

Heterologous expression of Bluetongue VP2 viral protein fragment in *Pichia pastoris*

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Abstract The partial VP2-encoding gene of Bluetongue virus serotype 23 (BTV-23) was amplified using reverse transcription polymerase chain reaction (RT-PCR) and inserted into pPICK9K vector. Recombinant plasmid DNA was integrated into the genome of *Pichia pastoris* by electroporation and expressed protein was identified by SDS-PAGE. High-level secreted expression was achieved after selecting the *Mut*⁺ phenotype with multi-copy integrant in the recombinant yeast. The partial fragment of Bluetongue VP2 protein (BTV VP2) of approximately 45 kDa was secreted into the culture supernatant by the recombinant yeast when induced with methanol. Western and immuno dot-blotting methods confirmed the expressed BTV VP2 protein. The expressed protein has been demonstrated to be immunogenic in rabbits. A standardized method has been evolved for optimal expression and high-level production of the recombinant protein (284 mg/L). This is the first report demonstrating the possibility of mass production of BTV VP2 protein using *P. pastoris*.

Keywords Bluetongue virus serotype 23 · *Pichia pastoris* · Secreted BTV VP2 expression · Shake flask culture

Introduction

Bluetongue (BT) is an arthropod borne infectious and non-contagious viral disease of ruminants. While sheep, goats, cattle, buffaloes, camels, antelopes, and deer are all susceptible, sheep are most severely affected. Biting midges belonging to the species *Culicoides* are implicated in transmission [1]. BT is widespread in many parts of the world causing considerable harm to livestock. The double stranded RNA virus causing BT belongs to the genus *Orbivirus* and family *Reoviridae*. The Bluetongue VP2 protein forming the outer capsid determines the virus serotype [1]. The initial portion of VP2 protein consisting of three neutralizing epitopes is known to be conserved [2]. The current studies were intended to express this portion of BTV VP2 gene in a suitable expression system with a view to develop a potential and cost-effective subunit vaccine for efficient control of BT. *Pichia pastoris* is a methylotrophic yeast that has emerged as a highly popular expression host for the recombinant protein production on a large scale [3, 4]. This yeast can survive on methanol medium and uses methanol as its sole carbon source and energy. It also does not secrete high levels of endogenous proteins making the purification of the recombinant protein simple [5].

Complete BTV VP2 gene has been cloned and characterized [6]. But the possibility of expression of this partial gene sequence in yeast system has never

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been attempted. To the best of our knowledge, no studies have been undertaken so far to express this partial antigenic recombinant protein in any alien system. The current report is the first one demonstrating and standardizing the method for production of recombinant BTV VP2 preparing the necessary background for commercial production of subunit vaccine for BT. The current study demonstrates the complete procedure involving transformation, selection of recombinant colonies, and expression of the target protein followed by its purification.

Materials and methods

Isolation of BTV VP2 gene fragment

BTV serotype 23 propagated in Baby Hamster Kidney (BHK-21) cell line was used in the present study and initial part of BTV VP2 gene, i.e., 1,064 bp was isolated from the virus using the following gene-specific primers employing reverse transcription polymerase chain reaction (RT-PCR).

BTV VP2LT (*EcoRI*): 5' GCGAATTCGGTTAAA ATAGTGTCGCGATGGA 3' (Base pairs: -9 to +21 NCBI accession number:L46685)

BTV VP2RM (*HindIII*): 5' GTATCGGAAGCTTC AATCAT 3'. (Base pairs: +1047 to +1066 NCBI accession number:L46685)

Total RNA from BTV infected BHK-21 cells was extracted using Trizol reagent (Gibco BRL, USA) method and was further subjected to cDNA synthesis at 42°C for 60 min using Murine Moloney Leukemia Virus (M-MLV) reverse transcriptase enzyme with VP2 specific primers (Bioserve Biotechnologies, Hyderabad, India). The cDNA was amplified through PCR employing the same primers. PCR was carried out under the following conditions: 94°C for 45 s, 59°C for 40 s, 72°C for 50 s, for 35 cycles, and finally 72°C for 10 min.

Construction of recombinant pPICK9KBTVP2 secretion expression vector

Bacteria, Yeast, and growth media

The *E.coli* DH5 alpha strain, *E.coli* BL21 (Invitrogen, USA) used as hosts for DNA manipulations, and expression, respectively, were cultured in Luria–Bertani medium (Himedia, India) that was supplemented with 50 µg/ml ampicillin for the selection of transformants. The *Pichia pastoris* GS115 strain (Invitrogen, USA) was cultured in YPD medium (1% yeast extract,

2% peptone, 2% dextrose plus 2% agar in plates). Since GS115 has a mutant allele of the *HIS4* (*P. pastoris* histidinol dehydrogenase gene) and *AOX I* gene, it is a histidine auxotroph with a methanol utilization slow (*Muts*) phenotype.

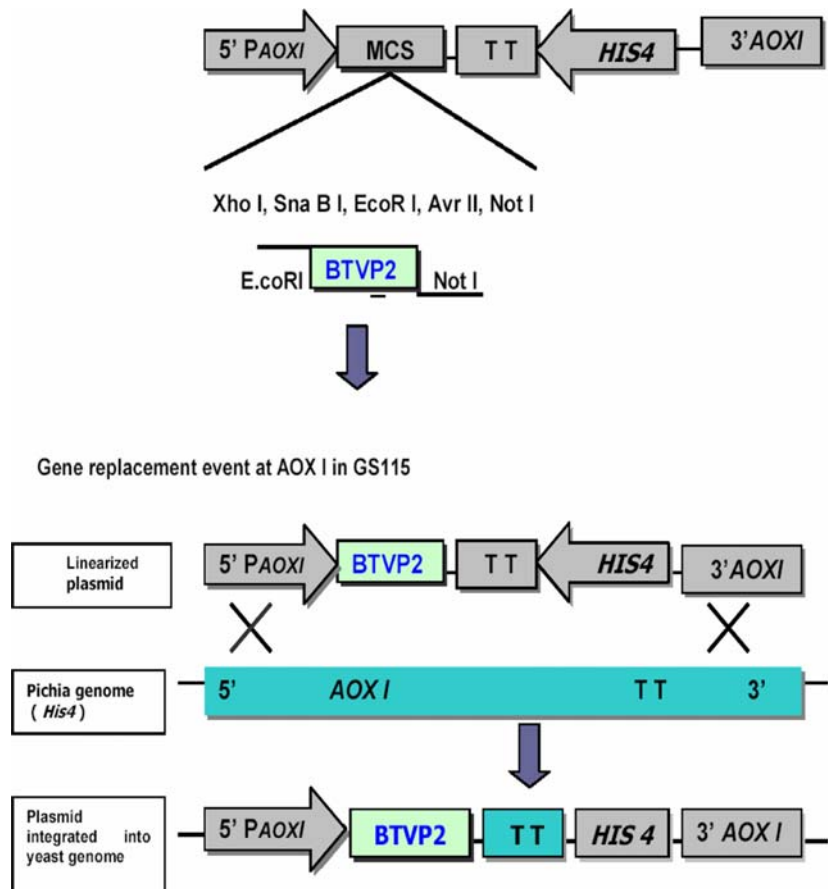
The BTV VP2 gene fragment was cloned into pET32(a) bacterial expression vector (Novagen, USA) at *EcoR I* and *Hind III* sites and the recombinant vector pET32(a)BTV VP2 was checked for VP2 protein expression in *E.coli* BL21 cells. The gene was further amplified from pET32(a) BTV VP2 chimeric plasmid using the same oligonucleotide primers except for changing the restriction site in the reverse primer (*Hind III* was replaced with *Not I*) and sub-cloned into pPIC9K yeast transfer vector at *EcoR I* and *Not I* sites under the control of *AOX I* promoter with *Saccharomyces cerevisiae* alpha factor secretion signal. Use of *EcoR I* and *Not I* recognition sequences in primers facilitated the insertion of BTV VP2 gene into pPIC9K yeast transfer vector. The resultant recombinant vector was named as pPIC9KBTVP2 (Fig. 1). The alpha secretory signal present in the vector upstream to the BTV VP2 gene was used to make the target protein to secrete into the medium. The recombinant vector was mobilized into *E.coli* DH5 alpha electro competent cells via electroporation at 1,700 V using Eppendorf electroporator [7]. The positive bacterial transformants were selected through restriction digestion of plasmid DNA using *EcoR I* and *Not I* enzymes and PCR analysis.

Transformation of yeast with pPICK9KBTVP2 and screening of *Pichia pastoris* expression strains through PCR and Southern hybridization (Dot-blotting)

Approximately 10 µg of recombinant expression plasmid pPIC9KBTVP2 was linearized by digesting with *Sac I* enzyme to get *HIS*⁺, *Mut*⁺ transformants in *Pichia pastoris* GS115 cells (Invitrogen) and used to transform competent *P. pastoris* cells by electroporation using Eppendorf electroporator at 1,450 V [7]. After transformation, cells were plated on SD-His plates (1.34% yeast nitrogen base, 2% dextrose, 0.01% complete amino acid supplement minus Histidine, 1 M sorbitol supplement, and 2% agar), and incubated at 30°C for 4–6 days until colonies appeared. The parent pPIC9K without insert, linearized with *Sac I* was also transformed as negative control.

The colonies obtained were re-streaked on fresh SD-His plates. Transformants bearing the chromosomally integrated copies of the pPICK9KBTVP2 were then detected by a genomic PCR assay using the

Fig. 1 Cloning strategy of partial Bluetongue virus VP2 gene in pPIC9K yeast transfer vector and integration event in *Pichia pastoris* (GS115): A gene replacement (omega insertion) event arises from a double cross-over event between the *AOXI* promoter and 3' *AOXI* regions of the vector and genome resulting in complete removal of the *AOXI* coding region (gene replacement)



BTVP2 gene primers BTVP2LT and BTVP2RM. Genomic DNA extracted from sixteen colonies was subjected to PCR along with appropriate control samples as described earlier [7]. All sixteen DNA samples were also subjected to Southern hybridization assay (Dot-blotting) using newly constructed BTVP2 non-radioactive digoxigenin probe. The DNA samples were heat denatured and spotted on nitrocellulose membrane. The transferred DNA samples were hybridized with VP2 specific probe and the hybridization reactions were detected using anti-DIG alkaline phosphatase [8]. Both transfer and hybridization reactions were performed using Hybri-max blotting and hybridization system (HybriBio, Hong Kong).

Optimization of BTVP2 protein expression in *Pichia* system

The colonies that were found positive with PCR and Southern hybridization assay were selected for induction. Three *His⁺ Mut⁺* colonies were inoculated into 50-ml buffered complex glycerol medium (BMGY) (1.34% yeast nitrogen base without amino acids [Hi-

media, India], 0.00004% biotin, 1% glycerol, 0.004% histidine in 100 mM potassium phosphate buffer, pH 6.0) taken in 250 ml conical flask along with negative control (*Pichia* transformed with pPIC9K without insert) and were incubated at 28°C in a shaker incubator at 250 rpm until the culture reached an A_{600} of 2–4. The cells were harvested by centrifugation at 3,000g for 5 min at room temperature and the cell pellet was suspended in buffered methanol complex medium-BMMY (1.34% yeast nitrogen base without amino acids, 0.00004% biotin, 1–2% methanol, 0.004% histidine in 100 mM potassium phosphate buffer, pH 6.0) so as to get an A_{600} of 1.0 in a conical flask for induction with proper aeration. Incubation was continued at 28°C on an orbital shaker (250 rpm) with the addition of methanol to achieve concentrations ranging from 0.5–2% (0.5%, 1%, 1.5%, and 2%) at every 24 h to sustain the induction [3, 9, 10]. Different induction periods ranging from 16–120 h (16 h, 24 h, 48 h, 72 h, 96 h, and 120 h) were also tested along with different methanol concentrations as above in replicates of three each to find the optimal expression conditions. After every induction with varied methanol concentration and incubation periods, the protein in

the culture supernatants were precipitated using 30% ammonium sulfate solution and were quantified using spectrophotometer (Nanodrop, USA) and were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Analysis of the expressed products through SDS-PAGE and confirmation of the expressed BTV VP2 protein through Western and immuno dot-blotting

The expressed protein samples were separated by electrophoresis on a 12% denaturing sodium dodecyl sulfate-polyacrylamide gel [7]. The secretory expression of BTV VP2 protein was confirmed with BT-positive serum through Western and immuno dot-blotting employing Hybrimax blotting and hybridization system using the new rapid protocol that was developed in the laboratory. Briefly, proteins were transferred from the gel onto nitrocellulose membrane in case of western blotting whereas for immuno dot-blotting, the samples were directly spotted on the membrane. After transfer, both the membranes were probed sequentially with 1:500 dilution of the BTV VP2 specific polyclonal antibody raised in rabbit (against the same partial protein expressed in *E.coli* by the authors in a separate study) and 1:1,000 dilution of horse radish peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG). Protein samples were visualized with ODD/H₂O₂ (O-Dianisidine dihydrochloride-hydrogen peroxide) chromogen-substrate solution [11].

Purification of the target BTV VP2 protein

Single colony of yeast transformant showing high level of secreted expression was cultured under optimal expression conditions and the culture supernatant was collected after methanol induction. The protein was precipitated using ammonium sulfate at 65% saturation and the pellets were dissolved in a minimum volume of 0.01 M phosphate buffer, pH 7.0. The concentrated sample was loaded onto Sephadex G-100 column and eluted with the same phosphate buffer. Immuno dot-blotting identified the elution fraction containing BTV VP2 fraction.

Immunization studies in rabbits and detection of serum antibodies through antibody capture ELISA

Three healthy male rabbits 18–20 week-old were immunized subcutaneously with 100 µg of purified BTV VP2 fraction protein in Freund's complete adjuvant at three sites, and two rabbits of the control group

were immunized with the expressed product of negative control *P. pastoris*. After two booster doses with the same samples using incomplete Freund's adjuvant at 15 days intervals, blood samples were collected from marginal vein after 12 days of second booster shot, and sera were separated. Antibody capture ELISA technique was applied to detect the antibodies against BTV VP2 partial protein [11]. Briefly, the purified BTV VP2 partial protein expressed in *Pichia* was coated overnight onto Nunc polystyrene microtitre plates (500 ng/well in 0.1 M sodium bicarbonate, pH 8.0) and the unreacted sites were blocked using 3% BSA. The captured proteins were reacted first with the above rabbit hyper-immune sera followed by horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G secondary antibody conjugate (Bangalore Genie, India) for 1 hour each. Enzymatic color development was done using ODD/H₂O₂ chromogen/substrate solution [11].

Results

Isolation of BTV VP2 gene and construction of pPIC9KBTVP2 recombinant vector

The 1,064 bp fragment of BTV VP2 gene was isolated through RT-PCR. The gene was first cloned in bacterial expression vector pET32(a) and its expression was confirmed in *E.coli* BL21 cells (data not shown). The gene was further subcloned into pPICK9K yeast transfer vector at *EcoR I* and *Not I* sites and the recombinant construct; pPICK9KBTVP2 was mobilized into *E.coli* DH5 alpha for its mass production before transforming in *Pichia*.

Transformation and selection of recombinant *Pichia* clones carrying foreign gene through PCR and Southern hybridization assay (Dot-blotting)

Transformation of *Pichia* through electroporation yielded 1.6×10^2 His⁺ transformants/µg of DNA. Three out of sixteen genomic DNA samples isolated from randomly selected yeast transformants amplified the expected 1,064 bp DNA along with known positive control whereas negative sample did not show any bands (Fig. 2). Southern hybridization assay performed for all sixteen DNA samples using BTV VP2 non-radioactive digoxigenin probe also resulted in positive signals in all the three samples that were previously found positive through PCR further confirming successful transformation event (Fig. 3).

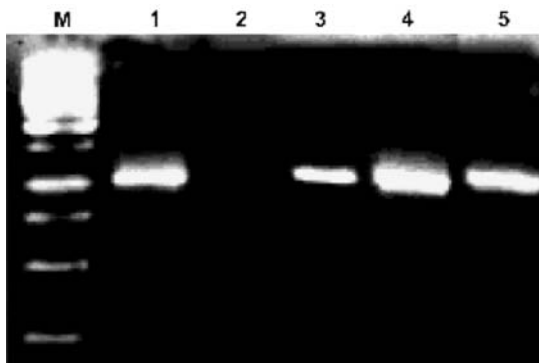


Fig. 2 Gel showing amplification of Bluetongue virus VP2 (BTV VP2) fragment (1,064 bp) through PCR from transformed yeast colonies: Lane M: Kilo bp marker (MBI Fermentas, USA). Lane 1: Amplification from positive control (pPICK9KBTVP2 plasmid DNA). Lane 2: Negative control (Genomic DNA from yeast transformed with pPICK9K without insert). Lane 3–5: Amplification from three different *Pichia* pPICK9KBTVP2 transformants

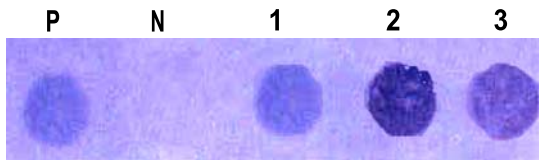


Fig. 3 Southern hybridization analysis (Dot-Blotting) of genomic DNA from *Pichia* transformants: P: Positive control (pPICK9KBTVP2 plasmid DNA). N: Negative control (Genomic DNA from yeast transformed with pPICK9K without insert). 1–3: Genomic DNA from three different *Pichia* pPICK9KBTVP2 transformants

Optimization of BTV VP2 protein expression in recombinant GS115 *Pichia* system and analysis of the expressed products through SDS-PAGE

All the three *His*⁺ *Mut*⁺ colonies that were found positive with both PCR and Southern hybridization were selected for inducing the expression of the target gene. In order to study the expression of BTV VP2 gene fraction, the optimal method and growth conditions necessary for expression were standardized. Out of three clones induced, only one clone showed expression of the expected 45 KDa protein after 72-h post-induction (Fig. 4), whereas there was no specific protein band detected in pPICK9K vector transformed yeast and uninduced positive transformant at that range. The protein bands were clearly visible only in case of the sample that was incubated for 72 h and no clear bands were observed before or after 72 h of incubation. Studies conducted to scale-up the expression level with increased induction period and methanol concentration revealed that 72 h of post-induction

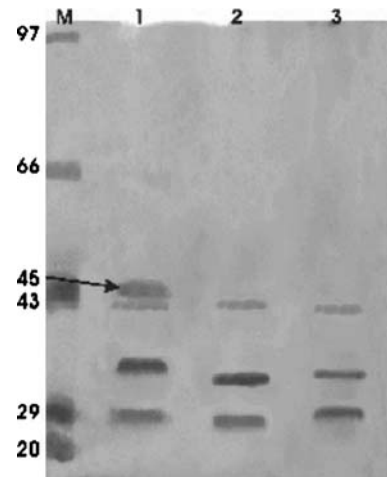


Fig. 4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%) showing expression of 45 KDa Bluetongue virus VP2 (BTV VP2) partial protein in *Pichia*; Lane M: protein molecular weight marker (MBI Fermentas, USA). Lane 1: secreted protein from *Pichia* transformed with pPIC9KBTVP2 (Induced). Lane 2: secreted protein from *Pichia* transformed with pPIC9KBTVP2 (Un-induced). Lane 3: secreted protein from *Pichia* transformed with pPIC9K (without insert)

incubation period with 1.5% methanol concentration is ideal for large amount of BTV VP2 protein expression (284 mg/L of the culture supernatant). However, the level of expression could not be further boosted above this scale with either increased methanol concentration or increased duration of incubation. BTV VP2 protein expression was observed even after six passages in the medium.

Western and immuno dot-blotting assay for the expressed protein

Ammonium sulfate precipitated protein from the positive transformant that showed expected band of 45 KDa through SDS-PAGE was analyzed through Western blotting to confirm the specificity of the expressed protein. Appropriate positive signal was obtained in case of the positive transformant, whereas the protein sample transferred from *Pichia* transformed with pPICK9K negative control did not develop any signal on the membrane (Fig. 5). Immuno dot-blotting test showed positive signals with precipitated protein before purification and in fractions that contained the BTV VP2 protein during gel filtration (Fig. 6).

Immunization studies in rabbits and detection of serum antibodies through antibody capture ELISA

The results from ELISA test showed that the serum from the rabbits immunized with purified BTV VP2

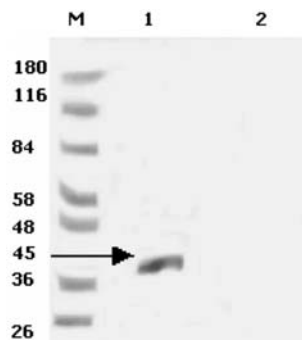


Fig. 5 Western blotting analysis for confirming the specificity of the expressed 45 KDa Bluetongue virus VP2 partial protein from *Pichia pastoris*: Lane M: Pre stained protein molecular weight marker (Sigma, USA). Lane 1: Positive *Pichia* pPIC9KBTVP2 transformant (Induced). Lane 2: Protein from *Pichia pastoris* transformed with pPICK9K (Without insert)

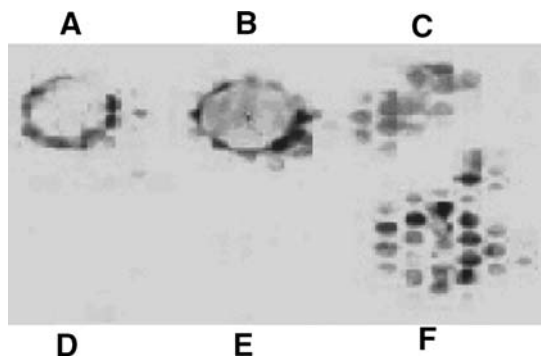


Fig. 6 Immuno dot-blotting analysis of the yeast purified BTVP2 protein through gel chromatography: A–C: Purified BTVP2 eluted fractions after gel chromatography. D: protein sample from culture supernatant of pPIC9KBTVP2 transformant (Un-induced). E: protein sample from culture supernatant of pPIC9K transformant (Without insert), F: Positive control (Purified bacterially expressed BTVP2 protein)

protein could react with recombinantly expressed BTVP2 protein coated in the well, while the serum from the rabbit immunized with the protein from *P. pastoris* transformed with pPICK9K negative control did not show any reaction.

Discussion

The *Pichia pastoris* system used in the present study is able to utilize methanol as its sole carbon source and has been widely used as a host for the expression of heterologous proteins. In this study, the partial length of BTVP2 gene was inserted towards the downstream of AOX1 promoter of the secretory expression vector pPICK9K and the chimeric construct was inte-

grated into the host chromosome through homologous recombination. The recombinant yeast can also perform many eukaryotic post-translational modifications in the target protein, such as glycosylation, disulfide bond formation, and proteolytic processing. As the target gene is integrated within the genome, it is difficult to lose the target gene when the recombinant yeast is cultured and passaged. Therefore, *P. pastoris* has been used successfully to express a wide range of heterologous proteins [9, 12, 13]. Heterologous expression in *P. pastoris* can be either intracellular or secreted. Secretion requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. Although all three positive colonies were selected for induction, only one colony showed expression of the target protein and the remaining two did not show detectable levels of the target protein in the gel for which reasons are not known. Hybridmax blotting and hybridization system employed in this work was efficient in screening large number of samples rapidly. The method developed for DNA transfer, hybridization, and color detection yields results very efficiently in less than 60 min.

SDS-PAGE and Western blotting analysis showed that a specific protein, whose molecular weight is approximately 45 KDa is expressed in *P. pastoris* and the expressed protein can react with positive BT serum. Gene expression was seen even after six passages in the medium, indicating good genetic stability of the introduced gene fragment within the recombinant yeast system. In order to increase the expression level, the expression conditions were optimized. Under the optimal condition, the expressed protein amounted to about 284 mg/L of the culture. The protein bands were clearly visible only in case of the samples that were incubated for 72 h and no clear bands were observed before or after 72 h of incubation. This could be due to the critical incubation period, i.e., 72 h required for biomass accumulation. Very faint but multiple bands were observed following 96 h and no bands were visible after 120 h of incubation indicating that host-specific proteases may be acting on the protein following prolonged incubation. The observed low level of expression may be partly attributed to the low copy number of the genes integrated within the yeast genome and partly to the nature of the protein [9]. The most important parameter for efficient expression in *Pichia* was found to be adequate aeration during methanol induction and hence the culture volume within the flask was kept as low as 20% of the total flask volume. It was also necessary to maintain the incubation temperature at 28°C with rotation of 250–300 rpm. With the use of fermenter and codon

optimization of BTV VP2 gene, the expression level can be further improved. The BTV VP2 protein contains neutralizing epitopes [14–17] and is a potential target for developing a subunit vaccine. Although we have demonstrated that the expressed partial BTV VP2 protein can elicit an immune response in rabbits, studies like serum neutralization, and BT viral challenges in sheep are required to confirm its action as a potential subunit vaccine. Since the ultimate goal of our study was recombinant production of BTV VP2 in *P. pastoris* that could be taken up for upscaling, we did not carry out extensive studies on vaccination in sheep. However, the recombinant protein expressed may have potential applications in developing serotype-specific diagnostics and also as a subunit vaccine, which needs further studies.

Conclusion

In conclusion, the partial BTV VP2 recombinant protein has been expressed for the first time in an alien expression system namely the yeast, *Pichia pastoris*. Our studies indicate that *Pichia pastoris* can be an excellent alternative for large-scale production of this recombinant protein. The pPICK9KBTVP2 recombinant vector constructed in the present study is suitable for secretory expression of the target protein and can be employed for commercial production of recombinant BTV VP2 protein for several reasons including the practical feasibility like ease of expression and purification of the recombinant protein.

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