cleavage and splicing. The smallest of them, known as ‘hammerhead’ from their characteristic secondary structure shape, recognize a minimal target sequence for cleavage. RNA cleavage specificity is mediated by the
ribozyme sequence, which is complementary to that of the target RNA, and flanking the catalytic sequence. A number of studies so far have demonstrated efficient cleavage of HBV mRNAs in \textit{in vitro} experiments using transfected cells or cell-free systems. Ribozyme activity has been demonstrated by targeting regions that included the encapsidation signal in pgRNA, HBx RNA and the poly A signal region of HBV. Ribozymes are presently in Phase I/II clinical studies.

4.1.6 HBsAg purification methods

Several methods like ultracentrifugation, chromatography or by a combination of chromatography methods were successfully applied to achieve $>95\%$ purity as mentioned by WHO reference standard. A process based on the integration of several chromatography steps has the advantage of being easily automated and scaleable. The downstream process for the purification of HBsAg was established first at laboratory scale using acid precipitation, diatomaceous earth, Immuno-affinity, colloidal silica, Triton X-100, hydroxyapatite, polyethylene glycol fractionation, ion-exchange and gel filtration etc. and further scaled up from 250 to 500 fold. The following are some of the strategies used for purification of HBsAg.

1] HBsAg of high purity was achieved by extraction in presence of surfactant followed by adsorption and desorption on colloidal silica at pH 8.8 and 11.0. Ion exchange with an optional gel extraction chromatography generated the 24 kDa polypeptide. [Pilliot \textit{et al.}, 1976].

2] HBsAg containing material was treated with an acid, optionally subjected to salting out with ammonium sulfate, and chromatographed with hydroxyapatite, by which a highly purified HBs antigen suitable for the preparation of HBV vaccine was obtained.
3] The supernatant of recombinant yeast producing hepatitis B surface antigen was disrupted in the presence of a polysorbate non-ionic detergent, clarified by addition of urea and adsorbed on colloidal silica from which the purified antigen is desorbed with a low ionic strength buffer supplemented with urea and a polysorbate non-ionic detergent.

[Wijnendaele et al., USPTO 4649192].

4] HBs antigen-containing material produced by a recombinant organism was processed for adsorption chromatography with silica, optionally followed by a gel filtration and further an adsorption chromatography with a hydroxyapatite, and then eluting the HBs antigen, preferably, with a buffer having a pH 9 or more which is incorporated with urea.

[Hamada et al., USPTO 47738926]

5] HBsAg from yeast was purified by initial acid precipitation followed by adsorption and disorption to diatomaceous earth matrix at pH 4.0 and 8.2. Immuno-affinity chromatography was used to scale up and this generated the 22 nm particles. [Agraz et al., 1993].

6] HBsAg secreted from Drosophila Schneider-2 cells was purified by polyethylene glycol [PEG] precipitation followed by successive ultracentrifugation and gradient fractionations with cesium chloride and sucrose [Deml et al., 1999].

7] HBsAg expressed in P.pastoris was purified by sequential steps of acid precipitation, diatomaceous earth matrix adsorption/desorption, and Immunoaffinity or Ion-exchange or size-exclusion chromatography methods. Partial purity was achieved
using diatomaceous earth matrix and >95% purity was achieved with either of the chromatography steps. [Hardy et al., 2000].

8) Plant-derived antibody for the immunopurification of the Hepatitis B surface antigen for human use was employed. Immunoaffinity purification methodology that uses plant-derived antibody from tobacco leaves and coupled to Sepharose CL-4B with high efficiency was used to generate 22 nm particles. The plant-derived antibody immunoaffinity matrix purification behavior was comparable to that of its mouse-derived monoclonal antibody homolog. [Valdes et al., 2003].

9) Recombinant HBsAg expressed in Bombyx mori larvae was purified by initial ammonium sulfate precipitation and then by size exclusion – Immunoaffinity chromatography. [Bachurina et al., 2005].

10) HBV from infected plasma was extracted to homogeneity in a single step using immobilized heparin chromatography –Hi Trap columns.[Zahn et al., 2005].
4.2 Materials & Methods

This section deals with the materials and methods that were applied for construction, expression, purification and characterization of HBsAg from *P. pastoris*.

4.2.1 Yeast strains

Parent yeast strains utilized for recombinant clone construction are listed in general materials and methods section. The following table details the *P. pastoris* strains generated during this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. pastoris</em></td>
<td>KM7- arg4 aox1::ARG4 HIS4</td>
<td>Prototroph host integrated with pB2ZB2 expression vector</td>
</tr>
<tr>
<td>pB2ZB2</td>
<td>Sh ble</td>
<td>pB2ZB2 expression vector</td>
</tr>
<tr>
<td><em>P. pastoris</em></td>
<td>KM7- arg4 aox1::ARG4 HIS4</td>
<td>Prototroph host cloned with recombinant expression vector pB2ZB2-HBsAg</td>
</tr>
<tr>
<td>pB2ZB2-HBsAg</td>
<td>Sh ble</td>
<td>pB2ZB2-HBsAg</td>
</tr>
</tbody>
</table>

Vectors

Parent yeast expression vectors utilized for recombinant *P. pastoris* clone construction are listed in general materials section. The following table details the expression vectors applied/generated in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pB2ZB1</td>
<td>Constitutive yeast expression vector</td>
<td>This study generated from PIB2 and pPICZαA</td>
</tr>
<tr>
<td>pB2ZB2</td>
<td>Constitutive yeast expression vector</td>
<td>This study generated from pB2ZB1</td>
</tr>
<tr>
<td>pB2ZB2-HBsAg</td>
<td>Recombinant pB2ZB2 expression vector</td>
<td>This study vector containing hepatitis B middle surface antigen gene</td>
</tr>
</tbody>
</table>
pB2ZB2-HBsAg NheI  Recombinant pB2ZB2-HBsAg vector  This study linearized with NheI enzyme
pB2ZB2-HBsAg SapI  Recombinant pB2ZB2-HBsAg vector  This study linearized with SapI enzyme

4.2.2 Antibodies

Antibodies against HBsAg were raised in house in mice as detailed in general methods section

<table>
<thead>
<tr>
<th>Name</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Anti- HBsAg (polyclonal)</td>
<td>Hepatitis B middle surface antigen</td>
<td>This study</td>
</tr>
</tbody>
</table>

Methods

4.2.3 Construction of expression vectors

- **pIB2** vector was digested with enzymes *ScaI* and *PstI* enzymes. Purified 3.8 Kbp fragment obtained constitutes GAP promoter, *His4* wild type allele and truncated ampicillin resistance gene.
- **pPICZαB** vector was linearized with *BglII* and end filled with *Pfu* polymerase at 72°C for 5 min in presence of 2mM dNTP’s. Linearized blunt ended pPICZαB vector was digested to completion with *PstI*. A 2.3 Kbp fragment thus purified constitutes 3’AOX transcription termination region, zeocin expression cassette operably linked to promoters TEF1, EM7 and to CYC1 transcription termination sequence and pUC Ori region.
- A 3.8 Kbp fragment of pIB2 vector and 2.3 Kbp fragment of pPICZαB vector were ligated using T4 DNA ligase at 16°C for 5 hrs.
- *E. coli* DH5α was transformed with the ligated product and screened for zeocin resistant colonies. The *E. coli*-P.pastoris vector was named as pB2ZB1.

- EcoRV, Sall and NtI enzymes were used to digest pB2ZB1 vector to confirm the molecular size.

- The expression vector was digested with *XhoI* and ligated as mentioned above. *E. coli* DH5α cells are transformed with the pB2ZB1- *XhoI* ligated product and screened for zeocin resistant colonies.

- Plasmid was extracted and the orientation of the constituents was confirmed by restriction digestion analysis with Sall, Nhel. The *E. coli*/P. pastoris shuttle vector was named as pB2ZB2.

### 4.2.4 PCR amplification of HBsAg gene with Taq polymerase

Hepatitis B ‘S’ gene is amplified with the following primers using cloned *Thermus aquaticus* DNA polymerase and *Pyrococcus furiosus* DNA polymerase.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg-F.P</td>
<td>GGAATTCATGGAGAACATCACATCAGG</td>
</tr>
<tr>
<td>HBsAg-R.P</td>
<td>CCGGATCCCTCGAGTTAAATGTATACCCACAGACAA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reaction Constituents</th>
<th>×1 μl</th>
<th>Reaction Constituents</th>
<th>× 1 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Primer (10 pM/ul)</td>
<td>0.5</td>
<td>Forward Primer (10 pM/ul)</td>
<td>1.25</td>
</tr>
<tr>
<td>Reverse Primer (10 pM/ul)</td>
<td>0.5</td>
<td>Reverse Primer (10 pM/ul)</td>
<td>1.25</td>
</tr>
<tr>
<td>dNTPs Mix (10mM/100ul)</td>
<td>2.5</td>
<td>dNTPs Mix (10mM/100ul)</td>
<td>2.5</td>
</tr>
<tr>
<td>10 x Taq PCR buffer</td>
<td>2.5</td>
<td>10 x Pfu PCR buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>Taq polymerase-(5u/ml)</td>
<td>0.5</td>
<td>Pfu polymerase-(2.5u/ml)</td>
<td>1.25</td>
</tr>
<tr>
<td>DNA (50 ng/ul)</td>
<td>2.0</td>
<td>DNA (50 ng/ul)</td>
<td>2.5</td>
</tr>
<tr>
<td>Autoclaved milliQ water-</td>
<td>15.5</td>
<td>Autoclaved milliQ water-</td>
<td>13.75</td>
</tr>
<tr>
<td><strong>Total Reaction volume</strong></td>
<td><strong>25.0</strong></td>
<td><strong>Total Reaction volume</strong></td>
<td><strong>25.0</strong></td>
</tr>
</tbody>
</table>
PCR reaction conditions were set as follows, the amplification was done for 30 cycles with each cycle comprising of:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>5 min</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>94°C</td>
<td>1 min</td>
<td>Denaturation</td>
</tr>
<tr>
<td>58°C</td>
<td>1 min</td>
<td>Annealing</td>
</tr>
<tr>
<td>72°C</td>
<td>1 min</td>
<td>Extension</td>
</tr>
<tr>
<td>72°C</td>
<td>7 min</td>
<td>Final extension</td>
</tr>
</tbody>
</table>

PCR with *Pfu* polymerase requires an extension time of 2 min/1 Kbp product

### 4.2.5 Construction of recombinant HBsAg expression vector

- Hepatitis B virus surface antigen (HBsAg) gene was designed and synthesized commercially. A 0.7 Kbp HBsAg gene was amplified by polymerase chain reaction (PCR) using two synthetic primers listed.
- Amplified HBsAg gene was digested to completion with *EcoRI/XhoI* enzymes and inserted at *EcoRI* and *XhoI* sites in pB2ZB2 vector, downstream of GAP promoter.
- Inframe cloning of HBsAg gene between promoter and terminator in the expression plasmid was confirmed by DNA sequencing and restriction digestion analysis.
- The resulting recombinant expression vector pB2ZB2-HBsAg was used as shuttle vector between *E. coli* and *P. pastoris*.

### 4.2.6 Transformation & screening of *P. pastoris* with pB2ZB2-HBsAg vector

- pB2ZB2-HBsAg plasmid was linearized with *NheI* enzyme (for histidine integration), *SapI* (for GAP promoter integration) purified and integrated in to
chromosomal DNA of *P. pastoris* using lithium chloride transformation method.

- Competent cells were transformed with ~10μg of linearized expression plasmid in presence of lithium chloride and PEG 3550. Cells were plated on minimal dextrose medium (MD) without histidine and incubated at 30°C for 3 to 4 days.

- Transformants harboring the HBsAg expression cassette integrated into chromosomal DNA were selected on MD without histidine medium.

- Transformants harboring the HBsAg expression cassette integrated into chromosomal DNA were also selected on YPDZ medium.

- *Pichia* transformants were then analyzed for HBsAg gene insert by direct colony PCR and confirmed by genomic DNA PCR amplification using pGAP-F.P/3AOX TT R.P.

### 4.2.7 Growth profile/ Time course experiments

- Recombinant clone of *P. pastoris* KM71- HBsAg integrated with plasmid vector pB2ZB2-HBsAg was inoculated in 10 ml YPD medium and incubated at 30°C for 24 hrs at 180 rpm.

- 1 OD$_{600}$ cells from 24 hr culture were inoculated in 25 ml YPD medium in a 150 ml flask.

- Aliquots were taken at periodic intervals ranging from 24-120 hrs and analyzed for growth and HBsAg expression by ELISA.
4.2.8 Production of HBsAg in 10.0 lit continuous fermentation

- Continuous culture was set up for the pB2ZB2-HBsAg clone in fermenter using basal salts medium (BSM).
- Initial shake flask cultivation was performed in YPD medium. A 150 ml shake flask containing 25 ml of YPD medium was inoculated with one vial of pB2ZB2-HBsAg clone (~1 mL, OD_{600} = 30) at 30°C with shaking at 200 rpm for 24 hrs, until a cell density of 18 OD_{600}. 24 hr culture was then inoculated in 2.0 Lit shake flask containing 500 mL of YPD medium and incubated to conditions as mentioned above.
- The above 24 hr culture was used to inoculate a 10.0 L fermentor (Bioengineering, Wald, Switzerland) with a 6.0-L working volume at an initial density of ~4.0 OD_{600}. BSM used for batch fermentation contained per liter deionized water, trace salts solution 4.3 mL.
- The fermentor was maintained at a pH 6.5, temperature of 30°C and agitation rate of 500 rpm. pH was controlled by the addition of phosphoric acid or liquor ammonia as needed. Dissolved oxygen (dO_{2}) levels were initially maintained at 35% air saturation by the introducing air in to the fermentor at a rate of about 0.8 volume per volume fermentor per minute (VVM).
- After ~24 h of batch culture, continuous fermentation was initiated as indicated by dO_{2} spike due depletion of initial glucose.
- Continuous feed medium contained (per liter deionized water) 300 g glucose, 13.35 mL H_{3}PO_{4} (85%) CaSO_{4}, 2H_{2}O 0.47 g, K_{2}SO_{4} 7.45 g, MgSO_{4}. 7H_{2}O 2.07 g, KOH 0.47 g, D-biotin 0.87 mg, and 4.35 mL trace salts solution.
### Basal Salt Medium composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>400 g</td>
</tr>
<tr>
<td>H₃PO₄ (85%)</td>
<td>26.0 ml</td>
</tr>
<tr>
<td>CaSO₄·2H₂O</td>
<td>0.9 g</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>18.0 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>14.0 g</td>
</tr>
<tr>
<td>KOH</td>
<td>4.0 g</td>
</tr>
<tr>
<td>D-biotin</td>
<td>0.87 mg</td>
</tr>
<tr>
<td>D. water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

### Trace salt solution composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO₄·5H₂O</td>
<td>6.0 g</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Fe (SO₄)·7H₂O</td>
<td>65 g</td>
</tr>
<tr>
<td>ZnCl₂·7H₂O</td>
<td>20 g</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.2 g</td>
</tr>
<tr>
<td>KI</td>
<td>0.8 g</td>
</tr>
<tr>
<td>D. water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

- Aliquots were taken daily for cell density OD₆₀₀, southern analysis and western analysis.

- Continuous culture was harvested daily and cells were pelleted by centrifugation (Sanyo RC3C) at 4000 rpm for 30 min and stored at -20°C until proceeded further. This process of culture removal and fresh medium replenishment was continued for 6 days.

#### 4.2.9 Preparation of soluble HBsAg extract

- HBsAg protein expressed intracellularly is obtained by yeast cell disruption.

- 1ml aliquot of shake flask culture was pelleted by low speed centrifugation (6000 rpm, 5–10 min) and washed twice with buffer (50 mM Tris, pH 8, 5 mM EDTA, 0.1 M NaCl).

- Washed cells were resuspended in lysis buffer (50 mM Tris, pH 8, 5 mM EDTA, 0.1 M NaCl, 0.1%, Triton X-100, 1 mM PMSF) containing glass beads
(0.5 mm) and lysed by 10 one minute bursts of vortexing at maximum speed, with chilling on ice for 1–2 min between bursts.

- Cell lysate was centrifuged at 10,000 rpm for 30 min. Supernatant was proceeded for western analysis.

- For fermentation culture cells corresponding to a density of 100 OD$_{600}$ was pelleted, washed as described above and lysed by Dynomill (KDL, Basel, Switzerland) for 2-3 cycles and proceeded for purification.

4.2.10 Western blot assay

- Yeast extracts and purified samples were treated with equal volume of sample buffer (50mM Tris-Hcl, pH 6.8, 10% glycerol, 8% SDS, 50% 2-mercaptoethanol, 0.0025mM bromophenol blue and 1M DTT) and subjected to 15% SDS-polyacrylamide gel electrophoresis and transferred on to nitrocellulose membrane.

- 1% skim milk blocked membrane was incubated with mouse anti-HBsAg (1:2500 diluted in PBS) for 1 hr.

- Mouse anti- HBsAg antibody was generated in house by injecting purified HBsAg.

- The membrane was washed with PBST and incubated with HRP conjugated goat anti-mouse IgG (1: 2500 diluted in PBS).

- Substrate solution (3',3'-diaminobenzidine, 10μl- 30% H$_2$O$_2$ in 10 ml PBS) was used to visualize the reactive bands.
4.2.11 Quantitative ELISA

- The concentration of HBsAg in the soluble cell extracts and purified protein was determined in triplicates using a quantitative HBsAg EIA PLUS system specific for HBsAg (ANI Labsystems, Finland), with positive control (HBsAg derived from human serum) and negative control (untransformed Pichia cell extract).

- The clarified extracts were diluted 500-fold with a buffer containing 1% horse serum as a carrier. Aliquots ranging from 10 to 100 μl were assayed as per the manufacturer’s protocol. The absorbance was measured in a multiskan ELISA reader (Labsystems, Helsinki, Finland).

- 50 μl of conjugate was pipetted into the bottom of Microstrip® wells.

- 100 μl of the negative control is dispensed into three wells.

- 100 μl of the positive control is distributed into two wells.

- 100 μl of specimen/sample is added into the Microstrip® wells mixed gently by pipetting and covered.

- Microstrip® wells are incubated at 37°C for 2-3 hrs without shaking.

- The wells were emptied and 300-400 μl of washing solution was added to each well and soaked for a min of 10 sec. The washing step was repeated five times and tapped a few times on a paper towel.

- 100 μl of substrate solution (TMB-3,3', 5,5'-Tetramethylbenzidine) was added into each well and incubated for 30 minutes at room temperature in a dark place.
Enzyme-substrate reaction is stopped by adding 100 μl of 0.5 M H₂SO₄ solution into each well.

Absorbance was measured at 450 nm within 30 minutes after stopping the enzyme-substrate reaction.

4.2.12 Southern Analysis

Genomic DNA was isolated from recombinant P. pastoris and from untransformed P. pastoris culture as per the standard procedure described above. Genomic DNA was quantified and 10μg was restricted with EcoRI and XhoI and electrophoresed on 0.8% agarose gel.

Resolved fragments were transferred to Hybond nylon membrane and probed with 0.7 Kbp HBsAg fragment radiolabeled with [α-32P] dCTP. A 0.7 Kbp fragment was generated by PCR amplification from pB2ZB2-HBsAg vector.

Labeling was performed using nick translation kit (GIBCO-BRL).

Subsequent to hybridization and washing, HBsAg gene integration to yeast chromosomal DNA and stability of the vector during continuous culture was visualized by autoradiography.

4.2.13 Purification of Hepatitis B surface antigen

Acidic two phase systems (ATPS) were prepared from stock solutions of PEG 6000 (50%), NaCl (25%). The phosphate stock solution consisted a mixture of K₂HPO₄ and NaH₂PO₄ at pH 7.0. Stock solutions were stored at 4°C. Before use the temperature of all solutions was equilibrated by standing at
23°C. PEG and NaCl were prepared in 0.05M potassium phosphate buffer pH 7.2.

- HBsAg protein extract was treated with equal amount of 5% PEG/NaCl, mixed thoroughly and equilibrated for 2hrs at 23°C. To speed up the phase separation, phase system was centrifuged at 3000 rpm for 5 min.

- The top phase (PEG rich) was collected and quantified for HBsAg protein. To the PEG phase 2% zinc chloride (ZnCl₂) was added and the resultant slurry was mixed for 2 hrs at 23°C to complete precipitation process.

- The separation of supernatant phase from precipitate phase was achieved in a Dupont Sorvall RC-5C centrifuge for 30 min at 10,000 rpm.

- Protein precipitate thus obtained was suspended in 1M Tris / 0.05 M EDTA pH 9.0 buffer, mixed thoroughly and allowed to stand by for 1 hr. The above mixture was centrifuged at 15,000 rpm for 30 min to separate supernatant and precipitate. The supernatant and precipitate were quantified for HBsAg protein.

- Supernatant with HBsAg protein in Tris buffer was dialyzed against 50 mM phosphate buffer pH 7.2. The dialyzed protein was loaded onto DEAE Sepharose column and eluted with NaCl gradient. Eluted protein fractions were quantified for HBsAg by ELISA.

- Each step of purification process was monitored by SDS-PAGE/silver staining.

4.2.14 Ultracentrifugation and gradient fractionation

- Sucrose density gradient ultracentrifugation was applied to further characterize HBsAg particles at a density of approximately 1.2 g cm⁻³.
• 250μl of HBsAg particles were layered on sucrose step gradient (20-50%) and centrifuged at 25,000 rpm for 18 h using a Beckman SW-28 rotor.

• The gradients were fractionated and checked for HBsAg by commercial HBsAg EIA PLUS system.

4.2.15 Electron microscopy

• 15-20μl of HBsAg sample was placed on to a copper grid coated with carbon film and incubated for 2 min.

• Excess sample was removed and the grids were stained with 2% uranyl acetate and examined in Hitachi H-7500 TEM (Transmission Electron Microscope).

• HBsAg purified by metal affinity precipitation-DEAE chromatography, sucrose density gradient centrifugation and commercial licensed vaccine (Engerix-B) were visualized.

4.2.16 Reverse phase High pressure liquid chromatography

• Purified HBsAg was applied to a C8 reverse-phase column (Supelco Silica C8; 50 × 4.6 mm) using ÄKTA explorer Workstation (GE healthcare Pharmacia Biotech) with solvent A as 100% water + 0.05% Tri fluoroacetic acid and solvent B as 100% Acetonitrile + 0.05% TFA.

• A flow rate of 1ml min⁻¹ within 30 min was maintained.

• The protein was monitored at an excitation wave length of 280 nm and an emission filter with 325 nm cut off.
4.2.17 Immunization of mice with rHBsAg

- Groups of three female BALB/c mice 12-16 weeks of age were primed with a single intraperitoneal (i.p.) injection of purified HBsAg particles to evaluate the immunogenicity of rHBsAg.

- The antigen preparations were either naked HBsAg particles or HBsAg adsorbed to Alum.

- The vaccine Engerix-B, 10μg/ml (GlaxoSmithKline Biologicals) was used for immunization comparison of HBsAg.

- Mice were bled from the retro orbital plexus or tail vein at intervals and antibody titers were measured in mouse serum using commercially available kit HBsAg EIA PLUS system (ANI Lab systems, Finland).
RESULTS
Results

4.3.1 Construction of pB2ZB series of expression vectors

2.3 Kbp fragment generated by digestion and end filling of pPICZαB [Fig.11A] vector was ligated to 3.8 Kbp [Fig.11B] fragment generated by digestion of PIB2 vector with Scal and PstI enzymes. E. coli were transformed and plated on LSLB medium. pB2ZB1 plasmid [Fig.11C] was extracted and digested to completion with EcoRV, Scal, and NotI to identify the nucleotide length and orientation. EcoRV and NotI digestion yielded a ~ 6.26 Kbp fragment, Scal digestion yielded ~ 4.0 Kbp and ~ 2.26 Kbp fragments. Digestion of the plasmid yielded fragments of expected size. pB2ZB1 expression vector can be used directly for transformation of P. pastoris but the multiple cloning site (MCS) region has restriction sites that are not unique and so was not ideal for cloning of gene of interest. XhoI digestion removed a 50 Kbp DNA fragment thereby establishing unique sites in MCS region. pB2ZB2 plasmid [Fig.11C],[Appendix A1] constructed was extracted, digested to completion with Scal and Nhel individually. Digestion of the plasmid with both the enzymes yielded ~ 6.21 Kbp product. Restriction digestion patterns of the above plasmids are supported by in silico restriction digestion analysis.

The pB2ZB2 plasmid contains the GAP promoter and transcription termination sequences of 3’ AOX TT respectively separated by a multiple cloning site for insertion of the foreign gene of interest, pUC ori region and truncated ampicillin resistance gene (non functional). It contains a wild type copy of histidinol dehydrogenase (HIS4) gene that can be used for linearized integration in to host genome and for auxotrophic selection of Pichia transformants. In addition, the vector
also possesses TEF1, EM7 promoter driven dominant selectable marker zeocin that is bi-functional in both *P. pastoris* and *E. coli* for selection of transformants.

**4.3.2 Construction of recombinant HBsAg expression vector**

Hepatitis B virus surface antigen (HBsAg) gene was amplified by polymerase chain reaction (PCR) using two synthetic primers HBsAg- F.P/R.P. A 0.7 Kbp [Fig.13 A] amplified product was purified and inserted in to the pB2ZB2 expression vector. Inframe cloning of HBsAg gene between in the pB2ZB2 expression vector was confirmed by restriction digestion analysis [Fig.13 B] and by DNA sequencing using pGAP F.P/3AOXTT R.P [Fig.14]. The resulting recombinant expression vector pB2ZB2-HBsAg [Fig.11D],[Appendix A3] was used between *E. coli* and *P. pastoris*.

**4.3.3 Generation of constitutive *P. pastoris* KM71- HBsAg clones**

pB2ZB2-HBsAg vector was used to transform host *P. pastoris* KM71. pB2ZB2-HBsAg being an integrative type of vector, two modes the promoter integration and HIS4 integration were used.

*HIS4* (*histidinol dehydrogenase*) gene integration

pB2ZB2-HBsAg vector has NheI unique site in HIS4 locus. Prior to transformation the pB2ZB2-HBsAg vector was linearized with NheI and purified. The host strain *P. pastoris* KM71 carries a loss of *AOX* 1 gene (Mut^S_), and is a histidine requiring auxotroph. Linearized pB2ZB2-HBsAg vector has both ends homologous to the his4 region of the Pichia genome, it can integrate into the his4 locus by a single crossover event, with concomitant elimination of the host his4 gene [Fig.12A]. Integration of linearized pB2ZB2-HBsAg vector at his4 locus generated a prototrophic strain that can grow on minimal media lacking histidine. Transformation with lithium
chloride/PEG was used to generate prototrophic transformants. Pichia transformants harboring the constitutive HBsAg expression cassette were selected by plating on MD His\(^{-}\) plates and incubated for 3–4 days at 30°C [Fig. 13 C]. The presence of HBsAg insert in these His\(^{+}\) transformants was confirmed by colony PCR using pGAP F.P/3AOXTT R.P [Fig. 13 E]. 120 transformants selected after PCR amplification were then screened for clone that has maximum productivity.

**GAP promoter integration**

A second strategy of generating transformants is by linearizing recombinant pB2ZB2-HBsAg vector with SapI restriction enzyme prior to transformation of Pichia. SapI, which cuts within the GAP promoter, facilitates the insertion of the pB2ZB2-HBsAg vector into the resident GAP promoter region of the host genome by a single crossover event with concomitant elimination of the host GAP promoter [Fig. 12 B]. Integration of linearized pB2ZB2-HBsAg vector at GAP locus generated a prototrophic strain that grew on minimal media lacking histidine. Transformation with lithium chloride/PEG was used to generate prototrophic transformants. Pichia transformants harboring the constitutive HBsAg expression cassette were selected by plating on YPD medium with Zeocin and incubated for 3–4 days at 30°C [Fig. 13 D]. The presence of HBsAg insert 0.7 Kbp in these His\(^{+}\) transformants was confirmed by colony PCR using pGAP F.P/3AOXTT R.P primers [Fig. 13 E].

Promoter Vs his4 integration

Transformation of *P. patoris* KM71 host with NheI linearized pB2ZB2-HBsAg at his4 locus and SapI linearized pB2SK at GAP promoter region was done as described. A comparative analysis of growth rate, expression levels of HBsAg,
Figure 11. Construction of dual selection vectors for HBsAg expression in *P. pastoris*. (A) Generation of Pst I-end filled fragment of pPICZαB expression vector (B) Sca I-Pst I digested fragment of PIB2 expression vector were (C) ligated generating pB2ZB2 expression vector. (D) 0.68 Kbp HBsAg gene is operably cloned downstream to GAP promoter generating recombinant pB2ZB2-HBsAg expression vector.

Figure 12. Integration of recombinant B2ZB2-HBsAg expression vector in to Pichia chromosome. (A) Integration of SapI linearized expression vector in to host GAP promoter region. (B) Integration of Nhel linearized expression vector in to host his4 locus.
Figure 11.

A.
B.

PIB2-ScaI/PstI fragment
PIB2-Scal/PstI fragment
C.

PIB2-Scal/PstI fragment
D.

B2282-EcoRlHind
6179 bp

EcoRl

HBS4

Amp (frag)
pUC Orig

ZeoCin

Cpt1 TT

pTEF1

pEM7

6x His

3' AOX TT

KpnI

HbsAg gene 667 bp

Sap I 5953

Nhe I 6882

EcoRl 672

pGAP

HbsAg

HindIII 1359

Net I 1371

BamHl 1798

3' AOX TT

B2282-HbsAg
6907 bp

HS4

Amp (frag)
pUC Orig

CmRc cmRc

6X His tag

DE3 1798

pTEF1

pEM7

ZeoCin

Cpt1 TT

EcoRV Z714

HP-1 5953

Nhe I 6882

HbsAg gene 667 bp

Sap I 4386

EcoRl 672

pGAP

HbsAg

HindIII 1359

Net I 1371

BamHl 1798

3' AOX TT

B2282-HbsAg
6907 bp

HS4

Amp (frag)
pUC Orig

CmRc cmRc

6X His tag

DE3 1798

pTEF1

pEM7

ZeoCin

Cpt1 TT

EcoRV Z714
Figure 12.

A.

Integration by Homologous Recombination in 1 P._pastoris KM71 host genome
B.

B22B2-HB-Sap1

Sap1 digestion

P. pastoris genomic DNA

Integration by Homologous Recombination into P. pastoris KM71 host genome
stability of pB2ZB2-HBsAg vector in clones generated by his4 and GAP promoter integrations were studied. Expression of HBsAg was observed consistently in both clones qualitatively and quantitatively. Plasmid stability was observed with no loss of activity and no significant difference in growth rate of *P. pastoris* KM71-HBsAg clone, compared with *P. pastoris* KM71-PIB2 and *P. pastoris* KM71 [Fig. 15 A], suggesting that the expression of HBsAg is not toxic to the host. As both the clones have a similar expression profile recombinant *P. pastoris* generated by his4 integration was chosen for further studies.

4.3.4 Growth, expression profile

Aliquots were taken at different time intervals and analyzed for HBsAg expression by western blotting and ELISA (HBsAg EIA PLUS, ANI Labsystems, Finland). GAP promoter driven expression was identified from day 1 and was consistently seen to the end of the experiment. Western blot analysis of pB2ZB2-HBsAg transformed *P. pastoris* clone has indicated the presence of an anti-HBsAg immuno reactive 24 kDa band is seen from 24-120 hrs indicating the constitutive expression of HBsAg [Fig. 15 B]. To check whether continuous expression of HBsAg might have any toxic effects on the host cells, the growth rate of cells expressing the plasmid pB2ZB2 -HBsAg, cells with parent plasmids PIB2, pPICZαB and untransformed Pichia strain were compared in YPD medium. *P. pastoris* expressing pB2ZB2 -HBsAg plasmid showed no significant difference of growth rate with *P. pastoris* controls [Fig. 15 A] at indicated time intervals. Stability of the integrated plasmid was checked by southern analysis using genomic DNA isolated at periodic intervals ranging from 24-120 hrs from continuous culture. A 0.7 Kbp band was
Figure 13. Amplification, cloning and selection of HBsAg gene in *P. pastoris.* (A) HBsAg (0.68 Kbp) gene was amplified by PCR using *Pfu* polymerase (Lane 1), 0.25-12.0 Kbp DNA marker (Lane 2). (B) B2ZB2-HBsAg clone confirmation by restriction analysis with EcoRI/Xhol enzymes (Lane 1). HBsAg amplified product (Lane 2), Linearized B2ZB2 vector with EcoRI/Xhol (Lane 3), 0.5-10.0 Kbp DNA marker. (C) Selection of Pichia transformants on Minimal Dextrose medium without histidine (Lane 1), Untransformed Pichia (Lane 2). (D) Selection of Pichia transformants on YPD medium with zeocin (Lane 1), Untransformed Pichia (Lane 2). (E) Colony PCR of Pichia transformants selected on MD-His medium (Lanes 1, 2, 3), YPD medium (Lanes 4, 5), Amplified product of B2ZB2-HBsAg vector (Lane 6), 0.5-1.0 Kbp DNA marker (Lane 7).
Figure 14. Chromatogram of pB2ZB2-HBsAg DNA sequence. Sequence within the GAP promoter was used as forward primer and sequence within the AOX TT was used as reverse primer.

Figure 15. Growth comparison and expression profile of *P. pastoris* KM71-HBsAg clone. (A) Expression of HBsAg has no toxic effect on the growth rate of the *P. pastoris* host. *P. pastoris* KM71 cells transformed with pIB2, pPICZαB, pB2ZB2 parent plasmids and recombinant expression vector pB2ZB2-HBsAg were cultured in YPD broth and aliquots were withdrawn at indicated time points up to 144 hrs. OD, recorded and plotted. (B) Western analysis of HBsAg protein in cell lysis supernatant of recombinant Pichia from 24-144 hrs (Lanes 1-6), Untransformed Pichia (Lane 7).
Figure 15.

A. 

![Graph showing culture time vs. OD600 for different strains of P. pastoris.]

B. 

![Image showing microscopy or agar plate with different samples labeled 1 to 7.]

- P. pastoris KM71
- P. pastoris KM71-pB2ZB2 HBsAg
- P. pastoris KM71-P1B2
- E. pastoris KM71-pPK7aB
visualized consistently in all the time courses and is consistent with the result of a single copy clone [Fig. 16 B].

4.3.5 Continuous fermentation

*P. pastoris* KM71-HBsAg clone was adapted to BSM and grown in continuous mode with an initial 0.75 volume exchanges per day and increased to 1.2 by day 3.6 cycles of continuous culture was established with an average $\text{OD}_{600}$ of ~300 [Fig.16 A]. The culture was fed continuously with 30% glucose medium for 6 days. The plasmid stability and expression of HBsAg in the continuous batch process was monitored by southern and western analysis [Fig.16 B, C]. This is the first report describing recombinant prototrophic constitutive *P. pastoris* high cell density fermentation for HBsAg expression. Expression and cell yields remained unchanged to the end of the fermentation.

4.3.6 Purification of HBsAg protein

*Characterization of Zinc phosphate matrix*

Preliminary results indicated that r-HBsAg was adsorbed by Zinc phosphate, when a solution of antigen was placed in contact with zinc phosphate at acid pH.

To determine the influence of concentration of Zinc phosphate and pH on adsorption capacity stability studies of rHBsAg obtained by cell lysis were performed. Adsorption capability of Zinc at 1%, 2.0%, 2.5%, and 3.0% [Fig. 17 A] was evaluated by ELISA of the supernatants clarified by centrifugation. $>85\%$ of rHBsAg was found unadsorbed at 1% concentration, however the increase in the Zinc concentration raised the adsorption capability with $<10\%$ of rHBsAg unadsorbed. 2.5% Zinc precipitation
Figure 16. Constitutive expression of rHBsAg in 10.0 L Fermentor culture. (A) Constitutive rHBsAg expression in continuous culture with *P. pastoris* host, pB2ZB2 vector. 30% glucose continuous feed medium was fed and high cell density fermentation was run successfully for 6 days. (B) Southern blot analyses of *P. pastoris* KM71-HBsAg clone. *EcoRI/XhoI*, digested genomic DNA was capillary transferred to nylon membrane and subjected to hybridization with a α<sup>32</sup>P-labelled 0.7 Kbp HBsAg gene fragment. Lane 1. *pB2ZBZ-HBsAg* vector restricted with *EcoRI/XhoI*, Lane 2. *P. pastoris* KM71-HBsAg genomic DNA extracted from pre fermentor culture. Lane 3-5. *P. pastoris* KM71-HBsAg genomic DNA extracted from continuous culture, days 2, 4, and 6. (C) Western blot assay of purified HBsAg from 6 fermentor cycles. Purified HBsAg protein were electrophoresed on 12.5% SDS polyacrylamide gel, transferred to nitrocellulose membrane, and detected using antibodies as described in the text. rHBsAg control (lane 1), Cycle 2 (lane 2), Cycle 3 (lane 3), Cycle 4 (lane 4), Cycle 5 (lane 5), Untransformed Pichia (lane 6). ~24 kDa protein was observed consistently from Cycle 1-6.
Figure 17. Adsorption & Desorption studies of HBsAg (A) Concentration of ZnCl₂ required for precipitation of r-HBsAg. X axis represents loaded r-HBsAg. Concentration of adsorbed/ un-adsorbed r-HBsAg was measured by ELISA. (B) Adsorption of r-HBsAg on ZnCl₂ mediated precipitate at different pH; X axis represents loaded r-HBsAg per 2% ZnCl₂. (C) Desorption of r-HBsAg from ZnCl₂ mediated precipitate using different eluting pH buffers in two elutions.
Figure 18. Purification and Characterization of rHBsAg by metal affinity precipitation and DEAE sepharose chromatography. (A) Electrophoresis profile of total proteins after each purification step of HBsAg. Samples were electrophoresed on 15% SDS-PAGE with Tris-glycine buffer. The gel was photographed on a white background to contrast the silver stained protein bands. Lanes, 1. cell lysis supernatant; 2. PEG-NaCl supernatant; 3. Protein after buffer exchange; 4. 200mM eluant after ion exchange chromatography; 5. sterile purified protein; 6,7 eluant after partial purification by novel matrix; M, molecular weight markers from top to bottom are 97,66,45,30,20,14 kDa respectively. Gel resolved bands indicated by arrow correspond to the HBsAg monomer (~ 25 kDa). (B) Biochemical characterization of S antigen. Purified HBsAg was compared with commercial vaccine Engerix B® by sucrose density gradient centrifugation. A. 16 fractions were collected and analyzed for HBsAg concentration by a commercial HBsAg EIA PLUS system. The indicated density profile (△) represents the mean values of three gradients. (C) SDS-PAGE electrophoreogram of rHBsAg eluted from sucrose density gradient centrifugation. Fractions 4 (Lane 1), 5 (Lane 2), 6 (Lane 3), 7 (Lane 4), 8 (Lane 5), 9 (Lane 6), 10 (Lane 7).
Figure 18.

A.

M 1 2 3 4 5 M 6 7

24.0 kDa

B.

Fracture

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

1.8

1.4

1.2

1.0

0.8

0.6

0.4

0.2

0

0

C.
Table 17. Purification chart process for 100 OD_{600} cells.

Concentration, final yield, and purity of hepatitis B surface antigen (HBsAg) after each purification step.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>HBsAg protein μg mL^{-1}</th>
<th>Volume (Lit)</th>
<th>Final yield (%)</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-NaCl Supernatant</td>
<td>39.0</td>
<td>11.0</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Unadsorbed HBsAg</td>
<td>2.95</td>
<td>10.5</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Desorbate</td>
<td>89.54</td>
<td>1.5</td>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>Conc. Desorbate</td>
<td>393.97</td>
<td>0.80</td>
<td>81</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Ion exchange column</td>
<td>756.6</td>
<td>0.375</td>
<td>73</td>
<td>&gt;95</td>
</tr>
<tr>
<td>Sterile Purified protein</td>
<td>550.2</td>
<td>0.5</td>
<td>70</td>
<td>&gt;95</td>
</tr>
</tbody>
</table>

* a The above data represents a mean of 3 purifications.

* b Purity was estimated by Tris-glycine SDS-PAGE- silver staining method.
Figure 19. Transmission Electron microscopy (TEM) and Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) of purified r-HBsAg. (A) Sucrose gradient isolated purified rHBsAg (B) Engerix B® C. and particles directly visualized after chromatography, were deposited on copper grids and stained with uranyl acetate for TEM examination. (D) RP-HPLC of purified rHBsAg on a C8 silica column. Purified rHBsAg was reduced and denatured prior to loading on to a reverse phase column. Purified rHBsAg is represented by a peak eluting at 13.68 min.
HBsAg protein measured the contaminants as endotoxin levels (110.2 ± 20.6 pg) by limulus amoebocyte lysate assay, host DNA (< 10 pg) by semi-quantitative DNA estimation that were within limits for recombinant DNA hepatitis B vaccine requirements.

4.3.7 HBsAg expression and assembly into particles

HBsAg purified by metal affinity precipitation-DEAE chromatography was further characterized by comparing with commercial vaccine Engerix-B® through ultracentrifugation and TEM. HBsAg levels in purified samples were determined by quantitative ELISA and SDS-PAGE analysis [Fig.18 B, C]. The HBsAg positive sucrose gradient fractions were pooled, dialyzed and observed for particles in TEM. 20-22 nm particles were observed in sucrose gradient fraction of Engerix-B® and in purified fraction obtained by precipitation and chromatography [Fig.19 A-C] indicating the efficient assembly of HBsAg particles. The purity of rHBsAg was 95% or more as assessed by C8 reverse-phase high-pressure liquid chromatography [Fig.19 D].

4.3.8 HBsAg immunogenicity

To examine the immunogenicity of HBsAg particles expressed constitutively and purified by metal affinity precipitation, groups of each 3 mice were immunized with a single i.p. injection of either 10 μg of ‘naked’ particles or alum adsorbed particles. For comparison, three mice were immunized with commercial licensed vaccine Engerix-B®. A group of non-primed mice served as negative control. Mice were bled at regular intervals of 1-6 weeks and anti-HBs titers were measured. All of
the animals immunized with HBsAg particles developed a substantial HBsAg specific antibody response. Mice immunized with naked HBsAg particles measured a mean titer of 590 mIU/ml. Adsorption of HBsAg particles to alum increased the immunogenic response to 10-fold [Fig.20]. A similar response was also observed in mice immunized with commercial vaccine Engerix-B®. No HBsAg-specific antibody response was detectable from sera of non-immunized mice.
Figure 20. Antibody response of BALB/c mice to HBsAg preparations. BALB/c mice were immunized with a single i.p injection of 10 µg of HBsAg in adsorbed or naked particle form and response was determined at regular intervals till 6 weeks. The mean values of three individual mice in each group are shown.
Figure 20.