

COMMENTARY

## Recent advances in the production of recombinant subunit vaccines in *Pichia pastoris*

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### ABSTRACT

Recombinant protein subunit vaccines are formulated using defined protein antigens that can be produced in heterologous expression systems. The methylotrophic yeast *Pichia pastoris* has become an important host system for the production of recombinant subunit vaccines. Although many basic elements of *P. pastoris* expression system are now well developed, there is still room for further optimization of protein production. Codon bias, gene dosage, endoplasmic reticulum protein folding and culture condition are important considerations for improved production of recombinant vaccine antigens. Here we comment on current advances in the application of *P. pastoris* for the synthesis of recombinant subunit vaccines.

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### Introduction

*P. pastoris* has become a highly successful expression system, due to its increasing popularity, which can be attributed to several factors such as high growth rate, the ease of genetic manipulation, high yield expression of heterologous proteins and the capability of performing eukaryotic post-translational modifications.<sup>1,2</sup> The *P. pastoris* system is much easier to perform and cheaper in cost compared with bacterial expression systems.<sup>3</sup> *P. pastoris* usually produces higher yield of recombinant proteins and is less demanding in terms of time and effort relative to complex eukaryotic systems.<sup>4</sup> At present, *P. pastoris* has been extensively applied for the commercial production of various foreign proteins.

Vaccines against viruses can be divided into 3 main categories: live attenuated, inactivated/killed, and subunit vaccines.<sup>5</sup> Live attenuated vaccines contain weakened viruses that still induce adaptive immune responses to both structural and nonstructural proteins.<sup>6</sup> The replication of live attenuated viruses should be sufficiently restricted to avoid pathological effects. Although live attenuated vaccines could induce strong and long-lasting immune responses, they might reverse to virulent wild-type strains that cause diseases in immunocompromised individuals.<sup>7</sup> Inactivated/

killed vaccines are relatively safe, but are less effective in eliciting protective immune responses.<sup>8</sup> Inactivated/killed vaccines may present production problems at commercial scale, in terms of growth of the virus to sufficiently high titers of economical yield, as well as hazardous containment issues for large scale growth of non-attenuated live virus.<sup>9</sup> Recombinant protein subunit vaccines are composed of at least 1 type of viral antigen that can be produced in heterologous expression systems. Recombinant protein subunit vaccines are significantly more secure than live attenuated and inactivated/killed vaccines. In addition, recombinant protein subunit vaccines can be scaled up in a more cost-effective manner compared with other types of vaccines. In the past 3 decades, there has been a trend toward developing subunit vaccine formulations that contain precisely specified antigenic components together with a potent adjuvant.<sup>10</sup>

Subunit vaccines must be efficacious, safe, and can be easily scaled up for mass production. The *P. pastoris* system is licensed to more than 160 companies in the biotechnology, pharmaceutical, vaccine and food industries.<sup>11</sup> *P. pastoris*-produced biopharmaceuticals or industrial enzymes have been approved by the United States Food and Drug Administration (FDA), indicating that the *P. pastoris* system is widely

accepted as safe and suitable for human use.<sup>12</sup> Additionally, *P. pastoris* is a useful system for both basic laboratory research and industrial manufacture, which provides an efficient platform for the high-level production of subunit vaccines.<sup>13</sup> Here we focus on new developments in the understanding and application of *P. pastoris* for the production of recombinant subunit vaccines against viruses.

### The general features of the *Pichia pastoris* expression system

All *P. pastoris* expression strains are derived from the wild type strain NRRL-Y11430. Auxotrophic mutants (GS115) and protease-deficient strains (SMD1163, SMD1165 and SMD1168) are frequently used.<sup>14</sup> The genome of *P. pastoris* contains 2 genes encoding alcohol oxidases, *AOX1* and *AOX2*.<sup>15</sup> With regard to the ability to utilize methanol, there are 3 phenotypes for *P. pastoris*, namely Mut<sup>+</sup> (methanol utilization wide-type), Mut<sup>s</sup> (methanol utilization slow) and Mut<sup>-</sup> (methanol utilization deleted).<sup>16</sup> Mut<sup>+</sup> strains containing intact and active *AOX* genes are characterized by a higher growth rate than Mut<sup>s</sup> strains.<sup>17</sup> Mut<sup>-</sup> strains are unable to grow on methanol as the sole carbon source due to the knock-out of both *AOX* genes.<sup>18</sup> The success of *P. pastoris* in the production of recombinant proteins is directly linked to the very strong and inducible promoter of the gene encoding the first enzyme of the methanol utilization pathway, alcohol oxidase 1 (*AOX1*).<sup>19</sup> The *AOX1* promoter (*P<sub>AOX1</sub>*) is repressed by glucose, glycerol or ethanol, and strongly induced by methanol, making *P. pastoris* a relatively tightly controlled expression system.<sup>20</sup> The use of *P<sub>AOX1</sub>* also holds advantages for over-expression of recombinant proteins.<sup>21</sup> Methanol is a highly flammable and hazardous substance.<sup>22</sup> Therefore, it is undesirable and unsafe to use methanol as the inducer for large-scale fermentations.

Alternative inducible or constitutive promoters, which can be induced without methanol but still reach high expression levels, have been used in the production of recombinant proteins. The promoter of the glutathione-dependent enzyme formaldehyde dehydrogenase 1 (*P<sub>FLD1</sub>*) is an attractive alternative to *P<sub>AOX1</sub>* as it allows the regulation of protein expression via induction with methanol or methylamine.<sup>23</sup> The *P<sub>FLD1</sub>* is transcriptionally efficient and shows similar regulatory properties to those of *P<sub>AOX1</sub>*.<sup>24</sup> Constitutive expression eases process handling, avoids the use of

potentially hazardous inducer and provides continuous transcription of the target gene. The glyceraldehyde-3-phosphate promoter (*P<sub>GAP</sub>*) provides constitutive expression of foreign proteins on glycerol, glucose and methanol media, which can reach the same expression levels as methanol-induced *P<sub>AOX1</sub>*.<sup>25</sup> Several constitutive promoters have also been used for the generation of recombinant proteins in *P. pastoris*, such as the promoters for phosphoglycerate kinase 1 (*P<sub>PGK1</sub>*) and translation elongation factor 1- $\alpha$  (*P<sub>TEF1</sub>*).<sup>26,27</sup>

When transformed into the host cells, the *P. pastoris* vectors integrate into the host genome to form a stably expressing clone.<sup>28</sup> To obtain high yield of foreign proteins, the optimal clone containing multiple copies of the target gene must be determined. Copy number can be assessed through colony screening using enhanced concentrations of antibiotic or real-time PCR.<sup>29,30</sup> The *P. pastoris* system is also highly suitable for large-scale growth and culture using bioreactors.<sup>31</sup> Moreover, the inexpensive growth medium makes *P. pastoris* an efficient and cost-effective expression system. Compared to other expression systems such as *Saccharomyces cerevisiae*, Chinese hamster ovary (CHO) cells and baculovirus/insect cells, the *P. pastoris* system usually produces higher levels of heterologous proteins.<sup>32</sup>

As a eukaryotic organism, *P. pastoris* possesses the capability to perform post-translational modifications including proteolytic processing, protein folding and glycosylation.<sup>14</sup> Foreign protein expression in *P. pastoris* can either be intracellular or secretory. One of the important advantages of using *P. pastoris* is its ability to secrete high levels of active heterologous proteins. Secretory expression of recombinant proteins requires the presence of a signal sequence in the vector.<sup>33</sup> The most commonly used secretion signal in *P. pastoris* is *S. cerevisiae*  $\alpha$ -mating factor leader sequence ( $\alpha$ -MF).<sup>34</sup> The  $\alpha$ -MF signal sequence has been proven to be the most effective in directing protein through the secretory pathway in *P. pastoris*. It has been reported that using  $\alpha$ -MF as the signal peptide can lead to the production of higher amounts of a foreign protein than using its native signal peptide.<sup>28</sup> Since *P. pastoris* secretes very low levels of native proteins, the secreted heterologous proteins constitute the majority of the total proteins in the medium.<sup>18</sup> The secretion of recombinant proteins into the medium contributes to avoiding toxicity from intracellularly accumulated materials as well as simplifying purification process.<sup>35</sup>

## Recombinant protein subunit vaccines

Currently available vaccines mostly consist of live attenuated or inactivated/killed viruses. Although these vaccine formulations have shown success and some diseases such as polio have been eradicated from most parts of the world, many issues still remain associated with these vaccines. For example, these vaccines are not always effective, and are needed to maintain cooling chain. In immunocompromised people, live vaccines may lead to severe disease.<sup>36</sup> Costs related to the production and administration makes these vaccines difficult to use for mass immunization of people.<sup>37</sup> Therefore, there is an urgent need to develop more effective subunit vaccines against infectious diseases. Recombinant protein subunit vaccines have gained more and more interest in recent years. Recombinant protein subunit vaccine is composed of 1 or a small number of microbial components that have been produced in heterologous expression systems.<sup>38</sup> This ensures that the subunit vaccine has a well-define composition.<sup>39</sup> Compared with other types of vaccines, the important advantage of subunit vaccines is their safety in that the components only contain recombinant proteins or synthetic peptides, without the involvement of infectious viruses.<sup>40</sup> Subunit vaccines usually do not induce side effects at the injection sites. These features have made subunit vaccine an attractive vaccine candidate.

An ideal subunit vaccine should be safe, stable and immunogenic in all immunized individuals.<sup>41</sup> The manufacture, purification and characterization should be efficient, and be inexpensive to enable widespread vaccine use.<sup>42</sup> The development of recombinant protein subunit vaccines depends on the knowledge of which viral antigens induce protective immunity in response to infection.<sup>43</sup> In addition, protein vaccines usually require correct folding for induction of immune responses.<sup>44</sup> The ability to produce large amounts of viral antigens for vaccine manufacture is important for subunit vaccine development. The large-scale production of antigens for industrial purposes has been enabled by recombinant DNA technology.<sup>45,46</sup>

Some recombinant viral antigens can spontaneously assemble into virus-like particles (VLPs) which are multiprotein structures without the incorporation of a viral genome.<sup>47,48</sup> VLPs is a potentially safe subunit vaccine that can induce immune responses

similar to those prompted by natural viral infection.<sup>49</sup> VLPs present repetitive high-density displays of epitopes and exhibit excellent adjuvant properties capable of inducing strong immune responses.<sup>50</sup> It has been found that VLPs are more stable and considerably more immunogenic than purified protein antigens.<sup>47</sup> Therefore, VLPs vaccines are considered as candidate alternative to whole-virus vaccines. At present, some VLPs vaccines have been licensed and commercialized. For example, 2 VLP-based vaccines produced in yeast, GlaxoSmithKline's Engerix-B® (hepatitis B virus, HBV) and Gardasil (human papillomavirus, HPV), are currently commercialized worldwide.<sup>51,52</sup> A large number of VLP-based vaccine candidates are undergoing preclinical or clinical evaluations, such as influenza virus and parvovirus VLPs.<sup>47,53</sup>

In order to verify vaccine efficacy, various parameters have to be monitored, such as levels of antigen-specific antibodies, the immunization dose, the type of immune cells responding and adverse effects.<sup>54</sup> Before a new vaccine enters the market, many obstacles have to be overcome. Research and development often last for decades until a vaccine is on the market. During this time, the antigen composition of the target pathogen might be adjusted, and vaccine dosage can also be increased due to lacking the long-term immunity after immunization.<sup>55</sup> The antigen that can stimulate protective immune responses is usually chosen as a vaccine candidate. After researchers develop a vaccine candidate, preclinical studies with cell cultures, tissues and animals will begin. However, animal experiments are still the main focus of preclinical studies.<sup>56</sup> Depending on the type of vaccine and route of administration, different animal models are used, such as mice, rats, dogs and primates.<sup>57</sup> Pharmacological and toxicological effects of vaccine candidates should be assessed before initiation of clinical studies. Nevertheless, new vaccine candidates usually fail in the preclinical phase, since they can not guarantee safety and efficacy. The clinical evaluation involves studies of effects of vaccine candidates on healthy volunteers for immunogenicity, efficacy and safety.<sup>58</sup>

## The use of *Pichia pastoris* in recombinant subunit vaccine development

*E. coli* is widely used in both commercial (for the generation of approximately 30% of biopharmaceutical products) and research laboratories (in the production

of >70 % of proteins).<sup>39,59,60</sup> Although *E. coli* has many advantages as a production system, generating recombinant proteins in this system can often result in inclusion body formation or low yields of foreign proteins lacking post-translational modifications.<sup>61</sup> Therefore, this is a general decline in the use of *E. coli* as a host cell. Eukaryotic microbes offer many of the benefits of eukaryotic host cells, while retaining the advantages of being microbial. Among eukaryotic microbes systems, *P. pastoris* is a promising platform to produce various foreign proteins. *P. pastoris* is capable of synthesising the protein in a form that mirrored its native conformation and immunogenicity. Therefore, *P. pastoris* has been widely used to manufacture subunit vaccine candidates. To date, a multitude of recombinant subunit vaccine candidates have been successfully expressed in *P. pastoris* (Table 1). The focus of this article is *P. pastoris*-derived subunit vaccine candidates against human viruses.

The success of trails on *P. pastoris*-produced recombinant Epstein-Barr virus (EBV) vaccine candidates has been reported. EBV is a ubiquitous human herpesvirus that is associated with the development of various malignancies such as nasopharyngeal carcinoma (NPC), gastric carcinoma (GC), Hodgkin lymphoma (HL) and Burkitt lymphoma (BL).<sup>62</sup> The viral glycoprotein gp350 is an attractive candidate for a prophylactic vaccine. The secretory form of truncated gp350 (gp350<sup>1-443</sup>, codons 1-443) has the potential to

be developed into EBV subunit vaccine.<sup>63</sup> Moreover, the recombinant protein was able to induce higher levels of specific antibodies than synthetic gp350 peptides.<sup>64</sup> EBV nuclear antigen 1 (EBNA1) is thought to be a promising antigen for EBV therapeutic vaccines. A previous study was conducted to investigate the immunogenicity of a truncated form of EBNA1 (E1ΔGA, codons 390-641) synthesized in *P. pastoris*.<sup>65</sup> The yield of the recombinant protein in *P. pastoris* (210.53 mg/L) was over 100-fold higher than that in *E. coli* (2 mg/L).<sup>66</sup> High immunogenicity of the recombinant E1ΔGA was also demonstrated by eliciting strong immune responses in mice. However, these results were not effective enough to use it as an EBV vaccine candidate. Further work is required to evaluate the protective efficacy of recombinant E1ΔGA.

Dengue, caused by 1 of 4 viral serotypes (DENV1-4), is one of the fastest spreading vector-borne diseases.<sup>67</sup> Envelope domain-III (EDIII) antigen of DENV-3 has garnered much attention as a promising vaccine candidate for dengue. *P. pastoris* was optimized for the secretory overexpression of EDIII.<sup>68</sup> The result suggested that casamino acids supplementation facilitated overexpressing *P. pastoris* cells to secrete more EDIII by reducing the proportion retained intracellularly. The purified EDIII antigen could induce neutralizing antibodies. The strategy described in this study enabled fulfilling the mounting demand for recombinant EDIII as well as laid direction to future studies on secretory

**Table 1.** A list of recombinant protein antigens produced in *P. pastoris* for use in the development of human subunit vaccines.

Pathogen/Antigen	Yield (mg/L)	Mode of expression*	Efficacy	Reference
Epstein-Barr virus envelope glycoprotein gp350	120.34	S	Recombinant gp350 <sup>1-443</sup> (codons 1-443) demonstrated strong immunogenicity	63
Epstein-Barr virus nuclear antigen 1 (EBNA1)	210.53	S	Recombinant E1ΔGA (codons 390-641) induced both humoral and cellular immune responses	65
Dengue virus (DENV-3) envelope domain-III (EDIII)	187	S	The purified EDIII antigen could efficiently induce neutralizing antibodies	68
Avian influenza virus (H5N1) neuraminidase (NA)	2	S	Recombinant NA elicited significant humoral responses in mice	70
Japanese encephalitis virus envelope (E) protein	19	S	Recombinant E protein could induce protective immunity in vaccinated mice	71
Human enterovirus 71 capsid protein VP1	500	S	Recombinant VP1 could protect neonatal mice from lethal virus challenge	73
Dengue virus (DENV-3) envelope ectodomain	15	I	DENV-3 E-based VLPs elicited predominantly neutralizing antibodies	74
Human papillomavirus type 16 major capsid protein L1	13.4-14.2	I/S	HPV VLPs were successfully produced	76,77
Coxsackievirus A16 P1 polypeptide and 3CD protease	8.3	I	CA16 VLPs induced high-titer serum antibodies and conferred complete protection against lethal virus challenge in neonatal mice	79
Human enterovirus 71 P1 polypeptide and 3CD protease	150	I	EV71 VLPs induced neutralizing antibodies and provided protection against lethal virus infection in neonatal mice	80
Hepatitis B virus surface antigen (HBsAg)	50	I	HBsAg-based VLPs were proven to be superior to currently licensed HBV vaccine (Engerix-B®)	81

\*I: Intracellular, S: Secreted.

expression of recombinant proteins in *P. pastoris*. The outbreak of the H5N1 highly pathogenic avian influenza had brought a serious threat to public health.<sup>69</sup> The neuraminidase (NA) of H5N1 was cloned into *P. pastoris* and the recombinant protein was purified.<sup>70</sup> The total yield of the purified rNA was 2 mg/L. Mice vaccinated with rNA exhibited a significant immune response. These results laid the foundation for fast and cost-effective development of vaccines for a circulating and newer strain of avian influenza.

Japanese encephalitis virus (JEV) envelope (E) protein expressed in *P. pastoris* was glycosylated.<sup>71</sup> The purity of E protein was greater than 95%. *P. pastoris*-produced recombinant E protein could stimulate immune responses that were capable of mediating significant protection against JEV infection. This study might provide helpful information for the development of subunit vaccines against JEV. Enterovirus 71 (EV71) is the major causative pathogen of hand, foot and mouth disease (HFMD).<sup>72</sup> High-level secretory production of EV71 capsid protein VP1 was obtained in *P. pastoris* (approximately 500 mg/L).<sup>73</sup> The recombinant VP1 could elicit protective immune responses in vaccinated mice. The *P. pastoris*-expressed VP1 is a promising EV71 vaccine candidate for industrial purpose. The successful expression of these viral antigens highlighted the potential of *P. pastoris* in developing safe, efficacious, and affordable subunit vaccines.

*P. pastoris* has also been developed as an innovative platform for the production of VLPs for vaccination purposes. *P. pastoris*-produced DENV-3 envelope-based VLPs not only preserved the antigenic integrity of the major neutralizing epitopes, but also were capable of eliciting neutralizing antibodies against DENV-3.<sup>74</sup> HPV L1 major capsid protein, which self-assembles into VLPs when expressed heterologously, is the main component of the currently available vaccines against cervical cancer.<sup>75</sup> However, the commercial HPV vaccines are expensive and still inaccessible to the majority of the population especially in economically disadvantaged regions. Therefore, this is a great need for the development of an alternative system for the production of HPV vaccines that can be provided by public health programs.<sup>76</sup> Recently, it has been reported that the gene encoding L1 was optimized and then inserted into the *P. pastoris* expression vector.<sup>76,77</sup> Intracellular HPV VLPs were successfully produced in *P. pastoris*, which facilitated the development of HPV vaccines.

Coxsackievirus A16 (CA16) can cause HFMD in infants and children.<sup>78</sup> *P. pastoris*-derived CA16 VLPs could induce high-titer serum antibodies and confer complete protection against CA16 lethal challenge in neonatal mice.<sup>79</sup> The yield of CA16 VLPs was 8.3 mg/L. However, the CA16 VLPs yield in *P. pastoris* could be further improved via optimization of regulatory elements or high-density yeast fermentation. An efficient production process that was developed using *P. pastoris* for VLPs containing EV71 P1 and 3CD proteins has also been reported.<sup>80</sup> EV71 VLPs were expressed at high levels up to 4.9% of total soluble proteins (150 mg/L) in *P. pastoris*. The EV71 VLPs could induce neutralizing antibodies against both homologous and heterologous EV71 strains, and conferred protection against lethal virus infection in neonatal mice. HBV surface antigen (HBsAg) has been used for a long time as a vaccine candidate. An enhanced method for the expression and purification of recombinant HBsAg VLPs in *P. pastoris* was described in a previous study.<sup>81</sup> Approximately 50 mg HBsAg VLPs with a purity of greater than 99% was obtained from 1 L culture broth. *P. pastoris*-derived HBsAg-based VLPs could elicit significantly high HBsAg-specific IgG titers as well as strong cellular immune responses. The results indicated that HBsAg-based VLPs had the potential as a superior vaccine to currently licensed HBV vaccine (Engerix-B®). These results demonstrated that *P. pastoris*-produced VLPs represented promising vaccine candidates with proven preclinical efficacy and desirable traits for manufacturing at industrial scale.

### Strategies to improve recombinant subunit vaccine production

To improve the yield of recombinant subunit vaccines in *P. pastoris*, it is important to optimize the gene sequence encoding the target antigen according to the codon bias of *P. pastoris*. The codon optimization of gene of interest usually results in a dramatic increase of protein expression levels in *P. pastoris* system.<sup>82</sup> For example, the yield of codon-optimized HBV e antigen (HBeAg) was approximately 5-fold greater than that of the wide type HBeAg.<sup>83</sup> Gene dosage of the foreign protein has a great impact on recombinant protein production. Screening of transformants containing multiple integration events is important for achieving high levels of recombinant protein. It has been

reported that increasing the gene copy number of recombinant hemagglutinin (HA) of swine origin influenza A virus (H1N1) enhanced the protein expression level by 5-fold.<sup>84</sup>

Not all recombinant proteins are efficiently secreted into the medium, and endoplasmic reticulum (ER) retention during high-level production can be a problem. Translation of a secretory protein is followed by translocation into the ER, which is mediated by a secretion signal peptide. When the recombinant protein fails to fold into its native state or protein expression exceeds the folding capacity of the ER, the unfolded or aggregated proteins may start to accumulate in the ER, thus triggering the unfolded protein response (UPR) and ER-associated degradation (ERAD) pathway.<sup>85</sup> The ER contains several chaperones and foldases such as the hsp70 member Kar2/Bip, calnexin, calreticulin and protein disulfide isomerase (PDI).<sup>86</sup> The co-overexpression of these ER proteins with the target protein was shown to significantly improve the yields of recombinant proteins.<sup>87,88</sup> ER stress and protein folding can affect the redox state of ER and the cytosolic redox balance, which have an impact on protein folding.<sup>89</sup> By altering the levels of redox active enzymes such as the antioxidant transcription factor Yap1 or glutathione peroxidase (GPX), the yield of the foreign protein could be improved.<sup>90,91</sup>

One of the major problems that hinders effective secretion and purification of heterologous proteins from *P. pastoris* is the degradation of the recombinant proteins by host-specific proteases.<sup>92</sup> High levels of host proteases are present in *P. pastoris*, and are readily induced by environmental stresses. Multiple approaches have been implemented to solve this problem, such as addition of protease inhibitors, peptone, casamino acids or specific amino acids, optimization of cultivation conditions (pH and temperature) and medium composition (nitrogen and carbon sources).<sup>92,93</sup> These approaches are usually limited in its effects. Genetic manipulation of the host proteases can efficiently reduce the degradation of recombinant proteins. Therefore, many protease-deficient yeast strains (SMD163, SMD165 and SMD168) have been developed.

Due to the availability of the genome sequence of *P. pastoris*, detailed understanding of the yeast's physiology, genetics and regulation has developed rapidly, which facilitated the development of improved strains

with customized properties for high yield production of recombinant proteins.<sup>94,95</sup> Furthermore, genetic engineering on *P. pastoris* has been utilized to optimize the host-cell background in order to produce proteins with proper post-translational modifications.<sup>96</sup> The optimization of culture condition and induction protocol are also essential to increase recombinant protein yields.<sup>97</sup> Various condition and induction parameters including media composition, temperature, pH, methanol concentration and induction time should be considered in designing an optimal production system to achieve high yield of the desired protein in an active form.

### Future perspectives

*P. pastoris* has shown excellent capacity of producing subunit vaccine candidates in sufficient quantity which is required for viable industrial applications. High purity of recombinant vaccine candidates with less contaminating protein could be obtained in *P. pastoris*. Strong immunogenic characteristics and improved stability due to the proper post-translational modifications and better protein folding performed by *P. pastoris* make this host superior to other expression systems. *P. pastoris* has become the most frequently used system for heterologous protein production. Recent approval of biopharmaceuticals produced in *P. pastoris* makes this system safe and suitable for the production of vaccine candidates. Thus, it can be assumed that the biopharmaceutical application of *P. pastoris* system will rapidly increase, adopting a place among the top 3 production systems besides *E. coli* and CHO cells. Advanced strains and process development will strongly increase the productivity of the *P. pastoris* system. Furthermore, achievements in the engineering of glycosylation, folding and assembly of complex proteins in *P. pastoris* will expand the range of target proteins to include those that are currently produced only in other expression systems.

### Conclusion

*P. pastoris* has been developed into one of the powerful and popular systems for the production of recombinant proteins of commercial interest. It offers various advantages as a host system including stable expression, high-level production, low cost and ease of genetic manipulation. More importantly, *P. pastoris* is capable of secreting a heterologous protein with its

native conformational structure and strong immunogenicity. These features along with improvements in its genetic manipulation for expression purpose have made *P. pastoris* an important expression system for the production of subunit vaccines.

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