Vaccine development against HBV infection through enhanced expression of Hepatitis B surface antigen in *Kluveromyces lactis*

BY

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Dedication

To Mamma for her love and support and for standing by me in my success and my failures

To Abbu for being my pillar of strength and for encouraging me throughout my life

To Nimra for just being her, I wouldn't have been the same, had it not been for her.

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Fatima Javed Mirza

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List of Acronyms

AS	Ammonium Sulphate
ASP	Ammonium Sulphate precipitation
Bp	Base Pairs
^{o}C	Degree Celsius
HBeAg	hepatitis B envelop Antigen
HBcAg	hepatitis B core Antigen
HBsAg	hepatitis B surface Antigen
HBV	Hepatitis B virus
HCC	Hepatocellular Carcinoma
Kb	Kilo base pair
K.lactis	*Kluyveromyces lactis*
mL	Milli liter
nm	nano meter

KD	kilo Dalton
PH	power of H^+
Rpm	revolution per minutes
RSCU	Relative Synonymous Codon Usage
μL	Micro liter
GRAS	Generally Regarded As Safe
FDA	Food and Drug Administration

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Abstract

Hepatitis B virus remains a leading cause of liver disease worldwide. According to an estimate approximately 350 million people worldwide have chronic HBV infection and that 1 million persons die each year from HBV-related chronic liver disease. HBV infection, caused by a small partially double stranded DNA virus belonging to *Hepadnaviridae* family is currently being dealt with vaccination and antiviral therapy. The present study aims to enhance the production of engineered Hepatitis B surface antigen as a recombinant vaccine using *Kluveromyces lactis* expression system, selected on grounds of its high efficiency and easy growth conditions. The engineered HBs Ag protein was separated, purified and concentrated using the techniques of Ammonium Sulphate precipitation and dialysis before being analysed using ELISA. In this study, it was ascertained that ASP followed by dialysis produced the maximum concentration of HBsAg amounting to 0.8 μg/mL. The vaccine developed so far now requires to be subjected to further more advanced concentration and purification techniques before being taken to the stage of pilot scale production and animal testing.

INTRODUCTION

Hepatitis B virus (HBV) infection, with over 300 million chronically infected patients worldwide is a global public health problem. Pakistan is highly endemic with nine million people infected with HBV along with a steady rise in the infection rate due to poor economic status of the country and the lack of proper health facilities (Ali *et al.,* 2011). Currently vaccination and antiviral therapy are serving to reduce the risk of HBV infection and its related liver diseases.

1.1 Hepatitis B Virus

HBV is the prototype member of the *Hepadnaviridae* family, comprising of hepatotropic DNA viruses that replicate via reverse transcription. Even though HBV has a genome of tiny size (about 3 kb) it is one of the most pathogenic, with an estimated 2 billion people having contracted the virus and 350 million or more being chronic carriers (WHO Fact sheet no. 204); chronic infection is correlated with a strongly increased risk for the development of severe, potentially lethal, liver disease (Ganem and Prince, 2004).

The virus is currently divided into eight genotypes (A-H) and four major serotypes. (*adr*, *adw*, *ayr* or *ayw).* In addition to the two more genotypes, which have recently been isolated from Vietnam and Japan and characterized through molecular and phylogenetic analysis. These have been assigned as I and J genotypes (Cassidy *et al.*, 2011).

1.2 Diversity of the HBV Genome

The spontaneous error rate of viral reverse transcription causes the HBV genome to evolve with an estimated rate of nucleotide substitution at 1·4 to $3{\cdot}2\times10^{-5}$/site per year (Okamoto *et al*.,1987) which has resulted in four major HBV serological subtypes (*adw*, *ayw*, *adr*, and *ayr*) and nine minor subtypes, identified by the antigenic determinants of HBsAg. Seven HBV genotypes (A to G) are defined by divergence of more than 8% in the entire HBV genomic sequence (Kao and Chen, 2000).

Table 1.1: Geographical distribution of HBV genotypes and serotypes (Kao and Chen, 2002)

Genotype	Serotype	Areas of Predominance
A	adw2, ayw1	Northwestern Europe, USA, Central Africa
B	adw2, ayw1	Taiwan, Japan, Indonesia, China, Vietnam
C	adw2, adrq+, adrq-, ayr	East Asia, Taiwan, Korea, China, Japan, Polynesia, Vietnam
D	ayw2, ayw3	Mediterranean area, India
E	ayw4	West Africa
F	adw4q-, adw2, ayw4	Central and South America, Polynesia
G	adw2	France, USA

1.3 Transmission of HBV

Hepatitis B virus is transmitted among people, via "sex, blood and needles" (Hepatitis B Foundation web site, www.hepb.org). Neonatal infection acquired from their infected mothers accounts for most chronic carriers of the virus in the world. Most transmission can be accounted for by person to person contact through parenteral routes (Guidotti *et al*., 1999).

HBV transmission in Pakistan mostly occurs through blood transfusions, parenteral drug use, local barber shops, unprotected sex and therapeutic injection administration by health care proffesionals (Ali et al., 2009; Usman et al., 2003)

1.4 HBV Remedy

Hepatitis B virus infection consists of three components: an infection source, a susceptible host, and an established route of infection. Thus prevention is the more effective way to control hepatitis B, rather than treating those who are already infected. The two major approaches to HBV remedy are interrupting the route of transmission and immunizing the susceptible host. Immunization and public education are two effective tools for the global control of HBV (Kao and Chen, 2002)

Hepatitis B virus treatment is recommended in case of liver damage which becomes evident through inflammation, fibrosis or cirrhosis. Available treatment options include antiviral drugs and interferon therapy. Antiviral drugs for the treatment of HBV infection have made significant advancement during the last decade. Oral nucleoside analogs which work by inhibiting viral replication or by

immunological control of viral replication improve the histology of liver and are effective against hepatocellular cellular carcinoma (Liaw et al., 2004). Nucleoside analogs are viral polymerase inhibitors and require to be administered for a long time to avoid viral relapse (Zoulim, 2004). The first nucleoside analog to be approved by FDA was Lamivudine, other nucleoside analogs include Adefovir, Entacavir, Telbivudine and Tenofovir (Zoulim, 2006).

Since 1992, subcutaneous administration of Interferon-α has become a successful treatment option for HBV infection. It has HBV inhibitory and host immune modulation properties (Perillo, 2009). It demonstrates the antiviral activity by interacting with the cell surface receptors on activation and cau sing viral RNA breakdown by enhanced expression of host cell genes (Rang et al., 2002)

1.5 Immunization options for HBV infections

Efficacious treatment options against HBV are continuously being researched all over the world for reducing the risk of this prevalent virus, but this human intervention is also increasing the mutation rate of the virus, resulting in a further increase in its genetic diversity. And this well-adapted virus continues to persist in our environment despite of all the strategies used for its control . The incidence of HBV infection among infants, children and adolescents has been reduced significantly in many countries through mass immunization programs. There are two major options for immunization against HBV, the active and the passive immunization.

Passive immunization involves the use of sterile ready-made anti hepatitis B antibodies named as Hepatitis B Immune Globulin (HBIG) and is recommended to be used for new borns of HBV infected mothers, needle prick of HBV infected needle, sexual exposure to HBV infected partner and liver transplantation (Hou et al., 2005).

Plasma-derived HBsAg subunit vaccines were the first commercially savailable HBV vaccines which are now largely being replaced by recombinant vaccines (Kane, 1995). A protective efficacy of 90–95% was observed in all these vaccines (Margolis *et al.,* 1995). The World Health Assembly of WHO has recommended that hepatitis B vaccine be incorporated into routine infant and childhood immunisation programmes, on the basis of disease burden and availability of a safe and effective vaccine, for countries with HBV carrier rates greater than 8% by 1995 and for all countries by 1997 (Kane, 1995).

In 1984 one of the most successful HBV immunization programs was started in Taiwan which significantly reduced the prevalence rates (Chen *et al*.,1987; Hsu *et al*., 1988; Ni *et al*., 2001). It not only saved the children from becoming HBV carriers but also protected them from HCC (Chang *et al*., 1997).

In Pakistan, four HBV recombinant vaccines are available- Engerix-B, Euvax-B, Heptis-B, Amvax-B which give >95% seroprotection (Hakim et al.,2009). The most effective HBV vaccines consitute recombinant HBsAg and comprise of the immune-dominant part known as the ‘a’ region which is shared by all the different strains of the virus (Norder *et al.,* 2004).

1.6 HBV Vaccines

HBV vaccine has been incorporated in the routine immunization program recommended by WHO since 1997. But its high cost becomes a major hindrance in addressing HBV in the developing countries (Goldstein and Fiore, 2001). Countries like Pakistan are in urgent need of producing the vaccine indigenously through economical and effective methods. Many expression systems like *Escherichia coli*, plant cells, fungi etc. are already being used for the production of HBV vaccine (Liu *et al.,* 2009; Romanos *et al.,* 1992).

This study was based on the premise that modification of genes through relative synonymous codon usage will lead to its increased expression using *Kluveromyces lactis* expression system. RSCU is a way of achieving high yield economically and is illustrated as the ratio of number of times a codon appears in a given gene with the number of expected occurences under equal codon usage. (Fox and Erill, 2010).

K lactis which is readily isolated from dairy products uses lactose as a carbon source and is an important microbial factory for the production of various useful proteins (Gellissen and Hollenberg, 1997). It also lacks the Crabtree effect (repressed respiration in aerobic condition) which is a characteristic feature of *Saccharomyces cerevisiae* and so allows high biomass production during fermentation (Gonsalez-Siso *et al*., 2009; Mulder *et al.,* 1995). It proves to be a promising host expression system for large scale production of recombinant HBV vaccine in the form of HBsAg as it is easier to grow, comparatively, and the growth conditions can also be easily altered to examine the most favorable ones

(Gellisen and Hollenberg, 1997). . The protein expression can also be enhanced by modifying the growth conditions in order to achieve a high yield at a more economical level (Feng et al., 2010).

Previously synthesized HBsAg expressed in synthetic pKLAC1 vector was used in this study with the following objectives:

- Extracellular expression of HBsAg by *K.lactis*
- Detection, separation and purification of the engineered HBsAg protein by ASP and dialysis
- Analysis of HBsAg through ELISA
- Determinion of the integration sites of the synthetic gene in the vector Pklac1.

REVIEW OF LITERATURE

Hepatitis B virus is a leading cause of liver disease worldwide affecting approximately 350 million people worldwide out of which approximately 1 million die each year. Pakistan is highly endemic with nine million people infected with HBV along with a steady rise in the infection rate due to poor economic status of the country and the lack of proper health facilities (Ali *et al.*, 2011). Currently vaccination and antiviral therapy are serving to reduce the risk of HBV infection and its related liver diseases.

The Hepatitis B infection is caused by the HBV belonging to the family of Hepadnaviridae. Discovered in the 1970's and named as the Australian antigen, the virus exits as Dane particles that are 42-47 nm in size sphere, filamentous or tadpole shaped particles (Dane *et al.*, 1970). Transmitted through the blood and body fluids, the disease is currently being treated using antivirals and interferons (Najafi *et al.*, 2013). Vaccines against HBV are available since 1982 but immunogenicity remains a major side effect with them which has resulted in a quest to develop Recombinant DNA vaccines using different expression systems (Emini *et al.*, 1986; Francis *et al.*, 1986). The advantages of Yeast expression system which favor its use over other expression systems include high efficiency at secreting foreign pharmaceutical proteins, resulting in increased biomass production in addition to comparatively easier and flexible growth conditions and requirements (Gellisen and Hollenberg, 1997).

2.1 Hepatitis B

Hepatitis B virus belonging to hepadnaviridae family of viruses is a small, enveloped, hepatotropic virus approximately 42 nM in diameter and consisting of ~3.2 kb partially double-stranded relaxed, circular DNA (rcDNA) genome within a nucleocapsid (core) which is surrounded by a lipid bilayer studded with complexes of viral glycoproteins (Block *et al.,* 2007; Seeger and Mason, 2000). The nucleocapsid encloses a single copy of viral genomic DNA and DNA polymerase that is covalently linked to the 5' end of minus strand DNA (Wang and Seeger, 1992; Wang and Seeger, 1993) along with cellular proteins, including chaperones and protein kinases (Hu *et al,* 1997; Block *et al.,* 2007).

Discovered in the 1970's and named as the Australian antigen, the virus exits as Dane particles that are 42-47 nm in size sphere, filamentous or tadpole shaped particles (Dane et al., 1970). Figure 2.1 shows the HBV replication cycle.

2.2 HBV Genome

The HBV genome is a partially double-stranded circular DNA of about 3.2kb (Liang, 2009) which encodes four overlapping open reading frames: S for the surface or envelope gene, C for the core gene, P for the polymerase gene, and X for the X gene (Robinson *et al.,* 1977; Lee *et al.,* 1997; Kao and Chen, 2000)

The S gene encodes the viral envelope and contains three distinct components, large, middle, and major (or small) proteins which are synthesized by beginning transcription with pre-S1, pre-S2, or S gene alone, respectively.

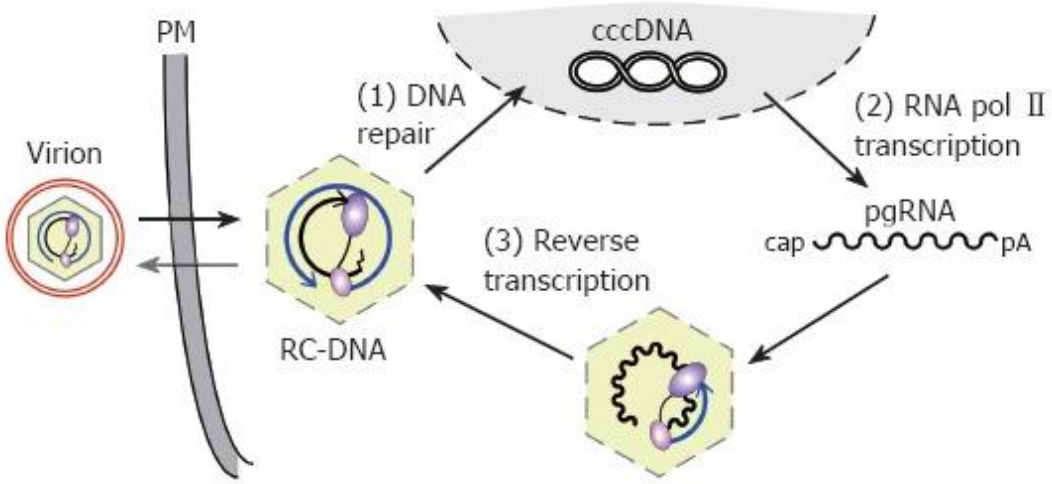

Figure 2.1: HBV Replication cycle; (1) Enveloped virions infect the cell, releasing RC-DNA containing nucleocapsids into the cytoplasm which is transported to the nucleus, and repaired to form cccDNA. (2) pgRNA is produced by the transcription of cccDNA by RNA polymerase II. (2) pgRNA is encapsidated, together with P protein, and reverse transcribed inside the nucleocapsid. (+)-DNA synthesis from the (-)-DNA template generates new RC-DNA. New cycles lead to intracellular cccDNA amplification; alternatively, the RC-DNA containing nucleocapsids are enveloped and released as virions. (Kao and Chen, 2000)

The core gene encodes for the Hepatitis B core antigen which is the nucleocapsid enclosing viral DNA and in case of an immune response elicited by the expression of peptides derived from HBcAg as well as envelope and polymerase proteins on the surface of liver cells, (Chisari, 2000; Jung and Pape, 2002) Hepatitis B e antigen (HBeAg) is secreted. It is a circulating peptide derived from the core gene, and serves as a marker of active viral replication.

DNA polymerase is encoded by the long P gene which also performs the reverse transcription function as the viral replication requires RNA intermediates. Two proteins, are encoded by the X gene, that have transactivation activities on HBV enhancer in aiding viral replication and may also play a part in hepatocarcinogenesis by transcativating other genes. Several enhancers and promoters are identified within the whole HBV genome in addition to these (Kao and Chen, 2002). Figure 2.2 shows the organization of the HBV genome.

2.3 Epidemiology of HBV

Different parts of the world have different prevalence rates of HBV infection. Areas are categorized into low, intermediate and high endemicity on the basis of age at the time of HBV infection (Hou *et al.*, 2005). More than 75% of the world's estimated 350 million carriers are located in Western Pacific and South East Asia. Hepatitis B is hyperendemic in most countries of this region, with carrier rates ranging from 5%-35%. Major differences in the pattern of infection have been reported within countries, towns, villages, and families (Gust *et al*, 1996)

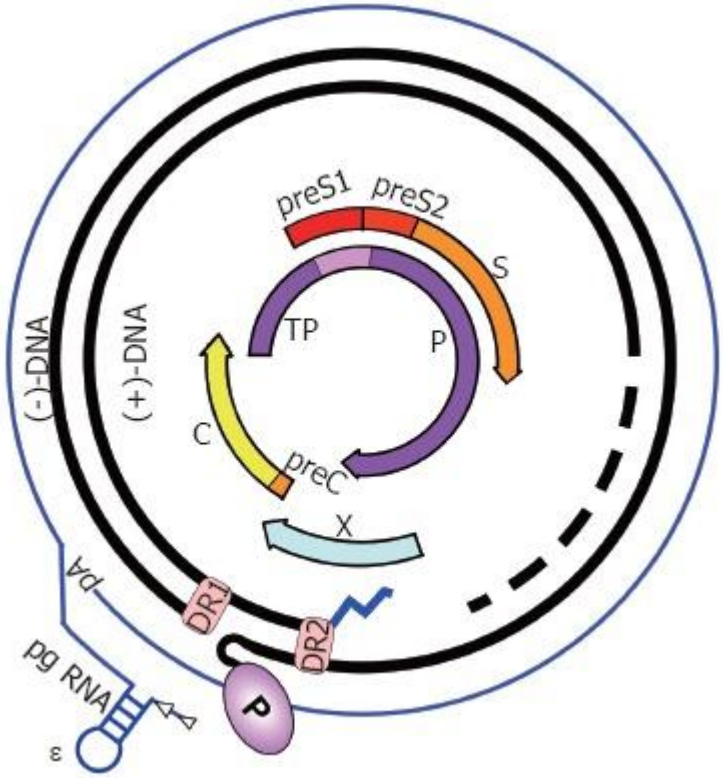

Figure 2.2: HBV genome organization. The thick black lines indicate partially double-stranded, circular RC-DNA, with P covalently linked to the 5´ end of the (-)-DNA, and the RNA primer (zigzag line) at the 5´ end of (+)-DNA. The heterogeneous lengths of the (+)-strands are shown by the dashed part. DR1 and DR2 are the direct repeats. The outer circle symbolizes the terminally redundant pgRNA with ε close to the 5´ end, and the poly-A tail at the 3´ end. The precore mRNA starts slightly upstream. The relative positions of the open reading frames for core (C), P, preS/S, and X are shown inside (Beck and Nassal, 2007).

Pakistan has a carrier rate of 3-5%, with estimated 7-9 million carriers of HBV especially concentrated in areas like Southern Punjab, Interior Sindh, District Tatta, Kurrum agency, Baltistan and some areas of Lahore. High endemicity and steady rise in infection rate can be attributed to lack of proper health facilities, poor economical status and less public awareness about the transmission of major communicable diseases (Ali *et al*, 2011)

Spontaneous error of the viral reverse transcriptase causes the evolution of HBV with an estimated nucleotide substitution rate 1.4 to 3.2 * 10-5/site per year which has resulted in four (adw, ayw, adr and ayr) major serological subtypes and eight (A-H) genotypes of HBV. (Okamoto *et al.,* 1987) These serotypes and genotypes have diverse geographical distribution (Kao and Chen, 2002).

2.4 Transmission of HBV

HBV is transmitted by the exposure of percutaneous and mucous membrane to infectious blood and body fluids containing blood (Alter, 2003). Currently, there are three recognized modes of HBV transmission: perinatal, sexual and parenteral/percutaneous transmission. Perinatal transmission of HBV from infected mothers to infants occurs through transplacental transmission in utero; natal transmission, postnatal transmission or through breast milk.

In low endemic areas of the world like North America, sexual transmission is a major source of infection with heterosexual transmission accounting for an increasing proportion. Parenteral/percutaneous transmission through HBV contaminated blood and blood products can occur with contaminated surgical

instruments and utensils during surgery, after needle-stick injuries, intravenous drug use, and following procedures such as ear piercing, tattooing, acupuncture, circumcision and scarification as well as nosocomial spread of HBV infection particularly in dialysis unit in the hospital (Hou *et al.,* 2005). There is a low risk of chronicity (less than 5%) for transmission through sexual contact, acupuncture, intravenous drug use, and transfusion (Hyams, 1995)

2.6 Treatment of HBV

Before the initiation of treatment, HBeAg status determination and a quantitative estimation of HBV DNA level in serum is recommended (Abbas *et al.,* 2009). There are two main classes of drugs aimed at the treatment of HBV, including the Antivirals which suppress HBV by interfering with the viral replication, and the immune modulators which aid the immune system against the virus (HBV WHO). In Pakistan the most commonly available drugs for the treatment of Hepatitis B are interferons and some nucleotide and nucleoside analogs like lamivudine, adefovir, entecavir, telbivudine, pegylated interferon and thymosin (Abbas *et al.,* 2009)

Currently interferons are being used for the treatment of chronic Hepatitis B as they are known to display a range of properties including antiviral, immunomodulatory as well as antiproliferative (WHO Fact sheet no. 204). The interferons are also known to activate the host pathways for enhanced expression of viral RNA chopping proteins (Rang *et al.,* 2002). The approved interferons include interferon alpha 2a and 2b. Peg-interferon is the pegylated form of interferon which has proved to be more effective in HBV treatment (Cooksley *et*

al., 2003; Janssen *et al.,* 2005). The interferon therapy is known to significantly reduce the HCC rate in patients with HBV related cirrhosis. However in high doses these may cause effects like fatigue, malaise, fever, and decrease in white blood cells and platelet count.

Antivirals like Lamivudine have strong inhibitory effects HBV polymerase and so HBV replication and are also well tolerated but the effects are reversed when the therapy is stopped (WHO Fact sheet no. 204). Several new agents like Ritonavir, Adefovir, famvir etc. are under development with promising data.

2.7 HBV Immunization

An effective vaccine is available against HBV for almost 20 years and attempts are being made by developed countries to attain universal vaccination (Parkin *et al.,* 1999) HBV vaccination induces protection in 95% of the recipients (WHO Fact sheet no. 204). Immunization against HBV is attained either by active or passive immunization methods. Passive immunization involves the injection of sterile anti hepatitis B antibodies called Hepatitis B immune Globulin (HBIG) (Hou *et al.,* 2005). However active immunization is a better way of prevention of HBV as it decreases the susceptibility of the host to the infection.

Since 1982, plasma derived vaccines are available which were later succeeded by recombinant yeast derived vaccines in 1986 due to their enhanced effectiveness (Emini *et al.,* 1986; Francis *et al.,* 1986). Currently recombinant HBV vaccines have replaced the other alternatives (Kao and Chen, 2002). These consist of purified subviral particles produced from yeast expressing recombinant

genes and confer long term immunity with a series of three injections (Xu *et al*,. 1985)

2.8 Production of HBV vaccines

HBV vaccine is the only approved vaccine against a major human cancer and is very effective against the transmission of HBV. Vaccines comprise of HBsAg and are either plasma derived or recombinant DNA in nature (WHO). The plasma derived vaccines Heptavax B and Hevac B were first manufactured and licensed in USA and France in 1981-82 (Liang, 2009; Robinson, 1994). The safety record of the plasma derived vaccines is impressive as more than 200 million vaccine doses have been distributed worldwide and only no major side effects except discomfort at the injection site in some people (Hollinger and Liang, 2001).

However in the 1980's genetically engineered vaccines replaced the plasma derived vaccines available on the market due to the possibility of manufacturing the vaccine in a shorter time period. Availability of new technologies, batch to batch consistency and continuous supply of material aided to the manufacturing of the vaccine in 12 weeks instead of 65 weeks (Hollinger and Liang, 2001; Robinson, 1994).

Recombinant DNA technology was used to synthesise the vaccine by expressing HBsAg using yeast (Saccharomyces cerevisiae, Kluveromyces lactis, Pichia pastoris) or mammalian (Chinese hamster ovary, CHO) cells into which the HBsAg gene has already been inserted using plasmids. The resulting transformed cells were then grown in large vessels causing the HBsAg to self-assemble into

spherical shaped immunogenic particles, exposing the highly immunogenic 'a' antigen. Then extensive purification steps were carried out to remove any cellular components followed by the addition of alum as an adjuvant. Studies reveal that the amount of HBsAg required per dose of the vaccine to induce a protective immune response may vary from 2.5µg to 40µg per adult dose depending on the different vaccine products.

However, due to the cost of these vaccines, they cannot be used much commonly in the developing countries like Pakistan resulting in a rapid increase in the incidence rate of HBV, and so the concern for such a prevention and treatment option, that is not only highly effective but also economical, is also rising. Different expression systems are hence being used, including bacterial, mammalian and plant cells, to aid in the production of the recombinant HBV vaccine (Romanos *et al.,* 1992).

2.9 Hepatitis B Surface Antigen as a vaccine candidate

Hepatitis B surface Antigen (HBsAg) is a lipoprotein found within the viral envelope, it is 22nm long and the smallest surface protein of the hepatitis B virus. The HBsAg particles can significantly differ in morphology and are abundantly produced during the early acute infection and continue to be produced in chronic disease (WHO, 2004). The particles consist primarily of a glycoprotein with 226 amino acids that carry the B-cell epitopes required for the induction of protective antibodies in humans and which confer immunity against hepatitis B infection (Hudu *et al.,* 2013).

Two adjacent regions of the HBV S open reading frame (S ORF), the pre-S and the S region encode the HBsAg proteins. The major protein constituent of the HBV envelope is the S protein comprising of 226 aa which is encoded by the S gene, the pre-S2 portion of the pre-S region encodes for the 'middle' gene consisting of 55 aa at the N-terminus and the whole ORF (pre-S1, pre-S2 and S, 389aa) encodes for a 'large' protein (Stibbe *et al*., 1983; Heermann *et al*., 1984; Neurath *et al.,* 1986). The density of epitopes of antigen determines the affinity between polyclonal antibody and multivalence antigen and the S proteins are known to have the highest density of epitopes against HBsAg among the three HBsAg molecules. (Neurath *et al.,* 1986; Milich *et al.,* 1985; Budkowska *et al*., 1985). S protein also has an advantage as a diagnostic antigen because of its higher reactivity to polyclonal HBsAb (Bo *et al.,* 2005)

HBsAg, approved in 1986 by the Federal Drug Administration (USA) became the the first recombinant protein-based vaccine, for human vaccination. Since the initial attempts to use *E. coli* based expression systems did not result in the formation of immunoprotective material while the mammalian based expression systems were too expensive for vaccine production, the yeast *Saccharomayces cerevisiae* was employed for commercial production of HBsAg from the viral gene encoding the 226 amino acids protein. The vaccines derived from the human plasma and recombinant mammalian cell are glycosylated (Stephenne, 1988; Kim *et al.,* 2009) while the HBsAg derived from yeast cells is not glycosylated neither from *S. cerevisiae* nor from the methylotrophic yeasts (Mcaleer *et al.,* 1984).

2.9 *Kluveromyces lactis* Expression system

The yeast *K. lactis* is widely studied for decades and is known to be safe for use in various food industry applications (Bonekamp & Oosterom, 1994). In the 1980s, a transformation system was established for *K. lactis* making it one of the first yeasts used for transformation (Das & Hollenberg, 1982), and leading to its development as an efficient host to be used for heterologous protein expression. *K. lactis*, as an expression host, is best known for its use in commercial production of the milk clotting enzyme bovine chymosin (Van den Berg *et al.,* 1990). It has also successfully been used to produce both secreted and intracellular enzymes on an industrial scale.

The absence of biohazard in the host organism is confirmed by the approval of *K. lactis*-derived proteins in the food industry, which is an important aspect, for the development of *K. lactis*-based oral vaccines (Krijger *et al.,* 2012).

One of the disadvantages of the use of *K. lactis* (and other yeast and fungal expression systems) in the production of therapeutics is that a non-human glycosylation pattern will be borne by the secreted proteins that become glycosylated which can adversely affect the half-life, tissue distribution and immunogenicity of a therapeutic protein. However, advancements in the area of humanizing yeast protein glycosylation pathways through the expression of human glycosylation enzymes in yeast (Gerngross, 2004) may prove to be promising for yeast therapeutic protein production, and may be applicable to *K. lactis* (Van Ooyen, 2006).

2.10 Protein concentration:

Proteins after being expressed in expression system need to be precipitated out in order to concentrate and purify the desired protein. In protein precipitation methods the proteins are converted into insoluble forms. Different precipitation techniques makes the protein insoluble by changing its charge, surface and concentration of solvent (Asenjo, 1990).

Purification methods depend on the physical and chemical properties of protein. Separation techniques may be based on solubility, density or molecular size of the protein (Nison, 2012) There are different methods that are used for protein precipitation including temperature, PH ionic precipitation e.g. Ammonium sulphate, sodium chloride, nonionic polymers (PEG), Metal ions (cu^{+2}, Zn^{+2}, $Fe^{+3)}$,and ligand-antibody interaction (Harrison, 1993). In this study the techniques of ASP and dialysis are used.

2.10.1 Ammonium Sulphate precipitation:

Ionic precipitation is a preferable method because it separates out protein without denaturing them. It separates the hydrophilic proteins on the basis of their solubility which depends on its charge an hydrogen bonding with the water molecule. At isoelectric pH net charge of a protein is zero, so the protein molecule is easy to aggregate and then precipitate. However if the pI is not known, salt precipitation can be employed which involves the use of high concentration of salt to precipitate protein by destroying hydrogen bond between protein and water molecule. Ammonium sulphate is used due to its high ionic strength and by its gradual addition proteins can be separated into a number of fractions (Nison,

2012). There are also several benefits of using ammonium sulphate precipitation including low heat of solution i.e. no heat is required, moreover it does not provide any intrusion in sedimentation of precipitated proteins (Deutscher, 1990).

2.10.2 Dialysis

Dialysis is a widely used process due to its simplicity, but it can be time consuming because the separation depends on diffusion. The techniques involves placing the sample inside a dialysis bag prepared from a tube made of semipermeable membrane. Most of the commercially available dialysis tubes, allow only small molecules whose sizes are less than 10 kDa to be removed from the sample to the surrounding medium. So this technique is used to remove salts from the protein solution hence also causing them to get concentrated (Nison, 2012)

MATERIALS AND METHODS

3.1 Revival of yeast cells:

Kluveromyces lactis cells were cloned with HBsAg gene. The se cells were obtained in vials of yeast medium and revived.

3.2 Yeast media preparation:

For the revival of already transformed cells, 1L of yeast culture medium, YP-Glu was prepared by dissolving 10 g yeast extract and 20 g peptone in 950 mL of autoclaved water. The media mixture was then autoclaved at 121°C, followed by the addition of sterile 40% glucose. The 40% glucose stock solution was prepared by dissolving 40g of glucose in 125mL of deionized water. The mixture was warmed to 50°C in order to aid dissolution and then later dispensed into different aliquots following syringe filteration.

3.3 Culturing of Yeast cells:

Culture tubes containing the transformed cells were obtained and vortexed for 15-30 seconds to allow the pellet to dissolve completely. Then 500µL of yeast culture was added in three of the four culture tubes already containing 3mL of yeast media. The fourth tube was kept as a control.

All the steps were carried out in laminar air flow cabinet in order to eliminate any chance of contamination. These culture tubes were then placed in the shaking incubator at 240 rpm and 30^0C for 96 hours.

3.4 Separating the protein from the culture:

The culture was harvested after 96 hours and was centrifuged at 4000rpm for 15minutes. Centrifugation was done in refrigerated centrifuge at 4^0C. The supernatant was carefully drawn into the new culture tubes and the pellet was discarded. For future use of the culture glycerol stocks were prepared by adding 200mL of glycerol in to the sample and it was than stored at -80 ^{0}C.

3.5 Ammonium Sulphate Precipitation (ASP):

Ammonium sulphate was used for ionic precipitation of protein which was carried out by the following methods:

3.5.1 Direct precipitation method:

For direct precipitation, 1mL of supernatant having protein was taken, and different quanitities of Ammonium sulphate were added to it according to the required saturation. Ammonium sulphate was added in small portions in microfuge tubes containing the culture and the tubes were vortexed to dissolve the AS. After complete dissolution sample was centrifuged in a refrigerated centrifuge at 14000rpm and 4^0C in order to maintain the temperature necessary for the stability of the protein.

The concentrations of Ammonium sulphate used for making different saturations are listed below in the table 3.1.

Table 3.1: Concentrations of Ammonium sulpahte for direct precipitation method.

Sr.NO.	% of AS saturation	Amount of AS in g
1.	30%	0.176g
2.	40%	0.243g
3.	50%	0.313g
4.	60%	0.390g
5.	70%	0.472g
6.	80%	0.56g

3.5.2 Serial precipitation method:

1n serial precipitation method, 10ml culture was taken initially and the calculated amount of Ammonium sulphate required for 10% ASP saturation was added to it. Amounts of Ammonium sulpahte required for each saturation was calculated from ENcor biotechnology Inc (http://www.encorbio.com/protocols/AM-SO4.htm). For next precipitation the amount of culture reduced by 1ml from the previous one was used. Same steps of vortexing and centrifugation were repeated for each concentration. Concentrations that were used in serial precipitations are shown in the table 3.2 below:

Table 3.2: Concentrations of ASP for serial precipitation method.

Sr.No.	Desired % saturation of ammonium Sulphate	volume of sample in mLs	Amount of Ammonium Sulphate in g
1.	10%	10mL	0.53g
2.	20%	9mL	0.49g
3.	30%	8mL	0.45g
4.	40%	7mL	0.40g
5.	50%	6mL	0.36g
6.	60%	5mL	0.31g
7.	70%	4mL	0.25g
8.	80%	3mL	0.20g
9.	90%	2mL	0.14g
10.	100%	1mL	0.07g

3.6 Protein purification by Dialysis method

Dialysis is a separation technique that works by diffusion, a process that results from the thermal, random movement of molecules in solution and leads to the net movement from areas of higher to lower concentration (until an equilibrium is reached). For dialysis, the dialyzing membrane used retained protein >12000 Da. First the membrane was prepared by wetting it with water and then the sample was loaded on to it and dialyzed for 1 to 2 hours at room temperature. Then the dialysis buffer was changed and dialysis was carried on for another 1 to 2 hours. The dialysis buffer is then changed again and dialysis was performed overnight at 4°C.

3.7 Analysis of HBsAg through ELISA

HBsAg Enzyme linked immunosorbent assay (ELISA) detection kit (Kehua Bioengineering, China) was used to screen and quantify the presence of HBsAg secreted by recombinant *K.lactis.* ELISA is basically an immunoassay based on sandwich principle. The ELISA kit consists of the antibody, specific for the analyte. This antibody is immobilised onto the solid phase, 96-well polystyrene microtitre plate wells. The stored centrifuged samples were syringe filtered in order to remove any cellular debris that may give false positive results. Each of these samples were pipetted into the coated wells. A total of 22 different samples were used. Apart from this, 75µL of negative control was pipetted into 2 wells and 50µL of positive control in another 3 wells. The very first well was used as a control and was left empty. The positive control contains HBsAg whereas the negative control is prepared from human blood found to be negative when tested for HBsAg.

Following the addition of all the samples into their respective wells, the ELISA plate was covered with a plate sealer and incubated for 1hour at 37ºC. Upon completion of the incubation period, 50 µL of the conjugate solution which is supplied along with the kit was added into each well excluding the empty well and further incubation of 30 minutes was followed at 37ºC again. Meanwhile, the concentrated washing solution was diluted to 1:25 using distilled water. The wells were then washed by adding 200µL of washing solution into each well and then inverting the plate onto a blot paper. This step was repeated 5 times in order to remove any unbound material.

A substrate solution was then added to produce a color change. The degree of color change is directly proportional to the amount of HBsAg present within the sample. The plate was then covered and incubated for another 15 minutes before the addition of stopping solution and mixing completely. To avoid contamination, care was taken not to touch the edges of the wells with the pipette tips when adding sample, conjugate or substrate. Microplate reader was used to read the absorbance of the solution in the wells at 450nm and 630nm. The corresponding results were thus recorded.

In order to get more accurate results, the ELISA procedure was repeated but this time vaccine injection, Engerix-B, was also included and used as a positive control. Serum diluent was added into the vaccine in order to dilute it 10-folds. This was pipetted into further 6 vials and subsequently added in to the wells. The picture below is used to show the sequence of the controls and samples which were added to the ELISA plate.

3.8. Bradford Assay

3.8.1 Preparation of Bradford Reagent

100 mg of Coomassie brilliant blue G-250 was dissolved in 50 ml of 95% ethanol. Then 100 ml of 85% phosphoric acid was added and final volume was made up to 1L with distilled water. The solution was then filtered twice with Whattman filter paper no.1 and stored at room temperature in amber coloured bottle.

3.8.2 Assay

2 mL of the Bradford reagent was added to 100 microliter of the lysate and incubated at room temperature for 10 minutes. Then the optical density was taken at 595 nm. Blank was adjusted by 2 mL of Bradford reagent having 100 microliter of NF water.

The protein concentration was estimated by drawing a standard curve with the help of different concentrations of bovine serum albumin treated with Bradford reagent and the optical density taken at 595 nm after incubating at room temperature for 10 minutes.

3.9 Determination of integration sites

3.9.1 DNA Extraction from the yeast cells

The protocol described by Harju et al, 2004 was used to carry out the process of DNA Extraction from yeast cells.

Firstly, 1.5mL of liquid culture of yeast grown for 96 h at 30°C in YPD was transferred into a microcentrifuge tube and the cells were pelleted by centrifugation

at 20,000 × g for 1-5 minutes. Then 200 µL of Harju- buffer prepared by mixing 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA was added. Then tubes were then immersed in a dry ice-ethanol bath for 2 minutes before being transferred to a 95°C water bath for 1 minute.

The last two steps were repeated followed by vortexing for 30 seconds. Then 200 µL of chloroform was added and vortexing was again done for 2 minutes. The tubes were then centrifuged for 3 minutes at room temperature, 20,000 × g.

The upper aqueous phase was transferred to a microcentrifuge tube containing 400 µl ice-cold 100% ethanol and then mixed by inversion or gentle vortexing. Then they were incubated at room temperature for 5 minutes. Alternatively DNA may be precipitated at -20°C to increase yield. Centrifugation was then carried out for 5 minutes at room temperature, 20,000 × g followed by the removal of the supernatant with a pulled Pasteur pipette by vacuum aspiration.

The pellet acquired was washed with 0.5 mL 70% ethanol and centrifuged for 5 minutes at room temperature, 20,000 × g. The supernatant was removed and the pellets were air dryed at room temperature or for 5 minutes at 60°C in a vacuum dryer before being resuspended in 25- 50 µL TE (pH 8.0) or water.

3.9.2 Analysis of DNA through Agarose Gel Electrophoresis

After preparing the 0.8% agarose gel in 1XTris Acetate buffer (TAE), it was run in the buffer composition in order to analyze the integrity of genomic DNA that was isolated from the recombinant yeast cells. 10X TAE buffer, having the pH 8.3, was prepared as a stock solution. This stock solution was prepared through the addition of 20 mL 0.5M EDTA, 48.5g Tris and 11.4 mL of glacial

acetic acid in 500 mL of distilled water. Then 1 L distilled water was used to adjust the volume of the solution. By diluting the stock solution with distilled water by 1:10, the working solution of TAE was made ready for the gel preparation. The agarose was dissolved by heating in microwave oven after adding 0.4 g agarose with 40 mL of 1X TAE for making the gel. After cooling the gel mixture to ~60 °C, stain gel was added to 5 µL of ethidium bromide (10mg/mL).1X loading dye, along with the 2 µL of DNA obtained from each sample - was run at a constant current of 60 mA for thirty to forty minutes in order to analyze. This gel was visualized under the ultra-violet light and photographed by gel documentation system, (Wealtec, Sparks, USA).

3.9.3 PCR Amplification for determination of integration sites

Multiple integrated cells are identified using PCR with Integration Primers 2 and 3. DNA extracted from yeast cells was used as template in a PCR reaction containing Integration Primer 2 and Integration Primer 3. A total reaction volume of 50 µL is recommended. Reaction mixtures containing cells were mixed by vortexing. Reactions were incubated at 95°C for 10 minutes, then for 2 minutes at 80°C when Taq DNA Polymerase (NEB #M0267 or NEB #M0273) should be added. Thermocycling is then performed consisting of 30 rounds of successive incubations at 95°C for 55 seconds, 50°C for 55 seconds, and 72°C for 1 min and 30 seconds, then a final incubation at 72°C for 10 minutes. 10µL of each amplification reaction is analysed on a 1% agarose gel. Cells harboring multiple tandem integrations of the expression fragment at the LAC4 locus in the *K. lactis* genome will result in amplification of a 2.3 kb product. The sequence of the intergration primers used are provided in the table 3.3.

Table 3.3: Sequence of Integration Primers used

SR. NO.	PRIMER CODE	SEQUENCE	Tm
1.	INT-1	TACCGACGTATATCAAGCCCA	60.6
2.	INT-2	ATCATCCTTGTCAGCGAAAGC	60.6
3.	INT-3	CAGTGATTACATGCATATTGT	54.8

3.9.4 Analysis of PCR Product by Gel Electrophoresis

In 1X TAE, 1% agarose gel was prepared and was run in the same buffer composition. In 40 mL of 1X TAE, 0.4g agarose was dissolved and was heated in the microwave oven. This mixture of gel was then cooled to ~60 °C. To stain the gel, 5 µL of ethidium bromide (0.5 µg/ml) was added upon cooling. Along with 1Z loading dye, 5 µL of PCR product was run on the gel in order to analyze. Afterwards, for almost 30-40 minutes, the gel was run at constant current of 60 mA and was visualized under ultra-violet light and photographed by gel documentation system. The PCR product was stored at -20 °C for further use.

RESULTS

4.1 Detection of Recombinant HBsAg

The HBsAg expressed by *K.lactis* was detected using ELISA (Enzyme linked immunosorbent assay) detection kit (Kehua Bioengineering, China).

Anti-HBs---HBsAg--- HRP complex was formed upon addition of the conjugate solution. Color development was observed for positive and negative results after the addition of 50µl of stopping solution. The blue color developed by the addition of enzyme substrate was turned to yellow after the addition of stopping solution whereas the wells containing samples negative for HBsAg remain almost colorless or develop only a low background color. Figure 4.1 shows the results of the

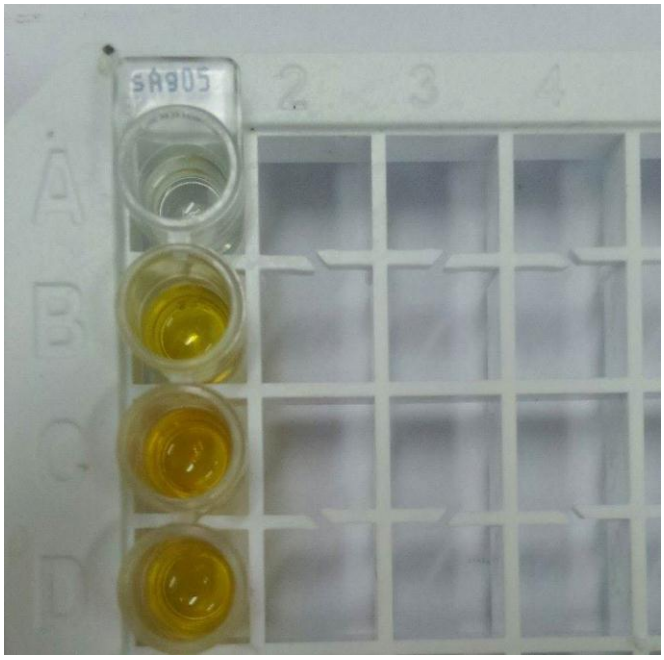

Figure 4.1: Sandwich Elisa of the secreted HBsAg. Well 1A contains negative control, well 1B contains positive control, Well 1C contains the dialysed sample while 1D contains dialysed sample subjected to ASP.

sandwich ELISA performed on dialysed sample and the sample subjected to dialysis and ASP. While Figure 4.2 shows the results of sandwich ELISA performed on samples purified using different concentrations of Ammonium sulphate when subjected to direct as well as serial method of precipitation. Maximum purification is attained at 30% ammonium sulphate concentration as maximum absorbance is recorded at this concentration.

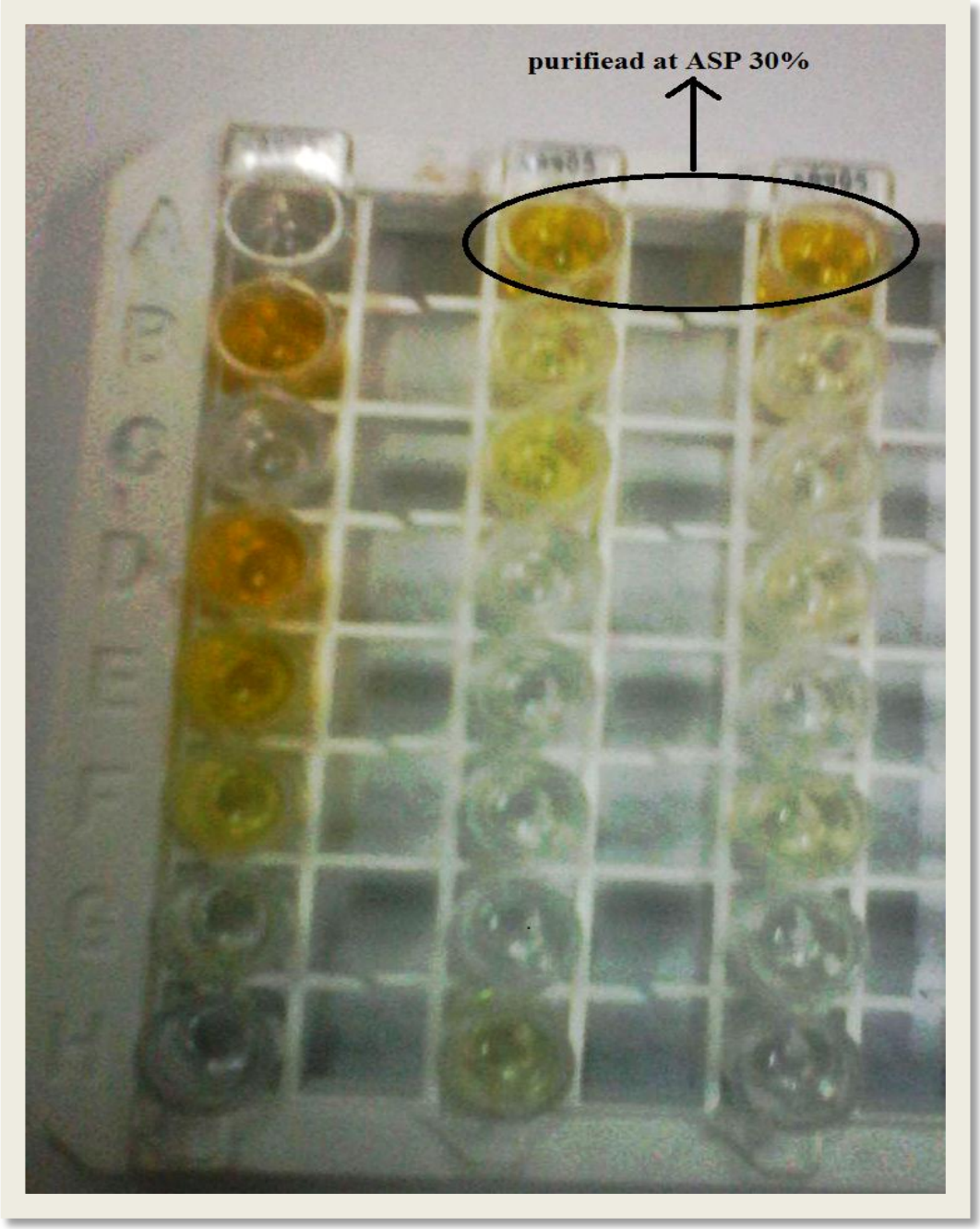

Figure 4.2: Sandwich ELISA for detection and quantification of Secreted HBsAg**.** Well 1A contains negative control, well 1B contains positive control, 1c contained cell culture expressing protein, and next five wells contain vaccine dilutions that were diluted five folds. Well 2B to 2F contained purified protein that was purified by 30% to 80% by direct Asp method. From 2F to 3H contain serial purified product of Asp.

Absorbance chart was obtained from multiple reader was analyzed in order to get an approximate concentration of protein purified by HBsAg. Following absorbance was obtained when sample were analyzed at 630nm wavelength. Table 4.1 shows the absorbance values of the vaccine dilutions

Table 4.1: Absorbance of vaccine dilutions used as standard

Sr.no.	**Sample**	**Absorbance**
1.	Negative	-.0003
2.	Positive	1.2
3.	Culture having protein	0.006
4.	Vaccine first fivefold dilution	2.52
5.	Vaccine first serial dilution	2.47
6.	Second serial dilution	1.98
7.	Third serial dilution	1.00
8.	Fourth serial dilution	0.213

Table 4.2 : Absorbance of samples concentrated with different percentages of Amonium sulphate using serial method

Sr.no.	Sample	Absorbance
1.	10%AS	0.002
2.	20%AS	0.177
3.	30%AS	1.700
4.	40%AS	0.104
5.	50%AS	0.052
6.	60%AS	0.102
7.	70%AS	0.008
8.	80%AS	0.086
9.	90%AS	0.008
10.	100%AS	0.013

Table 4.3: Absorbance of samples concentrated with different percentages of Amonium sulphate using direct method

Sr.no.	Sample	Absorbance
1	30%AS	1.661
2	40%AS	0.224
3	50%AS	0.430
4	60%AS	0.004
5	70%AS	0.010
6	80%AS	0.014

Table4.4: Absorbance of sample concentrated by dialysis

S.No	Sample	Absorbance
1	Dialysis	1.23

Table4.5: Absorbance of sample concentrated by ASP and Dialysis

S.No	Sample	Absorbance
1	ASP and Dialysis	2.259

Interpretation of Results:

ASP was most effective at the concentration of 30%, as maximum absorbance is observed at this concentration through ELISA reader under 630nm and 470 nm. The results are interpreted in the following graphical form shown in Figure 4.3 according to which ~0.16µg/ml-0.18µg/mL was obtained by this method.

Dialysis was comparatively less effective as the absorbance value acquired with it was 1.23 and graphical concentration estimation revealed a concentration of 0.064 µg/mL.

However ASP followed by dialysis proved to be the most effective technique as an absorbance value of 2.259 was acquired with it. And according to graphical estimation, it produced a concentration of HBsAg of around 0.8 µg/mL

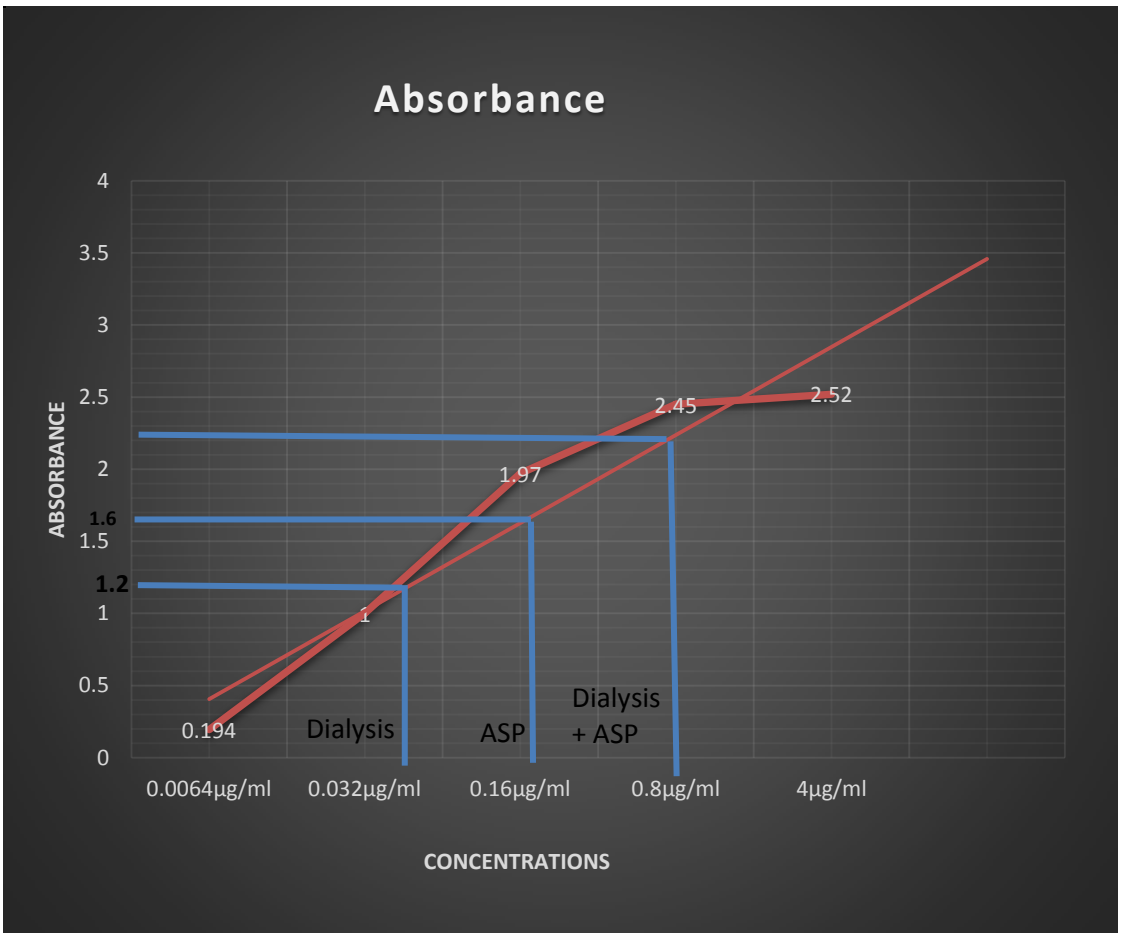

Figure 4.3: Graph depicting the absorbance values attained by ELISA of samples subjected to ASP, Dialysis and Dialysis + ASP

Absorbance of fivefold diluted vaccine at 630nmwas plotted against concentration in order to estimate the amount of HBsAg.

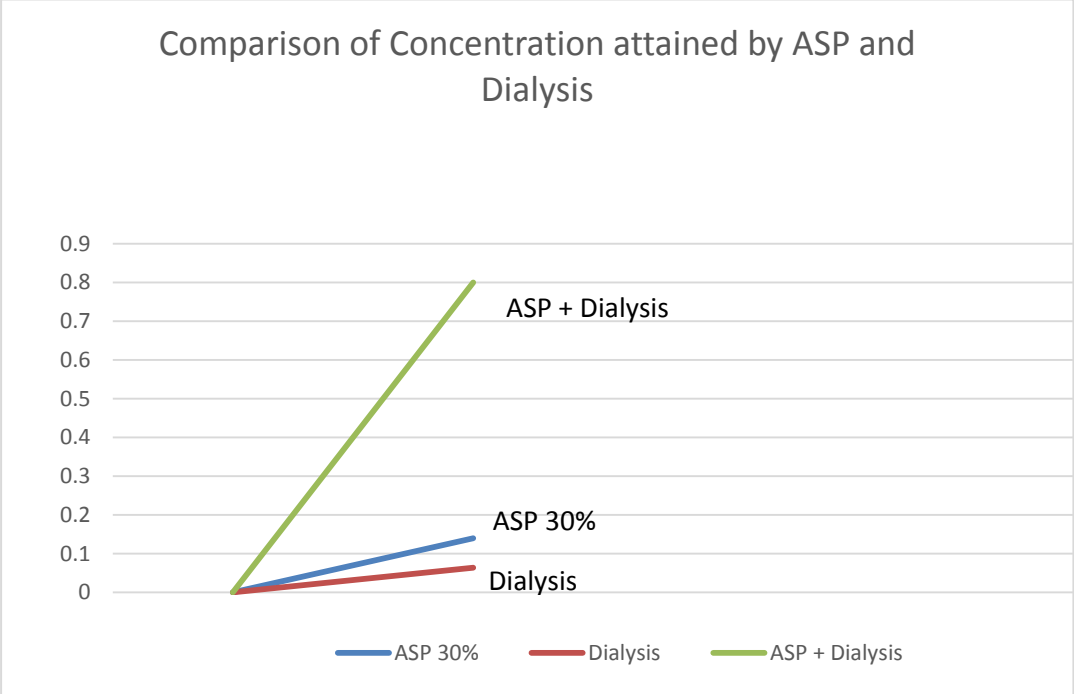

Figure 4.4: Graph comparing the concentration of HBsAg attained by ASP, Dialysis and ASP + Dialysis

4.2 PROTEIN PROFILING

4.4.1 Protein Quantification

Protein concentration of each sample was determined by plotting the absorbance value of the colored reaction product on the standard curve. The intensity of the colored product is directly proportional to the protein content of the sample. The figure 4.5 shows a Bradford standard curve plotted for five standard values.

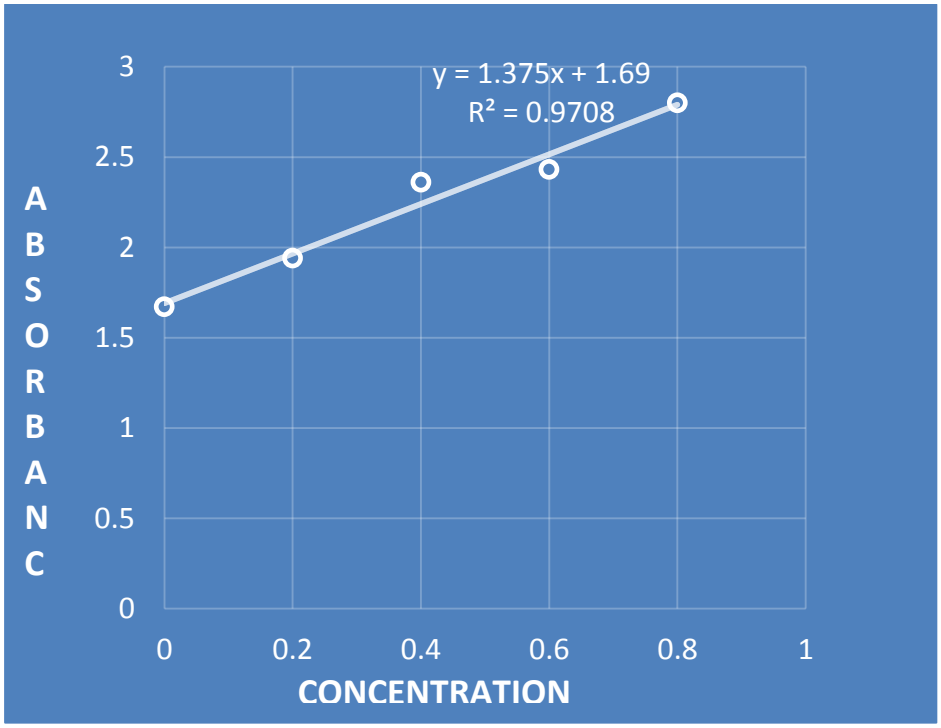

Figure 4.5: Bradford standard curve plotted for five standard values. Concentration was plotted on the x-axis (independent variable). Absorbance measured at 595 nm was plotted on the y-axis (dependent variable). This graph represents linear regression for the five standard points. The obtained linear regression value was 0.995 (R^2=0.9708).

4.3 Determination of Integration sites

PCR Amplification of DNA extracted from yeast cells

Appearance of bands adjacent to 2.3 Kb size on the ladder as shown in Figure 4.5 shows that multiple copies (up to 10) of the expression cassette are tandomly inserted into the genome during transformation of *K. lactis* cells.

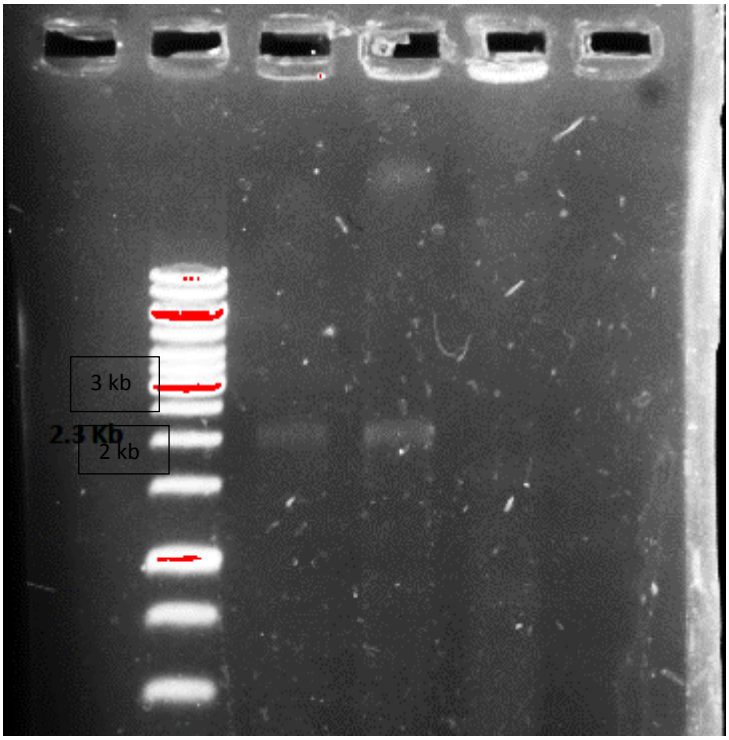

Figure 2.5: Agarose gel showing bands adjacent to 2.3 kb size on the ladder

Chapter 5

DISCUSSION

Development of the recombinant vaccine against HBV began with the designing of synonymous HbsAg gene and its sub cloning in Pklac vector for expression in *K. lactis* expression system. The HbsAg produced has been subjected to various analysis and concentration techniques so as to enhance the quality of the product.

Several methods are available for precipitation of proteins including PH, temperature, nonionic polymers (PEG), Metal ions (cu^{+2}, Zn^{+2}, $Fe^{+3)}$, and ligand – antibody interaction(Harrison, 1993). However, two concentration techniques that were used include Ammonium sulphate precipitation and Dialysis. The reason for selection of ASP over other methods was that it precipitates out the protein without disrupting the structure of the protein and the method is relatively simple and can be applied in a routine manner for the analysis of large numbers of plasma samples. Through AS proteins are precipitated out at different saturations levels due to change in their solubility. In one of the studies on ammonium Sulphate precipitation, the method was performed to precipitate recombinant adenovirus from culture medium in order to yield maximum virus titer from culture and it was precipitated out at 40% saturation of AS (Schagen et al., 2000). In another study conducted by Lei Jiang and colleagues for purification of sample prior to proteomic analysis they checked for different precipitation methods on plasma protein and maximum purification was obtained by fractional/serial precipitation

by ammonium sulphate in which ammonium sulphate was added serially (Jiang et al., 2004). In our current study we have done Asp by both serial and direct method and in both methods maximum amount of HBsAg was obtained at 30% saturation of AS. It suggests that at 30% saturation AS precipitate out HBsAg that is 24KD protein.

Dialysis is also a widely used method due to its simplicity, but it is time consuming because the separation depends on diffusion. This technique is normally used to remove salts from the solution of protein and to concentrate protein solution. The sigma dialyzing tubes that were used that retained protein > 12,000 Da. After dialysis, an ELISA analysis of the dialysed product and comparison with ASP product revealed that it was less effective than ASP for concentration of the protein.

A combination of ASP followed by dialysis produced the maximum concentration which was quantified using ELISA and a value of 0.8 micrograms per liter was determined.

Polymerase Chain reaction was used to ascertain if multiple copies (up to 10) of the expression cassette have tandomly inserted into the genome during transformation of *K. lactis* cells as strains harboring multiple integrations often produce more secreted protein. Multiply integrated cells were identified using whole-cell PCR with Integration Primers 2 and 3. Cells harboring multiple tandem integrations of the expression fragment at the LAC4 locus in the *K. lactis* genome resulted in amplification of a 2.3 kb product.

Further purification techniques can be applied in order to increase the yield of the protein so that it be tested on animal models before being taken to pilot scale for production.

CONCLUSION

HBs Ag secreted by Kluveromyces lactis which was engineered by cloning the HBsAg gene synonymous to the most prevalent HBV strain in Pakistan has been purified and concentrated using techniques of Dialysis and Ammonium sulphate precipitation so as to enhance its suitability for commercial scale production.

Also the multicopy integration of the expression cassettes into the genome is ascertained by performing PCR. From this study it can be concluded that the HBsAg is now purified and concentrated to be taken to testing on animal models before pilot scale production.

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