Evaluation of methylotrophic yeast *Ogataea thermomethanolica* TBRC 656 as a heterologous host for production of an animal vaccine candidate

Benjamas Chutiwitoonchai[a], Katanchalee Muensaen[a], Jaraspim Narkpuk[a], Peerada Promdonkoy[b], Kanokarn Kocharin[b], Amber R. Peswani[c], Colin Robinson[c], Lina Mikaliunaite[d], Niran Roongsawang[b], Sutipa Tanapongpipat[b], Peera Jaru-Ampornpan[a,#]

[1]Virology and Cell Technology Research Team, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani 12120, Thailand

[2]Microbial Cell Factory Research Team, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani 12120, Thailand

[3]School of Biosciences, University of Kent, Canterbury CT2 7NJ, United Kingdom

[4]Department of Biochemical Engineering, University College London, Gower Street, London, WC1E 6BT, United Kingdom

#Address correspondence to Peera Jaru-Ampornpan, Virology and Cell Technology Research Team, National Center for Genetic Engineering and Biotechnology (BIOTEC), Telephone: +66 2564 6700 ext 3395. Email: peera.jar@biotec.or.th

Running title: Production of swine vaccine candidate in *O. thermomethanolica*

Abstract

Multiple yeast strains have been developed into versatile heterologous protein expression platforms. *Ogataea thermomethanolica* TBRC 656 (OT), a thermotolerant methylotrophic yeast, has been shown to be suitable for production of several industrial enzymes. In this work, we demonstrated the potential of this platform for use in biopharmaceuticals manufacture. Using a swine vaccine candidate, porcine circovirus type 2d capsid protein, as a model, we showed that OT can be optimized to express and secrete the target protein at a respectable yield, competitive with other commercial yeast hosts. 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, thermotolerant methylotrophic yeast

Introduction

Due to low-cost cultivation, ease in genetic manipulation, and capability in 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]. Because successful production of each protein depends on multiple factors such as promoters, yeast hosts, complexity or glycosylation requirement of target proteins, 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 drug and vaccine development and bio-based industries, especially in low-income countries.

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 with superior performance at high temperature [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 some other high-valued protein-based biomolecules 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. Recombinant PCV2 capsid proteins produced by the baculovirus expression vector system remain one of the most successful commercial veterinary vaccines. More economical platforms such as bacteria and yeasts have been shown to produce potentially effective experimental PCV2 vaccines [10-13]. 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* [11-14].

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 [15]. Since the introduction of the first commercial vaccine in the mid-2000s, PCV2 has undergone genotype shift, with more dominant PCV2d strain completely replacing the first-discovered PCV2a, on which most of the commercial vaccines were based [16]. Despite some cross-protection, multiple pieces of evidence have suggested that a strain-matched vaccine could provide more effective protection, especially in experiments mimicking farm conditions [17, 18]. 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 [19]. Like the case of PCV2, retrospective serological surveys demonstrated 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 as an expression host for production of PCV2 and PCV3 subunit vaccine candidates.

We found that construct designs, codon optimization and culture condition optimization were crucial for successful protein expression. The construct containing an N-terminal deletion in the PCV2d capsid protein was expressed and secreted. 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 its potential as a viable, more cost-effective method to produce an updated PCV2 subunit vaccine.

Materials and Methods

Antigen designs

The amino acid sequence for PCV2d capsid protein was selected from the most frequent PCV2d representative from an extensive farm survey conducted in Thailand from 2009-2015 (accession number MF314329; [20]). PCV2d capsid protein codon optimization followed the published *P. pastoris*-based codon [11]. The amino acid sequence for PCV3 capsid protein was derived from the first PCV3 strain sequenced in Thailand (accession number MG310152.1; [21]). PV3 capsid protein codon optimization was based on the codon usage table for OT retrieved from the High-performance Integrated Virtual Environment-Codon Usage Tables (HIVE-CUTs) database described by Athey *et al.* [22].

PCV2d capsid protein fragment constructs encompassing known epitopes were designed based on the available information [23- 30]. 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 [31].

Construction of expression plasmids

Optimized DNA sequences for full-length PCV2d and PCV3 capsid proteins with flanking EcoRI and SacI restriction sites were synthesized by Synbio Technologies (New Jersey, USA). The coding sequences for the fragments, deletion in N-terminus (dN) or deletion in N- and C-termini (dNdC), were PCR-amplified from the synthesized gene using the primers possessing EcoRI and SacI restriction sites as shown in Table 1. Different constructs were then cloned into pOTNeo4 [32], modified to contain a SacI restriction site followed by a coding sequence for 6X-His tag at the C-terminus. All plasmids were verified by DNA sequencing.

Protein expression in O. thermomethanolica

One µg XmnI-linearized plasmids were transformed into *O. thermomethanolica* TBRC 656 by electroporation using the Bio-Rad gene Pulser system with a 2-mm cuvette with an electric field pulse: 5 kV/cm, 25 µF, 400 Ω. 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). Trial expression for each construct was performed on at least 20 positive clones. The cultures were grown in 5 ml YPD 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 OD600 = 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.

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-PCV2d-dN 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 1.8L working volume of BMGY medium in a 5-L bioreactor vessel for a 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 0.5g/h/L for 40 h followed by the simultaneous methanol induction and PCV2d-dN production stage in 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, glycerol batch and glycerol fed-batch, the temperature and pH were 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 PCV2d-dN. 5M 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 PCV2d-dN expression by Western blot analysis.

Purification of PCV2d-dN

The growth media was collected and filtered through a 0.45 μm filter (Merck, 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, 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, 250mM imidazole). All eluates were analyzed by

SDS-PAGE and Western blot. The eluates containing the major PCV2d-dN protein bands were concentrated and exchanged into storage buffer (1X PBS pH 7.4)

SDS-PAGE and Western blot analysis

Samples (clarified growth media or cell lysates) were separated on 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). The membrane was probed with a mouse monoclonal IgG anti-his antibody (R&D systems) or anti PCV2d capsid protein [33].

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 containing 30 µg of purified PCV2d-dN protein produced in OT or bacteria [33] or 200 µl PBS at a 1:1 volume ratio with complete Freud's adjuvant. On Day 14, mice were immunized again with 30 µg of purified protein or PBS mixed with incomplete Freud's adjuvant. 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 PCV2d-dN capsid protein produced in bacteria or 500 ffu (fluorescent focus units) of PCV2d virus [33] 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 for recombinant

protein or 1:200 for virus) 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 pAb Ms IgG (abcam) 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) 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). Data was analyzed with Prism9 (Graphpad software, San Diego, CA).

Table 1 Primers used in this study

Primer	Sequences
EcoRI-PCV2d-dN_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

Results

To assess the potential of the methylotrophic yeast *O. thermomethanolica* TBRC 656 (OT) in manufacturing high-value biotherapeutics, we chose to produce swine subunit vaccine candidates based on the capsid proteins from porcine circoviruses. The amino acid sequences of capsid proteins from representative PCV2d and PCV3 strains in Thailand were used for codon optimization [20, 21]. For PCV2d, the published PCV2b-based *P. pastoris*-optimized nucleotide sequence was modified to account for amino acids that are different between PCV2b and PCV2d [11]. For PCV3, codon optimization was based on the codon usage of *O. polymorpha*, which was evolutionarily closer to *O. thermomethanolica* [22].

As in some cases, smaller constructs could improve protein secretion and yields. Deletion of the N-terminus nuclear localization signal of the PCV2 capsid protein has been shown to improve expression in bacteria [27]. We also tried making a C-terminal truncation while preserving previously identified antigenic portions (Figure 1A; [23, 25-27]). Because of limited knowledge on PCV3 capsid, we made similar fragmental constructs based on the amino acid alignment with PCV2 capsid protein. In conclusion, for each protein, two other constructs were generated: ‘dN’ and ‘dNdC’ (Figure 1B). All successful OT integrants were confirmed with PCR.

Figure 2 summarizes trial expressions of these six constructs. For each construct, at least 20 positive integrants were grown in BMGY and induced for protein expression with 1% MeOH. 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 antibody. For all PCV2d capsid protein constructs, low levels of intracellular expression could be observed in some clones

(Figure 2 A-C). Notably, the PCV2d-dN construct could be observed intracellularly in most of the clones, while secreted PCV2d-dN could be observed in some clones, including Clone 2 and others (Figure 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 full-length and dN constructs showed signs of protein expression (Figure 2 D-F). The construct PCV3-dNdC showed very low expression in the cell lysate fractions from a few clones (Figure 2G). Therefore, the OT-PCV2d-dN integrant (clone number 2) with the highest expression level was selected for further work.

We further optimized the expression conditions by varying growth temperature, growth media pH and components of the growth media. When growth temperatures were reduced to 20 °C and 25 °C, larger expression of secreted PCV2d-dN capsid protein (hereafter termed "PCV2d-dN") was observed compared to the original 30 °C condition (Figure 3A). When compared at the same growth temperatures, BMMY media maintained at higher pH, especially in the range of 7.5 to 9.0, yielded larger expression of secreted PCV2d-dN (Figure 3B). Previous works have shown that addition of surfactants or antifoam matter could enhance recombinant protein yields in yeasts [34]. OT-PCV2d-dN 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 PCV2d-dN in the media, while AFE-1520 and PEG2000 mildly enhanced PCV2d-dN levels (Figure 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, optimization of expression conditions yielded the optimal conditions for PCV2d-dN secretion from the OT host: 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 (Figure 3D). The doublet bands were observed possibly belonging to PCV2d-dN and its slightly smaller cleavage product as also observed in the same construct expressed in *E. coli* [33].

Improvement in levels of secreted PCV2d-dN could involve several mechanisms, in particular enhanced expression/secretion of the target protein or increased stability of the target protein. We attempted to tease apart these possibilities by testing protein degradation in cell-free supernatants. The cell-free supernatant was taken from OT-PCV2d-dN grown at 30 °C in the pH 8 induction media and adjusted to indicated pH before further incubation at 30 °C. Samples were taken every 12 hours to assess the amounts of intact PCV2d-dN in the cell-free supernatant. Western blot analysis showed that, at lower pH of 5 and 6, most of the protein had degraded in 12 hours, and almost no protein was left by 48 hours (Figure 4A). At pH 7, degradation was not as pronounced, while at pH 8 most of the protein was retained (Figure 4A). Additionally, we tested the stability of PCV2d-dN at different temperatures. The cell-free supernatant from the optimal condition was stored at 20 or 30 °C incubators and sampled every 12 hours. While a large fraction of the protein remained intact after 48 h if stored at 20 °C, most of it disappeared between 24-36 h if stored at 30 °C (Figure 4B). Based on these data, we suspected that PCV2d-dN was degraded by OT proteases. We tested this by repeating the experiment with cell-free supernatant in the presence of protease inhibitor cocktail (PIC). In the presence of PIC, even at 30 °C storage after 48 h, almost none of the protein was degraded (Figure 4B).

Furthermore, adding the protease inhibitors into the original induction media could maintain accumulation of PCV2d-dN at normal growth pH and temperature (Figure 4D).

Next, we purified the target protein from the culture media and determined the yield. OT-PCV2d-dN 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). To remove incompatible components in the yeast growth media, the clarified media underwent ammonium precipitation and buffer exchange into Tris-buffered solution (pH 8.0) prior to incubation with Ni-NTA resin. In a side-by-side batch purification, eluates at equal volumes from both culture conditions were compared. While none of the protein could be detected from the original expression condition, highly-purified PCV2d-dN doublets could be observed with Coomassie staining after the Ni-NTA purification (Figure 5, top). Protein identity was confirmed by Western blotting with anti-PCV2 capsid protein (Figure 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/mL of yeast culture supernatant (Table 2). The purity was > 90%.

Large-scale production of PCV2d-dN 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 (Figure 6). When the glycerol concentration became the limiting factor for growth, determined by the increase in dissolved oxygen level, the methanol-feeding

phase commenced by feeding methanol at T = 44 h (Figure 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 (Figure 6, inset). As the fermentation progressed, the accumulation of PCV2d-dN was observed in the media from the start up to T = 83 h (Figure 6, inset). Between 83 h 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.

We next tested if PCV2d-dN produced in the OT system could induce antibodies in test animals. A group of six mice were immunized intraperitoneally with two doses of 30 μg purified PCV2d-dN, either from bacterial or yeast origins, mixed with adjuvant on Day 0 and Day 14. Compared with the mice injected with adjuvant only, anti-PCV2 IgG was observed on Days 28 and 35 in PCV2d capsid protein-based ELISA (Figure 7A). These antibodies also recognized the PCV2d virus particles as observed in the ELISA using the viral particles as the coating antigen (Figure 7B). Compared with the same construct produced in the bacterial system which has been shown to induce neutralizing antibodies in animals [33], the protein produced from OT exhibited almost identical antigenicity, suggesting its potential as an alternative platform for recombinant subunit vaccine production.

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*. 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 self-sustainable in bio-based industries is the cost associated with using commercial host systems.

Experimental PCV2 vaccines have been explored in multiple yeast platforms with different yields and characteristics [11-14, 35]. In this work, we demonstrated that the *O. thermomethanolica* (OT) platform, a methanol-inducible, thermotolerant system utilized in production of fungal-derived industrial enzymes [6, 9], can produce PCV2d antigen capable of inducing specific antibodies against PCV2d viruses. At optimal conditions, the OT system could yield upto 120 µg of purified recombinant PCV2d capsid protein per mL of yeast culture. Compared to other yeast hosts (Table 2), our platform offered a simpler purification scheme without cell lysis and a competitive yield after purification. Moreover, the trial run on the bioreactor showed feasibility in the scale-up process. The next step is to optimize growth and protein expression in minimal media to determine the yield and the cost of goods that more closely represent industrial scale production.

There are two critical challenges for the current OT system illustrated in this study. First, despite using the native OT alpha factor as the secretory signal which gave higher yield of the secreted proteins than the *S. cerevisiae* alpha factor [7], much of PCV2d-dN still accumulated

inside the cells. Despite theoretical capacity, heterologous protein secretion in yeast hosts varies widely depending on properties of the recombinant proteins and the host strains and the compatibility between the secretory signal, the secretory apparatus, and the sequence or structure of the recombinant protein [36]. 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 [36], or screening genome-wide for suitable secretion signals and fusion partners [37]. The second challenge is the excessive protease activities. Our results strongly suggest that secreted PCV2d-dN was degraded by OT proteases, and inhibition of these proteases whether by raising the pH condition, by lowering growth temperature, or by the presence of 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 such as optimizing fermentation conditions as explored in this and other works [38, 39]. For a more universal approach, many protease-deficient yeast strains have been developed through genetic manipulation [40- 42]. With improvements in these critical areas, we hope that the OT system can serve as a truly versatile heterologous protein production platform.

In summary, we reported the use of thermotolerant methylotrophic yeast *O. thermomethanolica* in production of a PCV2d swine vaccine candidate. The secreted and purified PCV2d-dN capsid protein was effective in inducing antibody response in animal models. Compared to other yeast-based recombinant protein production systems for PCV2

capsid protein, OT performed on par with, if not better, than those reported in the past (Table 2). To expand its potential into non-fungal recombinant protein production, the current OT systems can be improved on 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.

Table 2 Comparison of recombinant PCV2 capsid protein from different yeast platforms

Platform	Expression yield (μg/mL yeast culture)	Post-purification yield (μg/mL yeast culture)	Reference
P. pastoris (intracellular)	174	-	[11]
P. pastoris (secretion)	<140 (quantified total protein)	-	[35]
S. cerevisiae (intracellular)	Not quantified	Not quantified	[14]
H. polymorpha (intracellular)	Not quantified	10.8	[12]
K. marxiamus (intracellular)	1900	652.8	[13]
O. thermomethanolica (secretion)	Not quantified	120	This study

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Figure 1

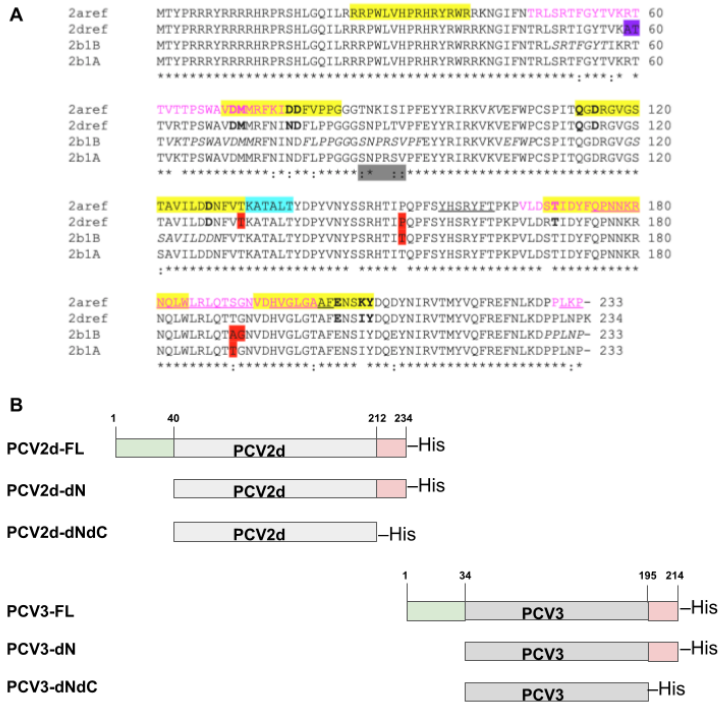

Figure 1 Antigen design for PCV2d and PCV3 subunit vaccines. (A) Alignment of the reference sequences for PCV2a (AF055392), PCV2b (AF055394 and AY678532) and PCV2d (AY181946). Immunodominant epitopes are in magenta [23]. Underlined, italicized or highlighted in yellow are linear epitopes identified by various past works [24- 26]. In bold are residues predicted to bind to antibodies by structural analysis [27]. Highlighted in red and purple are residues important in neutralization by antibodies [28, 29]. Highlighted in cyan is the PCV2-specific neutralizing epitope [30]. The gray box is the stretch that can be used to distinguish among different PCV2 subtypes [43]. (B) Schematics represent different constructs of PCV2d and PCV3 antigens tried in this work.

Figure 2

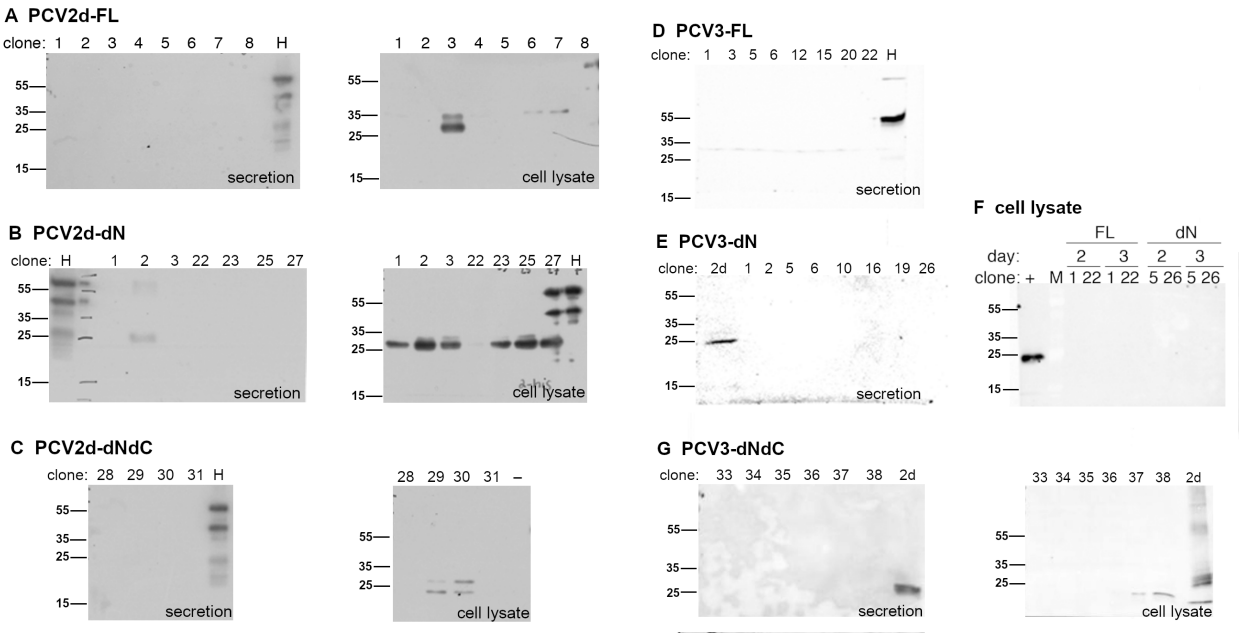

Figure 2 Trial expression of PCV2d (A–C) and PCV3 (D–G) antigen constructs in *O. thermomethanolica*. The his-tagged constructs in the secretion and cell lysate fractions were visualized by SDS-PAGE and Western blotting with anti-His antibody. ‘H’, unrelated his-tagged protein control. ‘2d’, OT-PCV2d-dN (clone 2). ‘M’, lane for protein marker.

Figure 3

Figure 3 Optimization of culture conditions for PCV2d-dN expression. Effects of culture temperature (A), pH of culture media (B) and additives (C) were investigated in shake flask culture of OT-PCV2d-dN. (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 SDS-PAGE and Western blotting with anti-His antibody.

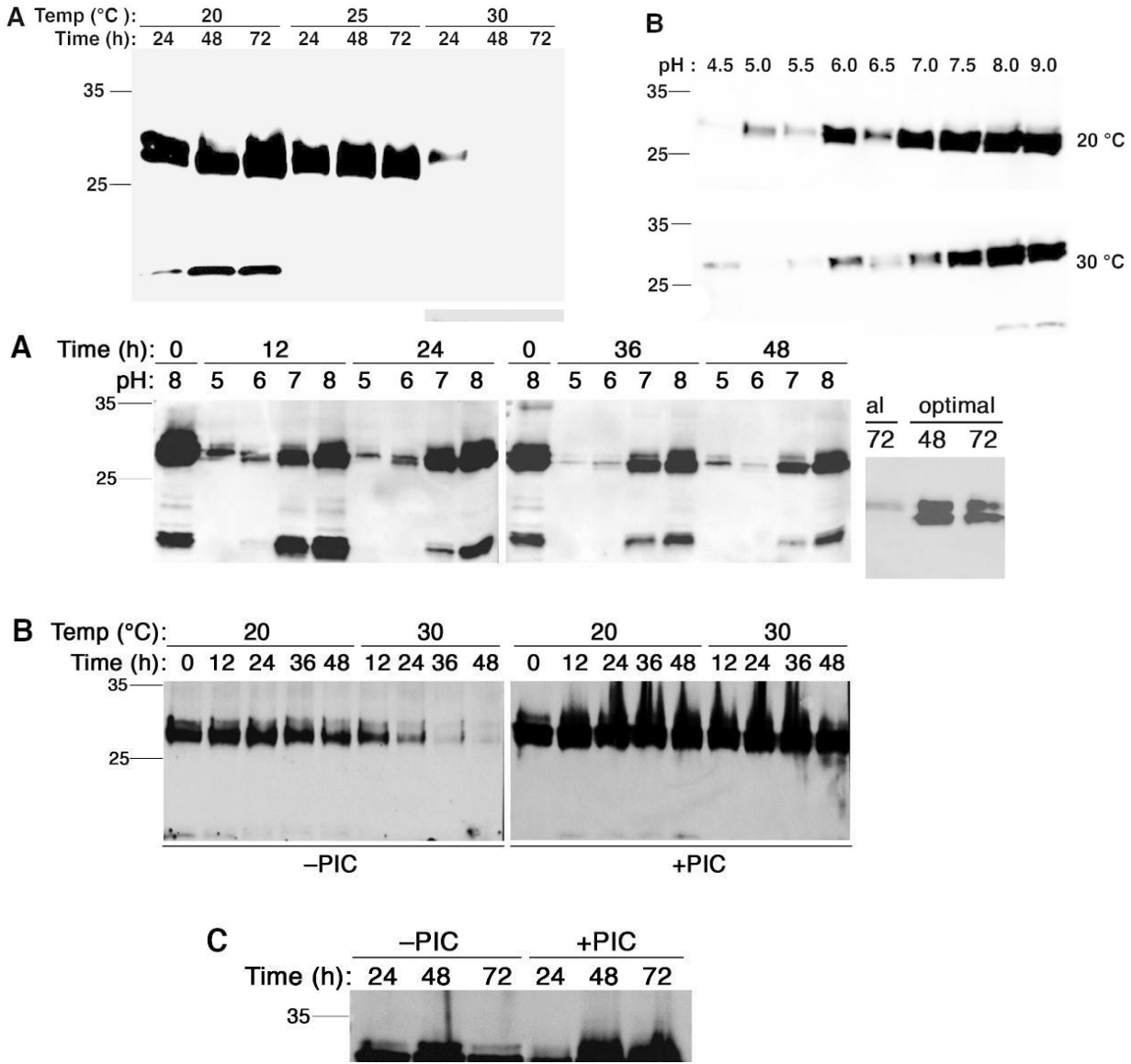

Figure 4

Figure 4 Secreted PCV2d-dN was degraded by OT extracellular proteases. Cell-free supernatant from OT-PCV2d-dN grown at 30 °C in the pH 8 induction media was adjusted to indicated pHat 30 °C incubation temperature (A) or to indicated incubation temperatures with or without addition of protease inhibitor cocktail (PIC) (B). Levels of leftover protein at different time points were visualized by SDS-PAGE and Western blotting with anti-His antibody. (C) PCV2d-dN was expressed at the sub-optimal condition in the presence or absence of protease inhibitor cocktail in culture media. Samples were taken at different time points and visualized by SDS-PAGE and Western blotting with anti-His antibody.

Figure 5

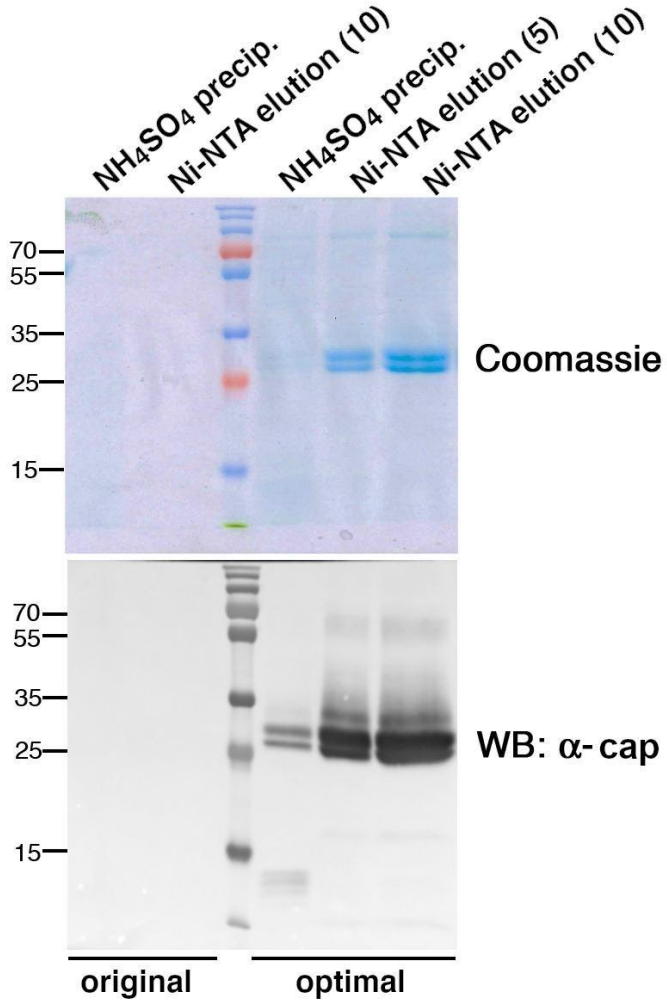

Figure 5 Purification of PCV2d-dN from equal amounts of OT-PCV2d-dN culture media from original and optimal conditions by ammonium sulfate precipitation and Ni-NTA resin. '5' and '10' indicated different amounts of eluates loaded on the gel.

Figure 6

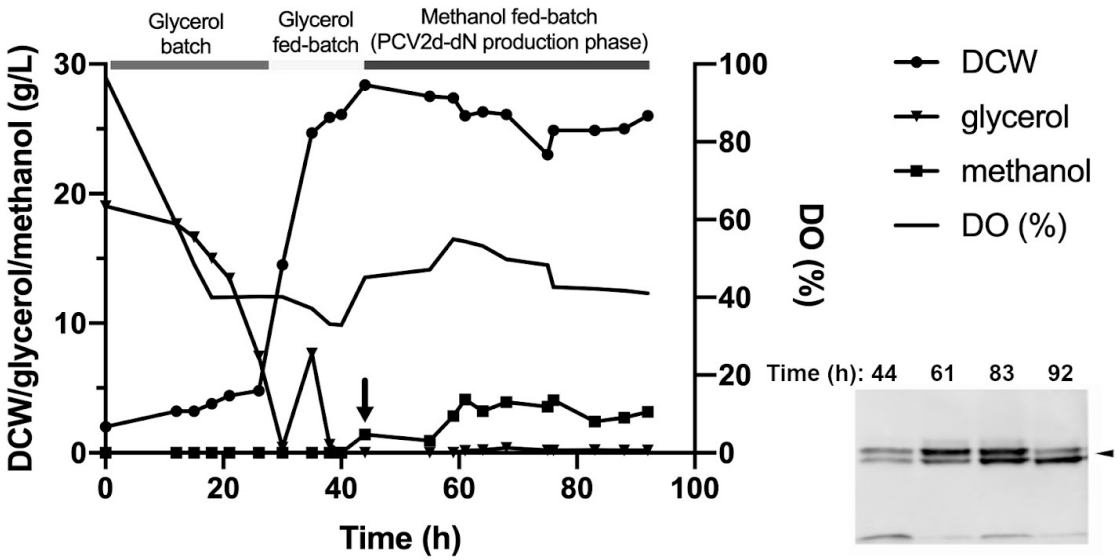

Figure 6 Growth and PCV2d-dN production by *O. thermomethanolica* under the methanol-inducible condition. Glycerol was used in batch and fed-batch stages. The methanol feed rate was continuously fed at 5 g/L in the production stage. Arrow indicates the start of methanol feed. Inset shows Western blot analysis of culture samples at different time points. Black arrowhead indicates the doublet bands characteristic of PCV2d-dN.

Figure 7

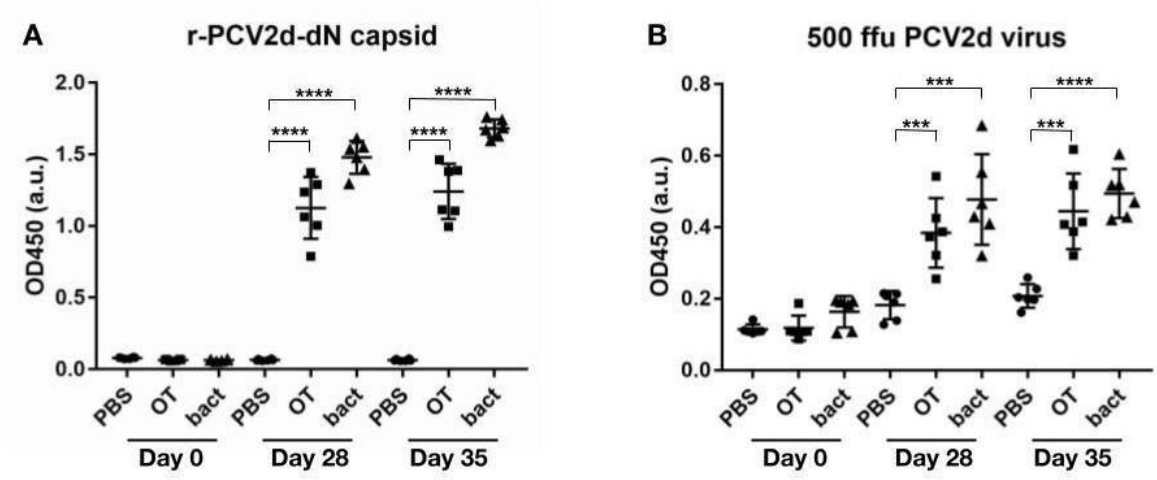

Figure 7 PCV2d-specific antibody response in mice immunized with PCV2d-dN antigen produced from OT yeast (squares) and bacteria (triangles; PMID: Peswani *et al.*, 2021) compared to the control group receiving PBS buffer (circles). The PCV2d-specific IgG was detected in mice sera (1:5000 and 1:200 dilution) by ELISA using PCV2d capsid protein (A) and inactivated PCV2d virus (B) as coating antigens. Each data point represents one mouse. Bars represent average values within groups. ***, $p < 0.0005$; ****, $p < 0.0001$.

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