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Heterologous expression of Cysteine Protease 8 from *Trichomonas foetus* in *Pichia pastoris*

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Abstract

Bovine trichomonosis is one of the most neglected venereal diseases of cattle. Trichomonas foetus, the causative organism was known over decades and is responsible for severe reproductive failure. Except for a few lab-based assays, to date, there are no point-of-care diagnostics developed to screen for the presence of infectious agents in cattle. In this study, we have identified cysteine protease 8 as a suitable antigenic protein for developing sero-diagnostics. A 960 bp Tf CP8 gene was cloned into methylotrophic Pichia pastoris X-33 by homologous recombination using a pPICZαA vector for recombinant protein expression. The traditional fed-batch method of induction with methanol resulted in inconsistent expression in 48h incubation, hence a novel single batch culture with 1% methanol induction for 24h was standardised and obtained optimal recovery of approximately 36 KDa recombinant protein secreted into media. To the best of our knowledge, this is the first report of cloning and expression of genes from Trichomonas foetus. This CP8 protein could be further optimised for developing lateral flow assays and ELISA as point-of-care tools.

Keywords: *Fed-batch culture, His Tag, methylotrophic yeast*

Bovine trichomonosis is one of the neglected venereal diseases in cattle. *Trichomonas foetus (T. foetus)*, the causative organism known for decades, is responsible for severe reproductive failure in cattle (Fernández *et al*., 2018). As per the recommendations of the World Organization for Animal Health (OIE Manual accessed on May 2019), several direct microscopic detection and staining techniques were developed for the detection of the causative agent (Bryan *et al*, 1999; Lun *et al.,* 1999). Polymerase chain reaction (PCR) based molecular detection assay was developed for the specific detection of *T. foetus* DNA (Casteriano *et al.,* 2016; Felleisen *et al*., 1998; Oyhenart *et al*., 2013). All the above procedures are found to be cumbersome, expensive and require technical

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expertise, not really suitable for point of care. Recombinant protein-based serological assays like lateral flow assay and ELISA would be more suitable as onsite screening devices by farmers and organized farms respectively.

In the process of searching for a suitable diagnostic target for *T. foetus*, multiple types of Cysteine proteases (CPs) were found to be involved in the pathophysiology of trichomonosis (Mallinson *et al.,* 1995; Thomford *et al.,* 1996; Singh *et al.,* 2005). Cysteine protease 8 (CP8) mediated uterine tissue damage is one of the most widely known mechanisms of pathogenicity in bovine trichomonosis (Sun *et al.,* 2012). Experimental treatment of CPs in cultures of bovine vaginal and uterine cells has been reported in the induction of apoptosis (Singh *et al*., 2004). Based on a few recently reported suitable antigenic proteins (Karli *et al.,* 2020), we have chosen to clone Cysteine protease 8 for the current study.

Materials and methods

Retrieval of CP8 gene sequence from NCBI

A 963 bp nucleotide sequence of *CP8* of *T. foetus* strain K was obtained from the whole genome shotgun sequence of scaffold number 148 submitted to NCBI GenBank with accession number MLAK01000493.1. In a 113675 bp scaffold, the gene was located at 105775-106737 with the locus tag of TRFO_16156.

Cloning of CP8 gene in Pichia pastoris X-33

The restriction enzymes map of the *CP8* gene was obtained by submitting the nucleotide sequence to NEBCutter V.2.0 from "New England Biolabs" with default parameters (Vincze *et al.,* 2003). The parameters such as analysis for "linear sequence" and restriction enzyme sites for all "commercially available specificities" were selected to modify the default analytical parameters. The pPICZαA shuttle vector (Thermofisher Scientific) was chosen for cloning in *E. coli* and expression in *Pichia*.

Amplification of CP8 gene from T. foetus using Polymerase chain reaction (PCR)

Primers for the CP8 DNA were designed using the freely available version Primer3Web V.4.0. The 960 bp CP8 gene (without stop codon) fragment was submitted with default parameters (Kõressaar *et al.,* 2018). The designed primer oligos (Table.1) were purchased with HPSF purity (Eurofins Genomics, India

The amplification reaction was set to a 50µL reaction mix with "GoTaq® Green Master Mix" (Promega, USA) with optimal concentrations of *Taq* DNA polymerase, dNTPs, $MgCl_2$, and 10X buffers (Green *et al.,* 2018). *T. foetus* Strain K DNA was kindly obtained from the Department of Genetics Universidad Nacional de La Pampa, Argentina. The details of the reaction set-up and reaction cycle are provided in Table.2

The PCR amplified sample was checked on 1% agarose gel electrophoresis (Lee *et al.,* 2012) by using 1kb molecular weight standard DNA (Genei labs, INDIA)

Cloning of CP8 into pPICZα A Vector

The CP8 DNA and pPICZα A vector were digested with restriction enzymes *EcoRI* and *Not1* sequentially. The ligation reaction was set in the 1:4 (vector: insert) ratio. (Thermo Fisher Scientific, USA). The reaction set-up for

Table 1. DNA Primer sequences for cloning CP8 gene into pPICZα A vector

Primer type	Oligo Sequence	Description
Forward Primer	GATCGAATTCATGTTTTCGTTCTTTGCTG	19 bases + Linker with *EcoRI*[a] Restriction site
Reverse Primer	GATCGCGGCCGCTGCGATTGGGATGCAGCT	20 bases + Linker with *Not1*[b] Restriction site

a. First restriction enzyme isolated from *Escherichia Coli* strain R. b. First restriction enzyme isolated from *Nocardia otitidis*. The respective linker sequences are highlighted in red.

Table 2. Details of PCR reaction setup and Thermal cycle reaction

PCR Reaction Mixture -50 µL	Thermal cycle Reaction
25µL - 2X master mix[a] 1µL Template DNA 1µL -Forward Primer 1uL-Reverse Primer 22uL water	Initial denaturation -95°C-5 min Denaturation 94°C -30 sec Annealing 54°C -1min Extension 72°C -1.20min Final extension 72-°C -7min Total cycles 35 cycles

[a]2X Master mix (Promega, USA) contains reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl2

Table 3. Cloning of *Cysteine protease 8* gene into pPICZαA Pichia expression vector

Restriction Digestion - *EcoR1*	Restriction Digestion - *Not1*	Ligation reaction
60µL réaction 50µL PCR product/pPicZα A 5µL 10X buffer 2µL - *EcoR1* 3µL - Water Incubated at 37°C -2h	60µL réaction 50µL -PCR product/ pPiCZα A 5µL 10X buffer 2µL - *Not1* 3µL - Water Incubated at 37°C -2h	20µL reaction mixture CP8 DNA - 12µL pPiCZα A double digested - 4µL T4 DNA ligase (5U/µL) - 1µL 10X ligase buffer-2µL Water- 1µL Incubated at 16°C overnight.

restriction enzymes digestion and ligation are summarised in Table.3.

The 10µL of ligation mix was transformed into *E. coli* DH5 alpha competent cells (Green *et al.,* 2013) and incubated at 37 °C with shaking for 1h at 200 rpm. Further, 50µL of the culture was plated on LB agar with 25µg/mL Zeocin along with a negative control and incubated at 37 °C in dark for 24h. Ten Colonies were picked and screened by PCR for *CP8* gene. A positive clone was cultured for plasmid isolated by the Alkaline lysis method (Green *et al.,*2016).

Cloning of pPICZαA-TfCP8 construct in *Pichia pastoris X-33*

Recombinant vector pPICZαA-TfCP8 was digested with *PmeI* restriction enzyme to obtain the linearised construct. A 50µL total reaction mix was set up by adding 5.0µL 10X buffer,0.5µL 100X BSA, 10µL vector DNA (up to 2µg) 2.0µL *PmeI* enzyme (Thermo Fisher Scientific, USA) and 32.5µL sterile double distilled water. The reaction contents were given a short spin and incubated at 37 °C for 2 h.

Based on the method developed by Kawai *et al.* (2010) with few modifications, *P. pastoris* X-33 cells (Thermofisher Scientific) were cultured in Yeast Peptone Dextrose media (YPD) at 30 °C, 300 rpm for 12h to reach an absorbance of 1 at 600 nm. Competent cells were prepared by using 100mM LiCl and transformed by sequential addition of 240µL 50% polyethene glycol, 36µL of 1M LiCl, 25µL of sheared salmon single-stranded DNA (2 mg/ml) and linearized plasmid DNA (5µg in 50µL sterile water). The contents were mixed vigorously and incubated at 30 °C for 30 minutes. Heat shock was given by incubating the cells in the water bath at 42 °C for 20–25 minutes. Cells were pelleted at 6000 to 8000 rpm and resuspended in 1 ml of YPD and incubated at 30 °C with shaking. After 4 h of incubation, we plated 100µL of cells on YPD plates 100µg /ml Zeocin in duplicates and incubated for 3 days at 30 °C. Ten colonies were screened by subculturing onto fresh YPD 100µg/ml Zeocin simultaneously by using colony PCR as per Table. 2 followed by separation on 1% agarose gel electrophoresis. The amplified fragments of DNA were visualised using a gel documentation system (GeNei Labs, India)

Induction of TfCP8 Protein expression in P. pastoris

Alcohol oxidase1 (*AlOX1*) was considered the strong promoter for protein expression in *Pichia.* Methanol was found to be the most efficient and cost-effective inducer of the *AlOX1* promoter. Initially, 360 ml of buffered minimal media (280 ml YP media was added with 40 ml of 1M potassium phosphate buffer pH 6 and 40 ml of 10X YNB,0.08 ml 500X Biotin) was prepared for initial seed culture as well as protein induction. A single colony of the *Pichia X-33* TfCP8 was inoculated in 40 ml of buffered glycerol minimal media (BGMY) (4 ml of 10X glycerol to 36 ml Buffered basal media) incubated at 30 °C with 300 rpm shaking for 24h to reach an OD.10 at 600 nm. The cells were resuspended in buffered methanol minimal media (BMMY) (4 ml 10X Methanol to 36ml basal media) incubated to get OD.1. The cells were collected and inoculated into 200 ml of BMMY (1% methanol) and incubated with shaking for 24 h. An aliquot of 2 ml of culture was taken and the cells and media were separated by centrifugation at 6000 rpm for 5 minutes. After every 24 h, 2 ml of methanol (to a conc. of 1%) was added to substantiate the loss by evaporation and consumption during the process. After 48h of incubation, all the cells and media were separated and stored at -20 °C (Weidner *et al.,*2010) for further analysis.

Protein was precipitated from the media by adding 250µL 100 % TCA to 1.0ml media. The tubes were incubated for 10 min at 4 °C followed by a high speed of 12000 rpm for 5 min. The pellet is washed twice with 200µL cold acetone and after drying boiled in 2X sample buffer for 10 min at 95 °C (Koontz *et al.,*2014). *Pichia* cells were lysed by the freeze-thaw method of cell lysis with few modifications (Harju *et al.,*2004). 20µL of lysate was boiled with 20uL of 2x Lamelli buffer and separated on 12% SDS PAGE along with the broad range protein molecular weight marker (Genei labs, INDIA) and the gel was stained with Coomassie blue (Smith *et al.,*1994).

Results and discussion

Cloning of CP8 gene from T. foetus into pPICZα A vector

CP8 gene of approx. 960 bp was amplified by PCR and visualised on ethidium bromide-stained gel in agarose gel electrophoresis (Fig. 2A) by using a UV transilluminator (GeNei Labs, India). The CP8 gene and *pPICZα A* vector were successfully double digested with *EcoR1* and *NotI* restriction enzymes (Fig. 2B). The transformed *E.coli DH5α* cells with recombinant vector appeared on LB agar with 25ug/ml Zeocin. No colonies were visible in the negative control (Fig.1:

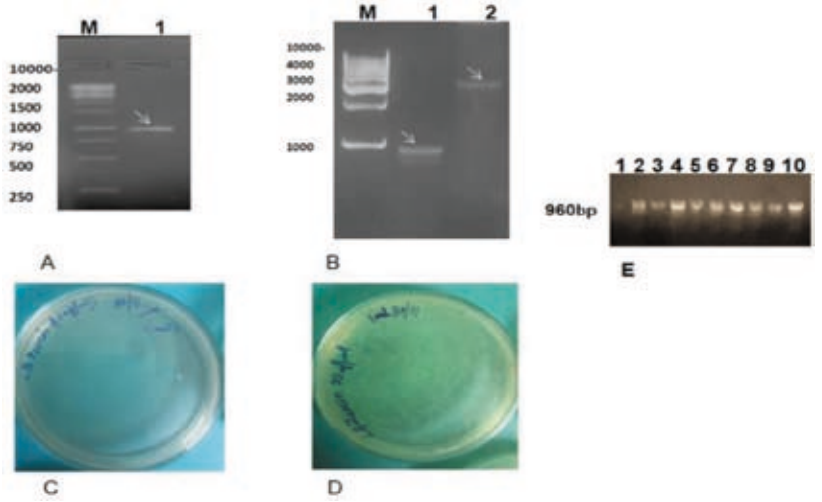

Fig.1. Cloning of Tf CP8 gene into *pPICZα A* vector and transformation into *E. coli* DH5*α* with Zeocin selection. a. Amplified *CP8* gene: M- DNA 1Kb ladder, 1- Amplified gene 960 bp. b. Preparation for Ligation: M- DNA molecular weight marker, 1- Double digested *CP8* gene. 2- Double digested *pPICZ α* A vector. c- Negative control for cloning d- transformed recombinant *pPICZα A-Tf CP8 positive colonies on LB Zeocin (25ug/ml*).

C-D). To the best of our knowledge, this is the first report of cloning *T. foetus* CP8 gene in the shuttle vector.

Generating recombinant strains of P. pastoris X-33 by homologous recombination with pPICZα A- Tf CP8

Recombinant clones appeared on YPD Zeocin 100ug/ml. No colonies were visible in the negative control. Lithium chloride mediated DNA transformation into *P. pastoris* resulted in the generation of positive clones with genomic integration. The event of homologous recombination with pPICZα A- Tf CP8 with the *Pichia* genome resulted in the generation of visible colonies after 3 days of incubation at 30ºC (Fig. 2: A-C). The positive clones were randomly selected and confirmed for CP8 integration by using PCR for the *CP8* gene (Fig. 3D).

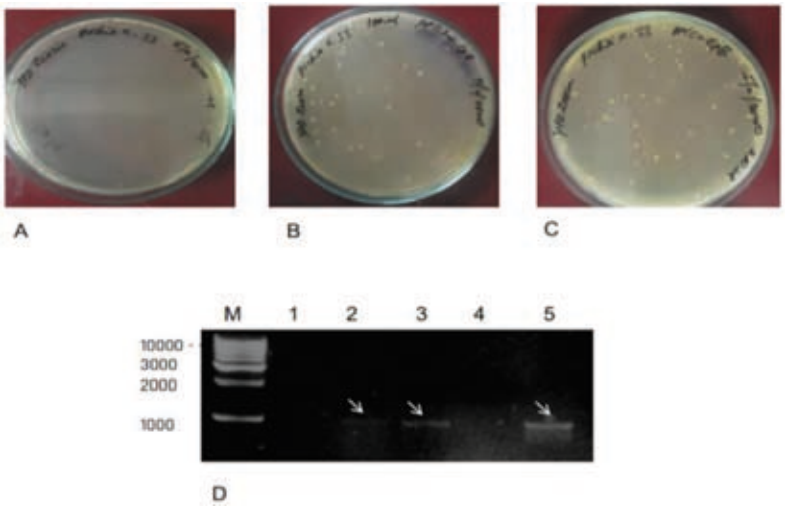

Fig.2 Transformation of linear *pPICZαA-Tf CP8* into *P. pastoris* X-33 and screening for recombinants of *P. pastoris* X-33 TfCP8. a. YPD Zeocin 100ug/ml Negative control b. YPD Zeocin 100ug/ml spread with 100 ul of transformed *Pichia* cells c. YPD Zeocin 100ug/ml spread with 100 ul of transformed *Pichia* cells d. Screening for genomic integration of Tf CP8 gene M-Molecular weight marker, 1-6 wells were loaded with PCR products to identify the positive clones of *Pichia* for the CP8 gene.

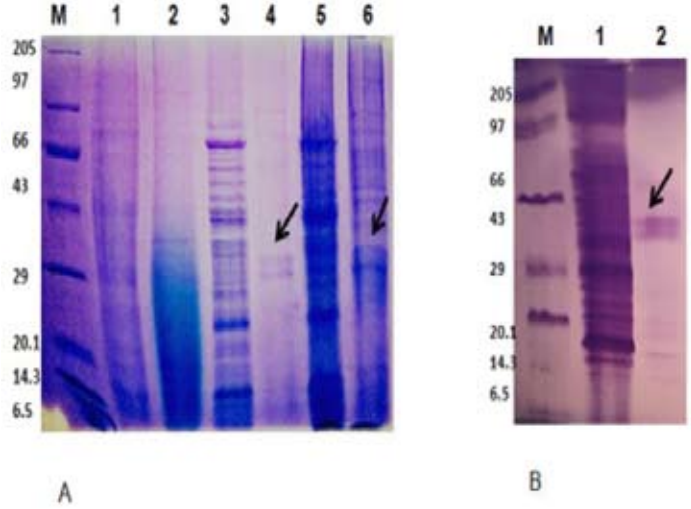

Fig.3. SDS-PAGE analysis of CP 8 expression in *Pichia Tf-CP8* culture induced with 1% Methanol a. Fed-batch culture: M-Protein molecular weight marker, 1- Uninduced cell lysate, 2. Uninduced Media,3. 1% methanol induced – 24h Cell lysate, 4. 1% methanol induced –Day 24h media, 5. 1% methanol induced –48 h Cell lysate, 6. 1% methanol induced – 48h media b. Batch culture: M-Protein molecular weight marker,1- 1% methanol induced – 24h Cell lysate.

Induction of CP8 protein expression in P. pastoris X-33 by using1% methanol

Induction with 1% methanol for 24h and 48h resulted in the identification of expressed CP8 protein of approximately 36 KDa in SDS-PAGE gel electrophoresis, followed by staining with Coomassie blue (Fig. 3A). The protein was expressed in both the cell lysate and media components. However, in the 24h induced media component almost the pure form of the secreted protein was visualised in the gel. When the induction period was extended for another 24h, we observed a massive reduction in cell division which resulted in several nonspecific proteins secreted in the media component. Based on these findings, we could achieve optimal protein expression with minimal impurities in the modified batch culture with approx. OD 10 cells, 1% methanol induced for 24 h (Fig.3B).

Pichia pastoris was the most popular heterologous eukaryotic host successfully employed for protein expression over the past two decades (Kurtzman, 2009; De Schutter *et al.,* 2009). However, the proteolytic degradation of expressed proteins and cellular lysis of the host cells have been the major challenge in optimal protein recovery. Endogenously secreted proteins were found to undergo lysis due to cytosolic proteases and secreted proteins have been reported to be lysed by vacuolar proteases in several instances (Zhang *et al.,* 2007). Development of the *Pichia* strains that are deficient in proteases (Gleeson *et al.,*1998; Salamin *et al.,* 2010), cloning and expression with multi-copy gene cassettes (Shu *et al.,* 2016) and using higher cell density culture fermentation with induction for 3-6 days were the few notable strategies developed for enhanced protein expression (Ahmad *et al.,* 2017)

In this current study, the successful expression of Cysteine protease 8 was a double-edged sword. CP8 was itself known as an extracellularly secreted virulent protein and was known to be an important factor in uterine tissue damage in cattle. Using the conventional culture method with prolonged incubation hours, CP8 secretion resulted in a drastic reduction in the cellular densities in cultures along with the secretion of several non-specific proteins into the culture media due to cellular leakage.

Hence, we have modified the traditional fermentation strategy, by generating parallel batch cultures with high cell densities and shorter incubation time to successfully attain the optimal protein recovery with minimal impurities secreted into media. This novel strategy of batch fermentation can be further optimised for the successful expression of heterologous toxic proteins in *Pichia*.

Conclusion

To the best of our knowledge, this is the first report on the cloning of *T. foetus CP8* genes and the synthesis of recombinant proteins. As CP8 was a toxic protein, the inability to use the same feed culture for continuous protein production as fed-batch culture was the major limitation. Hence, we have evolved with a suitable approach with massively grown seed culture for a single induction time to obtain the stable expression. This method can also be extended for the expression of any similar toxic proteins in *Pichia*. As the *Pichia* secretes very few proteins, this procedure offers a simple downstream process for protein purification.

In future works, the diagnostic specificity and immunoblot patterns need to be ascertained to enable this recombinant CP8 protein as a candidate to design suitable point-of-care diagnostics like ELISA and lateral flow assay Rapid kits for screening bovine trichomonosis. These proteins can also be further exploited for protein structure elucidation by X-ray crystallography followed by drug designing for the target.

Conflict of interest

The authors declare no competing interest

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