Evaluation of Methylotrophic Yeast *Ogataea thermomethanolica* TBRC 656 as a Heterologous Host for Production of an Animal Vaccine Candidate

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Abstract

Multiple yeast strains have been developed into versatile heterologous protein expression platforms. Earlier works showed that *Ogataea thermomethanolica* TBRC 656 (OT), a thermotolerant methylotrophic yeast, can efficiently produce several industrial enzymes. In this work, we demonstrated the potential of this platform for biopharmaceutical manufacturing. Using a swine vaccine candidate as a model, we showed that OT can be optimized to express and secrete the antigen based on porcine circovirus type 2d capsid protein at a respectable yield. Crucial steps for yield improvement include codon optimization and reduction of OT protease activities. The antigen produced in this system could be purified efficiently and induce robust antibody response in test animals. Improvements in this platform, especially more efficient secretion and reduced extracellular proteases, would extend its potential as a competitive platform for biopharmaceutical industries.

Keywords Porcine circovirus type 2 · Veterinary vaccine · *Ogataea thermomethanolica* · Heterologous expression · Methylotrophic yeast

Introduction

Due to low-cost cultivation, ease in genetic manipulation, and capability for post-translational modification and target protein secretion, yeast provides an attractive platform for production of high-valued recombinant protein biotherapeutics including human and veterinary vaccines [1]. Indeed, the first commercialized recombinant vaccine, the Hepatitis B vaccine, was produced in baker's yeast *Saccharomyces cerevisiae* [2]. Successful production of each protein depends on multiple factors, such as promoters, yeast hosts, folding and glycosylation of target proteins. Therefore, several yeast platforms with different properties have been developed to serve a wider need in heterologous protein expression. To date, the yeast species with efficient heterologous protein expression systems include *S. cerevisiae*, *Schizosaccharomyces pombe*, *Hansenula polymorpha*, *Pichia pastoris*, *Kluyveromyces lactis*, and *Yarrowia lipolytica* [3, 4]. Some of these strains are private and need licensing, creating an additional financial barrier in bio-based industries and vaccine development. Especially for biotech companies in low-income countries focusing on regional problems, an alternative production platform at lower cost is desirable.

Thermotolerant methylotrophic yeast *Ogataea thermomethanolica* TBRC 656 (OT) was isolated from soil collected in Thailand [5]. Based on its indigenous methanol-inducible promoter and secretion signal, it has been developed as a non-conventional host for production of industrial enzymes [6, 7]. The OT system has been tested

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for large-scale industrial enzyme production using high-cell density fermentation [8, 9]. Nevertheless, whether the OT system can be utilized for production of high-valued recombinant proteins of non-fungal origins remains to be demonstrated.

The subunit vaccine for porcine circovirus type 2 (PCV2) provides a good opportunity to explore the potential and pitfalls of the OT system in manufacturing high-valued recombinant proteins. Since its early proof of immunogenicity, the recombinant PCV2 capsid protein produced by the baculovirus expression vector system remains one of the most successful commercial veterinary vaccines [10, 11]. More economical platforms such as bacteria and yeasts have been shown to produce potentially effective experimental PCV2 vaccines [12–15]. For yeast, multiple strains have been tested for PCV2 capsid protein expression with varying degrees of success: *P. pastoris*, *S. cerevisiae*, *H. polymorpha*, *K. marxianus* [13–16].

PCV2 is associated with several swine diseases such as postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), and porcine respiratory disease complex [17]. Since the introduction of the first commercial vaccine in the mid-2000s, PCV2 has undergone a genotype shift, with the more dominant PCV2d strain completely replacing the first-discovered PCV2a, on which most of the commercial vaccines were based [18]. Several pieces of evidence have suggested that a matched strain of the PCV2 vaccine could provide more effective protection, especially in experiments mimicking farm conditions [19, 20]. Therefore, an update in commercial PCV2 vaccines is desirable. Furthermore, in 2016, a distantly-related circovirus was discovered in pigs suffering from PDNS and other symptoms and was designated PCV3 [21]. Like the case of PCV2, retrospective serological surveys demonstrated the widespread presence of PCV3 in pig farms in many areas of the world. Currently, there is no commercial vaccine for PCV3.

In this work, we evaluated the potential of non-conventional yeast *O. thermomethanolica* TBRC 656 (OT) as an expression host for production of PCV2d and PCV3 subunit vaccine candidates. We found that construct designs, codon optimization, and culture condition optimization were crucial for successful protein expression. From six different recombinant protein constructs that were originally designed and tried for expression, one construct showed promising results. This construct, containing an N-terminal deletion in the PCV2d capsid protein, was selected for further work. The protein antigen produced in this work was safe to use as a candidate vaccine in test animals and showed robust induction of PCV2-specific antibodies, suggesting the potential of an OT-based system as a viable, more cost-effective method to produce an updated PCV2 subunit vaccine.

Materials and Methods

Chemicals and Biochemicals

Chemicals were from the following sources: yeast extract, peptone, YNB (BD, New Jersey, USA), glycerol, methanol, potassium hydroxide (CARLO ERBA Reagents, Val de Reuil, France), biotin, Sigma-204 antifoam, G418, ammonium sulfate, imidazole, Tween-20, Tris–HCl (Sigma-Aldrich, Missouri, USA), PBS (HIMEDIA laboratories, Mumbai, India). Biochemicals and enzymes were from the following sources: EcoRI, SacI, PdmI (Thermo Scientific, Massachusetts, USA), bovine serum albumin (BSA; Merck, Massachusetts, USA).

Antigen Designs

The amino acid sequence for PCV2d capsid protein was based on the most frequently found PCV2d strain from an extensive farm survey conducted in Thailand from 2009 to 2015 (accession number MF314329; [22]). PCV2d capsid protein codon optimization was modified from the published *P. pastoris*-based codon [13]. The amino acid sequence for PCV3 capsid protein was derived from the first PCV3 strain sequenced in Thailand (accession number MG310152.1; [23]). PCV3 capsid protein codon optimization was based on the codon usage table for *Ogataea polymorpha* retrieved from the High-performance Integrated Virtual Environment-Codon Usage Tables (HIVE-CUTs) database described by Athey et al. [24].

PCV2d capsid protein fragment constructs were designed based on the available information on known epitopes [25–32]. For PCV3, there was no available epitope information. The N- and C-terminal deletions were based on alignment with PCV2d capsid constructs using Clustal Omega [33].

Construction of Expression Plasmids

Optimized DNA sequences for full-length PCV2d and PCV3 capsid proteins with flanking EcoRI and SacI restriction sites were synthesized (Synbio Technologies, New Jersey, USA). The coding sequences for the fragments, deletion in N-terminus (ΔN) or deletion in N- and C-termini (ΔNΔC), were PCR-amplified from the synthesized gene using the primers shown in Table 1. The expression vector is based on the original pOTNeo4, which contains the OT alcohol oxidase (OtAOX1) promoter, inside of which the PdmI (XmnI) restriction site for plasmid linearization is located, and the OT alpha factor, followed by the sequence for multiple cloning sites to assist restriction cloning [34]. We modified

Table 1 Primers used in this work

Primer	Sequences
EcoRI-rPCV2d-ΔN_F	CGGAATTCATGAACGGTATCTTCAACACCAG
PCV2d-SacI_R	CGGAGCTCCTTTGGGTTCAATGGTGGGTC
PCV2d-dC-SacI_R	CGGAGCTCAATGTTGTAATCTTGGTCGTA
EcoRI-PCV3dN_F	CGGAATTCATGACCGCCGGCACCTACTACAC
PCV3-SacI_R	TAGAGCTCCAGCACCGACTTGTATCTGATC
PCV3-dC-SacI_R	TAGAGCTCCTTCTCTGGCACGTAGATCG
Int_AOX TT_R	GAGTCGTAGTTGTCAATCATGACC
DAEA_F	CCAAGCTTACGATGAAATTCAACACTACTCTTC

the plasmid by adding a SacI restriction site and a coding sequence for the 6X-His tag at the C-terminus to create pOT-Neo4-His. Different constructs were cloned into pOTNeo4-His using EcoRI and SacI restriction cloning. Plasmids were propagated in bacteria strain DH5α. The resulting plasmids are summarized in Table 2. All plasmids were verified by DNA sequencing (Apical Scientific, Selangor, Malaysia).

Protein Expression in *O. thermomethanolica*

One μg PdmI-linearized plasmids were transformed into *O. thermomethanolica* TBRC 656 by electroporation using the following condition: 2-mm cuvette, 5 kV/cm, 25 μF, 400 Ω (Gene Pulser, Bio-Rad, California, USA). The transformants were selected on YPD agar supplemented with 200–400 μg/mL of G418 and verified for gene integration by colony PCR with primers DAEA-F and AOX_TT-R (Table 1). Although the theoretical integration site resides within the OtAOX1 promoter, random integration into the OT genome generally occurs, and we did not select for integration at any specific site. Trial expression for each construct was performed on at least 20 positive clones. The cultures were grown in 5 mL YPD (50-mL conical tubes) at 30 °C with shaking at 250 rpm until $OD_{600} = 15$. Then, cultures were inoculated in 20 mL of BMGY [100 mM phosphate buffer pH 6.0, 1% w/v yeast extract, 2% w/v peptone, 1% v/v glycerol, 1.34% YNB, 2 μg/L biotin] at $OD_{600} = 1$ and cultured at 30 °C with shaking at 250 rpm until $OD_{600} = 15$. Cells were harvested

and resuspended into 2 mL of BMMY medium [100 mM phosphate buffer pH 6.0, 1% w/v yeast extract, 2% w/v peptone, 1.34% YNB, 2 μg/L biotin, 1% v/v methanol] to induce protein expression at 30 °C with shaking at 250 rpm. During the subsequent 72 h, methanol was added to achieve a final concentration of 1% every 24 h. The supernatant and cell lysate samples were taken at different time points for SDS–PAGE and Western blot analysis.

Optimization of expression conditions was performed in shake flasks similar to the protocol mentioned above, except that the culture volume was increased to 20 mL in 250-mL flasks and the expression conditions or components of induction media were varied as indicated in each experiment.

Fermentation

Fed-batch fermentation was performed in a 5-L bioreactor (B. Braun Sartorius Ltd., Göttingen, Germany). The pre-culture was prepared by growing from glycerol stock of OT-rPCV2d-ΔN in 200 mL BMGY at 30 °C with shaking at 250 rpm for 30 h or until $OD_{600} = 15$–20. Then, the pre-culture was inoculated into 1.8 L working volume of BMGY medium in a 5-L bioreactor vessel for fed-batch fermentation. Foam was controlled by the addition of 0.01% Sigma-204 antifoam into the medium. Fed-batch fermentation was initiated by feeding glycerol with a constant feed rate at 0.5 g/h/L for 40 h followed by the simultaneous methanol induction and rPCV2d-ΔN production stage in

Table 2 Description of plasmids used in this work

Plasmid name	Description	Reference
pOTNeo4	OtAOX1 promoter-OT alpha factor-MCS-OtAOX1 terminator (Neo^R)	[34]
pOTNeo4-His	pOTNeo4-SacI-His$_6$	This work
pOTNeo4-PCV2dFL-His	pOTNeo4-PCV2d-His$_6$	This work
pOTNeo4-PCV2dΔN-His	pOTNeo4-*Δ2-40* PCV2d-His$_6$	This work
pOTNeo4-PCV2dΔNΔC-His	pOTNeo4-*Δ2-40,Δ212-234* PCV2d-His$_6$	This work
pOTNeo4-PCV3FL-His	pOTNeo4-PCV3-His$_6$	This work
pOTNeo4-PCV3ΔN-His	pOTNeo4-*Δ2-34* PCV3-His$_6$	This work
pOTNeo4-PCV3ΔNΔC-His	pOTNeo4-*Δ2-34,Δ195-214* PCV3-His$_6$	This work

which 100% methanol was fed continuously at a rate of 5 g/L until the end of fermentation (92 h). In this experiment, we employed a two-stage temperature and pH control strategy. In the first growth phase, both glycerol batch and glycerol fed-batch had a temperature and pH controlled at 30 °C and pH 6 to support growth and biomass accumulation. In the induction phase, the temperature and pH were controlled at 20 °C and pH 8 to enhance the production and secretion of rPCV2d-ΔN. 5 M KOH was used as a pH control reagent. Oxygen enrichment was applied to maintain the dissolved oxygen tension (DOT) at 40% throughout the fermentation. Samples were taken and analyzed for dry cell weight (DCW), residual glycerol, and methanol content throughout the cultivation. Culture samples were taken at indicated time points to monitor rPCV2d-ΔN expression by Western blot analysis.

Purification of rPCV2d-ΔN

The growth media was clarified and filtered through a 0.45 μm filter (Merck, New Jersey, USA). The protein was first precipitated out with 70% ammonium sulfate and then dialyzed with buffer A (100 mM tris–HCl pH 8.0, 10 mM imidazole). The sample was then loaded onto a Nickel Nitrilo-triacetic Acid (NTA) (Qiagen, Hilden, Germany) pre-equilibrated with buffer A. The column was washed with ten column volumes of buffer B (100 mM tris–HCl pH 8.0, 20 mM imidazole) and eluted with buffer C (100 mM tris–HCl pH 8.0, 250 mM imidazole). All eluates were analyzed by SDS–PAGE and Western blot. The eluates containing the major rPCV2d-ΔN protein bands were concentrated and exchanged into a storage buffer (1× PBS pH 7.4).

SDS–PAGE and Western Blot Analysis

Samples (clarified growth media or cell lysates) were separated on 15% or 12.5% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) prior to visualization with Coomassie Blue staining or Western blotting. For Western blot analysis, proteins were transferred to a nitrocellulose membrane using Mini Trans-Blot Cell (Bio-Rad, California, USA). The membrane was probed with a mouse monoclonal IgG His tag antibody (R&D systems, Minnesota, USA) or a rabbit polyclonal anti-PCV2d capsid protein antibody [35].

Mice Immunization

Animal experiments were conducted per the approvals of BIOTEC and Thammasat University IACUC (protocols numbers BT-Animal 20/2562 and 029/2562, respectively). On Day 0, six female BALB/c mice (6-weeks-old) per group were immunized intraperitoneally with 200 μL mixture of complete Freud's adjuvant and 30 μg of purified rPCV2d-ΔN protein produced in OT or PBS (1:1 volume ratio). As a comparison, another group of mice were immunized with the identical construct of rPCV2d-ΔN expressed in bacteria and purified by cation exchange chromatography [35]. On Day 14, mice were immunized again with 200 μL mixture of incomplete Freud's adjuvant and 30 μg of purified protein or PBS. Blood samples were collected on Days 0, 28, and 35, and sera were analyzed for PCV2d capsid-specific antibody response.

Enzyme-Linked Immunosorbent Assay

ELISA plates were coated with 5 ng of rPCV2d-ΔN capsid protein produced in bacteria or 500 ffu (fluorescent focus units) of PCV2d virus in carbonate buffer (pH 9.6) overnight at 4 °C. Wells were washed with PBS + 0.1% tween 20 (PBST) and blocked with 2% BSA in PBST for 1 h at room temperature. Mouse sera diluted in 0.5% BSA in PBST (1:5000 dilution for recombinant protein-coated plates or 1:200 dilution for virus-coated plates) were incubated in the plates for 2 h at room temperature. The plates were then washed three times with PBST and incubated with HRP-conjugated goat anti-mouse IgG (Abcam, Cambridge, UK) diluted at 1:5000 in 0.5% BSA in PBST for 1 h at room temperature. Following three washes with PBST, the plates were incubated with 3,3′,5,5′-tetramethylbenzidine (TMB; BioLegend, California, USA) substrate for 5 min at room temperature. The reactions were stopped with 2N H_2SO_4. Absorbance at 450 nm was measured with an ELISA plate reader (Synergy HTX Multi-Mode Reader, Agilent Technologies, California, USA).

Statistical Analysis

All graphical data were prepared with Prism9 (Graphpad software, California, USA). Values of means ± standard deviation (SD) were shown for each group. Statistical significance was calculated by one-way analysis of variance (ANOVA). p values < 0.05 were considered statistically significant.

Results

PCV2 and PCV3 Antigen Designs

In the initial phase, we designed several constructs based on the capsid proteins from porcine circoviruses, PCV2d and PCV3, found in Thailand. Since codon usage information for OT was incomplete, we decided to use the information from its close cousins such as *Pichia* or other *Ogataea* yeasts. For PCV2, the *P. pastoris*-optimized PCV2b capsid protein sequence had been published and shown to express

well in *P. pastoris* [13]. Therefore, we chose to modify from the published PCV2b sequence to account for amino acids that are different between PCV2b and PCV2d. The PCV3 capsid protein had not been attempted in any yeast platform. We then chose to codon-optimize based on the closest cousin whose codon usage information was available, *Ogataea polymorpha* (former name as *Pichia angusta* or *Hansenula polymorpha*). In some cases, smaller constructs could improve protein secretion and yields. We therefore made the N-terminal deletion based on the putative nuclear localization signal (NLS) of the PCV2 capsid protein. We also made an additional C-terminal truncation on the N-terminal deletion construct while preserving most of the previously identified antigenic portions (Fig. 1A). Because of the limited knowledge on PCV3 capsid, we made similar fragmental constructs based on the amino acid alignment with PCV2 capsid protein (Fig. 1B). In conclusion, for each protein, two other constructs were generated: 'ΔN' and 'ΔNΔC' (Fig. 1C). All successful OT integrants were confirmed with PCR.

Trial Expression Revealed an Appropriate Antigen Construct for PCV2d Capsid Protein

For each construct, at least 20 positive integrants were grown in BMGY and induced for protein expression with 1% methanol. Protein expression was checked on days 2 or 3 post-induction both in extracellular (secretion) and intracellular (cell lysate) fractions with Western blotting by anti-His tag antibody. For all PCV2d capsid protein constructs, low levels of intracellular expression could be observed in some clones (Fig. 2A–C). Notably, expression of the rPCV2d-ΔN construct could be observed intracellularly in most of the clones, while secreted rPCV2d-ΔN could be observed in some clones, including Clone 2 and others (Fig. 2B and data not shown). The other two constructs did not yield observable secreted expression in any of the confirmed OT integrants. For PCV3, none of the integrants for the full-length and ΔN constructs showed protein expression (Fig. 2D–F). The construct PCV3-ΔNΔC showed very low expression in the cell lysate fractions from a few clones (Fig. 2G). Therefore, the OT-rPCV2d-ΔN integrant (clone number 2) with the highest expression level was selected for further work.

Optimization of Expression Conditions for Secreted rPCV2d-ΔN

Focusing only on the yield of the secreted recombinant protein, we further optimized the expression conditions by varying growth temperature, growth media pH and components of the growth media and checked for improvement in the secretion fraction by Western blotting. When growth temperatures were reduced to 20 °C and 25 °C,

larger secretion of rPCV2d-ΔN capsid protein (hereafter termed "rPCV2d-ΔN") was observed, compared to the original 30 °C condition (Fig. 3A). When compared at the same growth temperatures, BMMY media maintained at higher pH, especially in the range of 7.5–9.0, yielded larger expression of secreted rPCV2d-ΔN (Fig. 3B). We next tested the effect of different surfactants on secreted rPCV2d-ΔN expression. OT-rPCV2d-ΔN were grown and induced for protein expression at 30 °C in growth media BMMY (pH 8.0) containing indicated additives, and protein secretion was compared at 72 h post-induction. Addition of Tween-20 and Antifoam-204 in BMMY significantly increased levels of rPCV2d-ΔN in the media, while AFE-1520 and PEG2000 mildly enhanced rPCV2d-ΔN levels (Fig. 3C). Triton X-100 did not improve protein expression. When the growth profile and total proteins were examined, it was found that cells did not grow as well and showed reduced total protein expression, suggesting that Triton X-100 might be toxic to the OT host (data not shown). In summary, the optimal conditions for rPCV2d-ΔN secretion from the OT host is BMMY + 0.5% Tween-20, pH 8, grown at 20 °C or 25 °C. When compared directly with the original expression condition, significant improvement could be observed (Fig. 3D). The doublet bands possibly belonging to rPCV2d-ΔN and its slightly smaller cleavage product were observed similar to the same construct expressed in *Escherichia coli* [35].

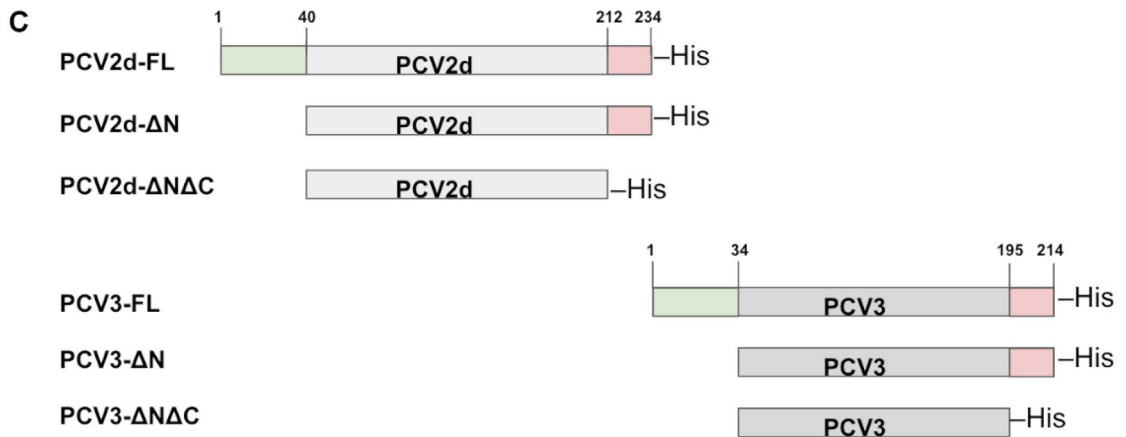## Modulation of OT Protease Activity is Critical for Secreted rPCV2d-ΔN Yield

Improvement in levels of secreted rPCV2d-ΔN could involve several mechanisms, in particular enhanced production/ secretion of the target protein (higher influx of rPCV2d-ΔN) or increased stability of the target protein in induction media (lower efflux of rPCV2d-ΔN). We attempted to tease apart these possibilities by testing protein degradation (efflux of rPCV2d-ΔN) in cell-free supernatants. The cell-free supernatant was taken from OT-rPCV2d-ΔN grown at 30 °C in the pH 8 induction media and adjusted to the indicated pH before further incubation at 30 °C. Samples were taken every 12 h to assess the amounts of intact rPCV2d-ΔN in the cell-free supernatant by Western blot analysis, compared to the starting time point. At the 12 h time point, we observed a large reduction in the level of the intact rPCV2d-ΔN protein at pH 5 and 6 conditions (Fig. 4A). At pH 7, degradation was not as pronounced, while at pH 8 most of the protein was retained (Fig. 4A). Additionally, we tested the stability of rPCV2d-ΔN at different temperatures. The cell-free supernatant from the optimal condition was stored at 20 °C or 30 °C incubators and sampled every 12 h. While a large fraction of the protein remained intact after 48 h if stored at 20 °C, most of it disappeared between 24 and 36 h if stored at 30 °C (Fig. 4B). Based on these data, we suspected that

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                                                          ▼
A   PCV2a    MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTFGYTVKRT 60
    PCV2d    MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTIGYTVKAT 60
    PCV2b1B  MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTFGYTIKRT 60
    PCV2b1A  MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTFGYTVKRT 60
             ***********************************************:.***:* *

    PCV2a    TVTTPSWAVDMMRFKIDDFVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGS 120
    PCV2d    TVRTPSWAVDMMRFNINDFLPPGGGSNPLTVPFEYYRIRKVKVEFWPCSPITQGDRGVGS 120
    PCV2b1B  TVKTPSWAVDMMRFNINDFLPPGGGSNPRSVPFEYYRIRKVKVEFWPCSPITQGDRGVGS 120
    PCV2b1A  TVKTPSWAVDMMRFNINDFLPPGGGSNPRSVPFEYYRIRKVKVEFWPCSPITQGDRGVGS 120
             ** ***********:*:**.*****:* :: ****************************

    PCV2a    TAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHSRYFTPKPVLDSTIDYFQPNNKR 180
    PCV2d    TAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHSRYFTPKPVLDRTIDYFQPNNKR 180
    PCV2b1B  SAVILDDNFVTKATALTYDPYVNYPSRHTITQPFSYHSRYFTPKPVLDSTIDYFQPNNKR 180
    PCV2b1A  SAVILDDNFVTKATALTYDPYVNYSSRHTITQPFSYHSRYFTPKPVLDSTIDYFQPNNKR 180
             :.********************** ***** ***** *************** ********

                                                ▼
    PCV2a    NQLWLRLQTSGNVDHVGLGAAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP- 233
    PCV2d    NQLWLRLQTTGNVDHVGLGTAFENSIYDQDYNIRVTMYVQFREFNLKDPPLNPK 234
    PCV2b1B  NQLWLRLQTAGNVDHVGLGTAFENSIYDQEYNIRVTMYVQFREFNLKDPPLNP- 233
    PCV2b1A  NQLWLRLQTTGNVDHVGLGTAFENSIYDQEYNIRVTMYVQFREFNLKDPPLNP- 233
             *********:.********:*****: ***:***:.*****************:*

B   PCV2d    MTYPRRRFRRRRHRPR---SHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTIGYTV 57
    PCV3     --MRHRAIFRRRPRPRRRRRRHRRRYARRRLFIRRPT--------AGTYYTKKYSTMNVIS 50
               :* : *** ***    *  :  *** :: :*▲        * : *:   *:.

    PCV2d    KKTTVRTPSWNVDMMRFNINDFLPPGGGSNPLTVPFEYYRIRKVKVEFWPCSPI-TQGDR 116
    PCV3     VGTPQNNKPWHANHFITRLNE--------WETAITFEYYKILKMKVTLSPVISPAQQTKT 102
               *   .. *:.: :  .:*:         :: ****:* *:** : *        * .

    PCV2d    GVGSTAVILDDNFVTKANALTYDPYVNYSSRHTITQPFSYHSRYFTPKPVLDGTIDYFQP 176
    PCV3     MFGHTAIDLDGAWTT-NTWLQDDPYAESSTRKV-MTSKKKHSRYFTPKPLLAGTTSAHPG 160
              .* **: **. :.*   . *  ***.: *:*:.         . *********:* **  . .

    PCV2d    NN----KRNQLWLRLQTTGNVDHVGLGTAFENSIYDQ------DYNIRITMYVQFREFNL 226
    PCV3     QSLFFFSRPTPWLNTYDPT----VQWGAL-LWSIYVPEKTGMTDFYGTKEVWIRYKSVL- 214
             :.     .*   **.      * *:    *** ▲   *:     ::::::..

    PCV2d    KDPPLNPK     234
    PCV3     --------     214
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C
PCV2d-FL      [1  40        PCV2d      212 234]–His
PCV2d-ΔN         [          PCV2d          ]–His
PCV2d-ΔNΔC       [          PCV2d      ]–His

PCV3-FL                   [1  34       PCV3       195 214]–His
PCV3-ΔN                      [         PCV3          ]–His
PCV3-ΔNΔC                    [         PCV3      ]–His
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◄**Fig. 1** Antigen design for PCV2d and PCV3 subunit vaccines. **A** Alignment of the reference sequences for capsid proteins from PCV2a (AF055392), PCV2b (AF055394 for PCV2b1A and AY67853 for PCV2b1B), and PCV2d (AY181946). Immunodominant epitopes are in magenta [25]. Underlined [26], italicized [27], or highlighted in gray [28] are linear epitopes identified by past works. In bold are residues predicted to bind to antibodies by structural analysis [29]. Highlighted in red and purple are residues important in neutralization by antibodies [30, 31]. Highlighted in cyan is the PCV2-specific neutralizing epitope [32]. The yellow box indicates a sequence that can distinguish among different PCV2 subtypes [36]. All indications are located in the protein sequence on which original works were based. Black triangles mark the positions where truncations were made. **B** Alignment of the sequences for capsid proteins from PCV2d and PCV3 used in this study. Black triangles mark the positions where truncations were made. Alignments in **A** and **B** were performed with Clustal Omega [33]. Symbols *, : and . indicate amino acids with full conservation, conservation between groups of strongly similar properties, and conservation between groups of weakly similar properties, respectively [33]. **C** Schematics for different constructs of PCV2d and PCV3 antigens tried in this work

rPCV2d-ΔN was degraded by OT proteases in the cell-free induction media. We tested this by repeating the experiment with cell-free supernatant in the presence of protease inhibitor cocktails (PIC). In the presence of PIC, even at 30 °C storage after 48 h, almost none of the protein was degraded (Fig. 4B). Furthermore, adding PICs into the original induction media could maintain accumulation of rPCV2d-ΔN at sub-optimal growth pH and temperature (Fig. 4C).

Purification of rPCV2d-ΔN

Next, we purified the target protein from the culture media and determined the yield. OT-rPCV2d-ΔN was grown in 50 mL of BMGY at a normal condition and then was exchanged into 50 mL of induction media at the optimal condition (BMMY, pH 8 with 0.5% Tween-20, 20 °C) for 72 h prior to harvest. To compare yield post-purification, another flask grown side-by-side was induced in 50 mL of induction media in the original condition (BMMY, pH 6, 30 °C). The clarified media containing secreted rPCV2d-ΔN was precipitated with ammonium sulfate and buffer exchanged into Tris-buffered solution (100 mM Tris–HCl pH 8.0, 10 mM imidazole) prior to incubation with Ni–NTA resin. In a side-by-side batch purification, eluates at equal volumes (10 μL) from both culture conditions were compared. While none of the protein could be detected from the original expression condition, highly-purified rPCV2d-ΔN doublets could be observed with Coomassie staining after the Ni–NTA purification (Fig. 5, top). Protein identity was confirmed by Western blotting with anti-PCV2 capsid protein antibody (Fig. 5, bottom). The purified protein concentration was determined by the Qubit protein assay, and the yield from this shake flask optimal condition was about 0.12 mg protein/mL of

yeast culture supernatant. The purity of the recombinant protein was over 90%.

Biofermentation of OT-PCV2d-ΔN

Large-scale production of rPCV2d-ΔN was attempted using fed-batch fermentation on a 5-L bioreactor. The glycerol batch stage was carried out until the dry cell weight (DCW) reached 4.8 g/L at $T = 26$ h. Subsequently, the glycerol fed-batch stage was initiated, and DCW increased sharply to 25.9 g/L at $T = 38$ h (Fig. 6). When the glycerol concentration became the limiting factor for growth, as determined by the increase in dissolved oxygen level, the methanol-feeding phase commenced by feeding methanol at $T = 44$ h (Fig. 6, black arrow) and continued until the harvest at $T = 92$ h. At the start of the induction, a small baseline expression could be observed (Fig. 6, inset). As the fermentation progressed, the accumulation of rPCV2d-ΔN was observed in the media from the start up to $T = 83$ h (Fig. 6, inset). Between 83 and 92 h, the protein level dropped slightly, possibly due to protein degradation by extracellular proteases and intracellular proteases released from dead cells over prolonged fermentation. The protein could be purified by Ni–NTA chromatography with a yield similar to the shake flask culture.

OT-Derived rPCV2d-ΔN is Immunogenic in Animals

We next tested if rPCV2d-ΔN produced in the OT system could induce antibodies in test animals. On Days 0 and 14, three groups of mice were immunized intraperitoneally with adjuvanted PBS buffer, adjuvanted OT-derived rPCV2d-ΔN protein (30 μg), or adjuvanted *E. coli*-derived rPCV2d-ΔN protein (30 μg). Compared with the mice injected with adjuvanted PBS buffer, significant induction of anti-PCV2 IgG was observed on Days 28 and 35 for mice immunized with bacterial or OT-derived rPCV2d-ΔN protein (Fig. 7A). These antibodies also recognized the PCV2d virus particles as observed in the ELISA using the viral particles as the coating antigen (Fig. 7B).

Discussion

Yeast heterologous protein expression systems have held a lot of promise in the manufacture of high-valued biopharmaceuticals. Host systems now have expanded from conventional Baker's yeast to a methylotrophic commercial host like *P. pastoris* to even more non-conventional newer host systems like *H. polymorpha* or *K. lactis* [3, 4]. This is because suitability of the host must be determined empirically for each protein. Another reason important especially for developing or transitioning countries that wish to be

Fig. 2 Trial expression of PCV2d (**A**–**C**) and PCV3 (**D**–**G**) antigen constructs in *O. thermomethanolica*. The his-tagged constructs in secretion and cell lysate fractions were visualized by Western blotting with anti-His tag antibody. 'H', unrelated his-tagged protein control. '2d', OT-rPCV2d-ΔN (clone 2). 'M', protein marker
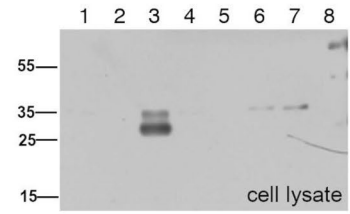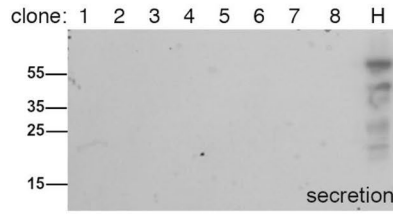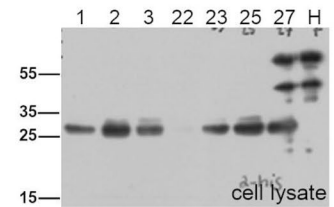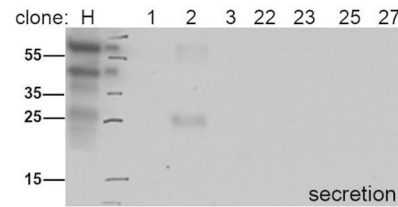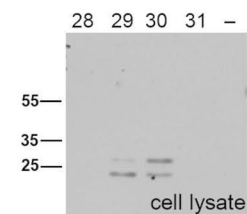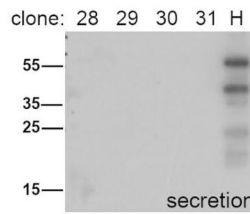
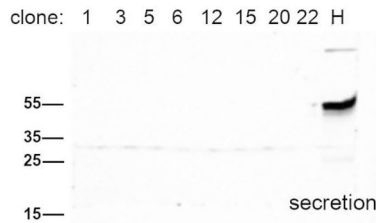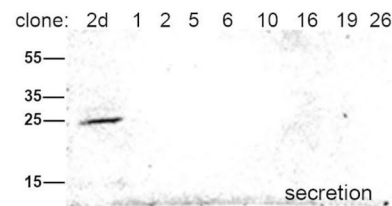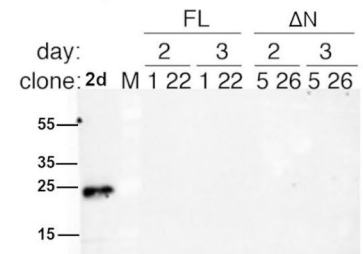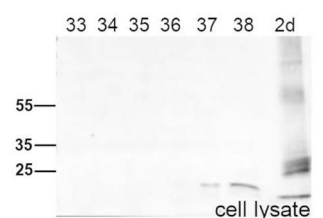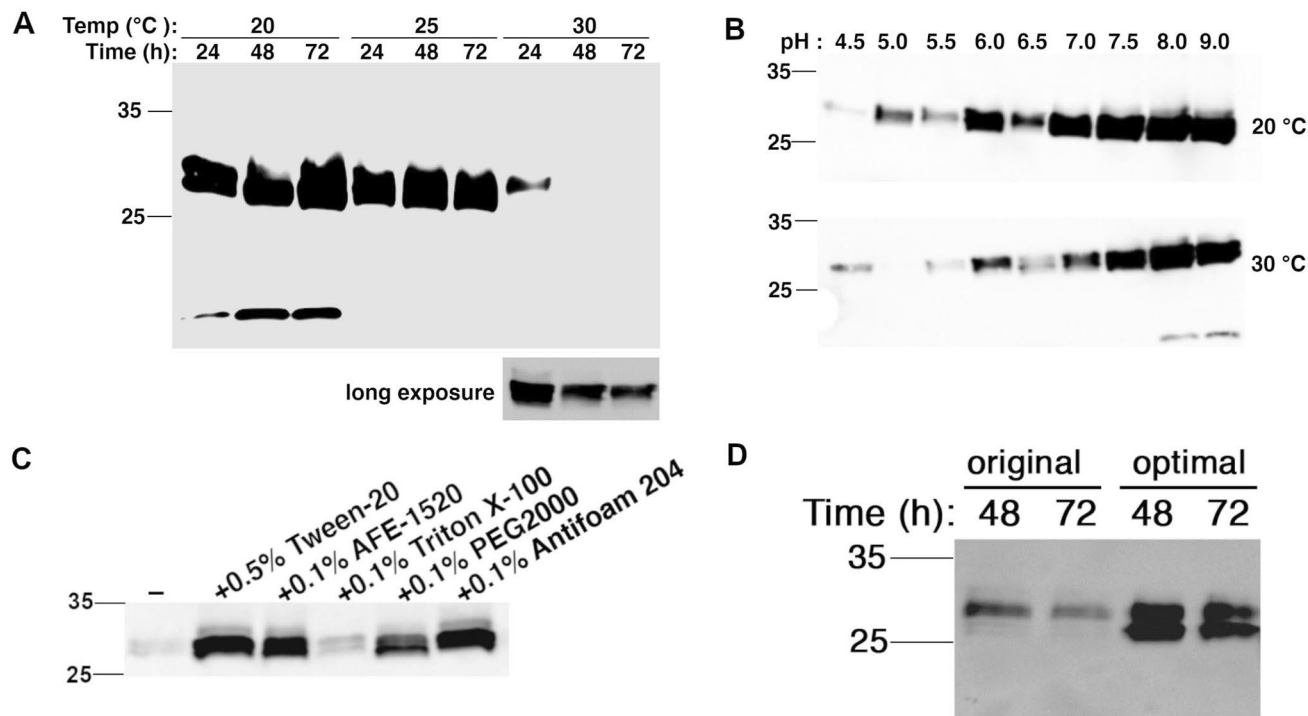**Fig. 3** Optimization of culture conditions for secreted rPCV2d-ΔN expression. Effects of culture temperature (**A**), pH of culture media (**B**), and additives (**C**) were investigated in shake flask culture of OT-rPCV2d-ΔN. **D** The finalized optimal condition (BMMY + 0.5% Tween-20, pH 8, grown at 25 °C) was tested side-by-side with the original culture condition. Samples of secretion fractions at 48 and 72 h post-induction were visualized by Western blotting with anti-His tag antibody
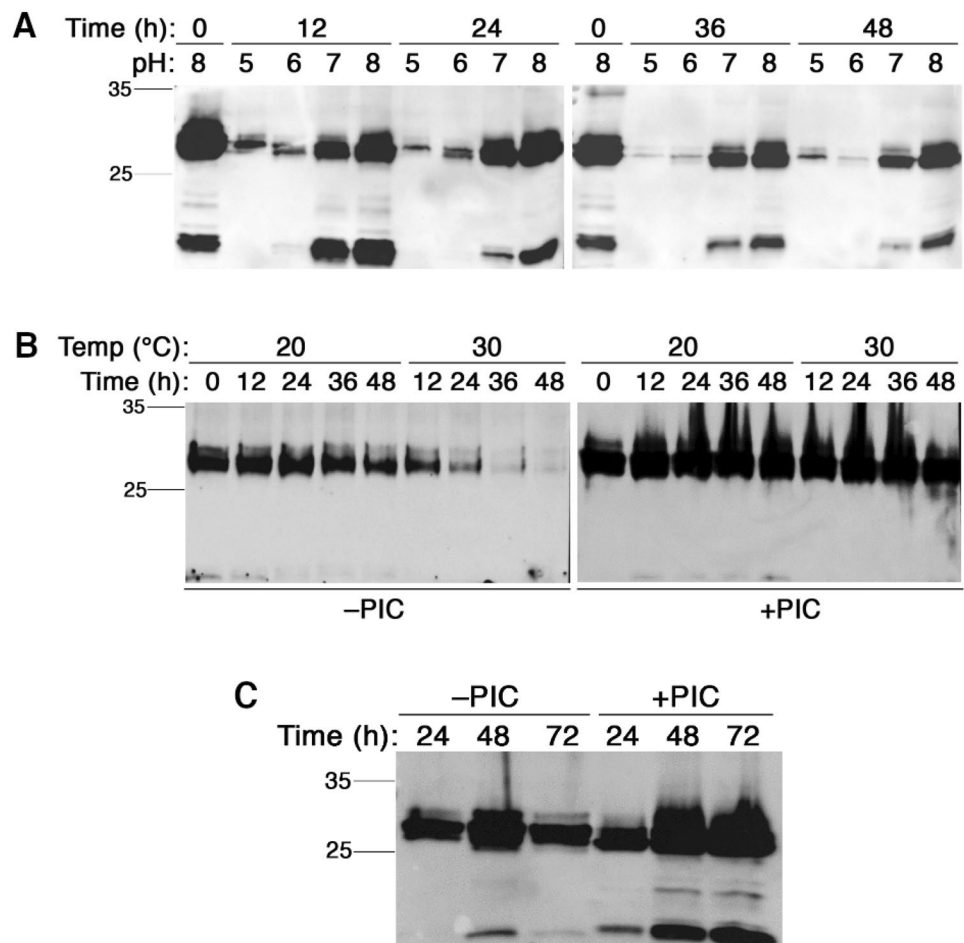
self-sustainable in bio-based industries is the cost associated with using commercial host systems.

Production of recombinant PCV2 and PCV3 capsid proteins has been explored in several microbial platforms (Table 3). In bacteria, codon optimization was necessary to get high yields, and deletion of NLS significantly increased expression of recombinant PCV2 and PCV3 capsid protein (Table 3, [12, 35, 37–39]). While successful production of recombinant PCV3 capsid protein has not been reported in yeast, different yeast platforms gave a wide range of yields and characteristics for recombinant PCV2 capsid proteins (Table 3). Similar to the bacterial platform, codon optimization was critical for high-yield expression. Different viewpoints existed for the importance of NLS deletion. For *K. marxiamus* and *P. pastoris*, full-length PCV2 capsid protein could be expressed to considerable yields [13, 15]. On the other hand, significant yield improvement was observed in the expression of the NLS-deleted constructs in *H. polymorpha* and our work with *O. thermomethanolica* (Table 3, [14]). As some of the expression vectors did not contain export sequences, these yeast platforms expressed the protein intracellularly and required cell breakage prior to purification [13–16]. Interestingly, Tu et al. failed to recover any secreted recombinant PCV2 capsid proteins from the *P. pastoris* supernatant, even though the recombinant protein was

fused to an alpha factor [13]. Another group later reported secretion of rPCV2 capsid protein from *P. pastoris* at a much lower yield (total protein content in supernatant at 140 μg/mL culture) [40]. Ours was the only platform that allows purification of rPCV2d-ΔN without the cell breakage step, with the yield of 120 μg purified protein/mL culture. Lastly, recombinant PCV2 capsid proteins purified from these yeast hosts exhibited different molecular weights, possibly due to distinct post-translational modification in each host. Some of these rPCV2 capsid proteins were shown to form virus-like particles (VLP) structurally similar to purified PCV2 viruses [14–16]. Based on the observation that the identical protein construct produced in *E. coli* did not form high-molecular weight structure [35], we expected that OT-derived rPCV2d-ΔN also did not form VLPs. Nevertheless, these antigens, including rPCV2d-ΔN from OT, could induce PCV2-specific antibodies in animal models, suggesting that these microbial platforms are suitable alternatives for PCV2 subunit vaccine production.

Besides the rPCV2d-ΔN construct, we attempted to produce other PCV2 and PCV3 antigens in OT. Trial expression results showed that PCV2-related constructs were more likely to express than PCV3-related constructs. The same trend was observed in bacteria [35]. It is possible that PCV3 capsid protein is inherently toxic or much less stable. Within

the PCV2-related constructs, we found that the rPCV2d-ΔN construct gave the best results. This is consistent with previous works in both bacteria and yeast suggesting that the NLS portion impedes PCV capsid protein production in microbial platforms [14, 35, 37, 39].

There are two critical challenges for the current OT system as illustrated in this study. First, despite using the native OT alpha factor as the secretory signal, a noticeable portion of rPCV2d-ΔN remained inside the cells. As PCV2 capsid proteins tend to spontaneously form large aggregates or VLPs, as demonstrated by VLP purification directly from yeast cell lysates [14–16], this protein's export efficiency could be inherently low. On the other hand, heterologous protein secretion is still a major bottleneck for many yeast strains [41–43]. Protein secretion from yeast cells involves multiple steps, including correct folding, ER-to-Golgi transport and Golgi-to-membrane transport. Overexpression of heterologous proteins in yeast could cause stress for machineries involved in any of these steps and result in accumulation of heterologous proteins inside the cells. Besides optimization in expression conditions as explored in this work, efforts to enhance target protein secretion in the OT hosts

may include engineering the protein folding system in the ER [7], engineering the protein trafficking pathway [41, 44, 45], or screening genome-wide for suitable secretion signals and fusion partners [46]. The second challenge is the excessive protease activities. Our results strongly suggest that secreted rPCV2d-ΔN was degraded by OT proteases, and inhibition of these proteases, whether by raising the pH condition, by lowering growth temperature, or by adding protease inhibitors, could alleviate the yield problem. Although we could not pinpoint exactly whether these are extracellular proteases or contaminations of intracellular proteases released by cell lysis during fermentation, the problem is not specific to the OT system but rather a familiar problem with yeast hosts. Several approaches can alleviate the protease problems, including optimizing fermentation conditions (this work, [47–49]) and removing potential protease cleavage sites from the target proteins [50]. For a more universal approach, many protease-deficient yeast strains have been developed through genetic manipulation [51–53]. With improvements in these critical areas, we hope that the OT system can serve as a truly versatile heterologous protein production platform.

Fig. 5 Purification of rPCV2d-ΔN from equal amounts of OT-rPCV2d-ΔN culture media from original and optimal conditions by ammonium sulfate precipitation and Ni–NTA resin. '5' and '10' indicated different amounts (in µL) of eluates loaded on the gel
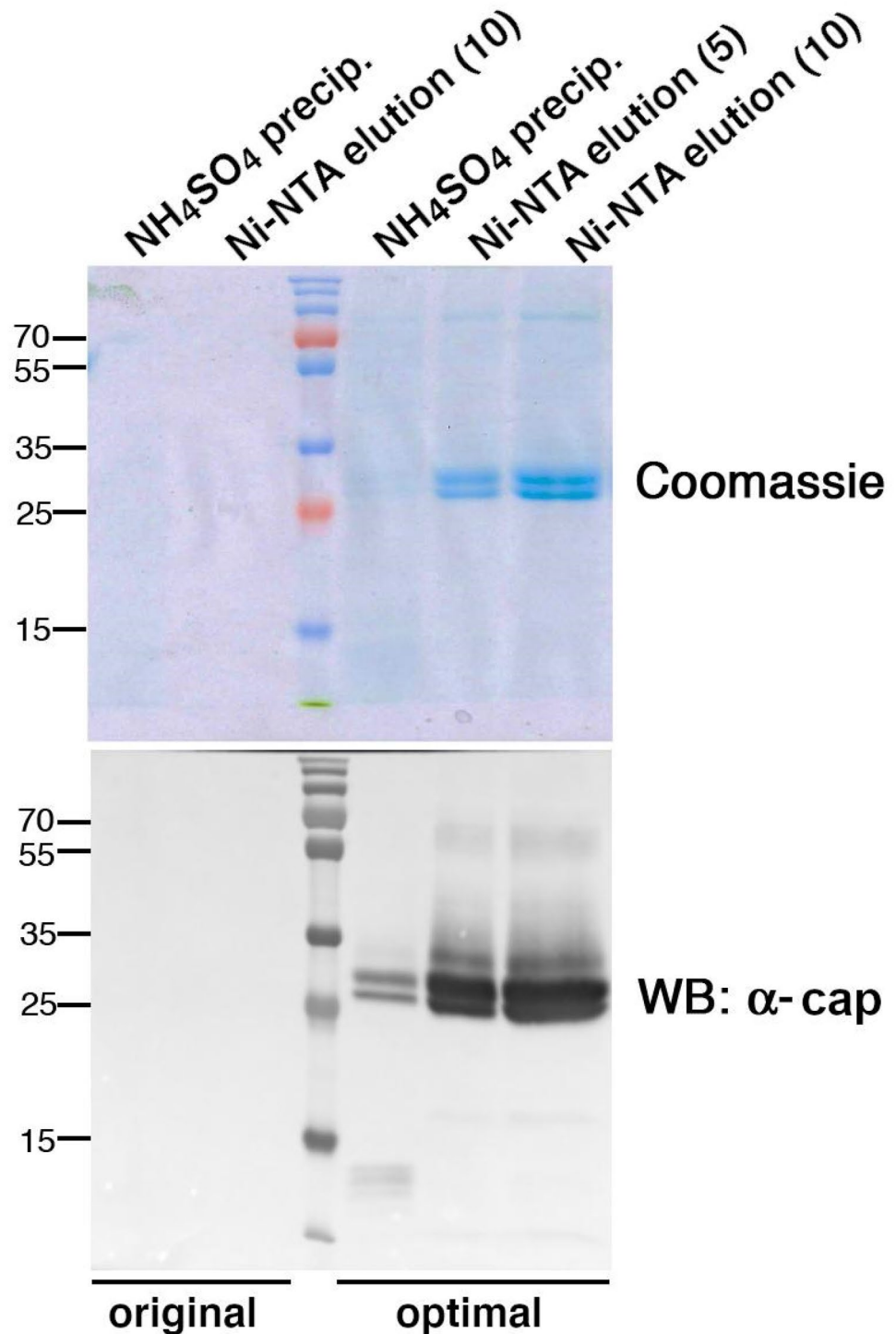

Conclusion

In this work, we explored the potential of methylotrophic yeast *O. thermomethanolica* in the production of high-valued biotherapeutics. After codon optimization, selection of appropriate antigen constructs and optimization of expression conditions, rPCV2d-ΔN capsid protein could be produced, secreted, and purified from the recombinant OT yeast. At optimal conditions, the OT system could secrete and yield upto 120 µg of purified recombinant PCV2d capsid protein per mL of yeast culture. OT-derived rPCV2d-ΔN capsid protein was effective in inducing antibody response in animals. To expand its potential into non-fungal recombinant protein production, we identified two important realms

Fig. 6 Growth and rPCV2d-ΔN production by *O. thermomethanolica* under the methanol-inducible condition. Glycerol was used in batch and fed-batch stages. The methanol feed rate was continuous at 5 g/L in the production stage. Arrow indicates the start of the methanol feed. Inset shows Western blot analysis of culture supernatant samples at different time points. Black arrowhead indicates the doublet bands characteristic of rPCV2d-ΔN
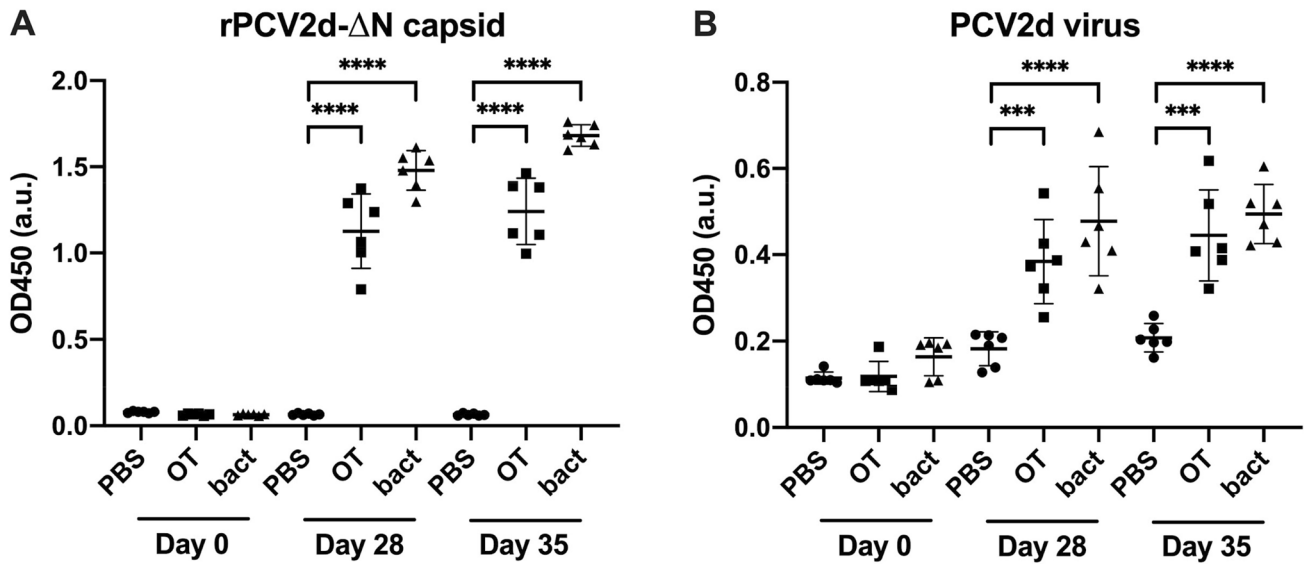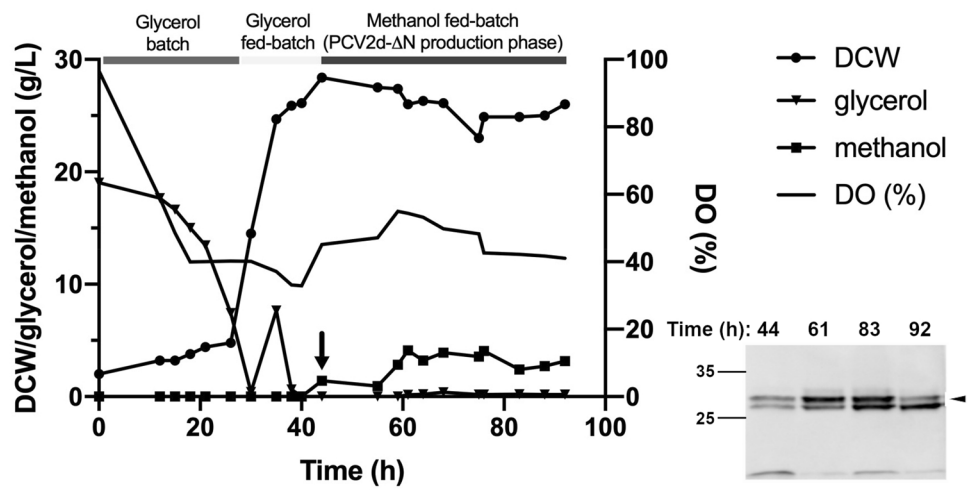
Fig. 7 PCV2d-specific antibody response in mice immunized with rPCV2d-ΔN antigen produced from OT yeast (squares) and bacteria (triangles) compared to the control group receiving PBS buffer (circles). PCV2d-specific IgG was detected in mice sera (1:5000 and 1:200 dilution) by ELISA using recombinant PCV2d capsid protein (**A**) and purified PCV2d virus (**B**) as coating antigens. Each data point represents one mouse. Average values ± SD were shown. ***$p < 0.0005$; ****$p < 0.0001$

Table 3 Yield comparison of recombinant PCV capsid proteins from different expression platforms

Platform	Genotype	Expression yield (µg/mL culture)	Post-purification yield (µg/mL culture)	References
Bacteria	PCV2b (FL)	Not quantified	50	[12]
Bacteria	PCV2d (ΔN)	Not quantified	1000	[35]
P. pastoris (intracellular)	PCV2b (FL)	174	–	[13]
P. pastoris (secretion)	PCV2b (FL)	< 140 (total protein)	–	[40]
S. cerevisiae (intracellular)	PCV2b (FL)	Not quantified	Not quantified	[16]
H. polymorpha (intracellular)	PCV2b (ΔN)	Not quantified	10.8	[14]
K. marxiamus (intracellular)	PCV2d (FL)	1900	652.8	[15]
O. thermomethanolica (secretion)	PCV2d (ΔN)	Not quantified	120	This study
Bacteria	PCV3 (FL and ΔN)	Not quantified	Not quantified	[38, 39]

for improvement: enhancing secretory efficacy and reducing protease activities. Nevertheless, a pre-pilot scale fermentation offered the first glimpse into using OT as a feasible, less cost-prohibitive vaccine production platform in the future.

References

1. Kumar, R., & Kumar, P. (2019). Yeast-based vaccines: New perspective in vaccine development and application. *FEMS Yeast Research*. https://doi.org/10.1093/femsyr/foz007

2. Valenzuela, P., Medina, A., Rutter, W. J., Ammerer, G., & Hall, B. D. (1982). Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature, 298*, 347–350.

3. Madhavan, A., Arun, K. B., Sindhu, R., Krishnamoorthy, J., Reshmy, R., Sirohi, R., Pugazhendi, A., Awasthi, M. K., Szakacs, G., & Binod, P. (2021). Customized yeast cell factories for biopharmaceuticals: From cell engineering to process scale up. *Microbial Cell Factories, 20*, 124.

4. Rebello, S., Abraham, A., Madhavan, A., Sindhu, R., Binod, P., Karthika Bahuleyan, A., Aneesh, E. M., & Pandey, A. (2018). Non-conventional yeast cell factories for sustainable bioprocesses. *FEMS Microbiology Letters*. https://doi.org/10.1093/femsle/fny222

5. Limtong, S., Srisuk, N., Yongmanitchai, W., Yurimoto, H., & Nakase, T. (2008). *Ogataea chonburiensis* sp. nov. and *Ogataea nakhonphanomensis* sp. nov., thermotolerant, methylotrophic yeast species isolated in Thailand, and transfer of *Pichia siamensis* and *Pichia thermomethanolica* to the genus Ogataea. *International Journal of Systematic and Evolutionary Microbiology, 58*, 302–307.

6. Promdonkoy, P., Tirasophon, W., Roongsawang, N., Eurwilaichitr, L., & Tanapongpipat, S. (2014). Methanol-inducible promoter of thermotolerant methylotrophic yeast *Ogataea thermomethanolica* BCC16875 potential for production of heterologous protein at high temperatures. *Current Microbiology, 69*, 143–148.

7. Roongsawang, N., Puseenam, A., Kitikhun, S., Sae-Tang, K., Harnpicharnchai, P., Ohashi, T., Fujiyama, K., Tirasophon, W., & Tanapongpipat, S. (2016). A novel potential signal peptide sequence and overexpression of ER-resident chaperones enhance heterologous protein secretion in thermotolerant methylotrophic yeast *Ogataea thermomethanolica*. *Applied Biochemistry and Biotechnology, 178*, 710–724.

8. Charoenrat, T., Antimanon, S., Kocharin, K., Tanapongpipat, S., & Roongsawang, N. (2016). High cell density process for constitutive production of a recombinant phytase in thermotolerant methylotrophic yeast *Ogataea thermomethanolica* using table sugar as carbon source. *Applied Biochemistry and Biotechnology, 180*, 1618–1634.

9. Boonchoo, K., Puseenam, A., Kocharin, K., Tanapongpipat, S., & Roongsawang, N. (2019). Sucrose-inducible heterologous expression of phytase in high cell density cultivation of the thermotolerant methylotrophic yeast *Ogataea thermomethanolica*. *FEMS Microbiology Letters*. https://doi.org/10.1093/femsle/fnz052

10. Nawagitgul, P., Morozov, I., Bolin, S. R., Harms, P. A., Sorden, S. D., & Paul, P. S. (2000). Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. *Journal of General Virology, 81*, 2281–2287.

11. Liu, L. J., Suzuki, T., Tsunemitsu, H., Kataoka, M., Ngata, N., Takeda, N., Wakita, T., Miyamura, T., & Li, T. C. (2008). Efficient production of type 2 porcine circovirus-like particles by a recombinant baculovirus. *Archives of Virology, 153*, 2291–2295.

12. Wu, P. C., Chen, T. Y., Chi, J. N., Chien, M. S., & Huang, C. (2016). Efficient expression and purification of porcine circovirus type 2 virus-like particles in *Escherichia coli*. *Journal of Biotechnology, 220*, 78–85.

13. Tu, Y., Wang, Y., Wang, G., Wu, J., Liu, Y., Wang, S., Jiang, C., & Cai, X. (2013). High-level expression and immunogenicity of a porcine circovirus type 2 capsid protein through codon optimization in *Pichia pastoris*. *Applied Microbiology and Biotechnology, 97*, 2867–2875.

14. Xiao, Y., Zhao, P., Du, J., Li, X., Lu, W., Hao, X., Dong, B., Yu, Y., & Wang, L. (2018). High-level expression and immunogenicity of porcine circovirus type 2b capsid protein without nuclear localization signal expressed in *Hansenula polymorpha*. *Biologicals, 51*, 18–24.

15. Duan, J., Yang, D., Chen, L., Yu, Y., Zhou, J., & Lu, H. (2019). Efficient production of porcine circovirus virus-like particles using the nonconventional yeast *Kluyveromyces marxianus*. *Applied Microbiology and Biotechnology, 103*, 833–842.

16. Nainys, J., Lasickiene, R., Petraityte-Burneikiene, R., Dabrisius, J., Lelesius, R., Sereika, V., Zvirbliene, A., Sasnauskas, K., & Gedvilaite, A. (2014). Generation in yeast of recombinant virus-like particles of porcine circovirus type 2 capsid protein and their use for a serologic assay and development of monoclonal antibodies. *BMC Biotechnology, 14*, 100.

17. Meng, X. J. (2013). Porcine circovirus type 2 (PCV2): Pathogenesis and interaction with the immune system. *Annual Review of Animal Biosciences, 1*, 43–64.

18. Xiao, C. T., Halbur, P. G., & Opriessnig, T. (2015). Global molecular genetic analysis of porcine circovirus type 2 (PCV2) sequences confirms the presence of four main PCV2 genotypes and reveals a rapid increase of PCV2d. *Journal of General Virology, 96*, 1830–1841.

19. Huan, C., Fan, M., Cheng, Q., Wang, X., Gao, Q., Wang, W., Gao, S., & Liu, X. (2018). Evaluation of the efficacy and cross-protective immunity of live-attenuated chimeric PCV1-2b vaccine against PCV2b and PCV2d subtype challenge in pigs. *Frontiers in Microbiology, 9*, 455.

20. Opriessnig, T., O'Neill, K., Gerber, P. F., de Castro, A. M., Gimenez-Lirola, L. G., Beach, N. M., Zhou, L., Meng, X. J., Wang, C., & Halbur, P. G. (2013). A PCV2 vaccine based on genotype 2b is more effective than a 2a-based vaccine to protect against PCV2b or combined PCV2a/2b viremia in pigs with concurrent PCV2, PRRSV and PPV infection. *Vaccine, 31*, 487–494.

21. Palinski, R., Pineyro, P., Shang, P., Yuan, F., Guo, R., Fang, Y., Byers, E., & Hause, B. M. (2017). A novel porcine circovirus distantly related to known circoviruses is associated with porcine dermatitis and nephropathy syndrome and reproductive failure. *Journal of Virology*. https://doi.org/10.1128/JVI.01879-16

22. Thangthamniyom, N., Sangthong, P., Poolperm, P., Thanantong, N., Boonsoongnern, A., Hansoongnern, P., Semkum, P., Petcharat, N., & Lekcharoensuk, P. (2017). Genetic diversity of porcine circovirus type 2 (PCV2) in Thailand during 2009–2015. *Veterinary Microbiology, 208*, 239–246.

23. Kedkovid, R., Woonwong, Y., Arunorat, J., Sirisereewan, C., Sangpratum, N., Lumyai, M., Kesdangsakonwut, S., Teankum, K., Jittimanee, S., & Thanawongnuwech, R. (2018). Porcine circovirus type 3 (PCV3) infection in grower pigs from a Thai farm

suffering from porcine respiratory disease complex (PRDC). *Veterinary Microbiology, 215*, 71–76.

24. Athey, J., Alexaki, A., Osipova, E., Rostovtsev, A., Santana-Quintero, L. V., Katneni, U., Simonyan, V., & Kimchi-Sarfaty, C. (2017). A new and updated resource for codon usage tables. *BMC Bioinformatics, 18*, 391.

25. Lekcharoensuk, P., Morozov, I., Paul, P. S., Thangthumniyom, N., Wajjawalku, W., & Meng, X. J. (2004). Epitope mapping of the major capsid protein of type 2 porcine circovirus (PCV2) by using chimeric PCV1 and PCV2. *Journal of Virology, 78*, 8135–8145.

26. Shang, S. B., Jin, Y. L., Jiang, X. T., Zhou, J. Y., Zhang, X., Xing, G., He, J. L., & Yan, Y. (2009). Fine mapping of antigenic epitopes on capsid proteins of porcine circovirus, and antigenic phenotype of porcine circovirus type 2. *Molecular Immunology, 46*, 327–334.

27. Ge, M., Yan, A., Luo, W., Hu, Y. F., Li, R. C., Jiang, D. L., & Yu, X. L. (2013). Epitope screening of the PCV2 Cap protein by use of a random peptide-displayed library and polyclonal antibody. *Virus Research, 177*, 103–107.

28. Mahe, D., Blanchard, P., Truong, C., Arnauld, C., Le Cann, P., Cariolet, R., Madec, F., Albina, E., & Jestin, A. (2000). Differential recognition of ORF2 protein from type 1 and type 2 porcine circoviruses and identification of immunorelevant epitopes. *Journal of General Virology, 81*, 1815–1824.

29. Khayat, R., Brunn, N., Speir, J. A., Hardham, J. M., Ankenbauer, R. G., Schneemann, A., & Johnson, J. E. (2011). The 2.3-angstrom structure of porcine circovirus 2. *Journal of Virology, 85*, 7856–7862.

30. Saha, D., Huang, L., Bussalleu, E., Lefebvre, D. J., Fort, M., Van Doorsselaere, J., & Nauwynck, H. J. (2012). Antigenic subtyping and epitopes' competition analysis of porcine circovirus type 2 using monoclonal antibodies. *Veterinary Microbiology, 157*, 13–22.

31. Liu, J., Huang, L., Wei, Y., Tang, Q., Liu, D., Wang, Y., Li, S., Guo, L., Wu, H., & Liu, C. (2013). Amino acid mutations in the capsid protein produce novel porcine circovirus type 2 neutralizing epitopes. *Veterinary Microbiology, 165*, 260–267.

32. Mo, X., Li, X., Yin, B., Deng, J., Tian, K., & Yuan, A. (2019). Structural roles of PCV2 capsid protein N-terminus in PCV2 particle assembly and identification of PCV2 type-specific neutralizing epitope. *PLoS Pathogens, 15*, e1007562.

33. McWilliam, H., Li, W., Uludag, M., Squizzato, S., Park, Y. M., Buso, N., Cowley, A. P., & Lopez, R. (2013). Analysis tool web services from the EMBL-EBI. *Nucleic Acids Research, 41*, W597-600.

34. Tanapongpipat, S., Promdonkoy, P., Roongsawang, N., Harnpicharnchai, P., Wongwisansri, S., & Eurwilaichitr, L. (2018). Gene expression product for producing target proteins or bio-products from heat-tolerant yeast by methanol induction and non-induction including its process of product usage. WO2018236294A1.

35. Peswani, A. R., Narkpuk, J., Krueger, A., Bracewell, D. G., Lekcharoensuk, P., Haslam, S. M., Dell, A., Jaru-Ampornpan, P., & Robinson, C. (2022). Novel constructs and 1-step chromatography protocols for the production of Porcine Circovirus 2d (PCV2d) and Circovirus 3 (PCV3) subunit vaccine candidates. *Food and Bioproducts Processing, 131*, 125–135.

36. Cheung, A. K., Lager, K. M., Kohutyuk, O. I., Vincent, A. L., Henry, S. C., Baker, R. B., Rowland, R. R., & Dunham, A. G. (2007). Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. *Archives of Virology, 152*, 1035–1044.

37. Trundova, M., & Celer, V. (2007). Expression of porcine circovirus 2 ORF2 gene requires codon optimized *E. coli* cells. *Virus Genes, 34*, 199–204.

38. Wang, Y., Wang, G., Duan, W. T., Sun, M. X., Wang, M. H., Wang, S. H., Cai, X. H., & Tu, Y. B. (2020). Self-assembly into virus-like particles of the recombinant capsid protein of porcine circovirus type 3 and its application on antibodies detection. *AMB Express, 10*, 3.

39. Liu, B. Y., Gao, B., Liu, M. Z., Zhang, T. T., Liu, B. S., & Chen, Z. L. (2020). High repetitive arginine in the anterior of PCV3 capsid protein is a severe obstacle for its expression in *E. coli*. *AMB Express, 10*, 214.

40. Silva, J. G., Coimbra, E. C., Jesus, A. L., Mariz, F. C., Silva, K. M., Lobato, Z. I., Campos, A. C., Coutinho, L. C., Castro, R. S., & Freitas, A. C. (2014). Secretory expression of Porcine Circovirus Type 2 capsid protein in *Pichia pastoris*. *Journal of Virological Methods, 207*, 226–231.

41. Idiris, A., Tohda, H., Kumagai, H., & Takegawa, K. (2010). Engineering of protein secretion in yeast: Strategies and impact on protein production. *Applied Microbiology and Biotechnology, 86*, 403–417.

42. Gasser, B., Saloheimo, M., Rinas, U., Dragosits, M., Rodriguez-Carmona, E., Baumann, K., Giuliani, M., Parrilli, E., Branduardi, P., Lang, C., Porro, D., Ferrer, P., Tutino, M. L., Mattanovich, D., & Villaverde, A. (2008). Protein folding and conformational stress in microbial cells producing recombinant proteins: A host comparative overview. *Microbial Cell Factories, 7*, 11.

43. Zahrl, R. J., Gasser, B., Mattanovich, D., & Ferrer, P. (2019). Detection and elimination of cellular bottlenecks in protein-producing yeasts. *Methods in Molecular Biology, 1923*, 75–95.

44. Kruasuwan, W., Puseenam, A., Tanapongpipat, S., & Roongsawang, N. (2021). Multiplexed CRISPR-mediated engineering of protein secretory pathway genes in the thermotolerant methylotrophic yeast *Ogataea thermomethanolica*. *PLoS ONE, 16*, e0261754.

45. Kruasuwan, W., Puseenam, A., Phithakrotchanakoon, C., Tanapongpipat, S., & Roongsawang, N. (2021). Modulation of heterologous protein secretion in the thermotolerant methylotrophic yeast *Ogataea thermomethanolica* TBRC 656 by CRISPR-Cas9 system. *PLoS ONE, 16*, e0258005.

46. Bae, J. H., Sung, B. H., Kim, H. J., Park, S. H., Lim, K. M., Kim, M. J., Lee, C. R., & Sohn, J. H. (2015). An efficient genome-wide fusion partner screening system for secretion of recombinant proteins in yeast. *Scientific Reports, 5*, 12229.

47. Velez-Suberbie, M. L., Morris, S. A., Kaur, K., Hickey, J. M., Joshi, S. B., Volkin, D. B., Bracewell, D. G., & Mukhopadhyay, T. K. (2020). Holistic process development to mitigate proteolysis of a subunit rotavirus vaccine candidate produced in *Pichia pastoris* by means of an acid pH pulse during fed-batch fermentation. *Biotechnology Progress, 36*, e2966.

48. Charoenrat, T., Khumruaengsri, N., Promdonkoy, P., Rattanaphan, N., Eurwilaichitr, L., Tanapongpipat, S., & Roongsawang, N. (2013). Improvement of recombinant endoglucanase produced in *Pichia pastoris* KM71 through the use of synthetic medium for inoculum and pH control of proteolysis. *Journal of Bioscience and Bioengineering, 116*, 193–198.

49. Adivitiya, B., Mohanty, S., & Khasa, Y. P. (2021). Nitrogen supplementation ameliorates product quality and quantity during high cell density bioreactor studies of *Pichia pastoris*: A case study with proteolysis prone streptokinase. *International Journal of Biological Macromolecules, 180*, 760–770.

50. Spiegel, H., Schinkel, H., Kastilan, R., Dahm, P., Boes, A., Scheuermayer, M., Chudobova, I., Maskus, D., Fendel, R., Schillberg, S., Reimann, A., & Fischer, R. (2015). Optimization of a multistage, multi-subunit malaria vaccine candidate for the production in *Pichia pastoris* by the identification and removal of protease cleavage sites. *Biotechnology and Bioengineering, 112*, 659–667.

51. Prabhu, A. A., Boro, B., Bharali, B., Chakraborty, S., & Dasu, V. V. (2017). Gene and process level modulation to overcome the bottlenecks of recombinant proteins expression in *Pichia pastoris*. *Current Pharmaceutical Biotechnology, 18*, 1200–1223.

52. Tomimoto, K., Fujita, Y., Iwaki, T., Chiba, Y., Jigami, Y., Nakayama, K., Nakajima, Y., & Abe, H. (2013). Protease-deficient *Saccharomyces cerevisiae* strains for the synthesis of human-compatible glycoproteins. *Bioscience, Biotechnology, and Biochemistry, 77*, 2461–2466.

53. Ganatra, M. B., Vainauskas, S., Hong, J. M., Taylor, T. E., Denson, J. P., Esposito, D., Read, J. D., Schmeisser, H., Zoon, K. C., Hartley, J. L., & Taron, C. H. (2011). A set of aspartyl protease-deficient strains for improved expression of heterologous proteins in *Kluyveromyces lactis*. *FEMS Yeast Research, 11*, 168–178.