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...
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
the plasmid by adding a SacI restriction site and a coding sequence for the 6X-His tag at the C-terminus to create pOTNeo4-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 OD600 = 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, 1.34% YNB, 2 µg/L biotin] 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 OD600 = 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 1 | Primers used in this work |
| --- | --- |
| Primer | Sequences |
| EcoRI-rPCV2d-ΔN_F | CGGAATTCATGAAAGTGATCTTTCAACACCCAG |
| PCV2d-Sacl_R | CGGAGCTCCTTGGGTCAATGTTGGGTC |
| PCV2d-dC-Sacl_R | CGGAGCTCAATGTTGAATCTTGGTGCAGTA |
| EcoRI-PCV3dN_F | CGGAATTCAATGACCGCGCCACCTACTACAC |
| PCV3-Sacl_R | TAGAAGTCCACGACCTGGTGAATCTGATC |
| PCV3-dC-Sacl_R | TAGAGCTCTTCTGGCCAGCGTAGATCG |
| Int_AOX TT_R | GAGTCGAGTGGTCAATCATGACC |
| DAEA_F | CCAAGCTTACGTAATAATCATAACTCTTC |

| Table 2 | Description of plasmids used in this work |
| --- | --- |
| Plasmid name | Description | Reference |
| pOTNeo4 | OtAOX1 promoter-OT alpha factor-MCS-OtAOX1 terminator (Neo<sup>R</sup>) | [34] |
| pOTNeo4-His | pOTNeo4-Sacl-His<sub>6</sub> | This work |
| pOTNeo4-PCV2dFL-His | pOTNeo4-PCV2d-His<sub>8</sub> | This work |
| pOTNeo4-PCV2dΔN-His | pOTNeo4-Δ2-40 PCV2d-His<sub>6</sub> | This work |
| pOTNeo4-PCV2dΔNAC-His | pOTNeo4-Δ2-40,Δ212-234 PCV2d-His<sub>6</sub> | This work |
| pOTNeo4-PCV3FL-His | pOTNeo4-PCV3-His<sub>6</sub> | This work |
| pOTNeo4-PCV3ΔN-His | pOTNeo4-Δ2-34 PCV3-His<sub>6</sub> | This work |
| pOTNeo4-PCV3ΔNΔC-His | pOTNeo4-Δ2-34,Δ195-214 PCV3-His<sub>6</sub> | 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 flu (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 H2SO4.

**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, BMGY 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 BMGY (pH 8.0) containing indicated additives, and protein secretion was compared at 72 h post-induction. Addition of Tween-20 and Antifoam-204 in BMGY 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 BMGY + 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...
**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.
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. marxianus 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.
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.

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