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VIRUS-LIKE PARTICLE VACCINE MANUFACTURING FROM YEAST

By

Ananya

A REPORT

Submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In Chemical Engineering

MICHIGAN TECHNOLOGICAL UNIVERSITY

2020

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This report has been approved in partial fulfillment of the requirements for the Degree of
MASTER OF SCIENCE in Chemical Engineering.

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Abstract

Influenza virus has an intrinsic nature to undergo mutations. Hence, there have been struggles to combat this disease since the time when the worst influenza pandemic first hit in 1918. Vaccines have proved to be effective in controlling the spread of the influenza A virus by providing herd immunity. However, the contemporary design of influenza A virus based on egg and cell-culture is not efficient in tackling influenza A virus transformation. Virus-like particles (VLPs) have established themselves as a potential platform for future vaccine candidates. VLPs have all the credentials to supplant contemporary vaccine designs without compromising on immunogenicity. For expression of VLPs, yeast has all the definitive potential to develop new-generation influenza A vaccines. This report describes how to use *Hansenula polymorpha*, a yeast, to make VLPs of influenza H1N1 virus.

1. Introduction

With the emergence and re-emergence of influenza viral strains, every so often there is a viral outbreak. Several influenza pandemics have occurred in the past [1]. Many such influenza outbreaks in the past have made people contemplate that influenza pandemics could happen again in the future [2]. Influenza viruses are one of the greatest threats to mankind as they cause a very contagious disease that has the potential to infect large populations [3]. Vaccines have proved to be a consummate approach to combat situations like these. Currently, most influenza vaccines are developed in eggs and some are developed in cell-culture. However, the contemporary strategy to design vaccines suffers from various pitfalls. During a pandemic situation, it would take approximately one year to make nearly 2.5 billion doses of vaccines (assuming vaccines will have only one hemagglutinin antigen), which would only cover 40% of the world population [4]. This downside calls for an advanced technology that can manufacture vaccines with high immunogenicity and simultaneously meet the public demand at low cost.

Virus-like particles (VLPs) are a very attractive alternative process to design vaccines. VLPs are nanostructures that impersonate real viruses without being infectious. VLPs are constructed using recombinant DNA technology. This technology can create unique nanostructures by altering and combining genetic materials from different sources [5]. Genetically modified VLPs can generate immune responses to fight against viral infections. These recombinant antigens require an expression platform that allows the proteins to self-assemble into VLPs. The self-assembly of VLPs happen at the plasma membrane when the glycoproteins come together with the matrix proteins and congregate to form VLPs. VLPs imitate real virions so that they are both useful and practical to be used as a vaccine candidate to provide immunity [6].

Yeast has emerged as an exceedingly attractive expression system to produce VLPs. Yeast is a eukaryotic organism that has the potential to express different types of proteins, including viral proteins. Moreover, its simplicity, unicellular nature, fast growth, and inexpensive

media requirements give an edge to this system over other expression systems [7]. In the past, yeast has been successfully used to produce HIV type1 gag virus-like particles [8]. Now, yeast is being seen as a prominent system of choice for vaccine production [7]. *Saccharomyces cerevisiae* and *Pichia pastoris* are the most used yeast expression system and have been extensively studied to synthesize recombinant proteins [9] .

Hansenula polymorpha (*H. polymorpha*), another type of yeast, is now gaining a considerable amount of attention. *H. polymorpha* is now being explored more as an expression platform because of its success in the past to express VLPs of hepatitis B [10]. *H. polymorpha* grows at a very high cell density and does not require expensive media. *H. polymorpha* efficiently performs post-translational modification and their fermentation attributes are highly favorable for heterologous protein synthesis [11]. Besides, *H. polymorpha* is one of the most heat resistive yeast [12]. In specific, *H. polymorpha* has been accorded as “generally recognized as safe” (GRAS) reputation and is considered safe for the production of recombinant proteins [13].

The goal of this report is to understand the synthesis of VLPs from yeast.

More precisely:

- The need to switch from live attenuated or inactivated influenza A virus vaccines to VLP-based vaccines.
- Understanding the structure of influenza A virus, the budding process and its pathogenicity, which determine the virulence factor.
- Exploring yeast as an expression system and comprehending its genetic modifications.
- Understanding the fermentation process and formation of VLPs in yeast cell.

This report is also proposing a method to produce enveloped virus-like particles (eVLPs) of influenza A virus using *H. polymorpha* as an expression system. *H. polymorpha* were engineered with viral and capsid proteins. This report starts with an introduction of this project as chapter 1. The literature review constitutes chapter 2, which states the background of this research and why this research is required. The material and methods have been described in chapter 3. The conclusion and future work are mentioned in chapter 4.

2. Literature Review

2.1 Vaccines

Vaccines have significantly improved our potential to reduce deaths caused by viral diseases. Many of these viral diseases like influenza A are highly infectious, contagious and ubiquitous. They have significant potential to cause worldwide pandemics [14]. There have been more than a dozen cases during the last century when the world succumbed to an influenza pandemic outbreak. The worst influenza A pandemic was in the year 1918, which killed approximately 50-100 million people worldwide [1]. The 21st century has also witnessed an influenza pandemic outbreak in 2009 because of a new strain of influenza A H1N1 virus [15]. However, the impact of H1N1 flu threat was effectively managed and mitigated with the help of vaccines. Vaccines not only helped in averting the spread of the H1N1 flu, but also assisted in controlling the acuteness of the viral disease [15]. While vaccines play a critical role in the control of viral diseases, there still remains a significant challenge to create vaccines [16].

One of the major challenges in the creation of vaccines is the way they are currently being designed. The process of development of vaccines tremendously affects their availability. Though the 2009 H1N1 pandemic was successfully controlled with the aid of vaccines, it equally highlighted our inability to develop vaccines quickly [15]. The 2009 influenza pandemic vaccines were shipped within three months of the pandemic announcement. By the time the vaccines were supplied, pandemic waves had already subsided. Moreover, many undeveloped countries were left deprived of the pandemic vaccines. This disappointing approach to tackle the problem demands the execution of superior innovation [17]. The shortages of vaccines worldwide also underscore the questionable nature of our vaccine development process. Therefore, developing vaccines using a new technique to make them available in a timely manner without compromising safety, immunogenicity and effectiveness, is a global need [18].

The immunogenicity and effectiveness of any vaccine is determined by four-factors (Figure 2.1). 1) The presentation of known antigen in the vaccine evokes the acquired immune response, 2) adjuvants or immune potentiators in the vaccines trigger the intrinsic immune response, which in turn stimulate the antigenic response, 3) stabilizers to maintain vaccines integrity and 4) an efficient delivery system targets the antigen and adjuvant to the correct cells in the body [4].

Figure 2.1 Factors determining the effectiveness of vaccines. (This image is created in biorender) [4].

Antigens are foreign molecules that trigger our body to induce immune response to destroy the virus (Figure 2.2). There are two types of immune responses; innate and adaptive. The innate immune response is non-specific, whereas the adaptive immune response is antigen-specific [19]. The adaptive immune response is further divided into two types of immune response, humoral and cell-mediated. There are two important lymphocytes cells in the immune

system which are vital part of the adaptive immune response: B-cells and T-cells, which fight against a viral invasion. The B-cell helps in the production of antibodies while T-cells help to trigger B-cells to produce antibodies. T-cells are also responsible for directly killing infected cells. The humoral immune response depends on the production of antibodies to kill the virus. The antibodies present on the B-cells attach to the antigens. After the attachment, the B-cell is activated by the T-cell to produce plasma cells. The plasma cells then generate antibodies against the antigens to destroy the virus. This process also produces memory B-cells, which provide immunity against any future viral invasion. However, sometimes antibodies are not efficacious in fighting against a viral infection. Therefore, the body triggers the cell-mediated immune response with the help of T-cells, which destroy the infected cells entirely [20].

Figure 2.2 Immune response against antigens. (This image is created in biorender) [20].

To increase the adaptive immune response to a vaccine, adjuvants are used in the formulation of vaccines. Adjuvants can either be added to the vaccine or occur through the design of the antigen. Adjuvants trigger the activation of antigen-presenting cells, therefore triggering T-cell and B-cell maturation, and inducing a stronger adaptive immune response [21]. When adjuvants are mixed with vaccines, they positively enhance the potency of vaccines. However, incorporating adjuvants increases the complexity of the vaccine development process.

It requires the testing of multiple formulations prior to vaccine release. Therefore, to decrease the development time of a vaccine, the adjuvant should be incorporated into the design of the vaccine antigen [4].

There are three types of conventional vaccines; live, attenuated vaccines; inactivated vaccines; and sub-unit vaccines (Table 2.1). Currently, the majority of vaccines are either live, attenuated or inactivated. Though conventional vaccines have proven to be effective in controlling some infectious diseases, we have yet to make a vaccine against every communicable disease [22].

Table 2.1 Conventional vaccines and their immunogenicity [4].

Type of vaccine	Example vaccine	Type of immune response
Live, attenuated	Yellow fever, Rotavirus, Influenza	Cell-mediated immune response and humoral response
Inactivated	Influenza, Hepatitis A, Polio	Cell- mediated immune response and humoral response
Sub-unit vaccines	Hepatitis B	Humoral immune response

Live, attenuated vaccines are weakened pathogens that are adapted to produce in non-human cells. These vaccines are very close to the infectious pathogen and therefore evoke a strong immune response. The production procedure of this vaccine is straightforward. The pathogens are grown either in cell culture or eggs until they reach high density. Once the desired density is obtained, the pathogens are collected from the supernatant and are subsequently purified. Vaccines manufactured in this style have a high yield and are economical [4]. Also, live, attenuated vaccines provide both humoral and cell-mediated immune response. Vaccines for measles, yellow fever, and rotavirus are a few examples of live, attenuated vaccines. However,

safety is the main issue with live, attenuated vaccines. Identification of attenuated strains is not often feasible and there is chance the pathogen will become virulent again [23].

In inactivated vaccines, the pathogenic organisms are inactivated without altering the efficacy of their antigens. This method is preferred when the organism to be inactivated is not extremely pathogenic. The production platform for these vaccines is similar to the live, attenuated vaccines. However, the purification process is different as the pathogens undergo an additional chemical treatment step using a chemical like formaldehyde to inactivate the virus [4]. The live viruses can also be inactivated by heat treatment [24]. Inactivation of organisms has been used to manufacture several viral vaccines like influenza and hepatitis A [4]. But, these vaccines are not as effective as live, attenuated vaccines in providing immunity because they fail to develop a strong cellular immune response. Moreover, they need adjuvants, like aluminium salts, to increase the vaccine effectiveness [23].

Subunit vaccines are a third conventional way of producing vaccines. Subunit vaccines involve using only a part of a virus to evoke an immune response. There are two main methods to create subunit vaccines. The antigen of interest can be isolated directly from the virus, which requires removing other parts of the virus. However, the safety of these vaccines depends on the degree of detoxification and purification of pathogenic organisms. Also, the large scale production of pathogenic organisms can make this process unsafe [23]. The second type of subunit vaccine is developed using genetic engineering. These vaccines use genetic engineering to synthesize the specific antigen in cell culture. The current hepatitis B vaccines are developed using this technique. However, for this vaccine type, multiple doses are required to provide strong immunogenicity [4].

Some of the major drawbacks of current vaccines are their susceptibility to revert back to being pathogenic, their laborious production process, and failure to induce proper immune response [25]. Besides, they also fail to consider factors that account for the challenge in combating infectious disease. Factors like the emergence of new pathogens, mutation of existing strains, climate conditions, varied population and age distribution are needed to be contemplated for the effective vaccine development process [7, 26]. Moreover, the reoccurrence of pandemics

has proven the inefficiency of the conventional process in meeting public demands, as in the case of influenza virus.

2.2 Influenza A virus, Virus Budding and its Pathogenicity

Influenza viruses belong to the Orthomyxoviridae family. Influenza viruses are enveloped viruses containing single-stranded, negatively charged RNA [27]. These viruses have segmented genomes containing up to seven or eight segments. Influenza viruses can be divided into three different types; influenza virus A, B and C, (Figure 2.3). The structure of influenza A and B viruses are similar, with eight RNA segments and two major surface proteins. Influenza C viruses have a different structure, as they have seven RNA segments and only one major protein on the surface. The host and pathogenicity of these viruses are different from each other. It has been noted that only influenza A and B viruses affect humans. However, it is the influenza A virus that poses maximum risk to the human population [1].

Figure 2.3 Influenza virus types. Influenza virus A and B are structurally similar (Two major surface proteins protrude from the surface and eight vRNA) whereas influenza C is structurally different (one surface protein and seven vRNA). (This image is created in biorender) [26].

Influenza A virus (Figure 2.4) consists of two surface proteins, hemagglutinin (HA) and neuraminidase (NA). The antigenic relationship of the two glycoproteins in influenza A viruses

are subclassified into different types [28]. HA has 16 known subtypes (H1–H16) and NA has 9 known subtypes (N1–N9) [29]. All the subtypes are unique, and any possible combination of HA and NA proteins are possible. Influenza A virus also has two matrix proteins M1 and M2. The M1 protein is present beneath the lipid envelope and the M2 protein is present on the virus envelope [28]. The influenza A virus also has three polymerase proteins PB1, PB2, and PA, two non-structural proteins NS1 and NS2 and one nucleoprotein (NP). The NP, PB2, PB1 and PA proteins help in replication and transcription of viral RNAs (vRNAs) inside the nucleus. RNP is the ribonucleoprotein, which helps in transcription. Replication of the genome happens when all eight vRNA segments encoding the proteins are present [30].

Figure 2.4 Structure of Influenza A virus. Two surface proteins are hemagglutinin (HA) and neuraminidase (NA). M1 and M2 stands for the matrix protein. NS2 is the non structural protein. Repinted from [31] with permission.

For influenza virus replication (Figure 2.5), the HA protein first links with sialic acid on the host cell. This attraction triggers the infection and the virus becomes attached to host cell receptors present on the surface of the cell [29]. The virus enters the host cell via receptor-mediated endocytosis. When the virus is inside the host it is transported to low pH endosomes. In the acidic environment the HA protein is stimulated and undergoes surface alteration. The surface alteration of HA proteins induces fusion of the viral and endosomal envelopes. The virus is then transported to the cytoplasm. At some point, the low pH of the endosomal membrane energizes the M2 proteins to release hydrogen ions to make the virus interior acidic. The acidic

environment weakens the interaction between the M1 matrix protein and the RNPs. This separates the RNPs from the virus and the RNPs are discharged into the cytoplasm [32]. The RNPs are then transported to the nucleus by viral proteins, where replication and transcription happens with the help of RNA polymerase proteins. The transcription of viral RNA leads to the synthesis of messenger ribonucleoprotein (mRNP) [33, 34], which are responsible for the translation of viral proteins. Whereas, the replicated viral ribonucleoprotein (vRNP) forms the genetic material of offspring virus. The viral proteins H1, N1 and M2 are synthesized in the endoplasmic reticulum from mRNA. The viral proteins are then trafficked to the Golgi complex to form mature proteins. The mature viral proteins H1, N1 and M2 are then transported to the plasma membrane by the trans-Golgi network [35]. Afterwards, vRNP is also transported to the plasma membrane. The viral proteins and matrix protein (M2 protein) envelope the M1 and vRNP near the plasma membrane, where they eventually assemble and bud off the surface [36].

Figure 2.5 Influenza A virus replication process. Reprinted from [32] with permission.

Several factors determine the pathogenicity of influenza virus. The presence of these pathogen markers increases the intensity of the influenza virus virulence. The HA proteins are the major contributor in the viral pathogenicity [16]. Another indicator of pathogenicity is the

presence of a coding sequence for protein PB1-F2. Past studies have shown PB1-F2 increases virulence of the strains. Also, influenza virus that caused a pandemic in the past has these virulence markers [37]. Besides, the HA protein receptor and HA protein cleavage causes the release of vRNPs, are important virulence markers. Moreover, HA governs host preference and assists in virus entry [1]. Therefore, the HA proteins mostly determine the strain type to be used in vaccines.

2.3 Current influenza A vaccines and their Downsides

Current influenza vaccines are typically trivalent and quadrivalent. The trivalent influenza vaccines contain two influenza A strains and one influenza B strain. The quadrivalent influenza vaccines contain two influenza A strains and two influenza B strains or three influenza A strains and one influenza B strain. The influenza virus strains are revised every year by the World Health Organisation (WHO) [38]. Currently, most influenza A vaccines are made in eggs, but some are also made in cell culture (Figure 2.6). The egg-based platform is used to produce both activated and inactivated vaccines. However, cell culture is only used to produce inactivated vaccines.

Figure 2.6 Comparison of egg-based and cell culture-based influenza vaccines. (This image is created in biorender) [39].

Live, attenuated influenza vaccines are manufactured in eggs using a cold-adapted technique [24]. This method uses two different techniques to weaken the virus. In the first technique, the viral strains are adapted to grow at a temperature of 25°C in chick kidney cells. This process attenuates the virulence of the virus, as the virus does not prefer to grow at body temperature, near 37°C [40]. The selected strains are grown in multiple passages in eggs under reduced temperatures, which helps to weaken the strains. Once the influenza strains are cold-adapted, they do not easily multiply in the lungs [24]. Live, attenuated vaccines impersonate natural infection and develop both humoral and cellular immune responses, unlike inactivated vaccines. Live, activated vaccines are delivered through nasal administration and are very effective for children in the age range of 2-17 years, but not for people with a weak immune system [16].

To produce inactivated influenza vaccines in eggs, embryonated eggs are inoculated separately with the three influenza strains (H3N2, H1N1 and B). After inoculation, the virus multiplies in the allantoic fluid of the egg [41]. After virus replication, the allantoic fluid is

collected and column chromatography is used to concentrate and purify the fluid [16]. After purification, chemical like β -propiolactone is used to inactivate the virus. [41]. Approximately three eggs are needed to produce a single dose of vaccines, one for each strain in the vaccine. Moreover, the time frame to produce vaccines in eggs is around 6 months. Inactivated vaccines provide humoral immune response. However, they do not successfully induce a cell-mediated response. The mode of administration of inactivated vaccines is parenteral [4]. The success rate of the effectiveness of inactivated vaccines is 60-80% among children and young adults. However, the success rate is very low among the old age population (above 60) who are more vulnerable to the influenza virus [16].

Although the yield of strains is very high in egg-based vaccines, vaccines developed using eggs have several limitations: 1) The process is very time-consuming and chances of contamination are extremely high [41], 2) The supply of the vaccines depends on the supply of the eggs, which can be interrupted during the time of epidemic or diseases [42], 3) many people are allergic to eggs and the vaccines can give rise to life-threatening allergic reactions [41], and 4) sometimes the glycoproteins that are manufactured in the eggs are structurally different from the original virus, which can make the vaccines ineffective against the real virus [42].

Recently, some of the disadvantages to egg production are being overcome by cell culture-derived vaccines. The cell lines, such as monkey kidney cells and canine kidney cells, are infected with the influenza virus. After infection, the virus is allowed to grow in a bioreactor. After a certain amount of time, the supernatant containing the viruses is collected. The supernatant is then purified to isolate the antigens, which are then formulated into vaccines [41, 43]. This system has many advantages over egg-based system: 1) cells can be grown in bioreactors, which can increase the production of vaccines [41], 2) the production process is controlled and systematized, 3) higher purity of vaccines can be produced [44], and 4) allergies from the egg-based vaccines can be avoided. However, they suffer from frequent pathogen contaminations. Some of the viral strains may have difficulties growing in cell culture. Also, the cell culture produced influenza vaccines are more expensive than egg-based vaccines [45].

The current vaccine design strategies are not sufficient to methodically tackle future influenza pandemics and ensure global safety because of their limitations. Also, the old methods

of designing influenza A vaccines are not competent enough to satisfactorily meet the current worldwide requirement for influenza A vaccines [15]. Hence, it is critical to develop new and coherent technologies that can successfully target viral outbreaks by boosting the vaccine delivery system [26].

2.4 Newer vaccine technology

A lot of effort is being put into designing new vaccines. Currently, the two areas that are being focused on are knowledge of the virulence factors and understanding the needed immune response [46]. The study of these two areas will assist in designing antigens to induce the needed immune response [4]. Novel techniques like gene-based vaccines and virus-like particles (VLPs) are now being explored to change the ways in which vaccines are designed [23].

2.4.1 Gene-Based Vaccines

Gene-based vaccines are one of the new ways to design vaccines to provide immunogenicity against viral diseases. Unlike conventional vaccines, gene-based vaccines only comprise DNA or messenger RNA (mRNA) of the virus, which is used to express the antigens in the vaccine recipients, to induce an immune response (Figure 2.7). Both, DNA-based vaccines and mRNA-based vaccines can be directly constructed by extracting the genes that encode a specific protein of the virus [47]. Vaccines designed this way eliminate the need to inactivate or attenuate viruses.

Figure 2.7 Immune response of gene-based vaccines. (This image is created in biorender) [48].

The DNA-based vaccines are designed in the following steps (Figure 2.8). First, the gene of interest is selected from the virus. Second, the gene of interest is encoded in a bacterial plasmid. The designed plasmids are then transformed into *E.coli* either chemically or through electroporation. The bacterial cell culture is then grown to high cell density to produce multiple copies of plasmids. The plasmids are then extracted and purified [49]. The purified DNA plasmids are then formulated into vaccines and delivered directly as naked DNA in the vaccine recipient. The introduction of plasmid DNA vaccines in the recipient causes the formation of messenger RNA in the nucleus by the process of transcription. The sequence of messenger RNA is then translated to synthesize the antigens in antigen-presenting cell. The expressed antigens then induce the necessary adaptive immune response [50].

Figure 2.8 Development of DNA-based vaccine. (This image is created in biorender) [49].

However, in RNA-based vaccines (Figure 2.9), the RNA of the desired proteins are first transcribed *in vitro* by utilizing specific DNA sequences from the plasmid DNA. The DNA is degraded using an enzyme called deoxyribonuclease (DNases). The synthesized RNA is then extracted and purified. After purification, they are directly introduced into the person as naked RNA. The RNA sequence is then used to synthesize the antigens within the person receiving the vaccine. The synthesized antigens trigger the immune response to fight against viral invasion [51]. Advantages and disadvantages of both the vaccines are compared in the Table 2.2.

Figure 2.9 Development of RNA-based vaccine. (This image is created in biorender) [51].

Table 2.2 Advantages and disadvantages of DNA-based and RNA-based vaccines [47].

Vaccine type	Advantages	Disadvantages
DNA-based vaccines	<ul style="list-style-type: none">• Produces both humoral and cell mediated immune response• DNA plasmids are stable• Mode- nasal, oral, intradermal, intramuscular• Hepatitis B, HIV	<ul style="list-style-type: none">• Safety concerns- causes disruption of the DNA sequence
RNA-based vaccines	<ul style="list-style-type: none">• Produces both humoral and cell mediated immune response• Safe to use as injecting RNA does not disrupt the DNA sequence• They enter the cytoplasm and not into the nucleus of the cell• Mode- nasal, oral, intradermal, intramuscular• Example HIV 1, rabies virus	<ul style="list-style-type: none">• Less stable than plasmid DNA• High cost

Both DNA and RNA vaccines use a fragment of the gene encoding the proteins to synthesize the required antigens in the vaccine recipient. However, the site of translation is different for both the vaccines. DNA vaccines are transported to the nucleus to synthesize antigens while RNA vaccines are transported to the cytoplasm. This technology is also a faster and effective way to design vaccines. Besides, these vaccines can induce both humoral and cellular immune responses. However, gene-based vaccines have several drawbacks like a low level of antigen expression and relatively low immunogenicity [26, 52].

To overcome the limitation of poor immunogenicity DNA based vaccines can also be delivered using viral vectors. Unlike the plasmid DNA, the DNA is introduced into the host cell using vectors that contain the DNA encoding the antigens. These viral vectors are engineered to carry the genes that express the surface protein. The viral vectors are mostly obtained from viruses like adenovirus and alphavirus. Both the viruses have a substantial ability to infect many

host organisms [52]. Viral vector-based vaccines are superior to DNA plasmid vectors in inducing strong immune responses. However, vaccines based on viral vectors have more intricate manufacturing processes. They experience genetic instability, design of the vector is complex, and a high degree of purification is required to guarantee safe vaccines [4, 8].

Gene-based technology is being used to design influenza A vaccines. This design technique has been used in many pre-clinical trials. Most of the pre-clinical trials express HA antigens. Besides, gene-based vaccines have not yet been accepted to be used in humans. Moreover, its low immunogenicity needs to be balanced with the addition of adjuvants, which may cause immunotoxicity. Furthermore, sometimes, the induction of immunity is also undermined as these vaccines fail to express enough antigens [53].

2.4.2 Virus-Like Particles and Assembly

Virus-like particles (VLPs) are another novel route to design vaccines. VLPs are nanostructure particles with a membrane that closely resemble viruses with respect to shape and size (Figure 2.10) [54]. The general size range of VLPs is 22-200 nm [55, 56].

Figure 2.10 Comparing virus with VLP. Real virus has viral genome and VLP has no viral genome, which make them non-infectious. (This image is created in biorender) [54].

Vaccines based on VLPs are very promising as VLPs are particulates that are a suitable size to be taken up by cells [56]. The particle size of the VLP is one of the pivotal characteristics in determining its effectiveness as a vaccine. Unlike the virions, VLPs do not have genetic material, which makes them non-infectious [55]. VLPs have an inherent capability to self-

assemble, which makes them highly organized particles [14, 57]. The epitopes that are found in virus-like particles are repetitive, have a high density, and display the same as in the infectious viral particle [55]. The structure of real virions are efficiently imitated by VLPs and they can produce strong humoral and cellular immune responses (Figure 2.11) [7]. VLPs do not necessarily require adjuvants to stimulate the immune response, as they are naturally self-adjuvating and can strengthen the weak immunogenicity of proteins and peptides. Moreover, the immunogenicity of VLPs is high compared to other sub-units and gene-based vaccines because they are strong stimulator of both T and B cell immunity responses [56].

Figure 2.11 Immune response of VLP-based vaccines. (This image is created in biorender) [48].

The morphogenesis of VLPs are similar to the real virus and, like viruses, can be enveloped or non-enveloped. Non-enveloped viruses exit from the host cell via cell lysis causing the death of the cell. However, enveloped viruses are produced by the process of budding. There

are three classes that address the minimal driving force behind the budding process of enveloped viruses: 1) viral membrane proteins drive the bud formation. 2) inner structural proteins drive the bud formation. 3) requirement of glycoprotein and viral core protein for bud formation. Therefore, the budding process can be attributed to any of the three mentioned factors or a combination of them [58]. Virus budding can happen at the plasma membrane or intracellular membranes (Figure 2.12), such as the Golgi, endoplasmic reticulum and nuclear envelope. When the virus particle buds from the plasma membrane, the particles accumulate at the plasma membrane and are directly released to the extracellular space. In contrast, when budding occurs at the intracellular membrane, the particles are accumulated in the lumen of cell organelles. In this case, the virus particles are carried to the plasma membrane via secretory vesicles prior to release into the extracellular space [58].

Figure 2.12 Different paths of budding process for viruses. Budding of viruses happening at plasma membrane, golgi complex, endoplasmic reticulum and nuclear envelope. Reprinted [58] with permission.

Influenza virus VLPs are enveloped VLPs (eVLPs) that are surrounded by a lipid membranes. There are two steps involved in the self-assembly process of eVLPs. First, the inner structural proteins are developed and second, the lipid membrane is formed during budding. For the construction of influenza VLPs, three proteins, HA, NA and M1, from influenza A virus are needed. HA protein is important for designing VLPs, as antibodies are generated against them to

make the virus ineffective. The other surface protein NA is also needed, as it is the second dominant surface protein and is used to impersonate real virus. However, the presence of these two proteins is not sufficient for the construction of influenza VLPs. Therefore, the presence of M1 protein, which is a capsid protein is imperative [59]. Capsid proteins have the potential to self-assemble. When the capsid proteins are encoded with the viral proteins into plasmids and inserted into the host, together all of them self-assemble to form VLPs [60].

2.5 Construction of VLPs and Different Types of Host Expression

Molecular cloning is an important part of recombinant DNA technology (Figure 2.13). The first step of VLPs construction involves selecting the antigen gene(s) to be cloned. The second step is inserting the gene encoding the antigen into a plasmid/vector for microbial transformation. The vector is inserted into the expression system through transformation. Expression system provides a platform for the antigens to express and assemble. The fourth step is screening. After transformation, screening is done to check the correct transformation process. Screening helps in checking which cells took the plasmids with the correct sequence of the gene encoding the antigens. The fourth step is putting the cells into an appropriate expression system to create a VLP [61, 62].

Figure 2.13 Steps of VLP Recombinant DNA technology. (This image is created in biorender) [59].

Different types of expression systems can be used to manufacture VLPs [63]. Choice of expression system is a combination of multiple factors. First, their ability to perform post-translational modification and synthesize standard length proteins. Post-translational modification incorporates process like glycosylation, formation of disulphide bonds and correct protein foldings. These attributes regulate the efficiency of the system. Second, easy manipulation of the expression system for orderly discharge of the VLPs. Third, their ability to secrete contamination-free products to make the purification process of the VLPs less challenging. Fourth, their capability to be economically scaled up for industrial use [6]. Different types of expression systems are compared in the Table 2.3.

Table 2.3 Comparison of different types of expression system [9], [64].

Expression system	Advantages	Disadvantages
Bacterial system	<ul style="list-style-type: none"> • Production of non-enveloped VLPs • Production cost is low • Fast production and expression rate • Easy scaling up production 	<ul style="list-style-type: none"> • No enveloped VLPs production • Incorrect protein folding • Solubility is low • Lipopolysaccharide contamination
Mammalian system	<ul style="list-style-type: none"> • Produces both enveloped and non-enveloped VLPs • Efficient and high quality VLP production • Post translational modifications, copy the parental virus very well 	<ul style="list-style-type: none"> • High production cost • Low yield • adventitious factor contamination
Insect system	<ul style="list-style-type: none"> • Produces both enveloped and non-enveloped VLPs. • Growth rate is fast. • Post translational modifications possible. • Large scale production possible. 	<ul style="list-style-type: none"> • Production cost is high. • Baculovirus contamination and time-consuming production.
Yeast system	<ul style="list-style-type: none"> • Produces both enveloped and non-enveloped VLPs. • Low production cost, high production speed and scalability. • Post translational modifications possible 	<ul style="list-style-type: none"> • Possibility of protein misfolding in high density cell culture.

	<ul style="list-style-type: none"> • Low contamination problem 	
Plant cell	<ul style="list-style-type: none"> • Produce both enveloped and non-enveloped VLPs • Production cost is low • Scalable, fast • Post translational modification • Low contamination problem 	<ul style="list-style-type: none"> • Time consuming • Glycosylation in plants is different than animal cell

The bacteria cell has been the most widely for the production of non-enveloped VLPs. VLPs that are simple in structure and have only one or two proteins are efficiently expressed in bacteria [55]. Bacteria as a host system do not require expensive culture media, support high cell density culture, and are easy to scale up [65]. *E. coli* has been extensively used for the production of vaccines like low-cost HPV vaccines and Hecolin, a hepatitis E vaccine [65]. However, bacteria lack the ability to perform complex post translational modifications such as glycosylation and hence it is not a preferred system for the production of eVLPs. Moreover, they experience protein insolubility, endotoxin contamination and they fail to express protein above 60kD [9, 14, 66].

Mammalian expression is a favorable choice to produce both non-enveloped and enveloped VLPs. Their post-translational modification is exceptional [14]. Also, the proteins synthesized in the mammalian cell have biochemical properties and structure similarities to human proteins [67]. CHO cells and human embryonic kidney cell line have been most exploited to produce eVLP [68]. These cell line can grow to a very high cell density, can synthesize glycoproteins, they support correct protein folding and can be scaled up [69]. Mammalian cell lines have been used in the past to synthesize influenza virus VLPs [63]. However, the mammalian system has many disadvantages for example, the production cost is very high, the media is expensive, they are difficult to transfect, they have an increased susceptibility to pathogen contamination, and the purification steps are complicated [68].

Baculovirus/insect cells are eukaryotic systems which are used to synthesize both enveloped and non-enveloped VLPs. The insect cell line has been widely used to produce high-quality eVLPs because of their ability to perform post-translation modification and glycoprotein

folding [70]. They produce high cell density cultures and have high protein expression levels. Also, multiple protein expression is possible in this system [71]. *High Five*TM and *Sf9* are most used insect cell line as they are easy to manipulate and can attain high density at a temperature of 27°C [14]. VLPs like HPV L1 vaccines have been produced in insect cell [63]. The baculovirus system is used where the genetic code to make the VLP is in the system itself. The cells are infected with this virus that can only infect insect cells and not mammalian cells. One major disadvantage of this system is removing the baculovirus from the culture, which makes the purification process intricate. Also, DNA contamination is another drawback of insect cell cultures. Therefore, conventional purification procedures like ultracentrifugation are unable to provide high grade purification for this system. [71].

Plant cells are another expression system which can produce both enveloped and non-enveloped VLPs [64]. Plant cells have ability to perform post-translational modification, however the glycosylation in plants is different than animal cells, which may give rise to complications during protein synthesis [14]. This expression system is very cost-effective and has the potential to be scaled up [72]. They have a low risk of contamination, as they do not permit human or mammalian pathogen growth. [64, 72]. HIV-1 VLPs has been produced using plant cell [64]. Although this technique is cheap, its very time consuming and the protein expression level is not up to commercialized standard [72].

Yeast, as an expression system, can be used for the production of enveloped and non-enveloped VLPs. They are easy to cultivate; they support extremely high growth rates and have a low level of contamination [12]. Besides, genetic manipulation are possible in yeast, which makes them heterologous host organisms to synthesize proteins [73]. Yeast expression systems also support the production of recombinant glycoproteins. Yeast can perform complex post-translational and its genetics are more superior compared with other eukaryotic expression systems which make their manipulation simple [74]. Moreover, yeast-based systems can be used to produce VLPs at large scale because of its high fermentation attributes [75].

Each of the expression systems has certain advantages and disadvantages as discussed above. However, it appears that yeast can be a preferred expression system because it supports all the attributes for a functional VLP production. Also, yeast display adjuvant characteristics

and hence can naturally induce an immune response. Besides, the chances of contamination is lower in yeast, making their purification process simpler. These characteristics make them suitable host organisms for the production of VLPs [7].

2.6 Yeast Manipulation

Cells require genetic modification to express recombinant proteins. Factors like cloning procedure, choice of vectors, selection of promoters and markers and cultural conditions play a consequential role in determining the expression of proteins. Therefore, to assess conditions that will give the optimum protein production, it is essential to amalgamate all factors together and simultaneously screen for the best combination for protein production [76]. The manipulation of yeast starts with the selection of the antigen sequence, followed by cloning and vector design.

An efficient cloning is a prerequisite for a successful genetic modification. Cloning can be done in several ways and there are multiple factors that decide the choice of cloning procedure. Factors like speed, efficiency, cost and feasibility are some of the important elements that are considered while selecting a cloning method. However, there are two mostly used cloning methods; restriction enzyme cloning and polymerase chain reaction-based cloning [77].

In the restriction enzyme method (Figure 2.14) the antigen gene and plasmids are cut using the same restriction enzyme, and then combined to produce a recombinant plasmid. The first step in the restriction enzyme method is the selection of restriction enzyme that will be used to cut the antigens and plasmids. There are more than 3000 restriction enzymes, such as ECOR1, HindIII, Alu I, etc.[78]. Each of the restriction enzymes is unique and sequence-specific. Restriction enzymes efficiently determine the correct palindromic sequence of the antigens [79]. The second step of cloning is digestion. The antigen gene and donor plasmids are digested with restrictive enzymes and buffer. These enzymes cut the preferred antigen genes at the restriction sites, which produces a sticky end. The amount of restriction enzymes used is proportional to the amount of DNA of antigens. The same enzyme is used to cut the recipient plasmids. The third step of cloning is isolation. The antigens and donor plasmids are isolated using gel purification. The fragments are run on the agarose gel along with a known DNA ladder, which helps in locating the desired antigen based on its expected size following digestion. After running gel

purification, the antigen DNA is cut from the gel. This step helps in extracting the antigen DNA from the plasmid backbone. After extraction, DNA is purified and the purified DNA is then ligated into the recipient vector using DNA ligase. However, sometimes, instead of flanking the antigen DNA, restriction enzymes may cut within the antigen DNA. Also, there are chances of DNA inserting into the plasmids in wrong orientation. In this case PCR based cloning can be used [77].

Figure 2.14 Restriction enzyme cloning. (This image is created in biorender) [77].

PCR based cloning simultaneously adds restriction sites to the antigens while making several copies [80]. PCR reaction happens at series of temperature and four segments are needed for the reaction to happen; antigens, primers, nucleotides and polymerase. PCR uses primer to create compatible ends of antigens with respect to the recipient plasmid. These primers are synthesized oligonucleotides of DNA and have a specific sequence. Primers compliment the two ends of the DNA by matching the forward and the reverse strands. An enzyme, DNA polymerase, which is extremely heat resistive is used for the amplification of the gene of interest. With the help of specific nucleotides, these polymerases are linked to the primers that are

attached to the gene of interest [81]. After running the PCR, the PCR product is isolated and purified. The purified antigen DNA is used for restriction enzyme digestion. Which is followed by isolation, gel purification and ligation. This method is effective and fast compared to the restriction cloning method [80].

The construction of compatible plasmid/vector is also an important part of yeast manipulation (Figure 2.15). The vector has an autonomously replicating sequence (ARS), which helps in maintenance of plasmids inside the yeast [82]. ARS contains the origin of replication that allows the plasmid to multiply independently [83]. The gene encoding the antigen is cloned in a vector cassette. The expression cassette is an integral part of the vector which is fused with regulatory sequences that instruct the cell to synthesize proteins. These cassettes are comprised of multiple sites including marker and promoter. The promoters and selectable markers are required for the propagation into the host organism [10]. There are five types of plasmids/vectors used in the transformation of yeast cell Autonomously Replicating Plasmids (ARS), Yeast centromere Plasmids (YCps), Yeast Integrating Plasmids (YIp), Episomal Vectors (YEP plasmids) and Yeast Linear Plasmids (YLp). YCp and YEp are the most used plasmids in the transformation of yeast because they give firm stability to the recombinant strains [12].

Figure 2.15 A general constructed vector with selection marker, ARS sequence, expression cassette, gene of interest (This image is from an open access article) [84].

Selectable markers play a very significant role in the transformation process by signaling what cells have been transformed. Many plasmids suffer from unstable inheritance. Hence, they do not get stably integrated into the host cell [85]. Markers are used to discard the cells that have lost the desired plasmid [86]. The exogenous DNA is supplemented with these selectable markers to identify the stable integration [87]. Markers successfully allows mutation in yeast. Selection can be done in two ways positive and negative selection [12]. In positive selection, the cells that have the desired gene of interest grow. Positive selection can be done in two ways; antibiotic selection and auxotrophy selection. In antibiotic selection, the plasmid inserted has an antibiotic-resistant marker. However, antibiotic selection is not preferred for yeast cells because of their ability to naturally develop resistance. Hence, auxotrophy selection is more suitable. In auxotrophy selection, organisms are incapable to synthesize the compound which supports their growth. Henceforth, to accomplish the selection process, minimal media is used to grow the cell. The minimal media is deficient in a specific nutrient provided by the plasmids that are pertinent for the cell to grow [88]. However, in negative selection, cells that have lost the gene of interest survive [12].

The next step in the genetic manipulation of yeast is the selection of promoter. The promoter is where proteins bind to the DNA to start transcription [12]. The promoter is responsible for the inducibility of the proteins and controls the protein expression level [6]. Constitutive promoters are always turned “on” and do not require an inducer to start the transcription process. However, inducible promoters need a specific activator to start the transcription process. The regulated promoter can distinguish between the growth phase of the cell and the expression phase [6]. Regulated promoters can transition from OFF to ON mode or ON to OFF mode. Figure 2.16 shows a regulated promoter, there is no transcription initially (OFF mode). When the inducer is added, it binds itself to the specific site of the DNA and starts the transcription process (ON mode). A strong, tightly regulated promoter saves the cell from undesirable transcriptions, which increases the burden on the cell and hence can affect the stability of the synthesized protein. Commercially used promoters are of two types; inducible promoters and constitutive promoters. Constitutive promoters are not regulated promoters and are in the ON mode all the time. Inducible promoters need an inducing agent like methanol to start the induction process [89]. It is essential that the promoter is compatible with the vector and

the cell as transcription is not the same for every yeast [6]. Once the vector is constructed with the antigens, promoters, and selectable markers the next step is introducing the vector into the host organism.

Figure 2.16 In the regulated promoter, there is no transcription initially and once the inducer is added, the activator binds with promoter and starts the transcription process (This image is created in biorender) [89].

Molecular transformation is a process of introducing the vector into the host cell. Yeast have cell walls surrounding their plasma membrane. The inner cell wall is made up of β -1,3-glucan and chitin. Mannoproteins and β -1,6-glucan make up the outer wall of the yeast. β -1,6-glucan links the inner and outer components of the cell wall and chitin helps in maintaining fiber insolubility [90]. Because of the thick cell wall surrounding the cell membrane, the introduction of any foreign gene of interest is not straightforward. Therefore, the cell wall needs to first be for molecular transformation to happen [91]. There are three different methods for molecular transformation of yeast, including spheroplast production, chemical transformation using monovalent cations, and electroporation.

In the spheroplast production method, the cell wall of the yeast is digested using enzymes. Enzymes like zymolyase or β -glucuronidase can be used to chemically alter the cell wall of the yeast cell [12]. These enzymes are used to hydrolyze the glycosidic bond which is responsible for the cell wall rigidity [92]. The transformation of spheroplasts happens in a medium containing sorbitol, enzymes, PEG, CaCl_2 and plasmids. Sorbitol, along with PEG maintains the osmotic pressure of the medium. When the cell wall is digested by the enzymes,

the host cell uptakes the plasmid. After transformation, selective plating of the cells is done using agar plate. Although this process of transformation is extremely efficient, the use of agar plates makes the screening process onerous [12]. These limitations have led to the development of other transformation methods.

Yeast transformation can also be performed using monovalent cations. Treating the yeast with metal ions like lithium, potassium, or sodium increases the permeability of the cell wall of the yeast. The transformation happens in the solution containing PEG and cations. The interaction between the negatively charged PEG, positively charged ions, and the yeast alter the charge of the yeast cell wall. This treatment makes the yeast cell more capable to take up the plasmids. The treatment with cations does not affect the viability of the cell. However, the effectiveness of transformation depends on the duration of the treatment [93]. Moreover, the efficiency of the transformation is less than the spheroplast method [12].

The third method used to perform transformation in yeast is electroporation. In this technique, a high electric field is applied by a pulse decay method. This causes the voltage to decay exponentially and rapidly. The electric field alters the cell wall by generating pores. The alteration caused by the electric field is temporary and the pore formation in the cell wall aids in the introduction of plasmids into the cells. However, this method requires individual optimization of the process for each yeast and the efficiency of the transformation depends on several factors like strain type, the strength of voltage, condition of the cell, etc. [94]

Currently, most of the transformation process is done either using monovalent cations or electroporation. These two processes are efficient and fast, which makes the transformation process relatively faster than the spheroplast method.

2.7 Types of yeast

The choice of yeast for molecular transformation depends on the individual abilities and functions of the yeast cell. Some of the types of known yeast are *Saccharomyces cerevisiae*, *Arxula adenivorans*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Pichia pastoris* and *Yarrowia lipolytica*. All yeast have specific vectors, promoters, and markers that effect the

expression level of the recombinant protein and the compatibility of the constructed plasmid with the yeast [12]. The selection of yeast can broadly be determined by the three factors;

- 1) Expression level. The likelihood of recognition of cloned genes increases when the expression level is high during the plate screening. This helps in selecting the favorable clone. [6].
- 2) Glycosylation ability. Using western blot technique, the platform provided for post translational modification of proteins by a yeast cell can be detected and compared to check efficiency of a yeast cell [6].
- 3) Transformation efficiency and stability of plasmid. The transformation efficiency of yeast is determined by how successfully it takes up the plasmids. And at the same time, a stable plasmid allows identification of correct transformation and later positive replication of cells [6].

S. cerevisiae, *H. polymorpha* and *P. pastoris* are the most used yeast expression platforms (Table 2.4) and have been utilized to synthesize approximately 30 VLPs [9].

Table 2.4 Advantages and disadvantages of different types of yeast expression

Yeast type	Advantages	Disadvantages	Reference
<i>Saccharomyces cerevisiae</i>	<ul style="list-style-type: none"> • Most studied system • Genetics very well know and can be easily manipulated 	<ul style="list-style-type: none"> • Low expression level • Low copy no. vector • Low yield and low-quality product • Plasmid instability • Not suitable for large – scale production • Weak promoters • Very strong fermenter 	<ul style="list-style-type: none"> • [12]
<i>Pichia pastoris</i>	<ul style="list-style-type: none"> • Strong and efficient promoters • Not a strong fermenter • Higher expression level 	<ul style="list-style-type: none"> • Not resistant to high heat • Lack of moderate promoters • Few selectable markers are available 	<ul style="list-style-type: none"> • [95]

	<ul style="list-style-type: none"> • Better host for heterologous protein • High cell density • Potential to be scaled up 	<ul style="list-style-type: none"> • Need methanol for induction 	
<i>Hansenula polymorpha</i>	<ul style="list-style-type: none"> • High expression level and stable integration. • Very strong and efficient promoter • Strains formed are meiotically stable • Extremely heat resistant • High cell density culture • Induction is possible without methanol as well. 	<ul style="list-style-type: none"> • Sometimes experience over glycosylation • Newer system to work with 	<ul style="list-style-type: none"> • [10] • [11]

S. cerevisiae is one of the most studied systems and has been used to a great extent for the cloning of genes [96]. *S. cerevisiae* fermentation is thoroughly understood and when provided with favorable conditions, this yeast can grow on a wide variety of carbon sources. *S. cerevisiae* is also suitable for genetic manipulation using the latest recombinant DNA technology. An extensive gamut of strains and many adaptable vectors are accessible for the transformation in *S. cerevisiae*. Hence, they are used most of the times for protein production [97]. However, *S. cerevisiae* has many limitations. Their protein production capacity is relatively low compared to other yeast expressions. *S. cerevisiae* cannot perform the complex post-translational modification. They also experience plasmid instability problem which results in no expression of recombinant proteins. Moreover, the attained cell density and yield of the product are not satisfactory for *S. cerevisiae* and hence they may not be suitable for large scale production [98]. Strong and regulated promoters are not widely available for *S. cerevisiae* which may increase the load on the cell and can result in the production of unfolded proteins [12]. Besides, *S. cerevisiae*

is a very strong fermenter and produce ethanol which can cause toxicity to produced proteins [99].

P. pastoris is a member of the methylotrophic yeast family. It can grow on many carbon compounds and does not require complex media. After *S. cerevisiae*, *P. pastoris* is the second most studied system and has widely been used for the production of recombinant proteins [63]. Unlike, *S. cerevisiae*, *P. pastoris* is not a strong fermenter which is one of their advantages. A strong fermenter can cause toxicity which can obstruct the cell growth [99]. *P. pastoris* can perform complex post-translation modification; the formation of disulfide bonds is also possible, and can also execute glycosylation. Also, factors like pH and carbon source that are essential to increase the heterologous protein efficiency can easily be manipulated. In minimal media, high-density cell culture is easily attainable, hence *P. pastoris* as the potential to be scaled at an industrial level [95]. Besides, the availability of strong promoter like AOX gives a high level of protein expression making *P. pastoris* a desirable host expression. However, they lack promoters which do not require methanol for induction. Which is one of their limitations as using methanol at industrial-scale can be hazardous. *P. pastoris* also lack availability of moderate promoters. Using strong promoters may overwhelm the cell and can result in the synthesis of unfolded proteins. Therefore, the use of moderate promoters which can induce without methanol is desirable [99].

Hansenula polymorpha is also a member of methylotrophic yeast family. It follows the methanol utilization pathway to produce heterologous protein. *H. polymorpha* is one of the most heat resistant yeast and grows well at high temperatures. Some strains can tolerate heat up to 50°C and are capable of producing high heat endurance proteins. *H. polymorpha* has diverse host strains and is versatile as an expression platform [12]. They efficiently perform post-translational modification and their fermentation attributes are highly favorable for protein expression. Besides, *H. polymorpha* protein production capacity is very high [6]. Compared to other yeast they can grow at a very high density in minimal media [100]. They have robust and strong promoters, formate dehydrogenase (FMD) and methanol oxidase (MOX), which are responsible for boosting the production of foreign gene expression and high level of recombinant protein expression [10]. *H. polymorpha* mostly uses strong methanol-derived promoters. Methanol is

very toxic and explosive, and it's not considered as a favorable chemical to be used at large scale. Yet, some of the *H. polymorpha* strain allows the use of constitutive promoter, like PMA1, which can induce in glycerol depression or glucose starvation without the supply of methanol. This is one of their major advantages [11]. With unique induction abilities, *H. polymorpha* also facilitates the production of recombinant proteins at industrial scale [100]. Henceforth, *H. polymorpha* makes a prominent candidate for expression of heterologous proteins.

All the above-mentioned yeast cell are capable of producing recombinant proteins. They all have successfully been used in the past for the synthesis of VLPs. *S. cerevisiae* has been used to produce HIV type gag 1 VLPs [63]. *S. cerevisiae* has also been exploited to synthesize influenza VLPs [97]. Bacteriophage VLPs and HBsAg VLPs have been produced in *P. pastoris* [63]. The strength of *H. polymorpha* has been taken advantage of to express VLPs of hepatitis B [10]. However, *H. polymorpha* is a relatively new expression system to work with and hence it was chosen to synthesize influenza A VLPs.

2.8 *H. polymorpha* and expression of proteins

Altogether there are three primary *H. polymorpha* strains; CBS4732, DL-1, NCYC495. All the other *H. polymorpha* strains are derived from the three parental strains. Among these strains, NCYC495 is not used for recombinant protein production, as a high growth rate is not supported on media containing methanol. Hence it doesn't have any robust promoters that are methanol-pathway derived for a strong induction. However, the other two strains CBS4732 and DL-1 grow well on media containing methanol and are mostly used for recombinant protein expression. As compared to other parental strain, DL-1 has many advantages like the growth rate is high, easily adjust to cultural media conditions, and the rate of occurrence of homologous proteins is high [12].

The vectors that are constructed for *H. polymorpha* or any other yeast is synthesized from bacteria and yeast sequences. General vector design for *H. polymorpha* (Figure 2.17) [101].

Figure 2.17 *H. polymorpha* constructed vector. It has an origin of replication and an antibiotic sequence, ampicillin. It also has the cassettes which is fused with the gene of interest, a promoter (FMD) and a terminator (MOX). In this vector, selective marker URA3 is used for the selection process. Reprinted [101] with permission.

There are two parts of the vector, the prokaryotic part and the eukaryotic part. The prokaryotic part consists of two portions, an origin of replication and an antibiotic resistance sequence. Both the prokaryotic parts aid in plasmid propagation in bacteria. Whereas, the eukaryotic part contains the components and sequences required for yeast transformation. To help with the propagation in yeast, there is a *H. polymorpha* autonomous replication sequences (HARSs) in the eukaryotic portion of the vector. Also, the eukaryotic part is equipped with multiple expression cassettes. These cassettes are comprised of three different sites, a cloning site for the antigens to be cloned, a promoter and a terminator. For the identification of correct integration, markers like URA3 and LEU2 are mostly used for *H. polymorpha*. Promoters like FMD, MOX and TPS1 help with the induction process during fermentation in *H. polymorpha* [10]. Once the required vector is constructed it is then inserted into the yeast [12].

Once the desired vector is constructed the cDNA of the antigens are inserted into the same vector using the earlier described cloning method. In our case, the RNA of the antigens (H1, N1, and M1) were derived from the influenza virus (Figure 2.18).

Figure 2.18 RNA of the proteins isolated from influenza virus for construction of VLPs. (This image is created in biorender) [59].

Figure 2.19 shows a general procedure of influenza H1N1 VLP development. The RNA of the desired protein is first isolated from the real virus. The RNA sequence of the antigen is difficult to be cloned. Therefore, to make the cloning process easy, RNA is first converted into complementary DNA (cDNA) using the enzyme reverse transcriptase. The cDNA is a double-stranded sequence and easier to clone into a vector [102]. The antigens are converted into the cDNA using reverse transcriptase. After digesting the vector, the PCR amplified cDNA is inserted into the vector. The antigens (H1 and N1) are introduced into the yeast cell by using the constructed vectors. The capsid proteins (M1) are also integrated into the yeast genome using the same vector by the process of transformation [59].

Figure 2.19 Shows the VLPs production process. (This image is created in biorender) [59].

The transformation of *H. polymorpha* is done in the same way as done for any other yeast. As described earlier, transformation can be done by the spheroplast method, use of monovalent cations, or electroporation. However, the vectors can randomly integrated or directed to a certain genomic location. In random integration, no specific location is targeted, and integration is done using *H. polymorpha* autonomously replicating sequence (HARSs). However, in target integration, defined gene loci are used for homologous recombination. Once the transformation is successfully done in *H. polymorpha*, mitotically stable strains are formed. These strains are comprised of multiple copies of the expression cassette. After transformation, the cells are screened for successful integration of plasmids on a selective media [101].

The preference of media depends on the choice of marker genes. Vectors are harbored with different selection markers like URA3 and plating of the cells is done on selective media. For example, if URA3 selection plasmid is used, the clone with the correct gene of interest will grow if the media is not supplied with URA3. Henceforth, the successful transformation is determined by the growth of cells in which the vectors are effectively integrated. The cells are

incubated at 37° C and colonies appear after 4-5 days. After the appearance of desired colonies, the colonies are transferred to the liquid media for induction to happen [12].

The presence of strong promoters like MOX or FMD is responsible for a high level of heterologous expression in *H. polymorpha*. A complex fermentation mechanism is followed which uses multiple energy sources for maximum expression level. The mechanism of fermentation that is followed by *H. polymorpha* is repression/depression. Usually, *H. polymorpha* grows on three carbon sources; glucose, glycerol and methanol. Glucose acts as a repression source, glycerol acts as a derepression source and methanol is an induction source of the promoters. The addition of these substrates is done in a fed batch mode or a continuous mode to get very high optical density and expression level [103]. First, the cells are grown in glucose to guarantee cells are grown to a desired optical density. Moreover, glucose acts as a repressive source for the promoter. Second, the cells are switch to media containing glycerol. Glycerol is a standard energy source for *H. polymorpha*, and it ensures that cells grow to high optical density. Third, the cells are supplemented with methanol which starts the induction process [104]. The limited supply of methanol in the end causes aerobic fermentation which results in very high expression level of proteins [105].

The path utilized by these promoters to use methanol for high expression level is called methanol utilization pathway (Figure 2.20). The utilization of methanol happens in two stages. The first stage of the reaction happens in peroxisomes, which is a subcellular organelle and the second stage of metabolic reaction take in the cytoplasm. The process starts with oxidation of methanol by alcohol oxidase. When methanol is oxidized, two products are formed; formaldehyde and hydrogen peroxide. In the next step, the enzyme catalase breaks hydrogen peroxide into water and hydrogen. The formaldehyde that is formed takes up two pathways; dissimilatory pathway and assimilatory pathway. In dissimilatory pathway formaldehyde is converted to carbon dioxide to yield energy. In the assimilatory pathway, formaldehyde is converted into the cellular carbon compound; dihydroxyacetone synthase (DHA) and glyceraldehyde-3-phosphate(GAP) to generate biomass. DHA is then converted into Dihydroxyacetone phosphate (DHAP). Later, DHAP and GAP react to form fructose-1,6-

biphosphate (FBP) [10, 105, 106]. The interaction of the promoters with the carbon sources plays a significant role in determining the expression level [103].

Figure 2.20 Shows the methanol utilization pathway. (Reprinted from [99] with permission).

Yeast has all the cellular factors to synthesize enveloped viruses [107]. However, yeast have cell walls surrounding the plasma membrane. Therefore, it was thought that the process of budding terminates at the cell wall. However, if the cell wall of the yeast cell is removed, budding can happen through spheroplast [108]. Spheroplasts are the cells without the cell wall which are spherical in shape and osmotically unstable [109]. As discussed above, in enveloped viruses, membrane protein or viral capsid protein or both can be the minimum driving force for the budding process [8]. Therefore, digesting the yeast with an enzyme like zymolyase in the media containing sorbitol causes the cell wall to break and hence aiding the budding of virus-like particles. However, the spheroplasts are not directly formed from the cell but it goes through a transient state where prospheroplast is formed first. Prospheroplast is also a cell without cell membrane and is osmotic pressure sensitive. When the yeast cell is digested with the enzymes the cell wall starts to get loosen up. Soon, the prospheroplast slowly makes its way out of the partially digested cell wall. Prospheroplast keeps its elongated shape regardless of the cell wall loss. The cell wall gradually gets digested and prospheroplast gets rid of its elongated shape and

transforms itself into a spherical structure, spheroplast [109]. These three antigens congregate together inside the yeast cell. The congregation happens at the plasma membrane and proteins form VLPs. The VLPs are released after the digestion of yeast cell walls. The cultural media is maintained osmotically stable using sorbitol to prevent the bursting of the spheroplast. The promoters help in the induction of the VLPs in the yeast cultural media containing methanol. Maintaining the yeast media culture integrity helps in the budding of the virus-like. The VLPs are then released into the supernatant [59]. The supernatant can be collected and VLPs can be purified to be formulated into vaccine.

Influenza virus vaccines development based on VLPs is efficient. This process is fast and economical. The traditional influenza vaccines manufacturing process takes approximately 6-9 months. However, influenza vaccines can be manufactured in 1-2 months using VLPs (Figure 2.21) [110]. A vaccine based on VLPs can drastically increase the production process by reducing the development time of vaccines. The research on VLP-based vaccines must be pursued to manifest the consideration of this technology.

Figure 2.21 Compares the influenza A vaccine development and production time of egg-based and VLP-based platform. (This image is created in biorender) [110].

3. Materials and Methods

3.1 Materials

The yeast cells for VLP production were received from Esperovax (Plymouth, MI). For yeast cell culture Peptone from meat, and bacteriological dextrose, were purchased Millipore Sigma (St. Louis, MO). Yeast extract (Alfa Aesar™) was purchased from Thermo Scientific (Pittsburgh, PA). Sorbitol, glycerol, adenine and methanol were purchased from Sigma Aldrich (St. Louis, MO). For SDS PAGE, NuPAGE NOVEX Gel 4-12% Bis-Tris Protein Gels, 1.0 mm, 12-well, NuPAGE MOPS SDS Running Buffer, NuPAGE LDS Sample Buffer and SeeBlue™ Plus2 Pre-stained Protein Standard were all purchased from Thermo Scientific (Pittsburgh, PA). For western Blot, XCell II Blot Module, NuPAGE Transfer Buffer were purchased from Thermo Fisher Scientific (Pittsburgh, PA). For protein detection, Non-fat dairymilk powder was purchased from Walmart (Houghton, MI). Goat Anti-Rabbit IgG Antibody (H+L) was purchased from Vector laboratories (Burlingame, CA). Peroxidase PI-1000,SK-4400 TMB Substrate Kit, Blue, 300 ml were ordered from Cole parmer (Vernon Hills, IL). Influenza A virus M1 (matrix protein) antibody was purchased from GenTex (Alton Pkwy Irvine, CA). Pierce™ 20X TBS Tween™ 20 Buffer was purchased from Thermo Scientific (Pittsburgh, PA).

3.2 Methods

3.2.1 Media preparation

Stock solution of 4xYP (yeast extract and peptone) media was prepared using 8 g yeast extract and 16 g peptone in autoclaved water in 200mL of autoclaved water. 200 mL of 50% (w/v) glucose stock solution, 2M of sorbitol stock solution, 200 ml of 50% of (v/v) glycerol solution and 25x adenine stock solution was prepared in autoclaved water. After the preparation, the solutions were autoclaved (121°C).

After autoclaving, the stock solutions were cooled down to room temperature and 100 ml of media containing sorbitol, YP media, glucose was prepared as per the Table 2.4 for Day 1 culture.

Table 3.1. Day 1 media preparation YPD

	2M sorbitol	4xYP	50% glucose	25x adenine
100 mL	50 mL	42 mL	4 mL	4 mL
Final conc	1M sorbitol	Approx. 2xYP	2% glucose	1x adenine

The media for day 2, containing sorbitol, YP media and glycerol was also prepared as per the Table 2.5.

Table 3.2 Day 2 media preparation YPG

	2M sorbitol	4xYP	50% glycerol	25x adenine
100 mL	50 mL	42 mL	4 mL	4 mL
Final conc	1M sorbitol	Approx. 2xYP	2% glycerol	1x adenine

3.2.2 Cell inoculation and induction of VLPs

Prepared media with 2% glucose (3 mL) was added in cell cultural test tube. The yeast cells from the YPD plate were inoculated. Starting $OD_{600} = 0.5$. The cells were then incubated over night at 37 °C and 250 rpm. The next day the OD was measured (the cell should grow with $OD_{600} = 8$). 3 ml of media from the stock solution of the 100 mL of YPG media, was added in the cell cultural tube. The preculture from the day one was inoculated in the 3mL of YPG media (starting OD= 1). The cells were incubated for 72 hours at 30 °C and 250 rpm. After 72 hours the cell culture was supplemented with 1% of methanol (30 μ L in 3 mL). The cell culture was incubated overnight at 30 °C and 250 rpm. Next day, the OD was checked. The cells should grow with $OD_{600} \approx 20$. The cell culture was harvested using Eppendorf 5810R Centrifuge at 1000 rpm for 10 minutes to separate the cells and supernatant. The supernatant was collected in a tube.

3.2.3 SDS-PAGE.

Running buffer 1X (1000 mL) was prepared using 20X MOPS SDS Running Buffer by adding 50 mL of it in 950 mL of ultrapure water. The gel used was NUPAGE Gel 4-12% Bis-Tris Protein Gels.

For 50 μ L of sample preparation, 12.5 μ L of NuPAGE LDS sample Buffer (4X) was mixed with 5 μ L of NuPAGE Reducing Agent (10X) and 32.5 μ L of supernatant. If 2-mercaptoethanol is used as a reducing agent, 1.25 μ L of it should be mixed with 12.5 μ L of NuPAGE LDS sample Buffer (4X) and 36.25 μ L of supernatant (total = 50 μ L). The prepared samples were heated for 10 minutes at 70-80 °C.

The gel cassettes were filled with SeeBlue™ Plus2 Pre-stained Protein Standard and the prepared sample. The chambers were filled with running buffer and the gels were run at 200V for 50 minutes. After electrophoresis the gel was carefully removed.

3.2.4 Western blot

The western blot was done using XCell 11 Blot module. 1X NuPage buffer was made by mixing 50 mL of 20X NuPage buffer in 950 mL of deionized water. The blotting pads and membrane were soaked in 1X NuPAGE Transfer Buffer until they were saturated. The gel, the nitrocellulose membrane, the filter were placed within the blot module. The assembly was secured between two halves of the blot module. The gel assembly was placed in the XCell 11 Blot module. The blot module was filled with 1X NuPage transfer buffer. The blot was run for 1 hour at 30 Volts. The proteins were transferred into the nitrocellulose membrane. The membrane was then tested for proteins.

3.2.5 Detection of proteins

The nitrocellulose membrane was blocked for 1 hour with 5% milk in Pierce™ 1X TBS Tween™ Buffer (TBST) at room temperature. 1X TBST buffer was made from 20X TBST buffer by mixing 50 mL of 20X TBST with 950 mL of deionized water. After blocking, the membrane was transferred to 10 mL of solution containing 1:2000 dilution of rabbit anti M1

antibody in 2.5% milk in TBST. The membrane was soaked overnight at 4°C on a shaker. Next day, membrane was transferred to 50mL 1X TBST and was vigorously washed for 5 minutes on a shaker for three times. The washing was repeated three times. After washing the membrane with 1X TBST, it was transferred to 10 mL of solution containing 1:1000 dilution of Goat Anti-Rabbit IgG Antibody (H+L), Peroxidase PI-1000, HRP conjugate. The membrane was soaked for 1 hour at room temperature on a shaker. After treating the membrane with secondary antibody it was quickly washed with water and then was put into 5 mL of SK-4400 TMB substrate.

4. Conclusion and Future work

4.1 Conclusion

Influenza vaccines based on VLPs have outstanding prospects to revolutionize the state-of-the-art of vaccine production. VLPs-based vaccines, impersonating real influenza virus, is a very safe alternative to the present day influenza A vaccines. Moreover, vaccines based on VLPs do not compromise on immunogenicity and shows probability of inclusive protection by inducing a cell-mediated immune response. This attribute of VLPs based vaccines can be very advantageous for the people who are in the high-risk category. VLPs based influenza A vaccines can conveniently overcome all the limitations of the contemporary designing method. It can also effectively reduce the production time and can successfully meet the public demand at low cost. Seasonal as well as pandemic influenza vaccines can be promisingly developed using VLPs of influenza virus.

The selection of a platform for synthesizing VLPs is also very critical in the development of VLP based vaccines. To make the purification process less cumbersome and to simultaneously minimize the downstream processing cost, it is requisite that synthesized VLPs are contamination free. Yeast cell being easy to manipulate emerges to be a foremost choice for the expression system. Moreover, yeast holds innate adjuvant characteristic which can intensify the immune response without the addition of any special immune potentiator. Yeast grows at a very fast rate and is extremely economical as compared to other expression systems.

H. polymorpha seems to be a promising candidate for the production of influenza A virus VLPs. They provide a highly favorable platform for heterologous protein synthesis. Because of its exemplary characteristics, *H. polymorpha* can be of considerable value in the synthesis of VLPs. Altogether, vaccines based on yeast bestow a silver lining for potent oral vaccines for influenza A virus to be ubiquitous in the coming future.

4.2 Future work

In the future, experiments will be performed to optimize the production of VLPs. Kinetics studies can help in understanding the parameters which affect the VLPs synthesis process. Experiments will be conducted to obtain the kinetics of cell growth and synthesis of VLPs. The production of VLPs of influenza H1N1 virus, from *H. polymorpha*, depends on glucose, glycerol and methanol. Experiments can be designed to optimize the concentration of all the three carbon sources on which the fermentation process depends. Moreover, mathematical modelling can be developed to get the relation between cell growth, cell viability and the consumption of carbon sources. This modelling can then ultimately be utilized to enhance the production of VLPs of influenza H1N1 virus. Besides, the kinetics will also help in understanding the repression/depression mechanism of *H. polymorpha* more precisely. Later, a simple model can be developed to optimize all the other important parameters on which the fermentation process depends. Parameters like pH, temperature, cell growth time and induction time can be optimized. Moreover, the induction time and expression level of *H. polymorpha* can be looked into more promptly by collecting the supernatant at a specific time after the addition of methanol.

In our method, we are targeting VLPs production of H1N1 influenza A virus in yeast. When formulated as vaccines these VLPs will induce immunity against the H1N1 strain of influenza A. This will help in averting any future viral infection when infected with a real H1N1 virion. However, as discussed, HA has 16 subtypes and NA has 9 subtypes. These subtypes can combine to form a unique strain of influenza A virus. In this case, vaccination with H1N1 strain type will not provide immunity against the novel strain. Therefore, this approach of synthesizing VLPs in yeast can be exploited more comprehensively to target any new strain of influenza virus. Yeast as an expression platform can be explored further and developed to control future influenza virus outbreaks. Besides, yeast cell has natural adjuvant characteristics which increase the effectiveness of VLPs when formulated as vaccines. Therefore, exploring this system for production of different types of influenza A antigens can be beneficial. Moreover, targeting a conserved domain of influenza A virus which does not undergo mutation and expressing it's VLPs using yeast can be explored in future.

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