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Molecular Cloning, Expression And Purification Of A Novel COVID-19 Vaccine Candidate Against Infectious Diseases In A Culture Of Escherichia Coli

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Abstract
Vaccination is one of the most effective medical interventions that has been introduced in the past few years. Vaccines are known to prevent 2.5 million deaths per year. Owing to new developing technologies, vaccines have the potential to make a huge contribution to the health of modern society by preventing many diseases of all age groups. There are many different types of vaccines based on the form of antigen used in their preparation. The present study puts emphasis on development of subunit-based vaccines against Covid-19. The subunit vaccines were constructed with two different approaches where one involved the use of Fc portion of the antibody tagged to the antigen while the other has a histidine tag for the ease of purification. The selected clones could then be transfected into mammalian cells to observe the protein expression.COVID-19, the global pandemic, infected and killed many human beings across the world. The sudden onset and global spread of the disease necessitated the development of an efficient vaccine for mass vaccination. This study provides the data for the expression and purification of a vaccine candidate against the SARS-CoV2 virus. The beauty of this vaccine is the employment of multiple epitopes targeting the structural and non-structural proteins of the virus, thus inhibiting the viral infection and replication. The study shows that the recombinant vaccine candidate is sequestered into inclusion bodies in E. coli. In order to maximize protein recovery, protein solubilization and refolding was optimized using mild chaotropic agents. Further, Anion Exchange Chromatography (AEX) was used as a negative chromatography to remove other protein impurities and recover the protein of interest in the flow-through. The Cation Exchange Chromatography (CEX) step provided pure protein, but the protein recovery was reduced. The final purified protein showed the presence of NSP9 and RBD when probed with antibodies against these epitopes. The study shows that a multiple epitope vaccine can be expressed in E. coli but further studies are required to prove the efficacy of the vaccine candidate.

Keywords: *Subunit vaccines, Multiple epitopes, Fc portion, Histidine, Expression, Refolding, Purification, Chromatography, and COVID-19.*

INTRODUCTION

Immunization is the foundation of public health for any nation. The first vaccination was done by Edward Jenner against small pox in a eight year old boy using cowpox lesion scratching from a milk maid[1–3]. Different types of vaccines are available against different disease-causing pathogens. Vaccines are usually classified into live attenuated and killed inactivated vaccines. Live attenuated vaccines have weakened but viable pathogen as antigens, while in killed vaccines; pathogens are inactivated by heat or chemicals. Over years, vaccines have been developed using specific biomolecules like proteins, DNA and mRNA[4–6]. Subunit vaccine makes use of the highly antigenic portion of viral and bacterial proteins, while conjugate vaccines are bacterial lipopolysaccharides covalently bound to an antigenic carrier protein to provide broad spectrum immunity. The protein-based vaccines can be produced using recombinant DNA technology. DNA and mRNA based vaccines have recently become strong candidates[5,6].

Recent pandemic of Coronavirus Disease 2019 (COVID-19) witnessed fast track research for developing a vaccine against its causative agent, severe acute respiratory syndrome coronavirus-2 (SARS CoV-2). Since 2019, more than 760 million people were infected and the virus killed 6.9 million deaths globally (https://www.who.int). Different approaches for targeting the virus were employed while developing vaccines for COVID-19 protein subunit vaccine mainly targeting either the receptor biding domain (RBD) or S1 subunit of complete spike protein of SARS-CoV2 virus, mRNA based vaccine, attenuated or inactivated viral vaccine and virus-like particle based approach to deliver SARS-CoV2 antigen[7].
While different types of vaccines are in pipeline of development, the work described in the dissertation thesis relates to the development of subunit-based vaccines against Covid-19. In the subunit vaccines, a smaller portion of a large antigenic protein is usually either natively purified or expressed in vitro either in bacterial cells or mammalian cell lines, purified and then administered to individuals. Here, the cloning of two different subunit vaccine candidates has been described. One of the vaccine candidates has the Fc region of the antibody tagged to the antigen, providing ease of purification and stability. The other candidate has a hexa-histidine tag to allow quick and easy purification by metal-affinity chromatography. Different molecular biology techniques like polymerase chain reaction (PCR), overlapping PCR, asymmetric PCR, digestion and ligation have been employed for generating the vaccine candidate clones. SARS-CoV2 is a positive stranded RNA virus and non-structural protein 9 (NSP9) is a RNA-binding protein in the RNA synthesis machinery[8,9]. A recent study has shown that targeting the NSP9 can hinder its binding to NSP12, and thus curbs viral replication and hence infection [10]. Further, NSP9 is well conserved among coronaviruses, with a sequence identity of 98% between SARS-CoV and SARS-CoV2[10]. This study focuses on developing a polymeric epitope vaccine using structural proteins and NSP9 of SARS-CoV2 virus. The present research work provides a detailed account of the expression of NSP9 and RBD based vaccine candidate in *Escherichia coli* (*E. coli*), and its purification. The recombinant subunit vaccine was expressed as inclusion bodies. Protein refolding, anion exchange chromatography (AEX) and cation exchange chromatography (CEX) were employed for purifying the recombinant subunit vaccine. The presence of epitopes was confirmed by western blot using commercially available antibodies and further testing in animal will be the path forward to test the *in vivo* efficacy of the vaccine candidate.

Materials and Methods

Preparation of media, antibiotics and buffers:

LB Broth HiVeg (LBV): 25 g of LB Broth was dissolved in 1 L of Lab Grade water, dispensed into two 1 L glass bottle and autoclaved at 121°C, 15 p.s.i. for 20 min. Once cooled, the medium was stored at R.T.

LB Agar: 30 g of LB Agar powder was dissolved in 1L of Lab grade water and sterilized by autoclaving at 121°C, 15 p.s.i. pressure for 20 min. Antibiotic Kanamycin was added when the solution temperature reached 50°C at the concentration of 50 µg/ml. Approximately25 ml of Agar mixture was poured into the plates and left to set. The plates were stored at 2-8 °C.

Kanamycin Stock (50 mg/ml): 500 mg of Kanamycin powder was dissolved in 10 ml of Milli Q water and filter sterilized in the Laminar Air flow unit. Aliquots were made and stored at -20°C. The final concentration to be used was 50µg/ml.

1% Agarose gel: Gel was prepared by adding 1 g of Molecular Biology grade agarose in 100 ml of 1X TAE Buffer and heating in the microwave till the agarose was digested. The mix was allowed to cool to ~50°C and 10 µl of 1000X SyBr Safe DNA gel stain was added, mixed and the gel was poured into the casting tray arranged with a comb having required number of wells. After solidifying, the gel was used to run the samples.

50X TAE Buffer: 242 g of Tris and 18.61 g of Disodium EDTA were weighed and dissolved in 700 ml of Lab grade water. 57.1 ml of Glacial Acetic Acid was added and total volume was adjusted to 1 L with lab grade water. The prepared buffer was autoclaved and stored at RT.

1X TAE Buffer: 20 ml of 50X TAE stock was diluted to 1 L with Lab Grade water. The 1X TAE solution has 40 mM Tris, 20mM Acetate and 1 mM EDTA, with a pH = ~8.6.
80% Glycerol: 80 ml of Glycerol was mixed with 20 ml of lab grade water and sterilized by autoclaving at 121°C, 15 p.s.i. for 20 min. The bottle was stored at RT.
Buffer PE of QIA quick Gel Extraction Kit: 24 ml of 96-100 % Ethanol was added to the provided concentrate of Buffer PE.
Buffer P1 (Resuspension Buffer of QIA prep Spin Miniprep Kit): Provided RNase A solution and Lyse Blue reagent (1:1000 ratio) added to Buffer P1, mixed thoroughly and stored at 2-8 °C.
Buffer PE (Wash Buffer of QIA prep Spin Miniprep Kit): 24 ml 96-100% of Molecular Biology grade Ethanol was added to the provided Buffer PE concentrate (6 ml) and stored at R.T.
Primer dissolution and working solution: Lyophilized primers were dissolved in TE buffer, mixed and incubated at RT for 20-30 minutes to get a final stock concentration of 100 µm. The working primer solutions were made by diluting stock solutions 1:10 in TE buffer.

Optimization of Expression level of Vaccine candidate:
The glycerol stock of *E. coli* strain carrying the expression construct used in the study was obtained from Aurigene Pharmaceutical Services Limited (APSL), Hyderabad. The stock was streaked on a Luria-Bertani (LB) agar plate to obtain isolated colonies. Kanamycin (50 µg/mL) was used as the selection pressure. Single colony was grown in 5 mL Kanamycin containing LB broth by incubating at 37°C, 200 RPM for 16-18 h. Next day, the overnight grown culture was used to inoculate 8 different flasks (F1-F8) containing 30 mL LB broth + Kanamycin each for optimizing time, temperature and Isopropyl ß-D-1-thiogalactopyranoside (IPTG) concentrations. All the flasks were incubated at 37°C, 200 RPM until the $O.D._{600}$ reached 0.65-0.75. The following table shows the scheme of incubation and IPTG added to each flask in order determine to optimal condition for expression.

Table 01: Conductions tested for optimizing expression level of protein

Flask ID's	Induction Temperature	Induction time period	IPTG concentration
F1	37°C	4 h	0 mM
F2	37°C	4 h	0.5 mM
F3	37°C	4 h	1.0 mM
F4	37°C	4 h	2.0 mM
F5	16°C	16-18 h	0 mM
F6	16°C	16-18 h	0.5 mM
F7	16°C	16-18 h	1.0 mM
F8	16°C	16-18 h	2.0 mM

At the end of induction period, $O.D._{600}$ was checked and 2.0 $O.D._{600}$ equivalent cells from all the flasks were sampled for expression analysis using SDS-PAGE. Briefly, required volume of culture was diluted to 500 µL using 250 mM Tris buffer, pH 7.5 and equal volume of 2X reducing Laemmli buffer (Bio-Rad) was added. The samples were heated at 90°C for 20 min, cooled on ice and centrifuged at 10,000 RPM, 4°C for 5 min. 15 µL of cell lysate was loaded onto 12% SDS-PAGE gel for determination of expression. In order to check for consistency of expression, six replicate flasks (F_I1 to F_I6) were cultured and induced with the finalized expression conditions, i.e., 37°C, 4 h and induction with 2 mM IPTG as described above. Other six flasks were cultured for uninduced controls ($F_{UI}1$ to $F_{UI}6$).

Localization of expressed protein:
Once the expression conditions were optimized, 500 mL culture was grown as described earlier and induced for protein expression. 10 mL of the culture was homogenized and used for determining the localization of expressed recombinant protein. Briefly, 10 mL of culture was harvested by centrifuging at 10000 ×g, 4°C for 15 mins. 1 mL of cell lysis buffer containing (50 mM Tris-HCl, 5 mM EDTA, 1 mM PMSF at pH 7, and a conductivity of 5 mS/cm) was utilized to resuspension the cell culture pellet, with

the help of Polytron homogenizer. Further, lysis was done using Panda plus using 12000 bar pressure, at 4°C. After 5 passes, lysate was centrifuged at 16500 ×g, 4°C for 60 min. The supernatant was collected in a different tube. The complete cell lysate, supernatant and cell debris pellet were analyzed on SDS-PAGE for localization of protein. 20 µL of the samples was loaded into each well. Gel was stained using Coomassie Brilliant Blue.

Large scale culture growth by fed-batch fermentation:

Pre-seed culture was grown by inoculating with glycerol stock, 50 µg/mL of Kanamycin in 100 mL of LB broth, for 16–18 hours, the flask was incubated at 37°C and 200 RPM.

After overnight growth, the $O.D._{600}$was measured and at an $O.D._{600}$ of about 4.2-5.0, seed flask for fermentor was inoculated. For seed flask, 40-60 mL of pre-seed culture was added into Kanamycin containing 200 mL of LB. The flask was incubated at 37°C, 200 RPM for 2-3 h until the $O.D._{600}$ reached >2.5. The composition of the fermentor media is described in Table1.

Table 2: Composition of fermentor medium

Chemical Composition	Concentration (g/L)	Chemical Composition (Trace elements)	Concentration (g/L)
KH_2PO_4	4.0	$FeSO_4.7H_2O$	40.0 mg
K_2HPO_4	4.0	$CaC1_2.2H_2O$	40.0 mg
$Na_2HPO_4.12H_2O$	7.0	$MnSO_4.H_2O$	10.0 mg
$(NH_4)_2SO_4$	1.2	$AlCl_3.6H_2O$	10.0mg
NH_4Cl	0.2	$CoC1_2.6H_2O$	4.0 mg
$MgSO_4.7H_2O$	2.4	$ZnSO_4.7H_2O$	2.0mg
Yeast extract	10.0	$Na_2MoO_4.2H_2O$	2.0mg
Glucose	10.0	$CuC1_2.2H_2O$	1.0mg
		H_3BO_3	0.5mg

Struktol (5%) was used as anti-foaming agent. Kanamycin (75 µg/mL) was added before seeding of fermentor and also 1 h before induction with 2 mM IPTG. Feed medium was composed of 60% Glucose, 40% Yeast extract and 2.5g magnesium sulfate per 100 g of Glucose. Fermentation parameters were set as follows: Temperature: 37°C, pH: 6.9, dissolved oxygen (DO): 40%, RPM: 200, Air: 0.5 lpm and overlay air: 0.5 lpm. Induction was started once the $O.D._{600}$ reached 80-90 and was carried out for 4-5 h. Sampling was done every hour for measuring $O.D._{600}$, pH and glucose levels. After 4-5 h of induction, the temperature of fermentor was reduced to 8-10°C before harvesting the culture. The culture was harvested by centrifuging at 7500 RPM, 4-6°C for 30 min. The obtained cell pellet was kept at -20°C for storage.

Isolation of inclusion bodies:

Resuspension of 2.5 g of cell pellet was done using a polytron homogenizer in 50 mL cold lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 1 mM PMSF at pH 7, and a conductivity of 5 mS/cm). Further, the cell lysate was prepared by passing through Panda plus homogenizer five times at 12000 bar pressure and 4°C. The sample was clarified using centrifugation at 16500 ×g for 60 min at 4°C. The cell pellet was weighed and resuspended in 50 mL cold wash buffer (50 mM Tris-HCl, 500 mM NaCl, and 1% of Triton X 100 at pH 7, and conductivity 48 mS/cm). Centrifugation was done at 16500 ×g for 60 min at 4°C. The washing step was repeated again and the resultant pellet was weighed and resuspended in 50 mL cold water for injection (WFI) to remove detergents and salts from the inclusion bodies. After centrifuging the mixture for 60 min at 4°C and 16500 ×g, the pellet, which consisted of inclusion bodies, was weighed. SDS-PAGE analysis was performed after every centrifugation step.

Solubilization and refolding of protein:
The pure inclusion bodies obtained after WFI wash were dissolved in 10 mL WFI and 40 mL solubilization buffer (2 M Urea, 50 mM Tris and 1 mM DTT, pH 12) by continuously stirring using a magnetic stirrer for 30 min at 4°C. Centrifugation of solubilized inclusion bodies at 14000 ×g for 60 min at 4°C was done to remove any insoluble aggregates. SDS-PAGE analysis was carried out for both supernatant and pellet. Based on SDS PAGE data, the supernatant was utilized for subsequent steps after filtration with 0.45 µm filter.
Protein refolding was done by diluting the filtered supernatant 10 times with refolding buffer (0.2 M Urea, 50 mM Tris, 10% sucrose, 10 mM EDTA and 250 mM of arginine, pH 7.2, Conductivity 18 mS/cm) and continuously mixing using magnetic stirrer at 4°C for 12-16 h. After 15 h, the aggregates and unfolded protein molecules were removed from the refolded protein by centrifugation at 14000 ×g, 4°C for 30 min. SDS PAGE analysis was performed for both supernatant and pellet. Based on SDS PAGE data, the supernatant was filtered through 0.45 µm filter and used for further steps. The pH and conductivity were measured for the refolded protein sample. Protein content was determined using the Pierce™ BCA protein assay kit (Thermo Scientific).

Anion exchange (aex) chromatography:
The refolded protein was purified using AEX column in the flow-through mode using AKTA Pure chromatography system (Cytiva). The strong anion exchanger resin, Q-XL Sepharose (Cytiva), was used. 5 mL resin was packed in XK 16 column. Column was equilibrated with 10 column volume (CV), i.e. 50 mL of equilibration buffer (0.2 M Urea, 50 mM Tris, 10% sucrose, 10 mM EDTA and 250 mM arginine, pH 7.2, Conductivity: 18 mS/cm), and then the refolded protein sample was loaded on the column. Post load washing was done using 5 CV equilibration buffer, followed by elution with 5 CV of elution buffer (0.2 M Urea, 50 mM Tris, 10% sucrose, 10 mM EDTA and 250 mM arginine, and 1 M NaCl, pH 7.2, Conductivity: 78 mS/cm). SDS-PAGE analysis was done to analyze the purity levels.

Concentration and Buffer exchange / Ultrafiltration and diafiltration (UFDF I):
Since the protein was obtained in the flow-through of AEX chromatography, the protein sample concentration and buffer exchange were done using a 100 kDa molecular weight cut-off tangential flow filtration cassette (low protein binding PES membrane). The protein sample was concentrated five folds, by reducing the volume from 500 mL to ~100 mL based on the $O.D._{280}$ values not exceeding 0.5 mg/mL. The transmembrane pressure (TMP) during ultrafiltration was kept constant at 0.25 Bar. Further, the diafiltration buffer (pH 7.2±0.3, conductivity: 5-7 mS/cm, 50 mM Tris, 10% sucrose, 0.01% Pluronic F68, and 250 mM arginine) was substituted for the original buffer.
After completion of diafiltration, TFF retentate volume was 80 mL, which was stored in a 125 mL PETG bottle. The cassette was flushed twice with diafiltration buffer (10 ml each) to recover protein remaining in the cassette. The TFF retentate was filtered using 0.45 µm filter. SDS-PAGE analysis was done to analyze the purity levels.

Cation exchange (CEX) chromatography:
The TFF retentate was purified using Cation exchange chromatography to remove impurities using SP Sepharose XL resin from Cytiva and AKTA Pure chromatography system (Cytiva).5 mL resin was packed in XK 16 column. Column was equilibrated with 10 column volume (CV), i.e. 50 mL of equilibration buffer (50 mM Tris, 10% sucrose, 0.01% Pluronic F68 and 250 mM of arginine, pH 7.2±0.3, Conductivity: 5-7 mS/cm). 100 mL TFF retentate was loaded onto the column and post load washing was done using 5 CV of equilibration buffer. 5 CV of elution buffer (50 mM Tris, 10% sucrose, 0.01% Pluronic F68, 250 mM arginine and 500 mM NaCl, pH 7.2±0.3, Conductivity: 43 - 46 mS/cm) was applied for elution of the protein.Elution fractions (7 fractions of 3 mL each) were collected based on $O.D._{280}$ values in the UV profile. SDS-PAGE analysis was done to analyze the purity levels.

Concentration and Buffer exchange / Ultrafiltration and diafiltration (UFDF II):
Pooled CEX elution fraction (21 mL) was used for this step. A 100 kDa molecular weight cut-off Amicon device was used. Concentration was performed by centrifugation at 1500 RPM, 4°C for 3-5 mins per cycle. The protein was concentrated to a concentration not more than 0.5 mg/mL. Further the buffer was exchanged with 50 mM Tris, 10% sucrose, 0.01% Pluronic F68 and 250 mM of arginine, pH 7.2, Conductivity: 6 mS/cm. After buffer exchange, pH and conductivity of the protein sample was measured and was observed to match with diafiltration buffer. The final volume of UFDF retentate was 5 mL. The sample was filtered through 0.45 µm filter, followed by sterile filtration with a 0.2 µm PES membrane syringe filter.

Western blotting:
Final purified protein (3 µg and 5 µg) was resolved on 12% SDS-PAGE gel with molecular weight marker in duplicates. The gel was briefly washed with water and then protein was transferred onto activated PVDF membrane. The transfer was done using 1X Tris-Glycine-SDS-Methanol buffer at a constant voltage of 30V for 16 h at 2-8°C. After completion of transfer, the membrane was blocked with 2.5% non-fat milk powder in 1X phosphate buffered saline with 0.05% (v/v) Tween 20 (1X PBST) for 30 min with gentle rocking. Once blocked, 5 mL 1X PBST was used to wash the membrane thrice for 5 min each on a rocker. The membrane was cut into two halves and incubated with two different primary antibodies (Anti-SARS-CoV-2 NSP-9 polyclonal antibody [In house, APSL], dilution 1: 1500 in 1X PBST and Anti-SARS-CoV-2 RBD polyclonal antibody [Gene Tex, Cat# GTX635692], dilution 1:1500 in 1X PBST) for 2 h at room temperature (RT) with gentle rocking. Excess and unbound primary antibodies were washed off by washing thrice with 1X PBST for 5 min each on rocker. Further, the membranes were probed with corresponding secondary antibodies (for NSP-9: Goat Anti-Mouse IgG HRP [Invitrogen, Cat# A16066], 1:5000 in 1XPBST and for RBD: Goat Anti- Rabbit IgG HRP [Dako, Cat# P0448], 1:5000 in 1X PBST) for 1 h at RT with gentle rocking. This was followed by washing thrice with 1X PBST for 5 min each on rocker to remove excess secondary antibody. The membranes were finally washed twice with 1X PBS for 5 min each on rocker and then developed with 3,3′-Diaminobenzidine (DAB, 0.05% w/v in 1X PBST) and H_2O_2(0.5% v/v) in dark. Once the expected bands appeared on the blot, the developing solution was drained; the membrane was washed in purified water and dried before scanning the image of the blot.

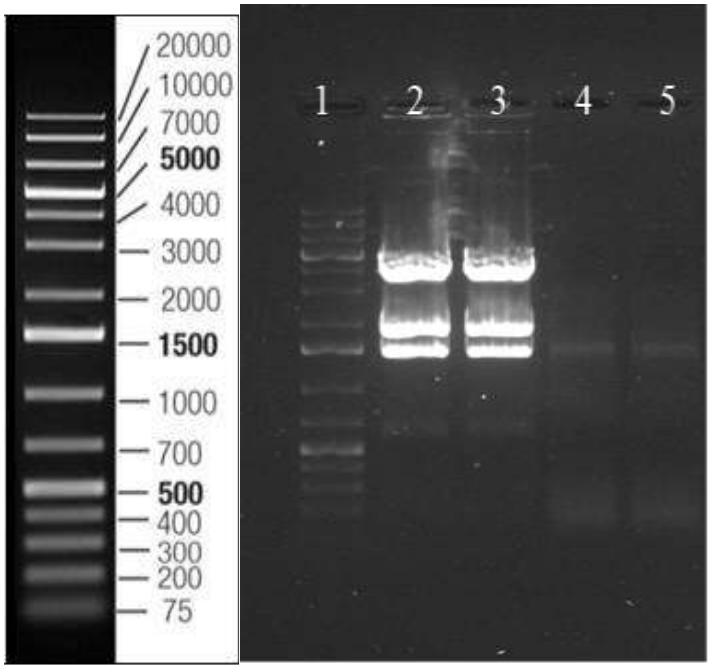
RESULTS

Cloning of VAX-1 protein:

VAX-1 protein has the antigen (Ag) conjugated to Fc fragment of an antibody. Fc is the fragment crystallizable region of the antibody which is the tail region responsible to interact with cell surface receptors called Fc receptors and some proteins of the complement system. Due to this, the antibodies can activate the immune system. The Fc fusion protein is vital for drug development due its important characteristics such as rapid purification, longer half-life and increasing immunogenicity of target antigens to elicit neutralizing antibody response. For cloning, overlap extension PCR method was employed. Primer sets were designed to have one primer in each set with some sequence complementary to the other counterpart.

Table 3: Specifications for the three PCR reactions for generating VAX-1 insert.

Sr. No.	Target	Primer set used	Template Plasmid	Amplicon length
01	Ag	Ag FP and Ag RP	APSL-Ag	700 bp
02	Fc	Fc FP and Fc RP	APSL-Fc	700 bp
03	VAX-1 (Ag-Fc)	Ag FP and Fc RP	Ag PCR + Fc PCR	1400 bp



Overlap extension PCR, Clone 3 were selected for further studies

Optimization of expression:

Different time-temperature and IPTG concentration combinations were used to analyze the level of protein expression as mentioned in materials and methods. Protein expression was analyzed by running crude cell lysates on SDS-PAGE. While the $O.D._{600}$ increased post induction, highest $O.D._{600}$ was observed in cultures induced with 2 mM IPTG (Table 2).

Table 4: Culture conditions and $O.D._{600}$ of cultures at the time of harvest after induction in expression analysis

Flask no.	Antibiotic	Temp (°C)	Rotation speed (RPM)	$O.D._{600}$ at Induction	IPTG Conc. (mM)	Induction time (h)	Induction temperature (°C)	$O.D._{600}$ at harvest
1	Kanamycin (50 µg/ml)	37±1	200	0.72	0	4	37±1	8.16
2				0.72	0.5	4	37±1	6.65
3				0.71	1.0	4	37±1	6.64
4				0.72	2.0	4	37±1	14.97
5				0.73	0	16-18	16±1	8.04
6				0.71	0.5	16-18	16±1	7.25
7				0.72	1.0	16-18	16±1	8.16
8				0.75	2.0	16-18	16±1	14.90

It was observed that induction with 2 mM IPTG resulted in highest expression (Fig. 1 Lane 4 and Lane 7) as compared to 1 mM (Fig. 1 Lanes 5 and 8) and 0.5 mM IPTG (Fig. 1 Lanes 6 and 9). Preliminary experiments showed that the extent of expression induction did not change with the change in temperature or time duration. Thus, the conditions of expression were finalized to be 37°C, 4 h and induction with 2 mM IPTG.

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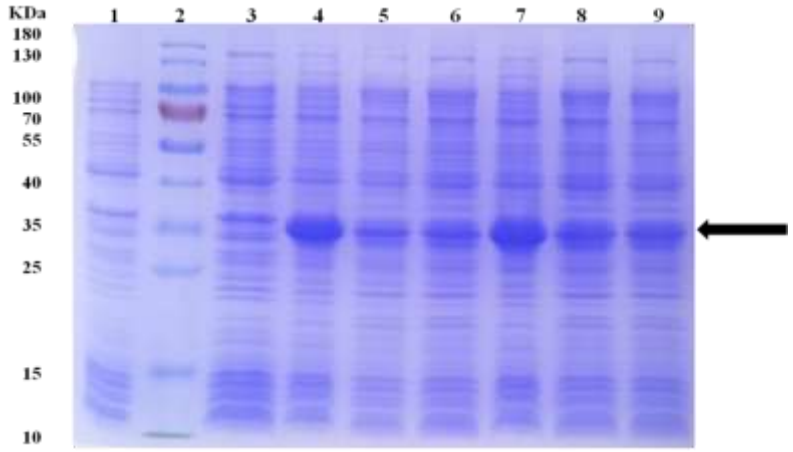


Figure 1: Determination of expression conditions with time-temperature combinations and IPTG concentrations. Lane 1: Culture lysate (uninduced, 37°C, 4 h); Lane 2: Molecular weight marker; Lane 3: Culture lysates (uninduced, 16°C, 16-18 h); Lane 4: Culture lysate (2 mM IPTG, 37°C, 4 h); Lane 5: Culture lysate (1 mM IPTG, 37°C, 4 h); Lane 6: Culture lysate (0.5 mM IPTG, 37°C, 4 h); Lane 7: Culture lysate (2 mM IPTG, 16°C, 16-18 h); Lane 8: Culture lysate (1 mM IPTG, 16°C, 16-18 h); and Lane 9: Culture lysate (0.5 mM IPTG, 16°C, 16-18 h).

Further, six replicate flasks were cultured to check for consistency. All the six flasks showed similar $O.D._{600}$ at the time of harvest (Table 3). SDS-PAGE analysis of crude cell lysates showed similar levels of expression of the protein of interest (Fig. 2 A and B).

Table 3: Culture conditions and $O.D._{600}$ of cultures at the time of harvest for replicate flasks

Flask	Antibiotic	Temp (°C)	Rotation speed (RPM)	$O.D._{600}$ at Induction	IPTG Conc. (mM)	Induction time (h)	$O.D._{600}$ at harvest
F_I1	Kanamycin (50 µg/ml)	37±1	200	0.72	2	4	14.25
F_I2				0.72	2		14.39
F_I3				0.71	2		17.98
F_I4				0.72	2		14.58
F_I5				0.73	2		15.01
F_I6				0.74	2		15.20

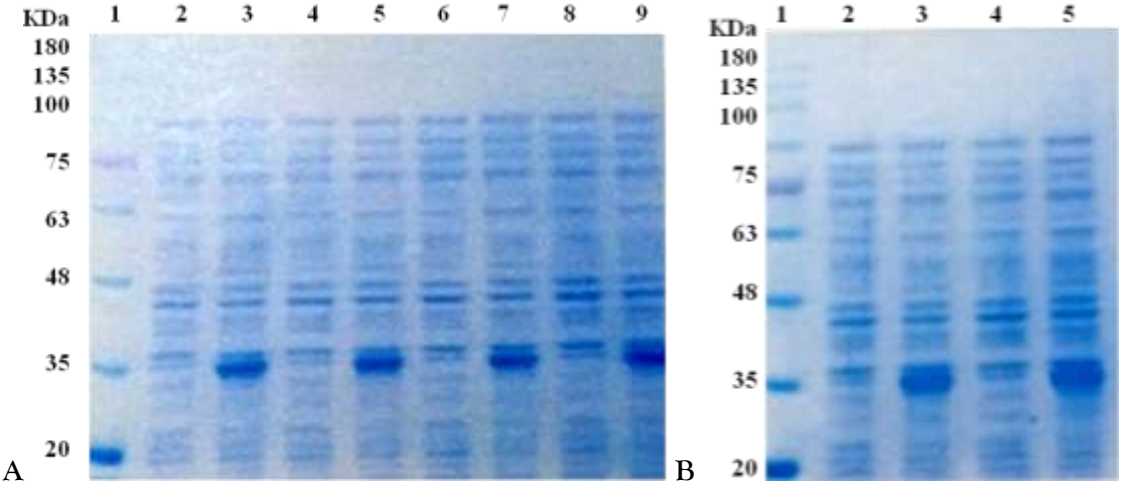


Figure 2: Consistency of protein expression in replicate flasks. Whole cell lysates (15 µL) were loaded for each sample **(A)** Lane 1: Molecular weight marker; Lane 2: Uninduced flask $F_{UI}1$; Lane 3: Induced

flask F_I1; Lane 4: Uninduced flask $F_{UI}2$; Lane 5: Induced flask F_I2; Lane 6: Uninduced flask $F_{UI}3$; Lane 7: Induced flask F_I3; Lane 8: Uninduced flask $F_{UI}4$; Lane 9: Induced flask F_I4. **(B)** Lane 1: Molecular weight marker, Lane 2: Uninduced flask $F_{UI}5$; Lane 3: Induced flask F_I15 Lane 4: Uninduced flask $F_{UI}6$; Lane 5: Induced flask F_I6.

Localization of expressed protein:

The localization of expressed protein was determined after culture and induction conditions were finalized. The protein was expressed as inclusion bodies and not as soluble, cytoplasmic fraction (Fig. 3).

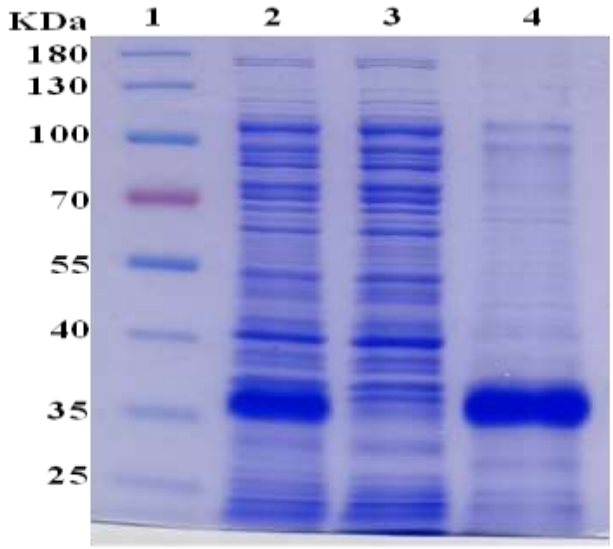


Figure 3: Localization of protein of interest in inclusion bodies. Lane 1: Molecular weight marker; Lane 2: Whole cell lysate; Lane 3: Supernatant; Lane 4: Cell pellet.

Once it was deduced that the protein was expressed and sequestered into inclusion bodies, fed-batch fermentation was carried out for a large-scale batch to optimize purification of the protein. The parameters measured (glucose levels, pH and $O.D._{600}$) are provided in Table 4. The values in bold indicate the time of induction of protein expression.

Table 5: Parameters of the fed-batch fermentation process. The highlighted data point marks the addition of IPTG and starts of induction process.

Time (h)	$O.D._{600}$	pH	Glucose	Time (h)	$O.D._{600}$	pH	Glucose
1	0.35	6.85	11.80	7	7	45.00	6.84
2	1.40	6.85	11.50	8	8	70.00	6.93
3	3.72	6.84	10.60	9	9	88.60	6.92
4	12.50	6.85	8.11	10	10	98.00	6.82
5	19.10	6.85	2.55	11	11	103.00	6.85
6	34.00	6.85	0.14	12	12	104.00	6.82

Inclusion bodies isolation and protein solubilization and refolding:

Although purification of expressed protein from inclusion bodies becomes easier, the refolding process can decrease the yield of active protein of interest[11]. The process was started with 2.5 g of cell pellet and the weight of inclusion bodies achieved after the final wash with WFI was 0.22 g. Figure 4A shows that pure protein is obtained after every washing step during isolation of inclusion bodies.

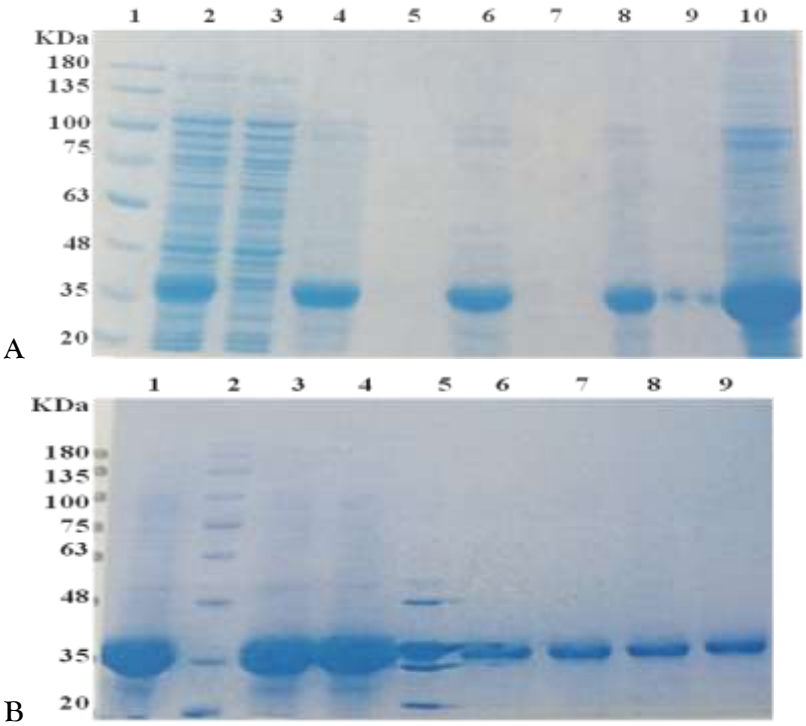


Figure 4: (A) SDS-PAGE profile of purification of inclusion bodies. Lane 1: Molecular weight marker; Lane 2: Homogenized Cell lysate; Lane 3: Supernatant; Lane 4: Cell pellet; Lane 5: Buffer wash 1 supernatant; Lane 6: Buffer wash 1 pellet; Lane 7: Buffer wash 2 supernatant; Lane 8: Buffer wash 2 pellet; Lane 9: WFI wash supernatant; Lane 10: WFI wash pellet. **(B) SDS-PAGE analysis of solubilized inclusion bodies and refolded protein.** Lane 1: Solubilized inclusion bodies; Lane 2: Molecular weight marker; Lane 3: Solubilized supernatant; Lane 4: Solubilized supernatant (0.45µm filtered); Lane 5: Solubilized pellet; Lane 6: Refolding output at 0 h; Lane 7: Refolding output at 15 h; Lane 8: Refolding output at 15 h supernatant; Lane 9: Refolding output at 15 h, supernatant (0.45 µm filter).

Further, using chaotropic agent like urea for solubilizing the protein and then refold it helped in achieving partially purified protein in the refolded structure (Figure 4B) and with minimal loss of protein as indicated by protein estimated using Bicinchoninic Acid (BCA) Protein Assay (Table 5). The percentage step recovery of protein after refolding was 90.76%.

Table 6: Details of volume, O.D.$_{280}$ and protein concentration by Bicinchoninic Acid (BCA) Protein Assay

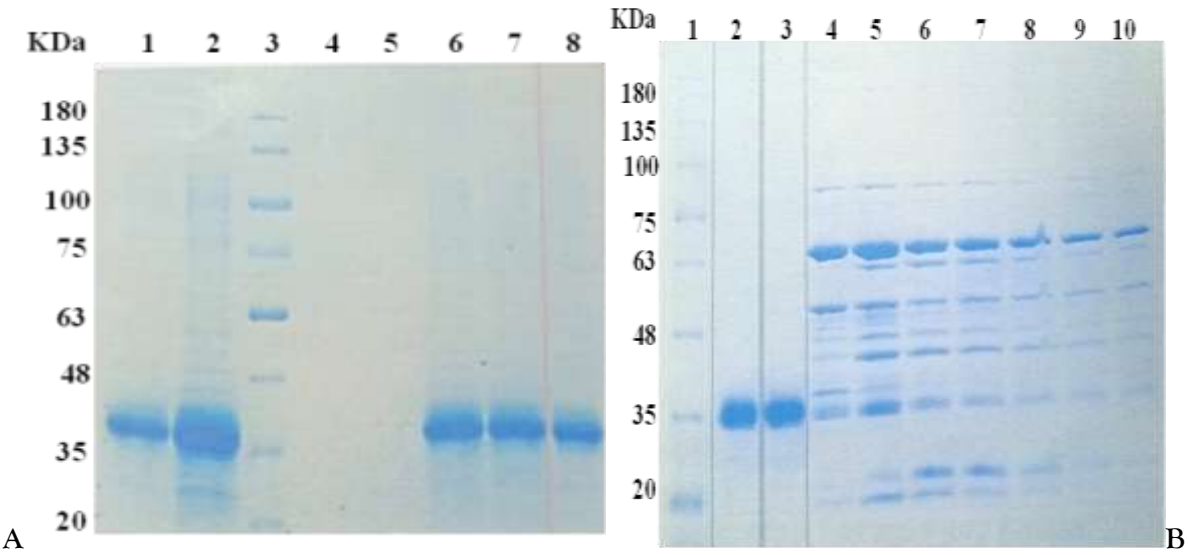
Sr. No.	Sample details	Volume (mL)	O.D.$_{280}$	Protein Concentration (µg/mL)	Total Protein (µg)	% step recovery
1	Filtered Solubilized supernatant	50	0.985	498	24900	NA*
2	Refolding output after 15 h	500	0.102	45.2	22600	90.76%
3	AEX Flow-through	500	0.105	43.7	21850	96.68%
4	UFDF Retentate I	100	0.490	216.8	21680	99.22%

*NA = not applicable

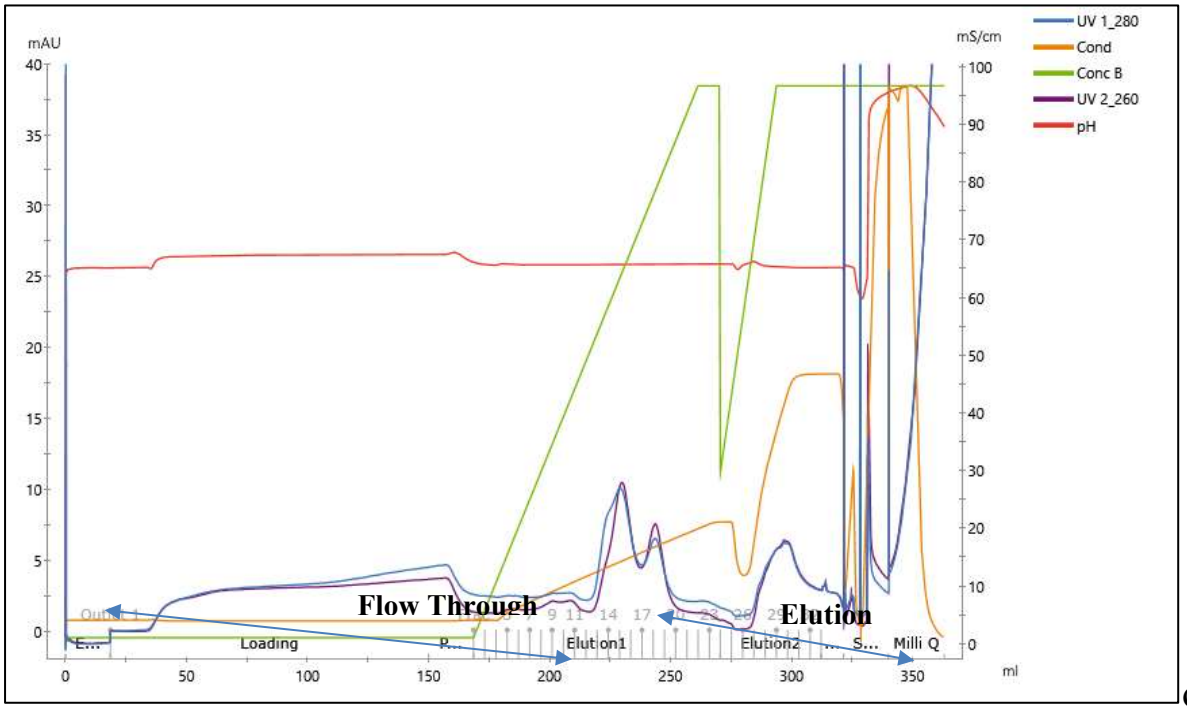
Purification of expressed protein using AEX and concentration of flow-through by UFDF I:

AEX chromatography step acted as a negative purification to remove extraneous protein impurities. Since the theoretical isoelectric pH (pI) of the protein is 8.32 and a pH of 7.2 was maintained throughout the AEX process, the protein remained positively charged and hence did not bind the resin and was

present in the flow-through. Figure 5A shows the presence of protein of interest in the flow-through, while other protein impurities were eluted later.



A B



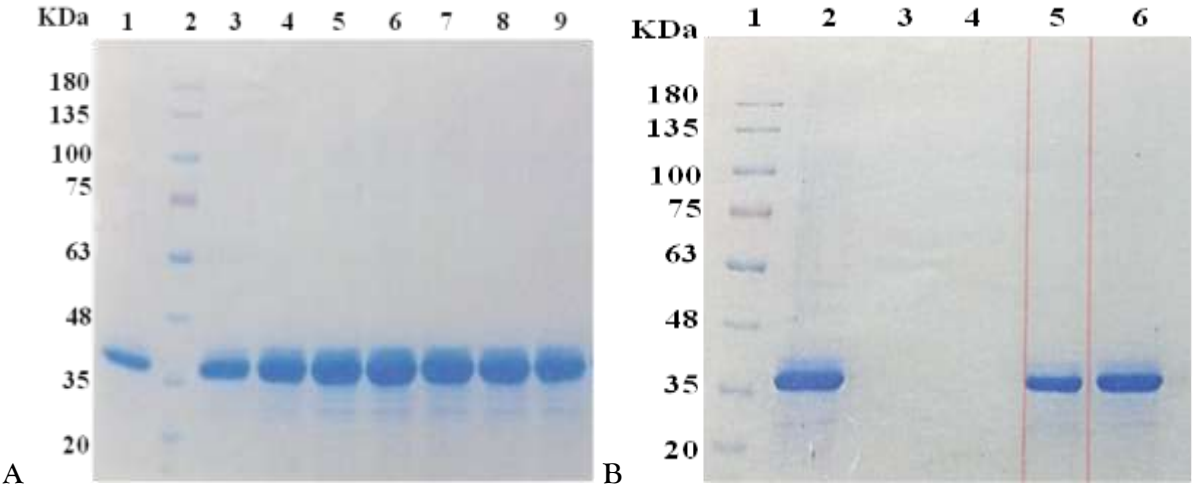
C

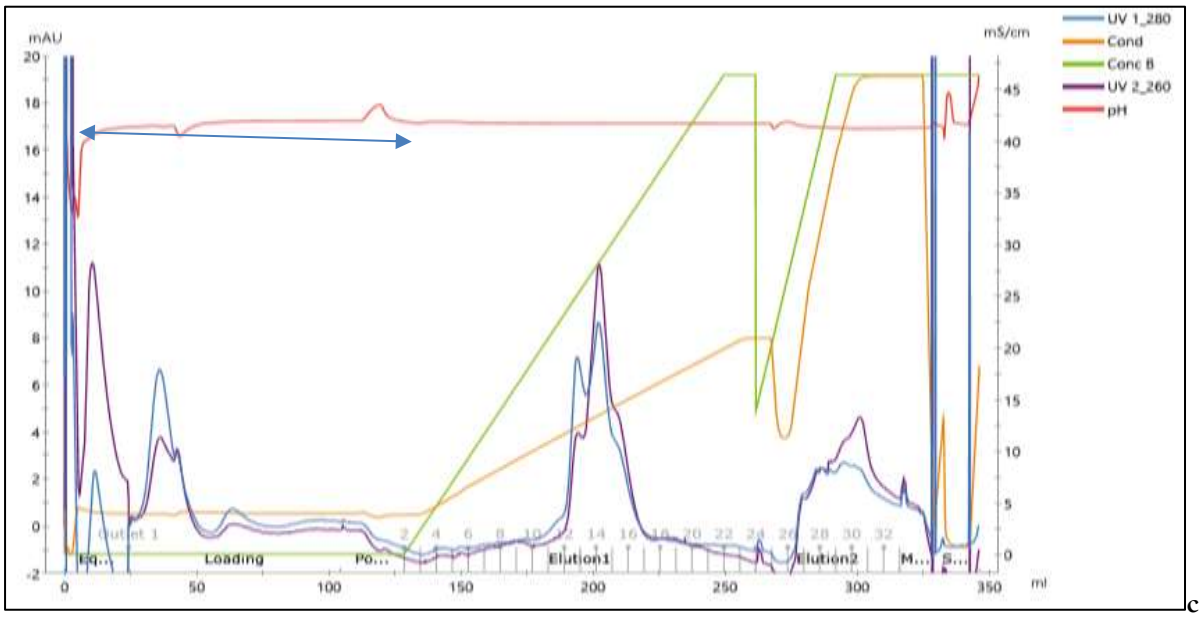
Figure 5: (A) SDS-PAGE analysis of AEX chromatography run. Lane 1: Molecular weight marker; Lane 2: AEX load; Lane 3: AEX flow-through; Lane 4: Elute fraction 1; Lane 5: Elute fraction 2; Lane 6: Elute fraction 3; Lane 7: Elute fraction 4; Lane 8: Elute fraction 5; Lane 9: Elute fraction 6; Lane 10: Elute fraction 7. **(B) SDS-PAGE analysis of protein after UFDF I.** Lane 1: AEX flow-through, Lane 2: Ultrafiltration I retentate, Lane 3: Molecular weight marker, Lane 4:

Ultrafiltration I permeate, Lane 5: UFDF I permeate, Lane 6: UFDF I retentate, Lane 7: UFDF I retentate after flushing, Lane 8: UFDF I retentate (0.45µm filtered). **(C) Chromatogram of AEX chromatography.** The chromatogram provides the detailed information about the A280, A260, pH and conductivity through the AEX chromatography run.As refolded protein from inclusion bodies is used as loading

material, the load and flow-through do not show much impurity for the volume (20 µL) loaded onto the gel (Fig. 5A Lanes 2 and 3). However, the elute fractions are more concentrated and hence show the presence of impurities when same volume (20 µL) is loaded onto the gel (Fig. 5A, Lanes 4 to 10). Figure 5C provides the chromatogram of the AEX chromatography run, with the profiles of absorbance at 280 nm (A280), conductivity, absorbance at 260 nm (A260) and pH.Since the volume of flow-through was high (500 mL), it was concentrated using UFDF. Concentration helps in preparing the sample for the next steps of purification and also allows exchange of buffers to the more compatible ones for protein stability and further purification methods. Figure 5B shows the SDS-PAGE analysis of the UFDF permeate and retentate. Further, protein estimation by BCA assay shows that AEX retained 96.68% of the protein in the flow-through and post UFDF I, the retentate showed a recovery of 99.22% (Table 5).

Purification of expressed protein using CEX and concentration of pooled elute fractions by UFDF II: Since the purified protein showed some high and low molecular weight impurities after AEX and UFDF I, CEX using SP Sepharose XL resin was employed to further purify the protein. Figure 6A shows the SDS-PAGE profile of elute fractions obtained after CEX chromatography. The protein elutes appear to be pure and devoid of impurities. The chromatogram for the CEX chromatography run is present in Figure 6C and 6D. However, the protein recovery from this step is only 11.41% (Table 6).





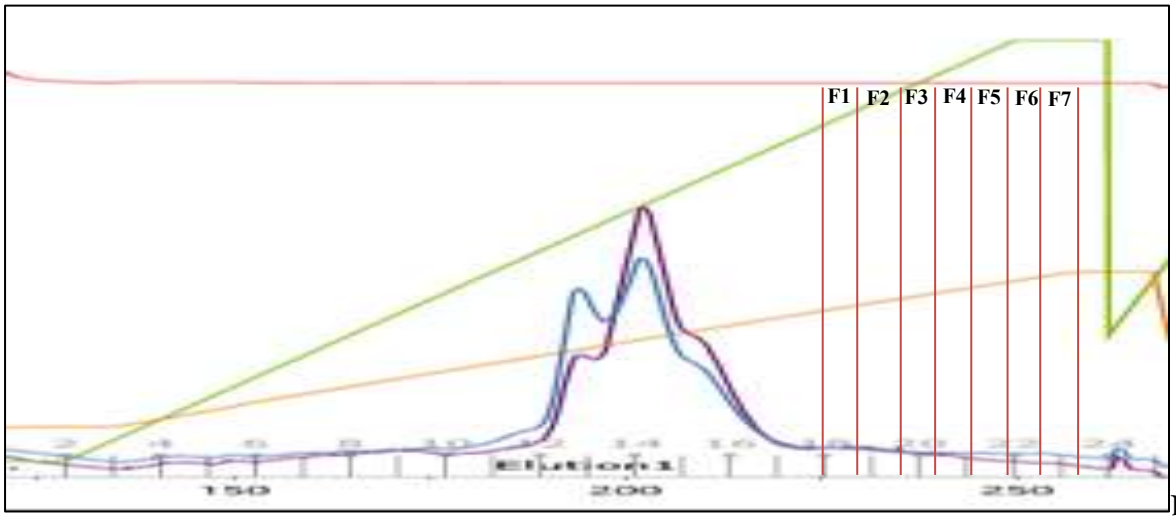


Figure 6: (A) SDS-PAGE profile of CEX chromatography run. Lane 1: CEX load; Lane 2: Molecular weight marker; Lane 3: CEX Elute fraction 1; Lane 4: CEX Elute fraction 2; Lane 5: CEX Elute fraction 3; Lane 6: CEX Elute fraction 4; Lane 7: CEX Elute fraction 5; Lane 8: CEX Elute fraction 6; Lane 9: CEX Elute fraction 7. **(B) SDS-PAGE analysis of protein after UFDF II.** Lane 1: Molecular weight marker; Lane 2: Pooled CEX elute fractions (UFDF II load); Lane 3: Ultrafiltration II permeate; Lane 4: Diafiltration II permeate; Lane 5: Final protein product (0.2 µm filtered); Lane 6: UFDF II retentate after flushing. **(C) Chromatogram of CEX chromatography.** The chromatogram provides detailed information about the A280, A260, pH and conductivity through the CEX chromatography run. **(D) Chromatogram of elution of CEX chromatography.** The chromatogram provides detailed information about the A280, A260 and conductivity for the elution step of the CEX chromatography run.

Table 7: Details of volume, $O.D._{280}$ and protein concentration by Bicinchoninic Acid (BCA) Protein Assay for CEX chromatography run and the percentage step recovery

Sr. No.	Sample details	Volume (mL)	$O.D._{280}$	Protein Concentration (µg/mL)	Total Protein (µg)	% step recovery
1	CEX Load	100	0.489	216.2	**21620**	NA*
2	CEX Flow-through	95	0.003	BQL**	NA*	NA*
3	CEX Post load wash	25	0.002	BQL**	NA*	NA*
4	CEX Elute Fraction 1	3	0.218	96.4	289.2	NA*
5	CEX Elute Fraction 2	3	0.320	141.5	424.4	NA*
6	CEX Elute Fraction 3	3	0.319	141.0	423.1	NA*
7	CEX Elute Fraction 4	3	0.340	150.3	451.0	NA*
8	CEX Elute Fraction 5	3	0.225	99.5	298.4	NA*
9	CEX Elute Fraction 6	3	0.226	99.9	299.8	NA*
10	CEX Elute Fraction 7	3	0.212	93.7	281.2	NA*
Total protein in pooled CEX Elute fractions					**2467.1**	**11.41%**

*NA= not applicable, **BQL=below quantification limit

The elute fractions of CEX were pooled and concentrated to ~5 mL using an amicon centrifugal device. UFDF II output was the final product (Fig. 6B) and was used for analytical characterization for correctness of epitopes by western blotting.

Western blotting shows the presence of expressed epitopes:
Since the vaccine candidate carries multiple epitopes like RBD and NSP9 of SARS-CoV2 virus, the purified protein was probed with polyclonal antibodies against RBD and NSP9 using Western blotting technique to analyze the presence and correctness of the required epitopes. In Figure 7A and 7B, positive bands for NSP9 and RBD can be observed when probed with in-house polyclonal antibody against NSP9 and the commercially available polyclonal antibody against RBD, respectively. However, both the blots also show the presence of bands corresponding to dimeric and trimeric forms of the protein. This could be attributed to the polyclonal nature of the primary antibodies used in the assay.

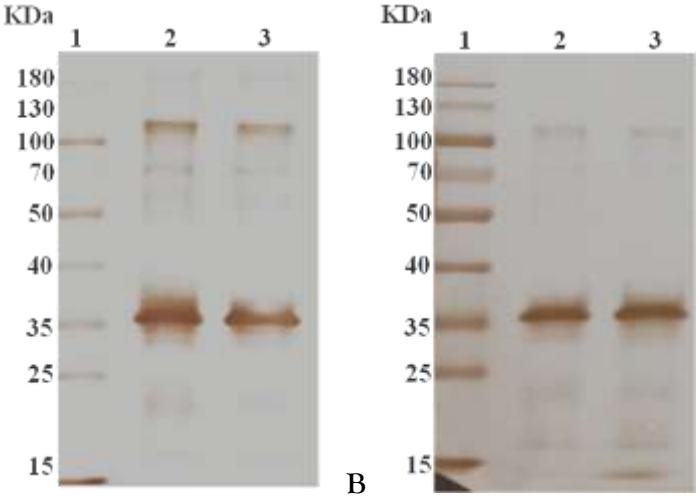


Figure 7: (A) Western blot for purified protein with anti-NSP9 polyclonal antibody. Lane 1: Molecular weight marker; Lane 2: 5 µg protein load; Lane 3: 3 µg protein load. **(B) Western blot for purified protein with anti-RBD polyclonal antibody.** Lane 1: Molecular weight marker; Lane 2: 3 µg protein load; Lane 3: 5 µg protein load.

DISCUSSION

Asymmetric PCR requires the right ratio of the two primers in the PCR reaction mix for the PCR to work in the direction required. Optimization of conditions for asymmetric PCR gave an idea about that. While cloning of very small fragments of DNA into big vectors, it is necessary to maintain the molar ratios instead of just adding a random volume of insert to avoid failure of ligations.COVID-19 was a global pandemic which has not been completely controlled. Newer cases of infection are being reported even today and mutant versions of the SARS-CoV2 virus are discovered almost every year. The global pandemic obligated fast track research and development of vaccine against the virus. Various research groups across the world were involved in developing a suitable vaccine for mass vaccination and increasing the immunity against the SARS-CoV2 virus[7,12]. This study presents the data for the design, expression and purification of another such vaccine candidate. The vaccine candidate is a multiple epitope protein-based vaccine using the most worked upon protein, RBD and a non-structural protein, NSP9 of SARS-CoV2. The study shows that the expressed protein of ~35 kDa is achieved as inclusion bodies and the expression localization does not change when induction temperature is reduced. This is not an unseen phenomenon with recombinant proteins[11]. Furthermore, as other research has recorded moderate chaotropic substances such as urea[11,13] were used to solubilize and refold the protein from the inclusion bodies in order to increase refolding efficiency and decrease protein loss (Table 6).
Protein purification was done using AEX and CEX to achieve the final purified protein. Throughout the AEX chromatography, pH was maintained at 7.2, below the pI of the protein (8.32). Thus, AEX acted as

a negative or flow-through technique and the protein of interest was recovered in the flow-through and the extraneous cellular protein impurities that bound to the resin were removed as elutes. The overall purification process showed good recovery of protein at each step, except the positive CEX where the recovery fell to ~11% (Table 7). However, the purity of the protein was good and further analysis using Western blotting with antibodies against NSP9 and RBD showed that the expressed protein had retained the epitopes of interest. The further studies to prove the efficacy of the vaccine candidate include *in vitro* and *in vivo* animal studies, which will be taken up in the future.

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CONCLUSION

While multiple vaccine candidates for COVID 19 have been developed, this study provides a novel candidate combining multiple epitopes in a single protein subunit vaccine. Multiple epitopes may lead to improved immune activation against COVID 19 infection. The study presents data regarding the expression and purification of a recombinant multiple epitope vaccine from *E. coli*.

Data Availability Statement
The manuscript incorporates all datasets produced or examined throughout this research study.

Ethics Approval Statement
Not Applicable

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Conflict of Interest
The authors declare that they have no conflicts of interest regarding the publication of this research article.

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