



Optimization of Culture Conditions for Expression of Anti-lipopolysaccharide Single Domain Antibody Clone in *Pichia pastoris*

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ABSTRACT

Background: Previously, a single domain antibody clone (dAbCl26) selected from phage display library of LPS-immunized Indian desert camel was expressed in prokaryotic expression system and found to inhibit the LPS-induced inflammatory cytokine both *in vitro* and *in vivo*. However, the level of expression in prokaryotic expression system was low and also there was problem of endotoxin contamination. *Pichia pastoris* has emerged as an alternative host and economical approach for the expression of recombinant protein as it has the ability to properly refold the protein, gives high yield and produce protein without endotoxin contamination. Encouraged by the earlier findings of *in vitro* and *in vivo* inhibition by dAbCl26, the expression of dAbCl26 was investigated in yeast expression system *P. pastoris*.

Methods: The dAbCl26 was cloned into the *Pichia* expression vector pPICZαA and transformed into *P. pastoris* strain X33. Mutant phenotypes of selected clones were assessed using agar plate methods. For expression of the dAbCl26 in *P. pastoris* strain X33, the final growth conditions were optimized in buffered minimal methanol media with induction at OD600 4.0 and methanol induction at 0.5%.

Result: Using the optimized conditions, a yield of about 4 mg/L of dAbCl26 was obtained in shake flask culture which is 2 fold higher than previously reported. The expressed protein was purified by using Ni-NTA chromatography and confirmed by Western blotting. The method developed in this study will be used as base for expression of other single domain antibodies and to scale up the expression on large scale. Based on its already reported functional properties, the *Pichia* expressed dAbCl26 can be a suitable candidate for further development as a therapeutic agent for endotoxaemia in animals.

Key words: dAbCl26, Expression, Phage display, *Pichia pastoris*.

INTRODUCTION

Different *in vitro* selection technologies such as phage display antibody provides a solution for the generation of recombinant antibodies (Chiu and Gilliland, 2016). Phage display technology has been found to be one of the most effective, robust, easy-to-perform methods to develop recombinant antibodies with high affinity and target specificity and has been utilized to generate single-domain antibodies or variable domain of heavy chain of heavy chain antibodies (VHHs), also widely known as nanobodies (Roncolato *et al.*, 2015; Homayouni *et al.*, 2016; Kazemi-Lomedasht *et al.*, 2016).

The Department of Veterinary Microbiology, LUVAS, Hisar, Haryana (India) had previously constructed a phage display library of single-domain antibodies of LPS-immunized Indian desert camel for diagnostic and therapeutic use (Singh, 2009; Banerjee *et al.*, 2020). From this library, one of the clone *i.e.* dAbCl26 was found to inhibit the LPS-induced inflammatory cytokine response in buffalo and murine macrophages *in vitro* (Gupta, 2014). Also, the dAbCl26 inhibited the LPS-induced TNF-α release in Swiss albino mice model. The dAbCl26 also neutralized the *E. coli* 0153 LPS in chicken embryo lethality model and was found to cross react with LPS isolated from different Gram

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negative organisms *i.e.* *P. multocida* B:2, *E. coli* 0153, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Klebsiella* spp. (Gupta, 2014; Banerjee, 2015). These findings suggested that dAbCl26 could be a novel candidate to prevent endotoxaemia and sepsis. However, utilizing prokaryotic expression systems for producing antibodies or other therapeutic proteins presents significant challenges. One major issue is the relatively low level of

protein expression in these systems. Additionally, the production process is often complicated by endotoxin contamination, as prokaryotic cells, particularly Gram negative bacteria, inherently contain LPS in their outer membranes. This contamination can pose serious risks, especially when producing treatments for conditions like sepsis, where endotoxins are a primary concern (Fux *et al.*, 2024).

To address these limitations, the *Pichia pastoris* expression system offers an efficient and economical approach for producing wide variety of heterologous proteins in a correctly refolded form with disulfide bridges (White *et al.*, 1994; Kurtzman, 2009; Anangi *et al.*, 2012; Swichkow *et al.*, 2022). Therefore, in this study, dAbCl26 was cloned and expressed in *P. pastoris*. Initially, a zeocin-resistant clone was selected and further optimized for culture conditions, including medium type, induction timing and methanol concentration, to maximize protein yield. After purification, the dAbCl26 was characterized by SDS-PAGE and Western blotting. By leveraging the unique properties of dAbCl26 and the advantages of the *P. pastoris* expression system, our aim was to create an efficient and scalable production method for this promising therapeutic agent.

MATERIALS AND METHODS

Ethics statement

Permission to work on *Pichia pastoris* was obtained from Institutional Biosafety committee (IBSC) vide reference no. LUVAS/ABT/2020/29 Dated 07.01.2020. The experimental work was carried out at Department of Veterinary Microbiology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana over a period extending from October, 2019 to November, 2020.

Strains and media used

The EasySelect™ *Pichia* Expression Kit (Thermo Scientific, K1740-01) was used for the expression of dAbCl26 in *P. pastoris*. *P. pastoris* X-33 strain, TOP10FA *E. coli*, *P. pastoris* vector (pPICZαA), zeocin, vector specific sequencing primers and media supplied with the kit were used in the present study.

Cloning of dAbCl26 in *P. pastoris* pPICZαA vector

The dAbCl26 gene (Acc no. KF990217) was cloned in *Pichia* vector (pPICZαA) in frame with *c-myc* and *His* tag. The dAbCl26 gene was amplified using gene specific primer (Table 1) by gradient PCR with annealing temperature ranging from 59-63°C. The PCR product was purified using Wizard® SV Gel and PCR Clean-Up System kit (Promega, A9281).

The vector pPICZαA and purified PCR product of dAbCl26 were double digested with *EcoRI* (Thermo Scientific, FD0274) and *XbaI* (Thermo Scientific, FD0684) restriction enzymes and the purified RE digested dAbCl26 was cloned into the vector pPICZαA keeping the insert:vector molar ratio to 3:1. The ligated gene product was transformed into chemi-competent Top10FA *E. coli* cells using *E. coli* transformation buffer set kit (Zymo Research, T3002). The Top10F' *E. coli* cells were then plated with 150 µl volume/plate on 90 mm low salt LB/zeocin (25 µg/ml) agar plates. Finally, plates were incubated overnight at 37°C. Next day, five transformed clones were randomly screened with gene specific primers and vector specific primers.

Confirmation of recombinant plasmid

For the confirmation of recombinant plasmid, recombinant vector plasmids (dAbCl26 + pPICZαA) and empty vector plasmids (pPICZαA) were double digested with *EcoRI* and *XbaI* enzymes. Meanwhile, the recombinant vector plasmid (dAbCl26 + pPICZαA) was linearized by single digestion with restriction enzyme *SacI* (Thermo Scientific, FD1134). This linearized plasmid was later used for transformation in *P. pastoris* strain X33. These digested products were resolved on 1.5% agarose gel for confirmation.

Transformation into *P. pastoris* strain X33

The recombinant linear plasmid (dAbCl26+pPICZαA) was chemically transformed into *P. pastoris* X-33 cells using *Pichia* EasyComp Transformation Kit as per manufacturer's protocol (Invitrogen, K173001). After transformation, the entire mixture was plated on the Yeast extract peptone dextrose (YPD)/zeocin (100 µg/ml) agar plates and incubated for 3 days at 28°C. From the plate, transformed clones were regrown on YPD plates supplemented with progressively increasing concentrations of Zeocin (100, 200 and 400 µg/ml). This step was undertaken to select the clone exhibiting hyper-resistance to Zeocin. The *Pichia* transformants surviving on 400 µg/ml Zeocin were screened by PCR using gene specific primers and vector specific primers. The PCR products were resolved in a midi gel horizontal electrophoresis apparatus using 1.25% agarose gel with 5 µg ml⁻¹ ethidium bromide in 1X TAE buffer at 5V/cm gel length and visualized in UV-transilluminator (*Spectroline*™).

Determining the Mut^r/Mut^s phenotype of selected clones

The PCR positive clones were screened for determining their Mut phenotype. To determine the Mut phenotype of the transformed clones, zeocin resistant *Pichia* transformants were selected. One half portion of the selected colony was picked and streaked on minimal dextrose (MD) agar plate

Table 1: Primers used for cloning of dAbCl26 into *Pichia* vector pPICZαA (Restriction sites shown in bold).

Primer name	Sequence (5'-3')	Tm (°C)
Forward primer	ccccGAATTCATGGATGTGCAGCTGCAGGA	67.5
Reverse primer	agagTCTAGAATCTCGAGGCTGGAGACGGT	67.8

and the remaining half was streaked on minimal methanol (MM) agar plate. The plates were incubated at 28°C for 2 days and the growth on MD and MM agar plates was compared. After confirming the Mut phenotype of the selected clones, Mut⁺ or Mut^S protocol was followed for inducing expression in *P. pastoris* strain X-33.

Optimization of culture conditions for expression of recombinant dAbCl26 in *Pichia pastoris*

Effect of different media on recombinant protein expression

A single recombinant *Pichia* colony was inoculated in 100 ml of Buffered glycerol-complex (BMGY) medium in 500 ml baffled flask and incubated at 28°C in shaking incubator at 270 rpm till OD600 reached 4.0. The cells were harvested by centrifugation at 6000 rpm for 7 min and re-suspended in 25 ml of different media: Minimal methanol (MM), Buffered minimal methanol (BMM), Buffered methanol-complex medium (BMMY) and BMMY supplemented with 2% Casamino acid. The culture was induced with methanol to final concentration of 0.5% (added after every 24 h). The culture supernatant was collected every 24h interval till 120 h. The cells were precipitated with 10% Trichloroacetic acid (TCA) and checked on SDS-PAGE for protein expression.

Effect of time of induction on protein expression

A single recombinant *Pichia* colony was inoculated in 100 ml of BMGY medium in 500 ml baffled flask in triplicate and incubated at 28°C in shaking incubator at 270 rpm till OD600 reached 2.0, 4.0 and 6.0 for each flask. The cells of each flask were harvested by centrifugation at 6000 rpm for 7 min and the pellet was re-suspended in BMM media and induced with 0.5% methanol (added every 24 h till 120 h). The culture supernatant was collected every 24 h interval till 120 h, precipitated with 10% TCA and checked on SDS-PAGE for protein expression.

Effect of methanol on induction of protein expression

A single recombinant *Pichia* colony was inoculated in 100 ml of BMGY medium in 500 ml baffled flask in triplicate and incubated at 28°C in shaking incubator at 270 rpm till OD600 reached 4.0 for each flask. The cells of each flask were harvested by centrifugation at 6000 rpm for 7 min and the pellet was re-suspended in BMM media and the flask was induced with 0.5%, 1% and 2% methanol, respectively. The methanol was added in the respective flasks after every 24 h till 120 h. The culture supernatant was collected every 24 h interval till 120 h, precipitated with 10% TCA and checked on SDS-PAGE for protein expression.

Purification of recombinant protein

The recombinant protein was purified using Ni-NTA purification system. The supernatant collected 96 h after induction was cleared by centrifugation at 7000 rpm for 10 min and was dialyzed against binding buffer (50mM NaH₂PO₄·H₂O, 300mM NaCl, pH 8.0) for 3 days with buffer changed after every 12 h. One ml of Ni-NTA agarose beads were settled down in column and washed with 5 volume of

binding buffer. These equilibrated Ni-NTA beads were incubated with dialyzed culture supernatant overnight at 4°C. Next morning, the culture supernatant packed in column was washed with 5 volume of washing buffer (binding buffer with 0.1% triton X). The 6xHis tagged protein was eluted with elution buffer (binding buffer with 300 mM imidazole). The eluates were collected in small fractions of 1 ml each and checked by SDS-PAGE.

Western blot analysis of *Pichia* expressed dAbCl26

For Western blotting, *Pichia* expressed dAbCl26 protein was resolved on 15% SDS-PAGE to obtain polypeptide profiles and the same were electro-blotted onto PVDF membrane (0.22 µm) in a semi-dry trans-blot apparatus (Atto® corporation, Japan) at 1.0 mA/cm² of the gel for 60 min at room temperature. The PVDF membrane was blocked in 3% BSA-PBST (0.05% Tween-20) blocking buffer for 1 hr in shaking incubator at room temperature. After washing with PBST (0.05% Tween-20), the membrane was probed with anti-His HRP conjugated antibody (1:5000 dilution) and developed in 3,3-diaminobenzidine tetrahydrochloride hydrate (Sigma, D5637) and hydrogen peroxide (DAB/H₂O₂) substrate solution.

RESULTS AND DISCUSSION

Cloning of dAbCl26 in *Pichia* expression vector pPICZαA

The recombinant plasmid (dAbCl26+pPICZαA) was transformed in TOP10FA *E. coli* competent cells for the purpose of storage and propagation of recombinant plasmid to get high copy number for the successful transformation in *P. pastoris* strain X-33. It had been well documented that for the optimization of protein expression, high copy number appeared to have dominant positive influence and to achieve the same *P. pastoris* plasmids were propagated in *E. coli* prior to transformation (Vogl *et al.*, 2018). Gradient PCR was conducted for the optimization of annealing temperature for the designed primers with the temperature range 59-63°C. It was observed that better amplification was obtained at the annealing temperature of 60°C. The vector pPICZαA and purified dAbCl26 PCR product were double digested, ligated and transformed in the competent TOP10FA *E. coli* cells and plated on the low salt LB/zeocin agar plates. After incubation at 37°C for 18 h, five randomly selected clones were found positive by PCR using both gene specific primers and vector specific primers, respectively. The gene specific primers showed a band of 400 bp while vector specific primers a band of 929 bp was observed.

Confirmation of the recombinant plasmid (pPICZαA + dAbCl26)

The recombinant plasmid and empty vector were double digested with *Eco*RI and *Xba*I enzymes. Double digested recombinant plasmid showed two bands, one band of 3.5 kbp and other of 400 bp, as expected of the insert. The empty vector pPICZαA also showed a single band of 3.5

kb. The single RE digested recombinant plasmid showed a single band of 3.9 kbp indicating that gene of interest was cloned in pPICZ α A vector (Fig 1).

Transformation and screening of recombinant clones transformed in *P. pastoris* X-33 strain

For transformation, the recombinant plasmid linearized with enzyme *SacI* was transformed into *P. pastoris* X-33 by chemical method. After 3 days incubation at 28°C, more than 50 colonies were observed on YPD agar plates containing 100 μ g/ml of Zeocin. These colonies were regrown on YPD plates supplemented with progressively increasing concentrations of Zeocin (100, 200 and 400 μ g/ml). This step was aimed at selecting clones that exhibit hyper-resistance to Zeocin, ensuring the selection of the most robust and genetically stable clones for subsequent protein production. This approach aligns with findings by Nordin

et al. (2011), who reported an intimate correlation between hyper-resistance against Zeocin and enhanced expression of foreign proteins in *P. pastoris*. Therefore, adopting this strategy was crucial for selecting hyper-resistant *P. pastoris* strains, which are likely to exhibit improved expression of the desired recombinant proteins. From YPD/zeocin (400 μ g/ml) agar plates, twenty-four clones were randomly selected and screened by PCR using both gene specific and vector specific primers, respectively. On agarose gel electrophoresis, out of twenty-four clones, 23 were found positive (400 bp) with gene specific primers (Fig 2) and 22 clones were found positive (~929 bp) with vector specific primers (Fig 2). This confirmed the integration of dAbCl26-pPICZ α A plasmid in *P. pastoris* genome.

Out of 22 positive clones (~929 bp), it was found that 21 clones showed two bands where one band corresponded to the size of AOX gene of ~2.2 kbp size and other band corresponded to dAbCl26 gene amplified with vector specific primers of size ~929 bp. This type of band pattern was found in Mut⁺ phenotype. Remaining one clone had only one band of ~929 bp which specified Mut^S phenotype. Based on PCR results, out of 22 positive clones, 21 clones were of Mut⁺ phenotype and only one clone was of Mut^S phenotype.

Confirmation of mut phenotype of the selected clones

The Mut phenotype of the positive clone was further confirmed by growing these zeocin resistant *Pichia* transformants on MD agar plate and MH agar plate. The Mut⁺ is the methanol utilization wild phenotype and has equal growth on the MM and MD agar plate while, Mut^S is the methanol utilization slow phenotype that has slow growth on MM plates than MD plates. Based on growth, it was found that Mut^S phenotype clone by PCR was also of Mut^S phenotype, since more growth was observed on MD agar plate as compared to growth on MH agar. Also, Mut⁺ phenotype clone by PCR was confirmed to be Mut⁺ phenotype as the growth was almost similar on both agar plates. Based on growth, it was found that out of 22 PCR positive clones 21 were of Mut⁺ phenotype and one was of Mut^S phenotype (Fig 3).

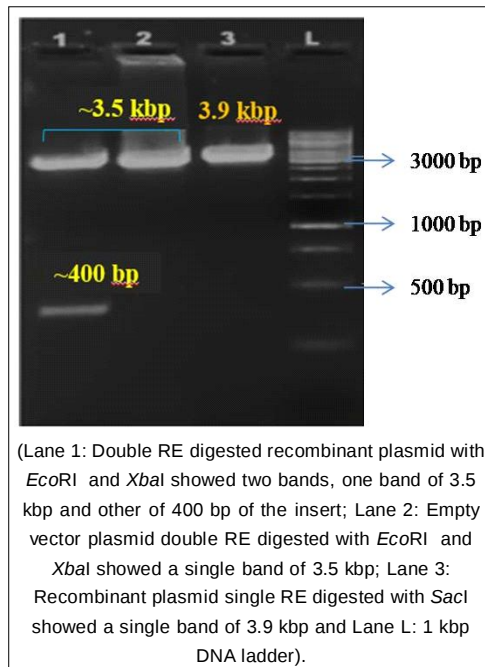


Fig 1: Agarose gel electrophoresis of RE digested products.

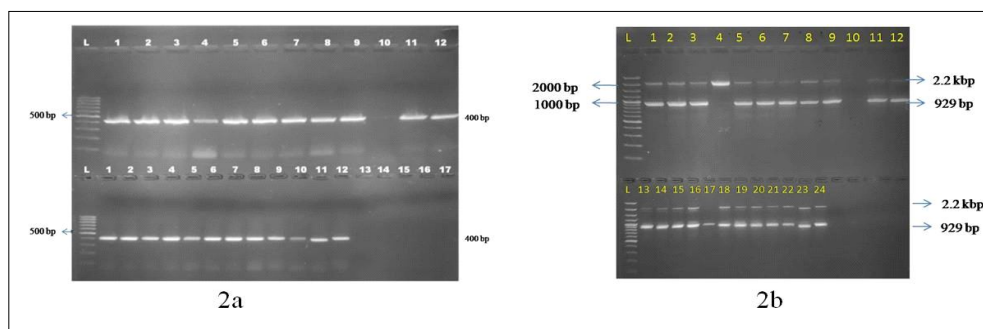


Fig 2: Agarose gel electrophoresis of PCR amplification of transformed *Pichia* clones using (a) Gene specific primers (*EcoRI* and *XbaI*); (Lane L: 100 bp DNA ladder; Lane 1-24: randomly selected clones with the band size of 400 bp); (b) Vector specific primers (5' AOX and 3' AOX); (Lane L: 100 bp plus DNA ladder; Lanes 1-24: Randomly selected clones with the band size of ~929 bp).

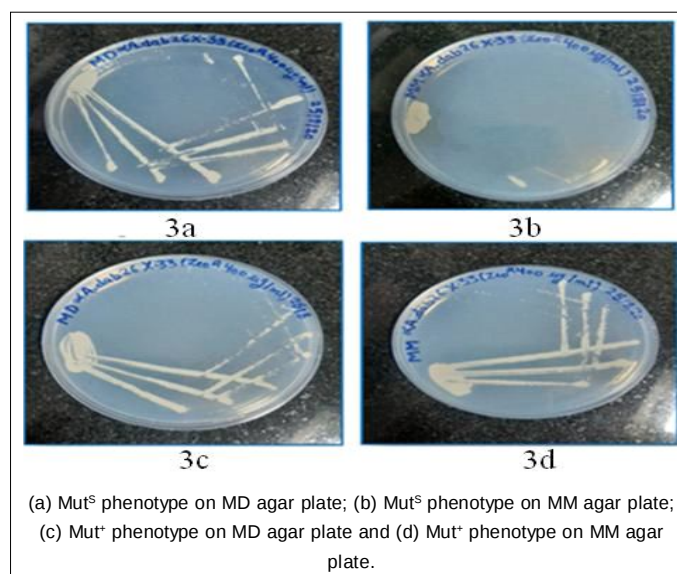


Fig 3: Confirmation of Mut phenotype of transformed clones.

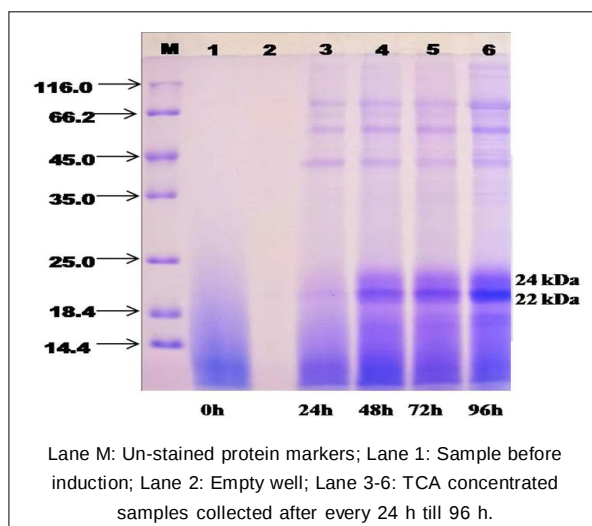


Fig 4: SDS-PAGE profile of recombinant protein expressed in *Pichia pastoris* strain X-33.

Optimization of culture conditions for expression of recombinant dAbCl26 in *P. pastoris* X-33

The expression level of heterologous proteins in *P. pastoris* can be significantly enhanced by adopting various strategies (Macauley-Patrick *et al.*, 2005; Yu *et al.*, 2010). In the present study, different conditions like type of media, biomass production and methanol induction, were optimized to obtain better expression results.

For optimization of effect of different media on protein expression, dAbCl26 clone was grown in different culture media like MM, BMM, BMMY and BMMY + 2% Casamino acid. Following induction with 0.5% methanol every 24 h over a 120 h period, the supernatant samples were analyzed by SDS-PAGE. A band of about 22-24 kDa was detected and it was found that BMM media showed

better expression of protein in comparison to other media used.

For optimization the effect of absorbance on protein expression, dAbCl26 in BMM media was induced with 0.5% methanol every 24 h till 120 h at OD₆₀₀ 2.0, 4.0 and 6.0 for each flask. The culture supernatants collected after every 24 h interval till 120 h were analyzed by SDS-PAGE. It was observed that protein expression was considerably higher when induced at absorbance of OD₆₀₀ 4.0 for 96 h than for other cultures when induced at absorbance of OD₆₀₀ 2.0 or 6.0.

For optimizing the effect of methanol induction on protein expression, dAbCl26 in BMM media was induced with 0.5%, 1% and 2% methanol, respectively every 24 h till 120 h at OD₆₀₀ 4.0. The culture supernatants collected after every 24 h interval till 120 h were analyzed by SDS-PAGE. It was observed that the expression of dAbCl26 was better after inducing with 0.5% methanol than other two samples induced with 1% and 2% methanol. Previous studies have indicated that suitable methanol concentrations for induction typically range between 0.1% and 3% (v/v) (Mu *et al.*, 2008; Zhang *et al.*, 2009; Minjie and Zhongping, 2013). These findings highlighted the importance of optimizing culture conditions for each specific protein of interest in *P. pastoris* to achieve maximum expression levels.

The final optimized conditions for expression of dAbCl26 in *Pichia* strain X-33 were BMM growth media, induction at OD₆₀₀ 4.0 and methanol 0.5%. The SDS-PAGE profile of expressed recombinant protein dAb26 in *Pichia* strain X-33 using the final optimized conditions has been shown in Fig 4. The size of protein expressed in *P. pastoris* was found slightly greater (~22-24 kDa) than that of the earlier expressed dAbCl26 protein in *E. coli* (~17 kDa). The increased size was probably due to the unique phenomenon of glycosylation by *P. pastoris*. Teh *et al.*, (2011) expressed recombinant human erythropoietin (rhEPO)

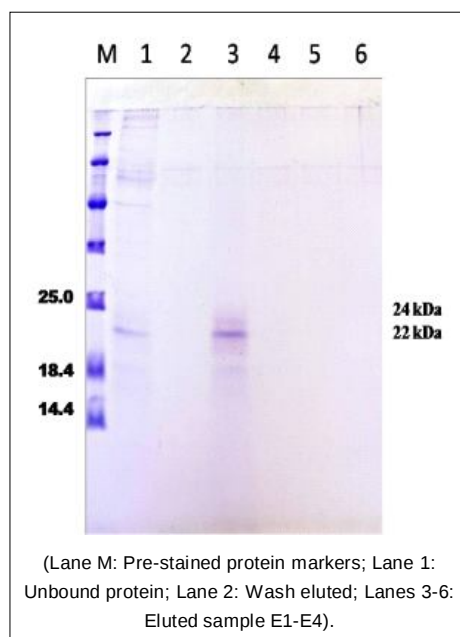


Fig 5: SDS-PAGE profile of recombinant protein purified by Ni-NTA column.

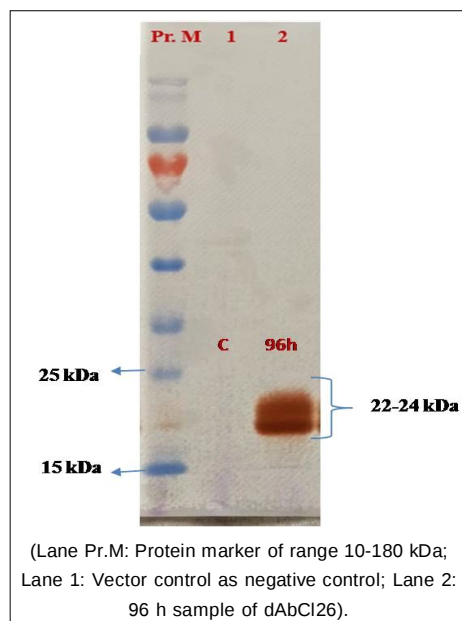


Fig 6: Western blotting of *Pichia* expressed dAbCl26.

gene in *P. pastoris* where the estimated molecular mass of the expressed protein ranged from 32 kDa to 75 kDa and the variation in size was attributed to the presence of glycosylation analogs.

Purification of recombinant protein

The culture supernatant collected 96 h after induction was purified using Ni-NTA purification system. The purified protein was seen as a band of approximately 22-24 kDa (Fig 5). Also, a total yield of 4 mg/L of the culture was

obtained which is two-fold higher as compared to previous reports (Gupta, 2014).

Western blot analysis of *Pichia* expressed dAbCl26

The expression of dAbCl26 was further confirmed by immunoblotting using anti-His HRP conjugated antibody (Invitrogen, R93125). A band of ~22-24 kDa was observed, thus confirming the results of SDS-PAGE analysis (Fig 6).

CONCLUSION

In conclusion, the work described here provided a feasible method to clone, express and purify the recombinant single domain antibody clone dAbCl26 in *P. pastoris*. Furthermore, the method developed in this study will serve as a foundation for expressing other single-domain antibodies and for scaling up expression to a large scale. Given its already reported functional properties, the *P. pastoris* expressed dAbCl26 presents itself as a suitable candidate for further development as a therapeutic agent for endotoxemia in animals.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors have no conflict of interest to declare.

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