Advantages of yeast-based recombinant protein technology as vaccine products against infectious diseases

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Abstract. The yeast expression system is widely used to produce functional recombinant proteins in the biopharmaceutical industry, such as vaccine products. The expression system choices using yeast as the host has many advantages. Various vaccines have been produced commercially using yeast expression systems. This review aims to explore the advantages of the yeast expression system in *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Hansenula polymorpha*, which emphasize vaccine products to prevent human infectious diseases. Selection of the appropriate expression system is carried out by identification at the genetic and fermentation levels, taking into account host features, vectors and expression strategies. We also demonstrate the development of a yeast expression system that can produce recombinant proteins, virus-like particles and yeast surface displays as a novel vaccine strategy against infectious diseases. The recombinant protein produced as a vaccine in the yeast system is cost-effective, immunogenic, and safe. In addition, this system has not introduced new microbe variants in nature that will be safe for the environment. Thus, it has the potential to become a commercial product used in vaccination programs to prevent human infectious diseases.

Keywords: vaccine, expression system, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*

1. Introduction

Every living organism needs proteins that will play a role in the cell cycle, cell signaling, inflammatory reaction and cell adhesion [1]. Many diseases in humans are caused by dysfunction of secretory proteins that can cause cell death [2]. Genetic and protein engineering is used to produce commercial proteins [1]. One of the hosts often used as a protein production system is yeast. The important biopharmaceutical products which produce in the yeast system are commercial vaccines against various diseases [3]. Yeasts are prominent eukaryotic hosts that produce functional recombinant proteins because of some advantages. The advantages are the ability to carry out appropriate post-translational modifications, easier genetic manipulation, produce high biomass, fast growth, free of pyrogen, and potentially express complex heterologous eukaryotic protein [3]. In addition, yeasts are able to modify in the extracellular medium, which is more efficient to reduce downstream purification costs [2]. Currently, there are most common yeasts that are used for recombinant protein expression systems, such as *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Hansenula polymorpha*. This review aims to explore those advantages of yeast-based technologies used for vaccine production that prevent human infectious diseases.
2. Principle of Yeast-based Recombinant Protein System

Proteins are the biomolecules with various functions in every organism's life, such as in biological roles, for research, therapy or disease prevention. However, the amount of protein in nature cannot meet the needs of these various functions, especially in the development of research that requires certain proteins with high purity and sufficient quantities in a short time and low cost producing. Recombinant protein is a synthetic protein that is engineered by genetic modification which is encoded by recombinant DNA and expressed in a system that supports the transcription and translation of that recombinant DNA into a commercialized-potential protein. Recombinant DNA is modified in order to control the target promoter for high level expression of target protein in choosing expression system [4]. Selecting the appropriate expression system is a key to successfully recombinant protein expression. It is considered by several factors such as target protein property, target application, protein yield, cost, and post-translational modification ability. The location and molecular weight are important things that should also be considered [5]. Proteins are most risky for denaturation caused by several factors, such as salt concentration, temperature changes, pH, organic solvents presence, surface and interface interactions that can form protein aggregates at lyophilization. Therefore, the genetic engineering process must be carried out carefully to produce a complete and functional protein [1].

The main method to produce recombinant protein is to transfer the vector that inserted the target DNA fragment to the host cell. *E. coli* (prokaryotic system) as a bacteria cell is the earliest used and most mature. *E. coli* has some abilities such as fast-breeding, easy and low-cost purification with high level expression, easy-to-modify genetic background, stable and broad range of application. But the prokaryotic system often expresses the incorrectly folded proteins in the cytoplasm and makes an insoluble protein aggregate, which is called inclusion bodies that causes a difficult purification process. Unfortunately, the post-translational modification process of prokaryotic is imperfect. Thus, a more sophisticated system is also being developed to allow impossible protein expression, such as glycosylated protein [6–8].

Yeast are eukaryotic cells that can be utilized as a heterologous recombinant protein expression system. It has been commonly used in genetic engineering since its complete gene sequence was released in 1996. It is considered a gripped creature and does not produce any FDA-approved toxins. In general, yeast is classified into two groups, namely non-methylotroph and methylotroph yeast. *Saccharomyces cerevisiae* (non-methylotroph yeast), *Pichia pastoris*, and *Hansenula polymorpha* (methylotroph yeast) are often used as a host for recombinant protein production [3]. One example of recombinant proteins utilization is vaccine products for infectious diseases prevention. The methylotrophic yeast is used widely nowadays because it has prokaryotic properties that can use the C1-compounds (such as methanol) to obtain energy and methylamine as a nitrogen source [3, 9]. The general principle of the recombinant protein production process is shown in Figure 1. It begins with constructing interest genes into recombinant plasmids to protein expression processes, including upstream processes. The protein purification and protein characterization including downstream processes [1].

![Figure 1. General process for production of recombinant protein. Adapted from Yunqi Ma et al. 2020 [1].](image-url)
The concept of recombinant protein production in the yeast system is according to the central dogma. The selected vector was cloned with the expressed target gene. Gene was transformed into the appropriate yeast cells using electroporation. Then, the transformants were plated in the medium and cultivated on a small scale. Cells that have high protein expression can be further produced on a larger scale. The cultivation process depends on the energy source provided through the culture medium [10]. The non-methylotroph yeast uses a fermentation system to obtain energy. Thus, its use as a recombinant protein production system needs to consider the source of glucose, the effect of pH, amount of dissolved oxygen, temperature, and osmolarity in the culture medium. It is known that decreasing the cultivation temperature can increase the productivity of Fab antibody fragments secreted by S. cerevisiae threefold in the cultivation of glucose-based bioreactors [8].

The expression system regulation in methylotroph yeast depends on the alcohol oxidase (AOX) promoter expressed by methanol induction. A high yield of heterologous protein can be obtained using this system. The AOX1 gene promoter is a very strong promoter that is tightly regulated by the carbon source present in the medium and is strongly inhibited by glucose and glycerol. But it is significantly induced when methanol is present as the sole carbon source. These characteristics enable P. pastoris to become a highly preferred strain for regulated bioreactor production. It can also be used as an efficient expression system for heterologous proteins. The methanol induction activates the transcription factors Mxr1 and Prm1. Both proteins will activate AOX1. AOX1 activation is also enhanced by Prm1 autoactivation and Mit1 activation by Prm1. Mit1 is the third transcription factor that activates AOX1. AOX1 expresses the alcohol oxidase enzyme, which enables the metabolism of methanol to produce energy. Methanol will be oxidized by alcohol oxidase, which resulting formaldehyde occurs in the peroxisome. Formaldehyde will enter the mitochondria and is a substrate source to produce energy in the Krebs cycle. Formaldehyde can also enter the pentose phosphate pathway via Xylulose 5 phosphate (Xu5P) to produce ribose as a backbone of nucleic acid (Figure 2) [9, 11, 12].

![Figure 2. Scheme of common methanol metabolism in methylotroph yeast. Adapted from Gaffar, 2010; Hiroya et al, 2011; Wang et al, 2016 [9, 11, 12].](image)

Two phenotypes can be expressed by methylotroph yeasts based on AOX regulation, namely MUT\(^+\) and MUTs. The MUTs imply the strain that can use methanol at a slower rate than MUT\(^+\) that use it at regular rate. The presence of AOX gene that is integrated into the genome of yeast plasmid determines the formation of those phenotypes. It is influenced by the feeding strategy and methanol induction in the medium. The MUT\(^+\) strain had a growth rate of 0.15 h\(^{-1}\) in methanol, while the maximum growth of
MUTs was 0.03 h\(^{-1}\). The optimized growth rates are in the range of 0.03-0.08 h\(^{-1}\), which can produce recombinant protein products up to 0.69 mg.h\(^{-1}\) [13].

3. *Saccharomyces cerevisiae* expression system

*Saccharomyces cerevisiae*, the non-methylotroph yeast, was first used as an expression host in the 80's in conventional biotechnology or fermentation such as wine and brew making. After the whole genome sequencing was discovered, it is often used as an expression system because of the broad range of pH tolerance and the elevated osmotic pressure resistance. In addition, it was used historically in nutrition industries at first, which claimed safeness [5, 14]. Other advantages of the *S. cerevisiae* system are scalable, able to perform most eukaryotic post-translational modification, protein folding, good expression level, endotoxin-free, and low-cost production [3].

One of the vaccine products that has been commercialized is called hepatitis B vaccine generation 2, which is produced from recombinant hepatitis B surface antigen protein using the *S. cerevisiae* system. Previously, the hepatitis B vaccine was produced from asymptomatic hepatitis B carrier patient's plasma, but there are limited supply and safety issues [15]. Because of those advantages, there are many vaccines developed using this system (Table 1). However, the glycosylation process is sometimes not suitable for O-linked oligosaccharides, including up to 200 mannose additions that occur in this process. This modification can result in protein misfolding and inhibit the release of proteins from the endoplasmic reticulum, reducing their production. In addition, an over-glycosylation N-linked site will cause decreased activity and receptor binding, which may trigger immune reactions [1, 8].

| Used strain | Antigen target | Targeted disease | Used vector | Expression strategy | References |
|-------------|----------------|------------------|-------------|--------------------|------------|
| 2085        | Dengue envelope domain III (scEDIII) | Dengue virus | pYEGLTB, pUC19 | Purified protein | [16]       |
| W303-1B     | HBsAg          | Hepatitis B virus | pYepDP1-8 | Virus-like particle (VLP) | [17]       |
| RAY3A-D     | Gag protein    | HIV-1/AIDS       | pRS423      | Virus-like particle (VLP) | [18]       |
| S. cerevisiae cell line VK1 | MSP3 (merozoite surface protein 3) | *P. falciparum/Malaria* | pYEpRPEU-3 | Purified protein | [6]        |
| EBY100      | Microneme protein 16 | *T. gondii/Zoonosis toxoplasmosis* | pCTCON2 | Yeast surface display | [19]       |
| W303α       | Rv1738, Rv2032, Rv3130, and Rv3841 | *M. tuberculosis/Tuberculosis* | pGI-100 | Purified protein | [20]       |

However, some disadvantages include not being suitable for high-density culture, lacking strong and strict regulation promoter, the glycosylation still different with mammalian, a tendency to hyperglycosylated protein, allergenic N-glycan structure, and low secretion efficiency above 30 kDa proteins [6, 21]. These limitations have resulted in developing alternative expression systems, including the methylotrophic yeasts such as *Pichia pastoris* and *Hansenula polymorpha*. 
4. **Pichia pastoris expression system**

*P. pastoris* was used first-time to produce Kallikrein inhibitor (Kalbitor) recombinant protein and approved by FDA in 2009 as potential host for biopharmaceutical production [22]. The advantages of *P. pastoris* system are simple culture condition with rapid scalable growth and high-level expression, low cost with extensive post-translational modification of proteins and protein folding, N-glycosylation higher eukaryotes than *S. cerevisiae*, and endotoxin-free. *P. pastoris* is well suited for expressing the disulfide-rich such as recombinant dengue envelope antigen and hepatitis B surface antigen. The resulting recombinant protein will be free of pyrogens, toxins, and viral inclusions because *P. pastoris* is a non-pathogenic organism [23, 24]. Furthermore, *P. pastoris* can secrete heterologous protein recombinant directly into the culture medium to facilitate purification in a downstream process. The yield is produced in the amounts of milligrams to grams [15].

The recombinant protein-based vaccine product in the *P. pastoris* expression system that has been used commercially is the hepatitis B vaccine. However, there are many vaccines developed using this system (Table 2). Recombinant protein expression can also be increased by utilizing multicopy transformants. For example, Hepatitis B has eight copies number expression cassettes [25]. The recombinant protein gp350 Epstein-Barr Virus used extracellular expression using the pPICZαA vector and the host strain *P. pastoris* GS115. However, because a bias codon in *P. pastoris* affects the expression of recombinant proteins, codon optimization was carried out on the N-terminal fragment of gp350 to increase the expression level [26]. The main disadvantages of *P. pastoris* are that methanol as an inducer has fire hazards, and glycosylation is still different from mammalian cells.

| Used strain | Antigen target | Targeted disease | Used vector | Expression strategy | References |
|-------------|----------------|------------------|-------------|---------------------|------------|
| GS115 (his4) | Hepatitis B surface antigen (HBsAg) | Hepatitis B | pAO815 | Virus-like particle (VLP) | [25] |
| GS115 (his4) | Non-structural 1 dengue serotype 1 | Dengue | pPICZαA | Recombinant protein | [27] |
| PichiaPink™ Strain 1 (Invitrogen) | Receptor-binding domain (RBD) | SARS-CoV-2 | pPink-a-HC | Recombinant protein | [28] |
| GS115 (his4) | gp350 EBV | Epstein- Barr Virus | pPICZαA | Recombinant protein | [26] |
| KM71H | Alkyl hydroperoxide reductase (AhpC) | Helicobacter pylori infection | pPICZA | Recombinant protein | [29] |
| GS115 (his4) | Gag protein | HIV type 1 | pHILS1 | Recombinant protein | [30] |
| GS115 (his4) | NLP-CSPCT, NLP-CSPR | Malaria (Plasmodium vivax) | pPIC9K | Recombinant protein | [31] |

5. **Hansenula polymorpha expression system**

The recombinant hepatitis B vaccine is the first commercialized products that express in *H. polymorpha* system. The hepatitis B virus S antigen (HbsAg) was formed into yeast-derived lipid membranes that are essential for the antigenicity properties of the HBsAg [32]. The methanol induction
is used to level up the recombinant proteins product and support the lipid membrane formation [32]. *H. polymorpha* has three main strains for basic research and biotechnology applications, namely CBS4732, DL-1, and NCYC495. It can use methanol as the only source of carbon and energy [33]. It has multicopy system integrase ability and a strong induction promoter. Thus, it has become a relatively popular host for heterologous protein production. Another advantage is the thermotolerant feature that allows ethanol fermentation at high temperatures. Xylose fermentation into ethanol can be carried out at high temperatures (48-50°C) [33].

Plasmids were established for phytase production in CBS4732-based strains containing pFPMT121 and a derivative of pMPT121 for anticoagulant hirudin production at industrial scales. The DL-1 strain as a recombinant host has plasmids (AMIpLD1, AMIpSU1, and AMpL1) that produce multiple copy numbers based on auxotrophic complementation. The *H. polymorpha* DL1 strain can be changed by selecting plasmids with different selectable markers for construction and transformation. The use of these plasmids allows generating transformants with plasmids integrated into low, moderate (<10), or high (up to 100) copy numbers depending on the host mutant strain [3, 34]. The vaccines which developed using this system shown in Table 3.

| Used strain | Antigen target | Targeted disease | Used vector | Expression strategy | References |
|-------------|----------------|------------------|-------------|--------------------|------------|
| DL-1        | HPV52L1        | Human Papilloma virus/cancer | pMOX-HARS | Virus like particle | [35]       |
| NCYC495     | HPV16L1-L2     | Human Papilloma virus/cancer | pHIPX4-HNBESX | Chimeric protein | [36]       |
| NCYC495     | sHBsAg         | Hepatitis B/Hepatitis | pHIPX4 | Purified protein | [37]       |
| ALU3        | Circumsporozoite protein | *P. falciparum/Malaria* | pFPMT121 | Virus like particle | [38]       |

### 6. The role of yeast system expression for vaccine products in disease control and environmental health

Disease control methods in the environment are an important concern, especially for diseases transmitted through vectors, such as mosquitoes. *Flavivirus* is a common genus that uses mosquitoes as vectors, such as Dengue virus, Japanese encephalitis virus and Yellow fever virus. In addition, the malaria parasite is also a disease that is transmitted through mosquitoes. In Indonesia, dengue infection is still one of *Flavivirus* that causes health problems. Dengue infection can cause mild to severe clinical manifestations with stages of dengue fever, dengue haemorrhagic fever (DHF), and dengue shock syndrome (DSS). Since 2011, dengue infection has spread in Indonesia, with the four serotypes of DENV also circulating [39].

Environmental control through vector control programs has not been able to prevent dengue outbreaks, which usually appear at the beginning and end of the rainy season in urban areas. In 2016, the number of DHF cases was 204,171 (incidence rate 78.85/100,000 population) with 1,598 deaths (CFR 0.78%). Most cases were found in West Java Province, followed by East Java and Bali. The government's environmental control program to prevent dengue infection is known as eradicating 3M Plus mosquito nests, which consist of draining and closing water reservoirs, recycling used goods, repairing clogged waterways, and preventing ways to minimize mosquitoes larvae breeding places [39–41].

The other alternative for dengue prevention is vaccination. The commercially available dengue vaccine is Dengvaxia with a live attenuated chimeric recombinant technology platform. It uses Yellow fever virus as a backbone with pre-membrane envelope gene substitution from the four serotypes of DENV [42]. Although, there are still no reported cases of Yellow fever virus infection in Indonesia, new surveillance is needed to prevent virulence in recombinant live attenuated vaccines. Therefore, the
Yellow fever virus as the vaccine backbone can introduce new variant viruses in the environment and become virulent, while the wild type of DENV still has not been controlled yet. The use of yeast expression systems to produce recombinant protein vaccines that we discussed previously have many advantages. In addition, this system does not use viral vectors, thus it will be safer for humans and environmental health.

7. Conclusions
The production of yeast-based recombinant proteins for various vaccines has been developed over the last two decades. Different types of vectors and host strains for protein expression have been developed, and the most commonly used yeast systems are *S. cerevisiae*, *P. pastoris* and *H. polymorpha* systems. One example of a vaccine that has been successful and can produce an optimal immune response is a recombinant protein vaccine for Hepatitis B. However, further optimization is needed for protein expression and secretion depending on the required interest product. This expression system is non-pathogenic, free of pyrogen, unlike the *E. coli* system with a production method that is easier to handle than the expression system in mammals. Moreover, the yeast system has post-translational modification ability so the resulting protein becomes functional. The media used for protein production is also cheaper, so it becomes cost-effective. The glycosylation process significantly affects the half-life and function of proteins that need to be efficiently controlled. Certain host strains such as *P. pastoris* have been attempted to produce humanized glycosylation of recombinant proteins that can be used for disease therapy. Yeast expression systems can produce recombinant protein, virus-like particles, and yeast surface display which can be developed into oral vaccines as a new strategy. The protein antigens expressed in the yeast system were also stable for up to 1 year at 2-8°C storage and at 23-25°C in the stationary phase yeast cell. In conclusion, the yeast expression system has good prospects in the future for vaccine products because it is safe, non-pathogenic, cost-efficient, easier, more stable handling, and can be developed into a new vaccine in the form of an oral vaccine. In addition, recombinant protein vaccines will be safer for humans and the environment than live attenuated chimeric recombinant vaccines because they do not introduce new viral or bacterial variants or parasites in the environment.

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Author’s contributions
Christina Safira Whinie Lestari and Gissi Novientri have contributed equally to this work.

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