Virus Like Particles in Yeast

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Several structural viral proteins can self-assemble to form a capsid without a viral genome. This property of viral proteins has been exploited for constructing virus-like particles (VLPs). The most important feature of VLPs is that they resemble the capsid of the original virus, but they are empty shells that do not contain the viral genome, and thus, they elicit an immune response without propagating inside the cells. VLPs have been produced in *Escherichia coli* and in mammalian, plant, insect, and yeast cells .

Keywords: virus-like particles (VLPs) ; Saccharomyces cerevisiae ; Pichia pastoris ; Hansenula polymorpha

1. General Considerations of Yeast as Expression System

Yeast is a eukaryotic organism that has emerged as an exceedingly attractive expression system to produce heterologous proteins and VLPs. As yeast strains used in the laboratory are nonpathogenic and nontoxic organisms, they are safe and can be manipulated without any special precautions. For this reason, they have been classed as generally considered safe (GRAS). They have several advantages over mammalian-based systems including rapid growth rates, ease of genetic manipulation, inexpensive and locally-available medium requirements, and well-established fermentative growth [1][2]. These features make yeast-based systems suitable for large-scale production. The genetic manipulation that can be performed on these organisms allows us to modify them in line with the heterologous proteins that need to be expressed. Yeast expression systems also support the production of one or more viral capsid proteins to form and display an extensive repertoire of antigenic sites including discontinuous epitopes and the production of recombinant glycoproteins [3]. Moreover, yeast has a low risk of contamination by adventitious agents. As a result, VLPs of several virus families have been produced in yeast (**Table 1**).

Table 1. List of virus proteins that have been expressed in yeast species to produce VLPs, grouped according to virus family.

Family	Virus Species
Hepadnaviridae	**Hepatitis B Virus, Hepatitis E Virus,**
Flaviviridae	**Hepatitis C Virus, Japanese Encephalitis Virus, Bovine Viral diarrhea virus, Tick-borne encephalitis virus, Zika virus**
Papillomaviridae	**Human Papilloma Virus 1, 6, 11, 16, 52, 58, Cottontail rabbit Papillomavirus, bovine papilloma virus 1,2, 4**
Picornaviridae	**Enterovirus D68, Enterovirus 71 and Coxsackievirus A6, A10 and A16, Poliovirus type I**
Nodaviridae	**Redspotted grouper nervous necrosis virus, Nervous necrosis virus**
Parvoviridae	**Porcine parvovirus, Adeno associated virus, Human Parvovirus 4, B19, Human bocaviruses**
Paramyxoviridae	**Sendai virus, Tioman virus, Human parainfluenza virus 2 and 4, Menangle virus, Nipah virus**
Circoviridae	**Porcine circovirus**
Retroviridae	**HIV**
Kolmioviridae	**Hepatitis Delta Virus**
Fiersviridae	**Cacteriophage Qbeta virus**
Sedoreoviridae	**Rotavirus**
Potyviridae	**Johnsongrass mosaic virus**
Polyomaviridae	**Human polyoma virus, hamster polyoma virus, bird polyomavirus, Goose hemorrhagic, Polyomavirus**

Family	Virus Species
Caliciviridae	**Norovirus, Rabbit hemorrhagic disease virus**
Bromoviridae	**Cowpea chlorotic mottle virus**
Birnaviridae	**Infectious bursal disease virus**
Secoviridae	**Grapevine fanleaf virus**
Togaviridae	**Chikungunya virus**
Iridoviridae	**Chinese Giant Salamander iridovirus**

Most information about the expression systems are taken from [4]. Virus classification was retrieved from https://ictv.global/taxonomy (accessed on 4 July 2023).

Traditionally, the yeast of choice in biotechnology has been *Saccharomyces cerevisiae*; however, other yeast strains are now successfully used to produce VLPs. They are grouped as non-conventional yeasts such as Pichia pastoris (*Komagataella phaffii*) and *Hansenula polymorpha* (*Pichia angusta*). The significant development of molecular tools, including synthetic promoters for fine-tuning of expression, engineered strains, and CRISPR/Cas9 technology, have driven the generation of strains more efficient in VLP production for different yeast species[5][6][7].

2. *Saccharomyces cerevisiae*

The budding yeast *S. cerevisiae* is one of the most studied genetic systems and has been used to a great extent for expressing and producing heterologous proteins for therapeutic or pharmaceutical purposes [8]. A great number of expression plasmids have been constructed in *S. cerevisiae*. These vectors offer a wide choice of promoters of varying strength and selection markers. In addition, these plasmids are usually shuttle vectors that can be propagated in yeast and bacteria, making them useful in gene cloning [9].

The first VLPs produced in *S. cerevisiae* and were composed of HBV proteins [10]. The production was so successful that it led to the approval by the FDA of the first yeast-based derived HBV virus vaccine in 1986. Over the years, VLPs of other viruses have been assembled in yeast, confirming that *S. cerevisiae* is a good model system for manufacturing VLPs [4] . Most VLPs purified from this yeast are assembled intracellularly and then purified after cell disruption. This procedure is inappropriate for enveloped VLPs because they originate from the budding of the membrane [11][12]. However, Sakuragi et al. demonstrated for the first time that enveloped virus can be produced in *S. cerevisiae* spheroplasts. Specifically, they produced VLPs of the human immunodeficiency virus (HIV), an enveloped virus, by budding out the particles directly in the medium [[13]. The release in the medium of VLPs allows the formation of enveloped VLP and makes purification easier and faster than that of VLPs assembled in the cytoplasm.

VLPs produced in *S. cerevisiae* are distributed worldwide as vaccines against infectious diseases; for instance, vaccines against HPV and *P. falciparum* are commercially available [4]. Using a novel engineered *S. cerevisiae*-based platform, a multigenic VLP from SARS-Cov-2, named PRAK-03202, was developed. Immunization of mice with PRAK-03202 induces an antigen-specific (spike, envelope, and membrane proteins) humoral response and neutralizing potential [14]. In addition, VLPs from yeast have also been used for virus research in order to study capsid assembly and related factors [15][16].

Although VLP production in *S. cerevisiae* has several success stories, some drawbacks are still ongoing. First of all *S. cerevisiae* has a lower efficiency than other yeasts, including *H. polymorpha* and *P. pastoris*, to secrete heterologous proteins [17], so all viral proteins are expressed intracellularly in relatively large amounts; this means that they can potentially produce misfolded aggregates that could be toxic for the yeast cells and consequently reduce VLP yield. Additionally, *S. cerevisiae* is unsuitable for high-density culture. This depends on its particular metabolism. The preferred carbon source of *S. cerevisiae* is glucose metabolized mainly by fermentation with ethanol production. This is because *S. cerevisiae* exhibits the so-called Crabtree effect: alcoholic fermentation in the presence of oxygen when the glucose concentration exceeds a certain threshold value, even under aerobic conditions. When the glucose concentration is restrictive, ethanol produced during fermentation is used as a carbon source, by a shift to a respiration mode. The shift from one carbon source to another, known as a diauxic shift, determines a growth slowdown necessary to adapt to the alternate carbon source [18]. Another limitation of using *S. cerevisiae* is the pattern of protein glycosylation that is different from mammalian cells. In this microorganism, N-glycosylation leads to hyper-mannosylated N-glycans (more than 100 mannose residues) and allergenic molecules because of the terminally added mannose attached by an α1,3 bond [19].

Synthetic biology, which has been focusing on the engineering of *S. cerevisiae*, could help to improve or optimize the expression level of viral proteins. Currently, many toolkits are commercially available to standardize methods and protocols in *S. cerevisiae* and other yeasts [20].

3. *Pichia pastoris*

The expression of heterologous proteins in *P. pastoris* leads to a higher yield of the protein in comparison to other yeasts, including *S. cerevisiae*. *P. pastoris* can grow to densities as high as 130 g/L of dry cell weight enabling the production of grams per liter of heterologous proteins [21]. Unlike *S. cerevisiae*, *P. pastoris* is a Crabtree-negative yeast, meaning that it metabolizes glucose by complete oxidation to carbon dioxide and water. *P. pastoris* is a methylotrophic yeast, because it can utilize methanol as its sole carbon and energy source. The first step of the methanol metabolism pathway is methanol oxidation by the enzyme alcohol oxidase (AOX) leading to the production of formaldehyde and hydrogen peroxide. In *P. pastoris*, two genes code for AOX: AOX1 and AOX2. Specifically, AOX1 is responsible for most of this activity and is expressed only in the presence of methanol as the sole carbon source and repressed by glucose. GAL1 expression in *S. cerevisiae* is induced by galactose and repressed by glucose, although a low level of expression occurs also in the presence of glucose. Most VPLs produced in *P. pastoris* are expressed under the control of pAOX1 [4]. Methylotrophic yeasts possess the capacity to secrete large quantities of correctly folded proteins. Secretion is an important step in VLP production because it simplifies purification, avoiding cell lysis, denaturation, and refolding of proteins. Secretion occurs also in other yeast species, but the advantage of *P. pastoris* is that it does not secrete proteases and only few endogenous proteins are released in the medium, thus facilitating subsequent purification. Moreover, comparing *P. pastoris* and mammalian genomes, the secretory pathway of *P. pastoris* resembles that of mammalian cells. This observation has been confirmed by structural analysis of Golgi apparatus, which, in this yeast species, is arranged in stacks and surrounded by a matrix that seems to fuse cisternae, as observed in mammalian and plant cells [22]. By contrast, in *S. cerevisiae*, Golgi cisternae do not stack and are individually scattered in the cytoplasm. One of the most frequently used secretion signals is the N-terminal portion of the pre-pro α-factor from *S. cerevisiae*. A success story of VLP secretion in *P. pastoris* is that of Norovirus (NoV) main structural protein VP1. VP1 was successfully expressed and secreted using the methanol-inducible promoter and the α-factor for secretion. NoV VLPs, purified directly from the culture medium, resulted in a total yield greater than 0.6 g/L, with a final purity product over 90%, and are capable of binding the Histo-Blood Group Antigen (HBGA) [23]. Compared to *S. cerevisiae*, *P. pastoris* has a shorter and less immunogenic glycosylation pattern, and for this reason is preferred over *S. cerevisiae* as a platform for producing glycosylated proteins. Several glycosylated VLPs have been produced in *P. pastoris.* The E protein forming the capsid of the Japanese encephalitis virus (JEV) is a 53 kDa protein containing a single potential carbohydrate attachment site. When expressed in *P. pastoris* for VLP production, it was secreted as a glycosylated protein and able to stimulate immune responses that protected mice against JEV infection [24]. Glycosylated VLPs inducing a potent immune response in mice were produced in *P. pastoris* for hepatitis E virus (HEV) [25]. The glycosylated envelope protein prE of several Dengue virus serotypes forms VLPs in *P. pastoris* and induces an immune response in mice [26][27][28][29] .

4.*Hansenula polymorpha*

Hansenula polymorpha (*Pichia angusta*) belongs to the facultative methylotrophic yeast species group. Compared to *P. pastoris*, *H. polymorpha* is thermotolerant: the optimal growth temperature is 37–42 °C. Growth at high temperatures can both reduce the contamination risk and promote the production of proteins requiring a temperature of 37 °C to maintain their biological activity [30].

As for other yeast species, the genome of *H. polymorpha* has been completely sequenced and several strong promoters have been identified and characterized, including strong methanol-inducible promoters of genes such as formate dehydrogenase (pFMD), methanol oxidase (pMOX), and dihydroxyacetone synthase (pDHAS or pDAS), which control the expression of enzymes belonging to the methanol utilization pathway. The shift from glucose to methanol causes the induction of the expression of these genes and the downregulation of those belonging to the glycolytic pathway. Notably, after 2 h of growth in methanol, pFMD is 347-fold upregulated (while pDHAS is 17.3-fold) compared to glucose growth [31]. This kind of promoter can be de-repressed by glycerol and, to a lower extent, by other carbon sources, while in *P. pastoris* the pAOX1/2 are not de-repressed but induced by methanol. Additionally, pMOX of *H. polymorpha* is also induced in a medium containing both methanol and glycerol [32]. Various genetic engineering tools and transformation protocols are well-established in *H. polymorpha*. The use of nanoscale carriers for DNA delivery is the most efficient method for *H. polymorpha* transformation [33], although other techniques are also widely used, such as the lithium acetate-dimethyl sulfoxide method [34] and electroporation (with the linearized vector) [35]. Contrary to *S. cerevisiae*, episomal plasmids are unstable in *H. polymorpha*, even when containing the *H. polymorpha* autonomous replicating sequences (HARS) for the

autonomous replication of the circular plasmids so integration plasmids are the most commonly used tool for the expression of VLP proteins [36]. Concerning post-translational modifications, N-glycans derived from *H. polymorpha* are similar to those of *P. pastoris* described in the previous paragraph and thereby are less hyper-mannosylated than the N-glycans produced by *S. cerevisiae* [37]. VLPs composed of glycosylated proteins have been effectively produced in *H. polymorpha* [4]. In the last decade, *H. polymorpha* has been widely studied for VLP production, leading to FDA-licensed and commercially available vaccines for HBV such as Hepavax-Gene® and Heplisav-B® .

As for the other yeast species, the bottleneck in producing VLPs is the purification method because viral proteins accumulate intracellularly [38]. *H. polymorpha* can be engineered to modulate the ER folding environment and, therefore, to secrete a heterologous protein by overexpression of calnexin[39], a key component of the quality control mechanism in the ER, or by the leader sequence of the α-factor [40]. However, secretion approaches must be further characterized, and these results could inspire further studies for the set-up of engineered H. polymorpha that can secrete the viral proteins for VLP assembly in the growth medium.

In **Figure 1**, the researchers compare the key features to consider when planning a platform for VLP production for the three species.

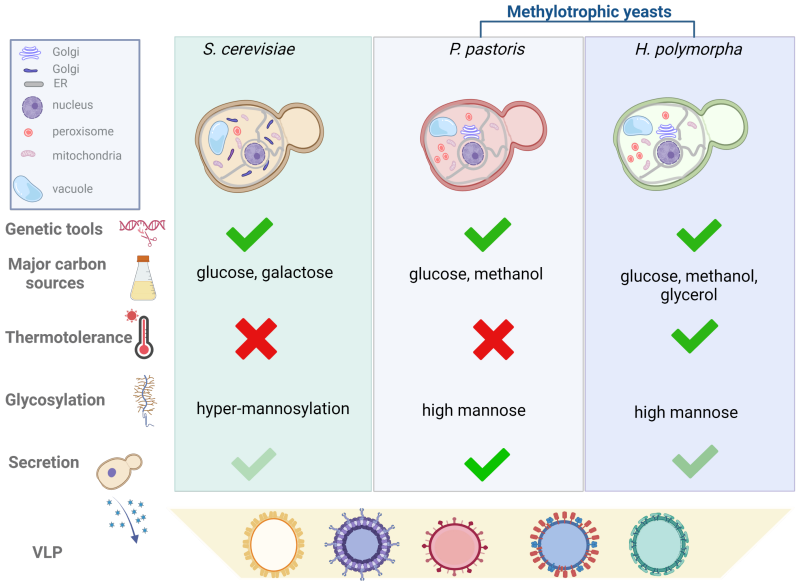

Figure 1. Comparison of yeast manipulation tools, features and parameters to be considered for the selection of the yeast species for VLPs production. Yeast cells of *S. cerevisiae*, *P. pastoris* and *H. polymorpha* are represented with the organelles to highlight the structural differences. Golgi apparatus of *S. cerevisiae* is organized in cisternae scattered throughout the cytoplasm; while in methylotrophic yeasts, Golgi apparatus is organized in stacked cisternae dispersed in the cytoplasm resembling those of mammalian cells. Methylotrophic yeasts are represented with a higher number of peroxisomes than *S. cerevisiae* to highlight their increase during growth in presence of methanol. Genetic tools such as mutant strains, plasmids, glycosylation humanized strains are developed for the three species. Carbon sources indicated in the figure are those preferentially used for heterologous protein expression; thermotolerance is referred to the ability to grow at temperature over 30°C. Hyper-mannosylation of proteins observed in *S. cerevisiae* means that the mannose chain is composed of more than 100 residues. Secretion in the culture medium, although occurring in the three species, has been harnessed mainly in *P. pastoris*. Created with BioRender.com.

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