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Review

Yeast and Virus-like Particles: A Perfect or Imperfect Couple?

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Abstract: Virus-like particles (VLPs) comprise viral structural proteins that self-assemble to form a particle similar to the native virus capsid. Since their discovery, they have been employed mainly as vaccines to prevent viral infection because they can elicit an immune response. Besides their use as vaccines, their application in cancer prevention and drug delivery is under intensive investigation. They can be produced in different systems such as bacteria, mammalian, plant, insect, and yeast cells. The main hurdle for their use is establishing a platform for production because many variables need to be considered. First, VLPs must be effective in the action for which they are constructed, depending on the nature of the VLPs. Second, the production platform must be suitable for safe and high-scale production. Yeast has been shown to be a valuable tool in VLP production, as it is able to express heterologous proteins efficiently and its manipulation is cheap and easy. Several species have been employed for this purpose. In the present review, we analyze the features of different yeast species and how they have been used to produce VLPs.

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

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1. Introduction

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. Although the number of articles dealing with VLP production has tremendously increased after the COVID-19 outbreak, the concept of VLPs was first postulated in 1955 when Fraenkel-Conrat and Williams observed that inactive proteins of the Tobacco mosaic virus were able to assemble into particles, producing lesions in the leaves similar to the virus [1]. Ten years later, VLPs were found in the liver cells of patients with infectious hepatitis [2]. Since then, VLPs have been produced in mammalian cells [3,4] and *Escherichia coli* [5], and have been identified in *Saccharomyces carlsbergensis* [6] and *Saccharomyces cerevisiae* during investigations of killer yeast strains [7,8]. Indeed, these kinds of viruses have been observed in several fungi, such as some *Penicillium* species, *Aspergillus foetidus*, and *Ustilago maydis* [9]. This observation has prompted researchers to use yeast to study genome replication and encapsidation [10,11]. Recently, VLP production has also started in plants [12,13] and insect cells [14].

The characteristics of VLPs make them a cheap and safe tool for producing vaccines and immunogens [15]. Several VLP-based vaccines are on the market and are used in humans, including Recombivax HB and Engerix-B for human hepatitis B virus (HBV), Gardasil, Cervarix, and Gardasil-9 for human papillomavirus (HPV), and Hecolin for hepatitis E virus (HEV), which are produced in bacteria, yeast, and insect cells [16], or in clinical trials, such as the seasonal influenza virus manufactured in plants [17]. In virology, they are widely used in investigations on virus assembly [18]. In addition, VLPs can be

exploited as carriers for the delivery of drugs and imaging substances in light of their empty inner space [19,20]. There is also growing interest in applying VLPs in cancer prevention and therapy [20,21]. Moreover, yeast VLPs have been exploited as a carrier for DNA and drugs. Examples of yeast-based VLPs used as carriers are HBV-VLPs filled with DNA plasmids or calcein with electroporation and then used to infect hepatocellular carcinoma cells and human carcinoma xenografts in mice [22]. VLPs applied in cancer prevention include vaccines against viruses involved in the pathogenesis of human cancer, such as HBV and HPV. On the other hand, VLPs exploited for cancer therapy are constructed with cancer-specific antigens capable of boosting the immune response against cancer cells. VLPs are generally produced in several systems, such as bacterial, plant, mammalian, insect, and yeast cells (Figure 1).

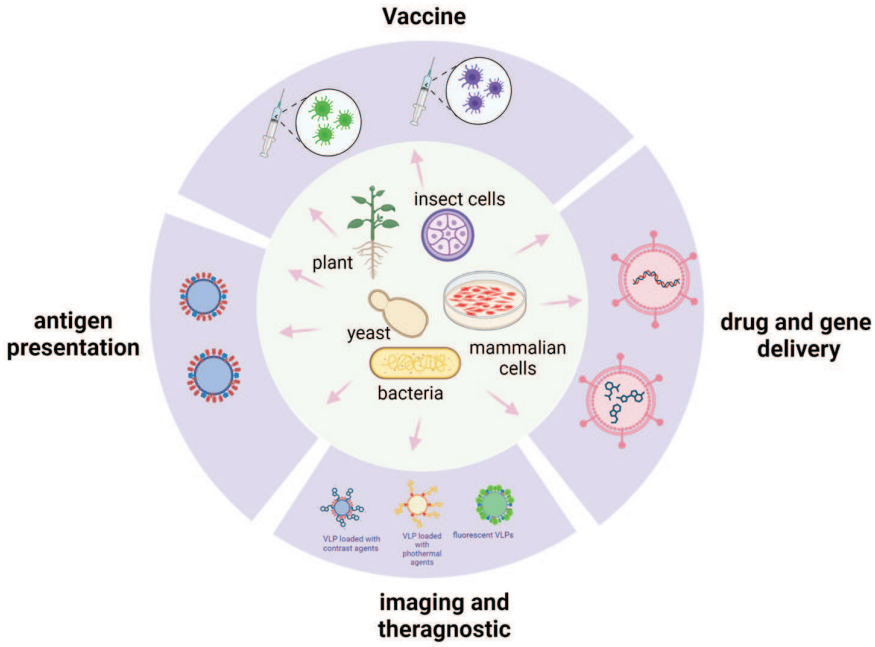

Figure 1. An overview of platforms for the production of VLPs and their applications. They are used in the manufacturing of vaccines, as carriers of drugs or nucleic acids; as nanocarriers for antigen presentation; conjugated with contrast agents for clinical imaging (for computed tomography, magnetic resonance imaging, and positron emission tomography), with photothermal agents for theragnostic applications, and with fluorescent dyes for optical imaging. Created with BioRender.com.

1.1. Types of VLPs

There are several critical steps to consider when a VLP production platform needs to be developed. First, we must consider the structural complexity of the virus of origin of VLPs, which can be classified as enveloped or non-enveloped (Figure 2). Enveloped viruses are made of single or multiple proteins surrounded by an envelope of the phospholipid bilayer derived from the host cell membrane, due to the budding or the secretory pathway. The specificity of the envelope is determined by the presence of viral glycoproteins [23]. Not all production systems can generate enveloped VLPs containing properly glycosylated proteins. Glycosylation is a post-translational modification (PTM) crucial for a protein to fulfill its biological activity and have the correct folding. This aspect is important in the case of heterologous protein expression because misfolded proteins can be promptly degraded by the ubiquitination pathway or retained in the endoplasmic reticulum (ER). The most suitable expression systems are those based on mammalian and insect cells that perform complex PTMs and the formation of an envelope. Bacteria do not have the enzymes to introduce PTMs, so enveloped VLPs cannot be produced in this organism. Yeasts can make most standard PTMs; however, glycosylation in yeast shows some differences from that occurring in human cells. Two main glycosylation types in eukaryotic cells (N-linked and

O-linked) occur in the ER or Golgi apparatus. While the biological role of O-glycosylation is poorly characterized, N-glycosylation is crucial for protein function. N-linked glycosylation consists of high mannose glycan (hyper mannose type) in yeast, whereas in human cells, in addition to high mannose glycan, hybrid glycans and complex glycans are attached to the nitrogen residues of asparagines (Figure 3).

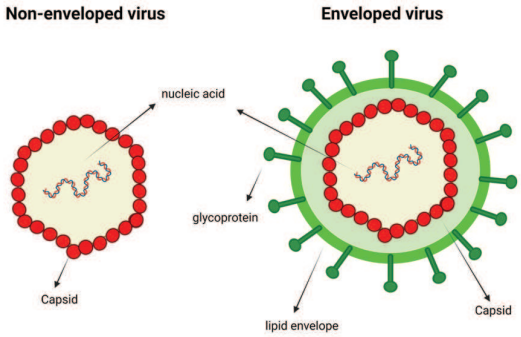

Figure 2. Schematic representation of non-enveloped and enveloped viruses. Created with BioRender.com.

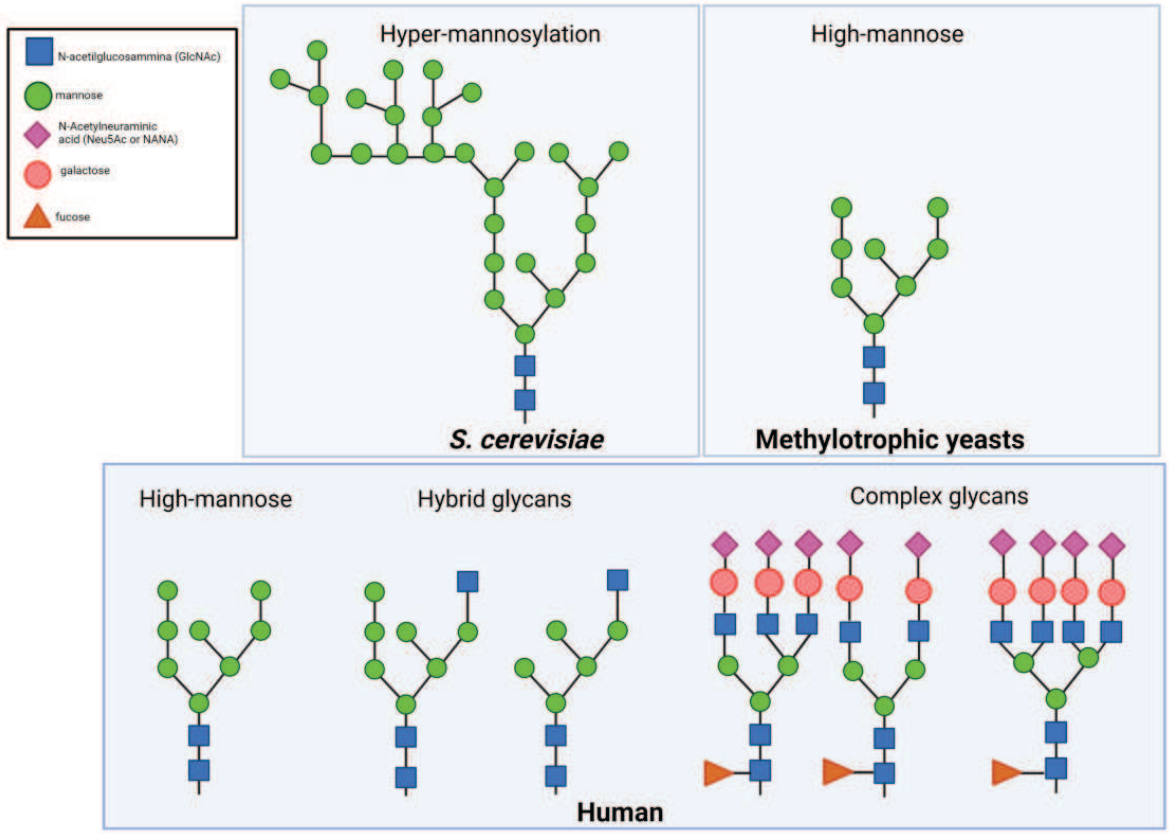

Figure 3. Representative N-glycan subtypes in *S. cerevisiae*, methylotrophic yeasts, and humans. Created with BioRender.com.

Formation of non-enveloped VLPs is more straightforward and occurs in the presence of nucleocapsid proteins. The simplest viruses are those with one type of capsid protein, while the most complex are composed of several proteins [15]. The problematic issue in producing VLPs resembling capsids consisting of multiple proteins is to maintain the native stoichiometry that is dependent on the concentration of each single protein. The expression of capsid proteins is tightly regulated by alternative splicing and translation start sites [24,25]. This condition must be reproduced in the expression system to build

VLPs able to enter inside the cell or stimulate an immune response. These types of virus are efficiently produced in mammalian and insect cells, bacteria, and yeast.

1.2. Critical Points in VLP Production

Purification is crucial in VLP production to ensure suitable efficacy and safety for clinical use. Generally, non-enveloped VLPs are not secreted; therefore, they must be purified from the cells by centrifugation, precipitation, ultrafiltration, and chromatography. For this reason, the solubility of VLPs is crucial. VLPs produced in bacteria may have low solubility due to aggregate formation; this also partially reduces the number of VLPs purified despite the high protein expression level. Another issue to be considered is the safety of the product. As the most significant use of VLPs is their administration as immunogenic drugs, safety issues occur in VLPs produced by bacteria because of endotoxins, and also in VLPs produced in insect cells because the expression of proteins occurs in a Baculovirus that, after VLP purification, has to be inactivated.

When VLPs are produced for commercial use, three other aspects must be taken into consideration: timing, scalability, and cost. During the pandemic, we have learnt how time is crucial to limit the diffusion of a virus. Thus, when a VLP-based drug has to be administered to many people, it is crucial to set up production strategies allowing a high yield of viral-based vaccines in a short time. If we consider that the development of a vaccine requires long phase 3 trials, for which companies must produce a large number of doses without knowing whether a vaccine is going to be efficacious and whether it will reach the market, it is important keeping costs as low as possible by mitigating cost/dose and setting up a flexible and scalable manufacturing platform as well.

Despite the fact that mammalian and insect cells represent the best solution to produce effective VLPs resembling the original virus, they are unsuitable for large-scale production because their manipulation is expensive and their growth rate is elevated and thus difficult to scale up. By contrast, bacteria and yeast culturing is cheap and easy, and their growth rate is short and thus scalable. In Table 1, we summarize advantages and disadvantages of the different systems used to produce VLPs.

Table 1. Comparison of features of the different VLP production platforms.

	Bacteria	**Yeast**	**Mammalian Cells**	**Insect Cells**	**Plant**
Production cost	Low	Low	High	High	Moderate
Type of growth media	Simple	Simple	Complex	Complex	Simple
Growth speed	Very high	High	Moderate	Moderate	Very slow
Production time	Low	Low	High	High	Very high
Expression yield	High	High	Low	Very high	Low
Secretion	No	Yes	Yes	Yes	Not applicable
Enveloped/ non-enveloped VLPs	Non-enveloped	Non-enveloped (enveloped only in some cases)	Enveloped/ Non-enveloped	Enveloped/ non-enveloped	Not applicable
Scalability	Easy	Easy	Very difficult	Very difficult	Difficult
Safety	Safe (possibility of endotoxins)	Very safe	Safe (possible contamination with human viruses)	Very safe	Very safe
Genetic manipulation	Very easy	Easy	Difficult	Difficult	Difficult
Glycosylation of proteins	Very different from humans	Different from humans (can be optimized)	Reproducing human glycosylation	Simpler N-glycosylation pattern compared to mammalian cells	Human-like glycosylation
PTMs	Lack of PTM system	Lack of complex PTM pathway	Complex PTM pathway	Complex PTM pathway	Complex PTM pathway

1.3. 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 [26–29]. 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 [16]. 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 2).

Table 2. 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
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 [30]. 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. 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 [31–33].

They are grouped as non-conventional yeasts such as Pichia pastoris (*Komagataella phaffii*) and *Hansenula polymorpha* (*Pichia angusta*).

This review aims to compare the features of yeast species that are essential for setting up a yeast-based VLP platform.

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 [34]. 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 [35]. Yeast promoters suitable for protein expression can be divided into two main classes: "constitutive" promoters can ensure a stable expression level; in contrast, "dynamic" or "inducible" promoters may drive substantial changes in the expression level in response to environmental stimuli. Strong constitutive promoters that drive high-level transcription are often used for heterologous proteins. These kinds of promoters include promoters of glycolytic genes, such as 3-phosphoglycerate kinase (pPGK1), glyceraldehyde3-phosphate dehydrogenase (pTDH3), triose phosphate isomerase (pTPI1), enolase (pENO2), and alcohol dehydrogenase (pADH1) [36–38]. Dynamic control of gene expression can be implemented by using inducible promoters. The bidirectional GAL1/GAL10 promoter (pGAL1/pGAL10, galactokinase/UDP-glucose-4-epimerase) is induced when yeast cells are grown in the presence of galactose [39], and the CUP1 promoter (pCUP1) can be induced by adding copper (II) [40]. There are also promoters that are induced when glucose concentration is low or absent (low-glucose inducible), including the high-affinity glucose transporter promoter (pHXT7) [38,41] and the alcohol dehydrogenase promoter (pADH2) [37,42]. The commercially available episomal plasmid, pYES2, which carries the promoter pGAL1, the origin of replication 2μ, and URA3 as selection marker, is one of the most commonly used systems to express heterologous proteins in *S. cerevisiae*. Presumably, using an inducible promoter is more appropriate than a constitutive one to avoid toxic effects due to the undesirable misfolding of heterologous proteins; therefore, pGAL1 is widely used in budding yeast to produce VLPs [30]. Plasmid stability is crucial and may profoundly affect protein expression. The endogenous 2μ plasmid has great stability in a complete medium; interestingly, the stability of 2μ derivative vectors in a rich medium strongly depends on the insert size [43]. The main promoters used for the expression of proteins of VLPs are reported in Table 3.

Table 3. Main inducible and constitutive promoters used to produce VLPs in the yeast species.

	Expression Promoters		
Type of Vectors	***Saccharomyces cerevisiae***	***Pichia pastoris***	***Hansenula polymorpha***
Replicative, Inducible	GAL1, GAL10, GAL7, GAL1/10, hybrid GAL10-PYK11, hybrid GAL10-CYC1		
Replicative, Constitutive	GAD, ADH/GAPDH, PGK1, TEF1, ADC1		
Integrative, Inducible		AOX1	MOX, FMD
Integrative, Constitutive		GAP, PGK1	

Replicative vectors may carry ARS or a 2 μ origin of replication. Integrative vectors are usually cut out with specific restriction endonulases and inserted into the genomic DNA of *P. pastoris* or *H. polymorpha*. Most information about the expression systems are taken from [30] and references within. Promoters derived from GAL1, GAL10, and GAL1/10 are inducible by galactose. The hybrid promoters are constructed by the galactose responsive DNA binding-domain and the transcription activation site of cytochrome C1 (CYC1) or pyruvate kinase gene (PYK11). GAL1: Galactokinase, GAL10: UDP-glucose-4-epimerase, GAL7: Galactose-1-phosphate uridyl transferase, PGK1: 3-phosphoglycerate kinase, ADH: Alcohol dehydrogenase, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, TEF1: Translational elongation factor EF-1 alpha, ADC: Arginine decarboxylase, AOX1: Alcohol oxidase, 1: GAP: Glyceraldehyde-3-phosphate dehydrogenase, MOX: Methanol oxidase, FMD: Formato dehydrogenase.

The first VLPs produced in yeast were made in *S. cerevisiae* and were composed of HBV proteins [44]. 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 [45–47]. 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 [48,49]. 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 [50]. 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 [30]. 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 [51]. In addition, VLPs from yeast have also been used for virus research in order to study capsid assembly and related factors [52,53].

Although VLP production in *S. cerevisiae* has several success stories, some drawbacks are still ongoing. Standardized methods to extract VLPs from *S. cerevisiae* are still required for pharmaceutical purposes. In *S. cerevisiae*, 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* has a lower efficiency than other yeasts, including H. polymorpha and P. pastoris, to secrete heterologous proteins [54,55]. However, the secretory pathway of *S. cerevisiae* has been successfully engineered and the secretion efficiency improved [56]. These strains could be useful for VLP production. VLP yield is also influenced by the fact that *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. Other non-fermentable carbon sources, such as lactate, acetate, or glycerol, can also be used by yeast. 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 [57]. Another limitation of using *S. cerevisiae* is the pattern of protein glycosylation that is different from mammalian cells. In this microorganism, N-glycosylation involves adding a high number of mannose sugars (more than 100 mannose residues), which often leads to hyper-mannosylated N-glycans. This modification can result in protein misfolding and prevent its exit from the ER, resulting in the loss of production titers [58,59]. N-linked carbohydrate chains are terminated by mannose attached to the chain via an α1,3 bond, which is allergenic. Synthetic biology, which has been focusing on the engineering of *S. cerevisiae*, could help to improve or optimize the expression level of viral proteins by choosing the most suitable molecular method and/or toolkit that can give contributions to standardizing the methodology. It is well known that synthetic metabolic pathways must be organized in cellular compartments to avoid interactions with other pathways and increase enzyme activity [60,61]. Currently, many toolkits are commercially available to standardize methods and protocols in *S. cerevisiae* and other yeasts [62]. All the standard parts of these toolkits are grouped according to plasmid backbone, promoters, and terminators, and are reported in a publicly accessible catalog (http://parts.igem.org/yeast, accessed on 4 July 2023). This catalog could be useful when VLPs from new viruses or newly identified viral variants must be studied or characterized.

3. *Pichia pastoris*

The history of *Pichia pastoris*, also named *Komagataella phaffii*, is more recent than that of *S. cerevisiae*. Investigations on the possibility of its use for the expression of heterologous proteins started in the early 1990s [63], and greatly increased after the sequencing of the entire genome in 2009 [64]. 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 [65]. Unlike *S. cerevisiae*, *P. pastoris* is a Crabtree-negative yeast, meaning that it metabolizes glucose by complete oxidation to carbon dioxide and water. The fermentative growth, typical of Crabtree-positive yeasts, generally results in reduced yields when yeast is used as a cell factory because it ensures fast growth at the expense of reduced protein synthesis.

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. To avoid diffusion inside the cells of the toxic hydrogen peroxide, this step is performed inside specialized organelles called peroxisomes. 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. The stringent regulation and strong activity of the AOX1 promoter (pAOX1) determine its wide use for recombinant protein production. Most VPLs produced in *P. pastoris* are expressed under the control of pAOX1 [30]. Although the production of heterologous proteins under the control of this promoter is high, and the growth in methanol represents an advantage in terms of costs, the use of large amounts of methanol poses considerable drawbacks, such as the risk related to its handling and storage and high oxygen consumption. To overcome these limitations, novel *P. pastoris* strains have been developed. One example is represented by the mutS (methanol utilization slow) strains that are deleted for AOX1, and exhibit a lower methanol consumption rate [66]. One strain of these has been used for the production of Dengue virus (DENV) VLPs [67]. The DENV capsid is composed by multiple copies of three different structural proteins (C, M, and E) surrounded by the host membrane. Although DENV is an enveloped virus, it has been shown that DENV structural proteins M and E, when co-expressed in heterologous hosts, form non-infectious VLPs [68,69], and that co-expression of protein E (E1 and E2) from two different serotypes results in bivalent VLPs able to elicit an immune response in mice [67].

Expression plasmids with constitutive promoters have been developed to avoid using methanol as a carbon source. The most well-characterized constitutive promoter, considered a reference standard for methanol-free expression systems, is the GAP promoter (pGAP). Additionally, other novel promoters have been developed, such as PDF and UPP promoters (pPDF, pUPP) [70,71]. When pGAP is used to express capsid proteins, yeast growth is more straightforward because no carbon source switch is required, and the hazards and costs related to the storage and delivery of large volumes of methanol are eliminated [72]. Constitutive promoters have been used for VLP production for viruses such as DENV [69,73], Coxsackievirus A16 (CA16) [74], high-level production of Hepatitis B surface antigen (HBsAg), and chimeric HPV-HIVL1P18 VLPs [75].

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 [76]. 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*. Several VLPs containing this signal are efficiently secreted; however, in some cases, pre-pro α-factor can induce the formation of aggregates, causing mislocalization and poor secretion. To address this issue, several attempts focused on modifying the α-factor with mutations, codon optimization, or inclusion of spacer sequences have been performed [77]. It has been shown that the replacement of the α-factor signal sequence with the Ost1 signal sequence, which directs co-translational translocation across the ER membrane, strongly increased the secretion of msGFP in *P. pastoris* [78]. Recently, Duan et al. identified new signal sequences showing higher secretion stimulation than the canonical α-factor [79]. In addition, as protein secretion is a complex process, a secretion library has been created to explore a wide range of secretion constructs [80]. Another strategy identified to improve protein secretion is the overexpression of genes involved in the unfolded protein pathway (UPR), such as Hac1p, chaperons Pdi and Ero1, or the ER lumen chaperone Kar2p, so far applied for the secretion of recombinant proteins and not for VLPs [81].

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) [82]. Secreted VLPs were also obtained for CA16 [74]. In this study, the authors developed a *P. pastoris*-based system for secretory expression of the VLPs for CA16 composed of VP0, VP1, and VP3 capsid subunit proteins. Secreted Cowpea chlorotic mottle virus (CCMV) VLPs were obtained with a yield of approximately 95 mg/L of CCMV and with a purity >90% [83]. Many other VLPs were obtained through secretion, such as VLPs for DENV, the enveloped Tick-borne encephalitis virus (TBEV) [84], HPV16 L1 capsid protein, [85,86] and Chikungunya virus (CHIKV) [87].

For the successful production of VLPs, it is crucial to know the role of glycosylation in virus biology. Viral glycoproteins can have a fundamental role in the enveloped virus assembly, in the entry inside the cells, as well as in the stimulation of the immune response. Thus, glycosylation must be taken into consideration during the design of the strategy of VLP production [88]. 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 [89]. Glycosylated VLPs inducing a potent immune response in mice were produced in *P. pastoris* for hepatitis E virus (HEV) [90]. The viral capsid is formed by the ORF2 protein harboring three glycosylation sites (Asn137, Asn310, and Asn562), with Asn310 considered the major site for glycosylation. The significance of the glycosylation of ORF2 of HEV is still not clear; however, it has been observed that mutations in the first two glycosylation sites prevent virion assembly, whereas mutation of the third site still allows virion particle formation and RNA encapsulation [91]. Gupta et al. demonstrated that the protein expressed in *P. pastoris* is glycosylated and can form VLPs inducing a potent immune response in mice [90].

The envelope protein prE of DENV is glycosylated. The glycosylation of this envelope protein occurs at Asn67 and has an important role in the transmission of DENV. Mutations in Asn67 glycosylation significantly suppress the replication of DENV and make the virus unable to produce new infectious particles [92]. Proper glycosylation of prE in *P. pastoris* has been shown for DENV-1 [93], DENV-2 [94], DENV-3 [95], and DENV-4 [96]. prE-proteins

expressed in *P. pastoris* were glycosylated and formed VLPs inducing an immune response in mice.

In order to create *P. pastoris* strains with a glycosylation pattern similar to mammalian hosts, several studies of genetic modifications of *P. pastoris* have been performed [97]. As a result, the Pichia GlycoSwitch system has been created (https://pichia.com/glycoswitch/, accessed on 4 July 2023). The Pichia GlycoSwitch® System consists of patents, strains, and vectors that are useful for engineering *P. pastoris* to make proteins with several near-homogenous glycoforms. This system can be beneficial for expressing glycosylated viral proteins that are not active with glycosylation typical of yeast.

4. *Hansenula polymorpha*

Hansenula polymorpha (*Pichia angusta*) belongs to the facultative methylotrophic yeast species group. Three main *H. polymorpha* strains are usually used for biotechnological purposes: CBS4732, first isolated in irrigated soil in Brazil in 1959; DL-1 also, isolated in the soil in 1973, and NCYC495, first isolated in spoiled orange juice in Florida in 1951 [98]. As with *P. pastoris*, it can grow at higher rates than *S. cerevisiae* on simple and defined media, leading to highcell-density fermentation. 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 [99].

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 down-regulation 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 [100]. 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 [101]. If the proteins of the viral capsomer are controlled by pFMD or pMOX, the expression can be easily controlled by changing the carbon source in the media. A methanol-inducible protein can be produced via two-step cultivation: a glycerol batch phase and a methanol/glycerol-fed batch phase (where the feed solution can be added continuously or in pulses). Adding glycerol directly in the first growing step allows us to skip the de-repression fed batch phase and to speed up the production. As for *P. pastoris*, the strong and constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (pGAP) is used for heterologous gene expression. The pGAP has a carbon-source- and phase-dependent activity that must be considered to develop an efficient fermentation process [102].

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 [103], although other techniques are also widely used, such as the lithium acetate-dimethyl sulfoxide method [104] and electroporation (with the linearized vector) [105]. 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 [106]. Therefore, episomal vectors are not the most suitable solution for heterologous gene expression in *H. polymorpha*. Another bottleneck in the genetic manipulation of *H. polymorpha* is the low frequency of homologous recombination (HR) after a double-strand break (DSB); this feature limits the exploitation of homologous integration of a foreign DNA fragment. To solve this problem, a recombinant strain knocked out for the *YKU80* gene, coding for a protein involved in the nonhomologous end joining (NHEJ) process after a DSB, was constructed. Consequently,

this *H. polymorpha yku80*Δ strain has a diminished probability of random integration but maintains other physiological characteristics [107]. The *H. polymorpha* integration plasmids (pHIP) are the most commonly used tool for heterologous gene expression. Many pHIPs differ in terms of the selective marker and the promoter (https://www.rug.nl/research/molecular-cell-biology/research/the-hansenula-polymorpha-expression-system, accessed on 4 July 2023) [108]. 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* (Figure 3) [109]. VLPs composed of glycosylated proteins have been effectively produced in *H. polymorpha* [110–112]. A recombinant strain carrying a deletion in two main yeast genes belonging to hyper-mannosylation pathways, α-1,6-mannosyltransferase (Δ*hpoch1*) and α-1,3-mannosyltransferase (Δ*hpalg3*) was constructed in order to produce human hybrid-type N-glycans. This strain is able to express the human gene encoding β-1,2- N-acetylglucosaminyltransferase I (GnTI), an enzyme that has a key role in glycosylation [113].

In the last decade, *H. polymorpha* has been widely studied for VLP production, leading to interesting results which we will summarize below. Moreover, *H. polymorpha*-derived VLPs are already used for FDA-licensed and commercially available vaccines. Specifically, Hepavax-Gene® (Greencross Vaccine Corporation, Seoul, Republic of Korea, http://dra.gov.bt/wp-content/uploads/2016/09/Hep-B-Vaccine-SPC.pdf, accessed on 4 July 2023) and Heplisav-B® (Dynavax Technologies Corporation, Berkeley, CA. USA) https://www.fda.gov/vaccines-blood-biologics/vaccines/heplisav-b (accessed on 4 July 2023) are commercially available vaccines for HBV.

Most common commercially available HBV vaccines are composed of the antigen HBsAg, but it has been demonstrated that the medium envelope protein PresS2-S can elicit a neutralizing response. Consequently, Xu et. al., expressed a codon-optimized truncated form of PreS2-S (26 amino acids of PreS2 C-terminus) fused to the N-terminus of HBsAg in *H. polymorpha*, under the control of pMOX in an integration plasmid, resulting in stable multiple copy insertions (an average of 60 copies). PreS2-S accumulated intracellularly, and was purified by cell disruption. They obtained morphologically uniform particles with ~99% of purity, more than that obtained with *P. pastoris* [114,115].

More recently, Wetzel et. al., used *H. polymorpha* to produce chimeric VLPs presenting four antigens from animal-infectious viruses. In particular, the membrane small surface protein (dS) of duck hepatitis B (DHBV) was selected as a scaffold for VLPs containing antigens derived from four animal-infecting viruses. The antigens were genetically fused to the dS and expressed under the control of pMOX of a stable genomic integrated plasmid. Chimeric VLPs with 95% purity and containing up to 33% of the fusion protein were obtained with a production efficiency of 40 mg of VLPs per 100 g of dry cell weight [116]. The same platform was used to develop three different types of chimeric VLPs, each displaying a different foreign antigen derived from *P. falciparum* [112].

Interestingly, *H. polymorpha* has successfully produced two-component human parvovirus B19 VLP with proteins in a different stoichiometry. The capsid of B19 is composed of two types of protein, the major capsid protein VP2 and the minor protein VP1. Specifically, VP1 and VP2 were co-expressed in *H. polymorpha* from two plasmids where they were cloned under the control of pMOX. Furthermore, another methanol-inducible expression promoter (pDAS) was adopted to improve the relative proportion of VP1 in the VLPs. Thereby, the authors produced two types of VLPs—the VLP with equally assembled VP1 and VP2 (VP1/VP2 VLP), and the VLP with an improved VP1 content (VP1h/VP2 VLP)—and demonstrated that VLPs with an increased VP1 content elicit a higher level of neutralizing antibodies against B19 than VP1/VP2 VLP [117]. As for the other yeast species, the bottleneck in producing VLPs is the purification method because viral proteins accumulate intracellularly [118]. *H. polymorpha* can be engineered to modulate the ER folding environment and, therefore, to secrete a heterologous protein [119]. The purpose of the study was to produce a recombinant truncated G protein (a glycoprotein generally localized in the envelope of the Rabies virus) to use it as a coating antigen in ELISAs and

thus obtain an inexpensive diagnostic tool. They expressed the protein in a *H. polymorpha* strain overexpressing calnexin, a key component of the quality control mechanism in the ER, shown to enhance the secretion of all types of heterologous protein [120]. A heterologous protein's secretion has also been performed with the leader sequence of the α-factor [102]. 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.

5. Comparison of VLP Production in the Three Yeast Species

In order to establish which is the best yeast to choose for developing a new VLP product, it is not easy to compare results reported in different publications. In most cases, VLPs produced in other yeast species can be composed of the same viral proteins, but also of different viral proteins. For instance, HEV VLPs, composed of the same viral protein, were produced in the three yeast species, with the only difference being that in *P. pastoris* were secreted; thus, the purification protocol was more straightforward than that used in other systems. Moreover, VLPs composed of different viral proteins have successfully been produced in *S. cerevisiae* and *P. pastoris* (Table 4).

Table 4. Summary of VLPS produced in more than one yeast species.

	Type of Antigens		
Virus	***S. cerevisiae***	***P. pastoris***	***H. polymorpha***
Hepatitis E virus	-HEV-3: full length capsid protein (ORF2, aa 1–660); 5′ and 3′ terminally truncated capsid protein (ORF2, aa 112–608) -Rat HEV: full length capsid protein (ORF2, aa 1–645); 5′ and 3′ terminally truncated capsid protein (ORF2, aa 112–608) [121]	ORF2, aa 112–608 [90]	ORF2, aa 112–607 (HEV genotype IV) [122]
Hepatitis B virus	-Surface protein (HBsAg) [123,124] -Viral M [preS2 + S] [125] -Wild-type and mutant C69R S genes [126]	-Surface protein (HBsAg) [115,127–129] -Core protein (HBc) [130,131]	-26 amino acids of PreS2 C-terminus (aa 120–145) [114]
Hepatitis C virus		-Core protein (HBc) [132,133] -CoreE1E2 protein, which consists of Core (269 nt–841nt) E1 (842 nt–1417nt), and E2 (1418 nt–2506nt) [134–138]	E2 Core Domain [111]
HPV 16, 11, 6, 58	Human papillomavirus major capsid protein L1 [139–144]	-Human papillomavirus major capsid protein L1 [85,86,145–149]	
Coxsackievirus	Capsid protein precursor P1 and the protease 3CD of Coxsackievirus A16 [150]	-Capsid protein precursor P1 and the protease 3CD Coxsackievirus A16 [74,151] -Capsid protein precursor P1 and the protease 3CD of Coxsackievirus A10 [152]	
Poliovirus	Capsid precursor protein P1 and the protease 3CD [153]	Capsid precursor protein P1 and the protease 3CD [154,155]	
Red-spotted grouper nervous necrosis virus	Capsid protein [156]	Capsid protein [157]	
B19 parvovirus	Minor-capsid protein VP1 and the major-capsid protein VP2 [158–160]		Minor-capsid protein VP1 and the major-capsid protein VP2 [117]

Table 4. *Cont.*

	Type of Antigens		
Virus	***S. cerevisiae***	***P. pastoris***	***H. polymorpha***
Bacteriophage Qβ	Coat protein (CP) [161]	Coat protein (CP) [161]	
Phage PP7 φCb5; Phages fr Phage SP	Coat proteins [161]	Coat proteins [161]	
Rotavirus		Capsid protein VP6 [162]	Capsid protein VP6 [162]

Some studies have focused on the comparison between yeast strains and *E. coli*. A yeast-derived VLP composed of recombinant bacteriophage Qβ coat protein (CP) was found to be as immunogenic as that produced in *E. coli*. Moreover, Qβ-VLP assembled in *S. cerevisiae* and *P. pastoris* were about 15–20% and 20–30% of the *E. coli* expression level, respectively [161]. The same group investigated the possibility of producing VLPs composed of other bacteriophages' CP proteins in yeast *S. cerevisiae* and *P. pastoris*. They compared the expression levels in *S. cerevisiae* and *P. pastoris* using episomal and integrative vectors, respectively. They observed that the amount of target protein varied significantly between strains. Specifically, efficient *P. pastoris* producer strains were selected for phage PP7 and φCb5 CPs; slightly better production of CP phages fr and SP was observed in *S. cerevisiae*. The solubility of most CP proteins was comparable in the two systems with exception of phage SP CP, which was completely insoluble when expressed in *S. cerevisiae* and partially soluble in *P. pastoris*. In another study, the expression level of rotavirus VP6 protein in *P. pastoris* and *H. polymorpha* was compared with that reached in *E. coli*. Codon optimized VP6 was expressed intracellularly under the control of methanol-inducible promoter. Results indicated that the highest VP6 concentration was obtained with *E. coli*; however, the expressed protein was found to be insoluble and consequently difficult to purify. On the other hand, in shake flasks, specific production of VP6 was approximately similar in both yeasts. The authors concluded that, when growing yeast cells in bioreactors, *H. polymorpha* outperforms both *P. pastoris* and *E. coli* [162]. In a recent study, *P. pastoris* and *H. polymorpha* were used to assemble VLPs of HPV. The level of L1 protein, expressed under the control of a methanol-inducible promoter, and production efficiency were both higher in *P. pastoris* than in *H. polymorpha* [163].

Overall, these studies suggest that performing VLP expression simultaneously in at least two species could facilitate identifying the best production platform. These studies also confirm that yeast can be a valuable tool in the production of VLPs to be used as antigens for vaccine development. Moreover, yeasts could be a potentially valuable system to generate VLPs to be used as a carrier for drug delivery, a strategy so far poorly explored [22,83]. It is a matter of setting up the best conditions. So far, these kinds of particles are mainly produced in *E. coli* and higher eukaryote platforms and are often assembled "in vitro".

The key question is what yeast species might be the best to produce VLPs. In Figure 4, we compare the key features to consider when planning a platform for VLP production for the three species. *H. polymorpha* has the advantage of growing at 37 °C, a condition that can be crucial for correct protein folding. On the other hand, secretion, which is important to facilitate purification, has been poorly investigated in *H. polymorpha*, so further studies are necessary to exploit this feature in this species.

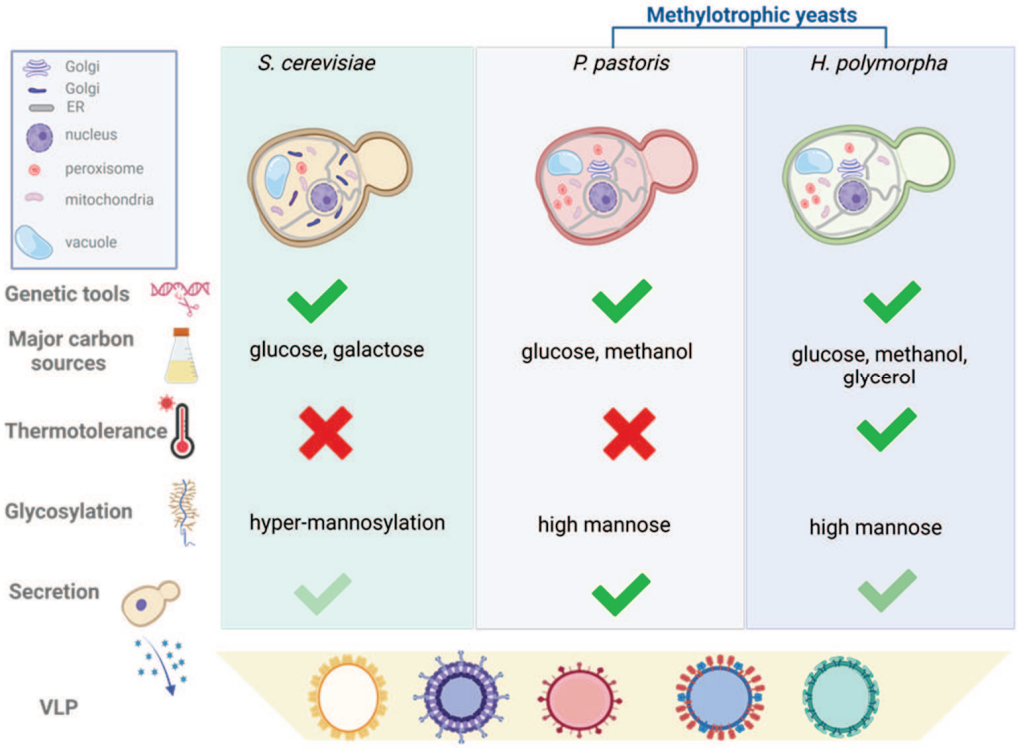

Figure 4. Comparison of yeast manipulation tools, features, and parameters to be considered for the selection of the yeast species for VLP production. Yeast cells of *S. cerevisiae*, *P. pastoris*, and *H. polymorpha* are represented with the organelles to highlight the structural differences. The Golgi apparatus of *S. cerevisiae* is organized in cisternae scattered throughout the cytoplasm; while in methylotrophic yeasts, the 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 the presence of methanol. Genetic tools, such as mutant strains, plasmids, and glycosylation humanized strains have been developed for the three species. Carbon sources indicated in the figure are those preferentially used for heterologous protein expression; thermotolerance refers to the ability to grow at a 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.

6. Conclusions

The SARS-CoV-2 outbreak highlighted the need to increase our knowledge of the vaccine production platform in response to the spread of the emerging virus. The yeast expression system has the same advantages as bacteria, in terms of growth rate and costs, and mammalian cells, because the proteins produced are safe and correctly folded.

So far, twenty-nine VLP-based vaccines have reached the market or have progressed to clinical trials [16]. Among them, ten were produced in yeast. In this analysis, we did not consider a VLP-based vaccine against HIV since it did not pass phase I clinical trial because it could not induce a significant humoral and cellular immune response [164,165]. Nine vaccines were produced in insect cells, six in bacteria, and only one in mammalian cells. Interestingly, eight out of nine yeast-based VLP vaccines were approved by the FDA for commercial use, suggesting that safety and costs have a high impact on the selection of the platform for VLP production.

In conclusion, the following aspects should be considered to set up a yeast-based VLP platform:

- The number of proteins to be expressed. Some proteins must be in a suitable ratio to assemble in functional VLPs;
- Primary assessment of heterologous protein expression. The evaluation of the effect of the protein is crucial; toxicity should be avoided; therefore, several mutants (protease deficient, high temperature tolerant, secretory efficient) should be considered;
- Inducible promoters and episomal plasmids are preferable; usually, high efficiency of DNA transformation is required. For this reason, *S. cerevisiae* and *P. pastoris* strains could be more suitable;
- Plasmid stability. In general, in *S. cerevisiae* and *P. pastoris*, episomal or centromeric plasmids are more stable than in *H. polymorpha*. In *H. polymorpha*, plasmids must be integrated into the genome to achieve high stability. Therefore, if the expression of the heterologous proteins confers toxicity when expressed in high copy number, the use of *H. polymorpha* expression system which consists of one single stable integrated copy of the gene is preferable.

The rationale behind the choice of the production strategy depends on the endpoint application of VLPs and yeast strains thanks to the available tools; its flexibility can be tailored to the type of VLPs. Therefore, we can conclude that VLPs, especially non-enveloped VLPs, and yeast are a "perfect couple".

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