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Cloning and Expression of Fimbrial Subunit Gene of *Dichelobacter nodosus* from Jammu and Kashmir, India and Purification of the Recombinant Protein

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Abstract

Objectives: The present study was aimed at cloning and expression of fimbrial subunit gene (*fimA*) of *D. nodosus* (serogroup B and E from J&K, India) in *E. coli*. Then the purification of the expressed fusion proteins was attempted.

Results: Sequence analysis of the cloned *fimA* genes, revealed that the serogroup B *fimA* gene had maximum homology with strains JKS-01B and serogroup E *fimA* gene with the strain JKS-O8E. The coding sequence of *fimA* gene (*cdsfimA*), encoding fimbrial subunit protein, was characterized and successfully expressed in *E. coli* BL21 (DE3), using pET32a (+) vector. Upon SDS-PAGE analysis, the estimated molecular weight of recombinant *D. nodosus* fimbrial subunit fusion protein (Thioredoxin-6x Histidine tagged) was approx. 35 kDa, which is very close to expected molecular weight. The exact identity of recombinant protein was confirmed by western blotting, using Ni-NTA conjugate antibody, directed against polyhistidine tagged recombinant fimbrial protein. The fusion protein was purified using Ni-NTA affinity chromatography and relatively purified fraction of the target protein was obtained in elution fractions (2nd & 3rd).

Major Conclusions: 1. The target protein was found to be toxic to host cells (BL21), as the transformed BL21 could not grow on LB/ampicillin agar without the addition of 1% glucose in the media. This was substantiated by the sizeable decline in viability and division of recombinant BL21cells post induction of expression (IPTG), as judged by decline in turbidity of induced control as compared to the un-induced control.

2. The recombinant *D. nodosus* fimbrial subunit proteins did not assemble to mature fimbriae in *E. coli*, after the observation that the expressed protein could not be released from the host without the use of detergent (SDS).

3. It was further elucidated that increasing the incubation time post induction of expression as well as repeated freeze thawing of cell lysate, increases the yield of recombinant protein as compared to the cellular milieu of recombinant host cell.

Future Prospectus: Further work needs to be continued to assess the protective immune response to the recombinant protein in laboratory animals and eventually formulate a recombinant vaccine using recombinant *fimA* protein to assess the immune response in host.

Keywords: Footrot; Recombinant vaccine; *fimA*; Expression; Affinity chromatography

Abbreviations

fimA: Fimrial subunit gene; PCR: Polymerase Chain Reaction; *cdsfimA*: coding sequence of fimbrial subunit gene; ORF: Open Reading Frame; NTA: Nitrilotriacetic Acid

Introduction

Footrot has emerged as one of the major constraints in sheep productivity and agriculture development in Jammu and Kashmir (India). The disease is almost endemic to the temperate region and has been extensively investigated. It has become one of the biggest challenges with huge economic impacts to the sheep farmers [1,2]. The high economic significance of the disease has stressed the need to explore the possibility of managing the disease by effective preventive and control measures.

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D. nodosus, the essential casual agent of footrot is an anaerobic, gram negative, rod shaped and heavily fimbriated bacterium. Various treatment regimens have been tried in the past with low degree of success, but vaccination against *D. nodosus* has been shown to be effective not only to prevent footrot but to treat sheep already presenting footrot lesions [3]. Footrot vaccines have been developed and evaluated over the last four decades to contain monovalent whole cell, multivalent recombinant fimbrial, and finally mono or bivalent recombinant fimbrial antigens [4]. Due to limitations of the conventional vaccines, the serogroup-specific commercially inactivated vaccine containing *D. nodosus* strains present in a geographical region has been used for successful control of virulent footrot in different countries including, Nepal and Bhutan [5,6]. However, inactivated vaccines are expensive and offer shorter duration of immunity. Further, the poor growth characteristics of *D. nodosus* due to its fastidious and anaerobic nature inhibit the large scale production and thus widespread use of these vaccines.

The fimbriae of *D. nodosus* are highly antigenic and considered as the major host protective immunogens [7]. Following the realization that protective vaccines only needed to contain fimbrial proteins, the next step in the development is to enhance the efficiency of production of the fimbrial antigens. Fimbrial antigenic protein of *D. nodosus* can be expressed in a less fastidious surrogate host having good growth characteristics. Monovalent vaccines based on recombinant fimbriae induce high levels of agglutinins and long lasting immunity. Fimbriae based vaccine is much more specific in protection than whole cell vaccine [8]. Further, the vaccines based on purified fimbriae avoid both dilution of effective immunogen by extraneous cellular antigen and the vaccinal lesions associated with administration of excess extraneous antigen as occurs with whole-cell vaccines [9]. However, the fimbriae are also associated with serogroup variation. So it is expected that area specific (mono or bivalent) fimbrial vaccines will remain the best option, until an antigen covering all serogroups is found [4]. Keeping this in view, the fimbrial subunit gene (*fimA*) of the predominant *D. nodosus* serogroup in J&K i.e., serogroup 'B' and additionally that of serogroup E were undertaken for cloning and expression in *E. coli*.

Materials and Methods

PCR amplification of full length *fimA* gene

The nucleotide sequence of the full length *fimA* gene of *D. nodosus* (serogroup B and E) along with the flanking regions was amplified using the published primers [10], given as: *fimA* fwd: GCGTCAGGCAACTGACTCTA; *fimA rev*: CCTCTCAAGAGAGAGGCTTTT. The amplification conditions were the same as for detection of *D. nodosus* [11].

Cloning of full length *fimA* gene in *E. coli*

Cloning of full length *fimA* gene of serogroup B and E *D. nodosus* was carried out in E. coli DH5-α using pTZ57R/T vector. Before taking up the ligation procedure, the PCR amplified products were subjected to PCR cleanup protocol using PCR purification kit (FAVORGEN Biotech Corp.). Ligation of the desired amplified gene into pTZ57R/T vector was carried out using (Instaclone PCR cloning kit, Thermo Fisher Inc.) as per the manufacturer's recommendations and mixture was incubated at 4^0C overnight in Thermo cycler (Eppendorf).

E. coli DH5-α cells (not>7 days old) were streaked on freshly prepared Luria Bertani (LB) agar and incubated for 24 hours at 37^0C. A well isolated DH5-α colony was made competent to take up foreign DNA as per the recommended procedure of the cloning kit. Before taking up the transformation step, Luria Bertani (LB) agar plate (not>2 days old), supplemented with ampicillin (Amp) to a final concentration of 50 μg/ml, were poured upon with 40 μl of X-gal (50 mg/ml stock solution) and 20 μl of isopropylthio-β-D-galactoside (IPTG) (100 Mm) and spread using L-spreaders. These were pre-warmed at 37^0C for 20 minutes before plating them with the transformation mixture. Transformation was achieved by transferring 2.5 μl of the ligation mixture (containing 14 ng of pTZ57R/T vector DNA) and 1 μl of super coiled control DNA (10-100bp) into two separate microcentrifuge tubes. These were chilled on ice for 2 min. 50 μl of the prepared competent cells was added to both tubes. The mixture was held on ice for 5 min and then 50 μl of the trans-formant mixture (DH5-α carrying *fimA* of *D. nodosus* serogroup B and E in pTZ57R/T vector) were placed on separate pre-warmed LB /Amp agar plates and spread using L spreaders. The plates were incubated overnight at 37^0C. plated on pre-warmed LB/Amp plates. After overnight incubation at 37^0C, blue and white colonies appeared on LB/Ampicillin plates. The white colonies were screened for the presence of *fimA* insert by colony touch PCR. The PCR positive clones were subjected to Plasmid extraction using FavorPrep plasmid extraction mini kit (FAVORGEN Biotech Corp). The product was screened for the presence of insert by fimA gene PCR. The retrieved plasmids were sequenced commercially from Agrigenome Labs, Kerala.

Expression of coding sequence of *fimA (cdsfimA)* in *E. coli*

PCR amplification and directional cloning of coding region of *fimA* gene into cloning vector: The primers for expression of ORF of *fimA*, which codes for the mature fimbrial subunit, were designed using the software (CLC MainWorkbench, CLC bio, Denmark). The primers used for the amplification of whole *fimA* gene were arbitrarily selected from end sequences of the gene segment attempted for expression. Care was taken in selecting the forward primer, so that 5′ end of the open reading frame gets ligated in frame with the ATG start codon of expression vector. The reverse primer was so selected, that the endogenous stop codon was retained in the insert. Further, the site for *BamHI* (G^GATCC) was incorporated in the forward primer and that for *Hind111* (A^AGCTT) in the reverse primer. The sequence of these primers is given as: *cdsrfimA* fwd: GCGG^GATCCTTCACCTTAATCGAACTCAG; *cdsrfimA* rev: GCCA^AGCTTCCTCTCAAGAGAGAGGCTTTT. These primers were used to amplify the coding sequence of *fimA* of *D. nodosus* (serogroup B and E) cloned in pTZ57R/T vector. The amplification conditions were evaluated and turned out to be essentially the same as for detection of *D. nodosus* [11], except for an increased annealing temperature of 60˚c for 30 sec. This was followed by purification of the amplified product was carried out using the PCR purification kit as described above. This was followed by directional cloning of the purified products in *E. coli* DH5-α using pGEM-T Vector (Promega), as recommended by the manufacturer. Plasmid was isolated from the PCR positive clones and screened for *cdsfimA* insert by double digestion, using restriction enzymes, *Hind111* and *BamHI*. The digest was screened by electrophoresis in 1% agarose gel. The retrieved plasmids were sequenced commercially.

Sub-cloning of the protein coding sequence *(cdsfimA)* in expression vector: The strategy was aimed at expression of thioredoxin and 6x Histidine tagged fimbrial subunit protein of *D. nodosus* in *E. coli* BL21 (DE3) using pET System expression vectors pET32a (Novagen). pET32a carry ampicillin resistance genes and restriction

sites for *BamHI* and *Hind111*. pGEM plasmid carrying *cdsfimA* inserts (serogroup B and E) were subjected to double digestion using restriction enzymes, *BamHI* and *Hind111* (Thermofischer Scientific), separately in 0.2 ml PCR tubes, according to the manufacturer's instructions. The components were mixed gently by pippeting and spun down. This was followed by incubation at 37°C for 15 min and heating at 80°C for 10 min to inactivate the enzyme. The incubation and heating steps were carried out in thermocycler (Eppendorf, Germany). The digested product was electrophoresed in 0.7% agarose gel in TAE. The *cdsfimA* insert was purified by agarose gel extraction method, using gel purification kit (FAVORGEN Biotech Corp.). The purified DNA was analyzed on 1% agarose gel.

The expression vector pET32a(+) was subjected to double digestion using same enzymes *(BamHI* and *HindIII)* in separate tube. The digested vector was further subjected to alkaline phosphatase treatment using FastAP Thermosensitive Alkaline Phosphatase (Thermoscientific). Both the double digested inserts and vector were purified prior to ligation by the method described earlier. The ligation of insert with the prepared vectors was carried out using T4 DNA ligase (Promega), as per the manufacturer's buffer and incubation conditions. The ligated mixture was used for the transformation of competent BL21 (DE3) cells, using TransformAid Bacterial Transformation Kit (Thermo Scientific), as detailed earlier. The *cdsfimA*-pET32a transformant mixture was plated over LB plates supplemented with ampicillin (50 μg/mL) and 1% glucose. After overnight incubation, recombinant colonies were screened for the presence of *cdsfimA* insert by colony touch PCR. Plasmid retrieved from PCR positive clones were screened for *cdsfimA* insert by restriction analysis with *BamHI* and *HindIII*. The Positive clones were sequenced commercially.

Expression of 6xHis-tagged recombinant fimbrial subunit protein in *Escherichia coli*

Single colony of transformant [*E. coli* BL2I carrying *cdsfimA* in pET32a] was inoculated in 5 ml LB broth containing 100 μg/ml ampicillin in a 50 ml tube. The culture was incubated for approximately 3 hours at 37°C in a shaking incubator (Eppendorf, Germany) at 300 rpm, until the OD600 reached 0.5-0.7. 1.5 ml of broth culture was taken immediately before induction (non-induced control), for Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The remaining broth culture was induced for expression by adding 100 mM IPTG (Merck, Germany) to a final concentration of 1.0 mM. The culture was grown for an additional 3 hours (in case of recombinant *fimA* of serogroup B) with shaking (300 rpm) and then the second 1.5 ml sample (induced control) was collected for SDS-PAGE analysis. To determine the effect of incubation (37°C) post induction upon extent of gene expression, overnight incubation (37°C, 300 rpm) post induction was carried out in the broth culture inoculated with BL21 carrying *cdsfimA* of serogroup E in pET32a.

Preparation of cell lysates for SDS-PAGE

For the preparation of crude cell extracts, 1.5 ml of each of the four grown broth cultures, two each from 3 hour and over nightly incubated (uninduced and induced) samples, were taken in seperate 2 ml eppendorf tubes and centrifuged at full speed (16900 g at 4°C) for 1 min to obtain a pellet. The supernatant was discarded and the pellet was re-suspended in 100 μl of 10% Sodium Dodcyl Sulphate (SDS) and refrigerated (-20°C) for 5-10 min to lyse the cells. The same sample incubated overnight post induction was repeatedly frozen and thawed several times after SDS treatment. After thawing the samples were again centrifuged at full speed for 1 min. 50 μl of the clear supernatant was taken from each tube and placed respectively into four new 2 ml eppendorf tubes. This was followed by addition of 50 μl of 2x Laemmli buffer containing loading dye to each tube and mixed by pippeting. After heating for 10 min at 95°C, the samples (10 μl each) were loaded in separate wells in 12% SDS-Polyacrylamide gel.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis of target protein

Protein samples were separated and analysed in SDS-PAGE as described by Laemmli [12]. This was performed at room temperature in 12% polyacrylamide gels, using the Mini-Protean tetra cell 4-gel handcasting system (BIO-RAD, China). 10μl of sample was loaded in each well along with 5 μl of pre-stained protein ladder (NEX-GEN-BlueAlpha, Puregene). The gel was run at 25 mA for aproximately 2 hours, stained with Coomassie Blue staining solution and destained with destain solution (40 ml methanol added to 7 ml glacial acetic acid and 53 ml distilled water to make a final volume of 100 ml), until the gel background was clear.

Western blotting

Recombinant *D. nodosus* fimbrial protein was resolved on 12% SDS-PAGE and transferred onto nitrocellulose membrane (0.45 μm, Sigma-Aldrich, USA). Expression of the recombinant protein was confirmed by probing the blotted protein with 1:1000 dilution of Ni-NTA HRP conjugate (Qiagen, Germany), as per the protocol provided by the manufacturer.

Purification of fimbrial subunit protein

For this the pellet obtained from the recombinant bacterial culture was treated with lysis buffer (CelLyticB, Cat #B7435, Sigma-Aldrich), as per the protocol. Then batch purification of trx-6xhis-tagged recombinant *D. nodosus* fimbrial subunit protein from *E. coli* BL21(DE3) was done under native conditions using Ni-NTA affinity chromatography as per QIAexpressionist™ manual (Qiagen, Germany). The supernatant was carefully removed and 5-15 μl was checked for the presence of the concerned protein by SDS-PAGE analysis. The supernatant was subjected to purification using Ni-NTA affinity chromatography. 1 ml of 50% Ni-NTA slurry was added to 4 ml cleared lysate and mixed gently by shaking (200 rpm on a rotary shaker) at 4°C for 60 min. The lysate-Ni-NTA mixture was loaded into a column with the bottom outlet capped. The bottom cap was removed and column flow-through was collected. The flow-through was saved for SDS-PAGE analysis. The column was washed twice with 4 ml wash buffer and the collected wash fractions were also analysed by SDS-PAGE. 0.5 ml elution buffer was used to elute the protein four times. The purity of the recombinant protein in different fractions was checked by electrophoresis on 12% SDS-PAGE gel after staining with Coomassie brilliant blue stain.

Results

PCR amplification of fimbrial subunit gene (*fimA* gene)

The pair of PCR primers designed to amplify the full length *fimA* gene of *D. nodosus* serogroup B and E, found specific to *D. nodosus* (BLAST), amplified (in silico) two products from whole genome sequence of reference, *D. nodosus* strain VCS1703A (Myers et al., 2007). One product of 597 bp was specific to *fimA*, while the other product of 16 bp being insignificant. All the samples detected positive for *D. nodosus* serogroup B and E were with the designed primer pair yielded an amplicon of approximately 600 bp (Figure 1).

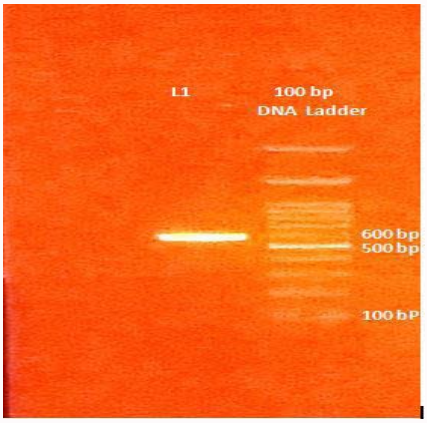

Figure 1: Amplified full length *fimA* gene of *D. nodosus* serogroup B.

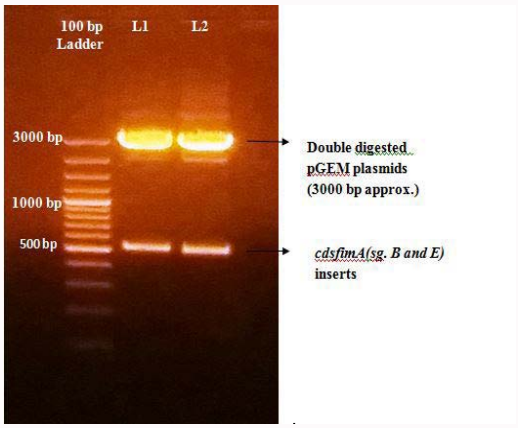

Figure 2: Restriction digestion Profiling (using *HindIII* & *BamHI*) of recombinant pGEM plasmids carrying *cdsfimA* inserts of *D. nodosus* serogroup B (L1) and E (L2).

Cloning of full length *fimA* gene

The Purified full length *fimA* gene amplicons of *D. nodosus* serogroup B and E (amplified directly from clinical samples) were cloned separately in *E. coli* DH5-α, using cloning vector pTZ57R/T. Success of the cloning was checked by screening the LB/Ampicillin agar plates for the presence of recombinant colonies. The clones with *fimA* inserts appeared white and were reconfirmed by colony touch PCR. Plasmids were isolated from two PCR confirmed recombinant clones, *E. coli* DH5-α carrying *D. nodosus* serogroup B *fimA* gene in pTZ57R/T, designated as FimB and *E. coli* DH5-α carrying *D. nodosus* serogroup E *fimA* gene in pTZ57R/T, designated as FimE. The sequence of these representative inserts, is shown in the appendix.

Basic Local Alignment Search Tool (BLAST) search of *fimA* sequence of clone FimB with the database of sequences (GenBank) showed that the sequence has maximum homology, 99.67%, with fimbrial subunit gene of *Dichelobacter nodosus* strain JKS-01B, from Kashmir (J&K, India) (Accession no.: GU362417). It also showed 99.45% homology with *Dichelobacter nodosus* strain Ind-Ap-MBNR1, from Andhra Pradesh, India (Accession no.: KT831900.1). Similarly *fimA* sequence of clone FimE showed maximum homology, 99.83%, with *Dichelobacter nodosus* strain JKS-08E, from Kashmir (J&K, India) (Accession no.: HM190159). It also showed 96.82% homology with *Dichelobacter nodosus* strain E-NZ2, from New Zealand (Accession no. : 52407.1).

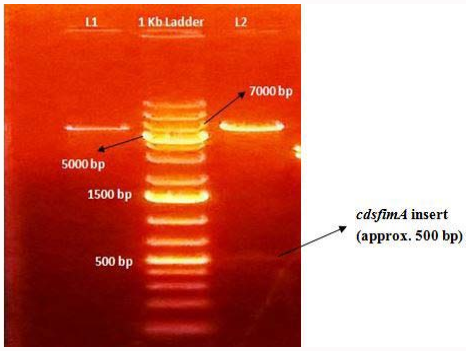

Figure 3: Double restriction digestion profiling (using *HindIII* & *BamHI*) of pET32 plasmid carrying *D. nodosus* serogroup B *cdsfimA*. L1: Recombinant pET32a plasmid carrying *D. nodosus* serogroup B *cdsfimA* insert (approx. 6400 bp); L2: Double restriction digested recombinant pET32a plasmid with liberated *cdsfimA* insert (approx. 500 bp).

Expression of *fimA* gene in *Escherichia coli* BL21(DE3)

The coding sequences of cloned *fimA* genes (serogroup B and E) of *D. nodosus* were attempted for expression. The designed primer pair for protein coding sequence of *fimA* gene *(cdsfimA)* was first used in PCR to amplify the coding sequence of *fimA* of both B and E serogroups. The purified PCR amplicons were then ligated directly to corresponding pGEM plasmids and the recombinant plasmids were transformed into competent *E. coli* DH5-α cells. The white colonies on LB/ampicillin agar were screened for the presence of *cdsfimA* insert by colony touch PCR and were further reconfirmed upon double digestion (*BamHI* and *HindIII*) of recombinant plasmids from the PCR positive to liberate *cdsfimA* insert (Figure 2). Orientation of insert in the vector plasmid was confirmed by sequencing of the extracted plasmid. The nucleotide sequence of cloned *D. nodosus* serogroup B *cdsfimA* gene (forward strand), found to be 522 bp, is shown in the appendix. The sequence showed that the coding sequence was intact and in the correct frame. The putative length of fimbrial subunit protein encoded from *cdsfimA* serogroupB is 155 amino acids as shown: sftlielmivvai igilaafa ipayndyi arsqaaegvsl adglkiri aenlqdgeckg pdadpasg avgnrd kgkyalaeikgdy dasktdagdpng ckveitygqgtaeg kisklitgkklvld qmvngsfi qgegtdladkfip navkkksk.

Both, pGEM plasmid carrying cdsfimA (serogroup B and E) and expression vector pET32a were double digested, using *BamHI* and HindIII. Further double digested pET32a(+) was subjected to alkaline phosphatase treatment. The cleaved inserts were gel purified and analyzed on agarose gel along with double digested pET32a(+) vector, to determine their respective concentrations. The concentration of the gel purified *cdsfimA* inserts of both serogroups was estimated to be approximately 20 ng/5 μl, while that of plasmid vector was approximately 80 ng/5 μl. Both double digested inserts were ligated into their corresponding expression vectors, pET32a(+), using DNA ligase (promega).

Sub-cloning in *Escherichia coli* BL21 (DE3): The *cdsfimA* gene of *D. nodosus* serogroup B and E after ligation with their corresponding expression vectors (pET32a) were transformed separately into competent *E. coli* BL21(DE3) cells and plated on to corresponding LB/Ampicillin agar plates supplemented with 1% glucose. After overnight incubation, very few colonies were obtained on LB/Ampicillin/glucose agar plate. However not all the clones were positive for the presence of *cdsfimA* insert by PCR. The colony touch PCR of positive clones showed amplification of *cdsfimA* and

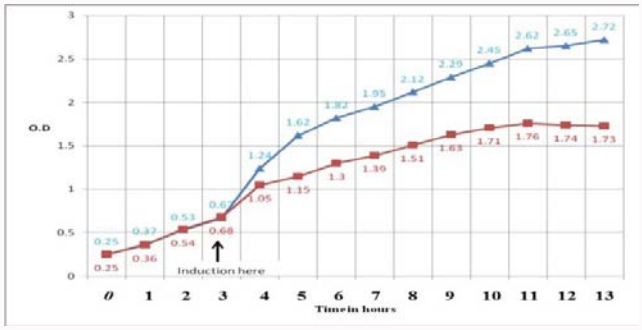

Figure 4: Cell growth (measured as the OD_{600}) of *Escherichia coli* BL21 harbouring pET32a at 37°C with induction (■) and without (▲) induction.

were reconfirmed for the presence of insert by restriction digestion profiling of the plasmids retrieved from PCR positive clones (Figure 3). The inserts were found to be in correct orientation inside the expression vector upon sequencing of pET32a plasmid carrying *cdsfimA* insert. The sequence of the complete reading frame of the vector insert construct shows correct orientation of insert with ATG and 6x-his tag upstream of the gene, is shown in the appendix. The deduced amino acid sequence of the protein to be expressed from vector insert construct is shown below.

trxmhhhhh hsftl ielmivva iigila afaipayn dyiars qaaegvsl adglki riaenlq dgeckgpdadp asgav gnrdkgky alaeik gdydasktdagdp ngckv eityg qgtaeg kisklitg kklvld qmvng sfiqgegtdlad kfip navkkksk*

trx: internal trx (thioredoxin) fusion tag, *hhhhhh*: internal 6x his(histidine) tag.

Expression of fimbrial subunit fusion protein: The recombinant fimbrial subunit protein was expressed by the *E. coli* BL21 host cells, atleast 3 hours after induction by 100 mM isopropyl-*β*-D-thiogalactoside (IPTG). However, there was a marked contrast upon the growth of recombinant cells prior and post induction. The broth containing *E. coli* BL21 cells carrying recombinant plasmid showed a uniform growth prior to induction, but after induction the growth was significantly reduced as compared to non induced control. There was discernible disparity in the OD600 in cells with and without induction (Figure 4).

SDS-PAGE analysis

The molecular mass of recombinant fimbrial subunit protein tagged with the fusion tag (Trx-His-S-tag), obtained by separation of different bacterial proteins from cell lysates through 12% SDS-PAGE technique was estimated to be approximately 35 kDa, which is very close to the expected molecular weight. Recombinant BL21 host cells expressing serogroup E fimbrial subunit protein, subjected to overnight incubation as well as repeated freeze thawing post induction, lead to increased yield of recombinant fimbrial subunit protein. The PAGE analysis of the target proteins is depicted (Figure 5).

Western blotting

The exact identity of the expressed protein was established by subjecting the recombinant *E. coli* BL21 cells to Western transfer analysis, along with negative control (uninduced recombinant *E. coli* BL21). Ni-NTA HRP conjugate (Qiagen) directed against Histidine-tagged proteins picked up a band at approx. 35kDa regions from the lanes loaded with cell lysate from the host cells induced to express the

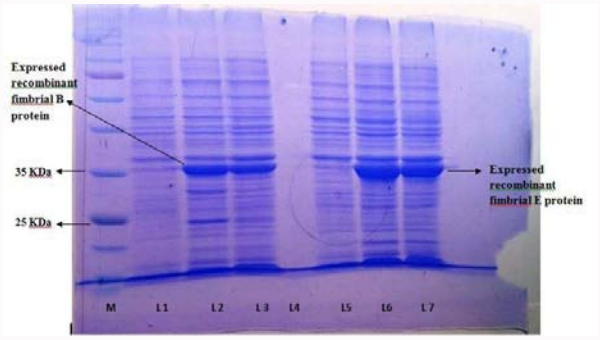

Figure 5: SDS-PAGE analysis of the target proteins. M: Protein Marker; L1: uninduced control-BL21 carrying *D. nodosus* serogroup B *fimA* gene; L2,L3: induced samples-BL21 carrying *D. nodosus* serogroup B *fimA* gene; L4 is blank, L5 depicts uninduced control-BL21 carrying *D. nodosus* serogroup E *fimA* gene; L6, L7 depicts: Induced samples-BL21 carrying *D. nodosus* serogroup E *fimA* gene.

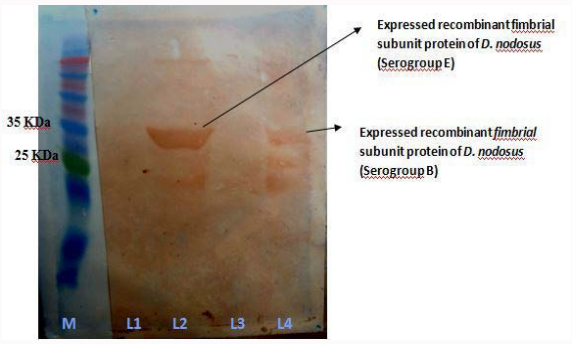

Figure 6: Western Blotting analysis of target proteins. M: Protein marker; Ll: Uninduced control- BL21 carrying *D. nodosus* serogroup E *cdsfimA*; L2: Induced whole cell lysate of BL21 (carrying *D. nodosus* serogroup E cds*fimA*); L3: Uninduced control- BL21 carrying *D. nodosus* serogroup B cds*fimA*; L4: Induced whole cell lysate of BL21 (carrying *D. nodosus* serogroup B cds*fimA*).

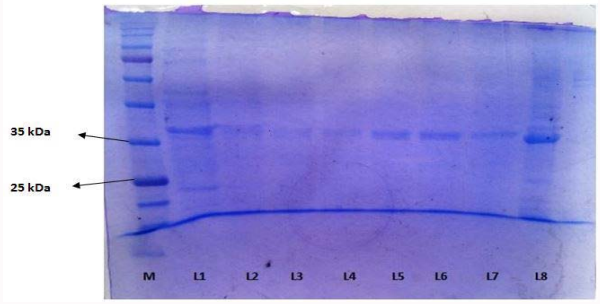

Figure 7: Purified recombinant fimbrial subunit protein of *D. nodosus* serogroup B, approx. 35 kDa (in L5, L6), using Ni-NTA affinity chromatography. M: Protein marker; L1: clarified cell lysate of BL21 carrying *D. nodosus* serogroup B *cdsfimA*; L2: wash fraction- 1; L3: wash fraction-2; L4: elution fraction-1; L5: elution fraction-2; L6: elution fraction- 3; L7: elution fraction-4; L8: Whole cell lysate of BL21carrying *D. nodosus* serogroup B *cdsfimA*.

serogroup B and E recombinant fimbrial protein. However, no such signal was produced in the lane from non-induced cell lysate. Thus, upon Western blotting, the expression of Trx-6x His tagged fimA fusion protein was identified by Ni-NTA HRP conjugate (Figure 6).

Purification of the target protein

After batch purification of Trx-6x-His tagged recombinant fimbrial protein of *D. nodosus* from *E. coli* BL21(DE3) cells using

Ni-NTA affinity chromatography, relatively purified fraction of the recombinant protein was obtained in the second and third elution fraction, as confirmed by SDS-PAGE analysis of different fractions (Figure 7).

Appendix

>Nucleotide sequence of *fimA* gene (serogroup B) of clone FimB was found to be 598 bp and its nucleotide sequence is given as;

GCGTCAGGCAACTGACTCTAAACAAGATG
ATATTTAAATGTTCACATTCTTAATAGGAGA
ATATGATGAAAAGTTTACAAAAAGGTTTTAC
CTTAATCGAACTCATGATTGTAGTTGCAATT
ATCGGTATCTTAGCTGCTTTCGCTATCCCTG
CATACAACGACTACATCGCTCGTTCACAAGC
TGCTGAAGGCGTTAGTTTGGCTGATGGTTTA
AAAATCCGCATCGCTGAGAACTTGCAAGACG
GCGAATGTAAAGGACCGGATGCAGATCCAG
CATCTGGTGCTGTTGGCAACCGAGACAAAGG
TAAGTATGCCTTAGCAGAAATTAAGGGTGAC
TATGATGCATCAAAGACAGACGCTGGCGATC
CGAATGGTTGTAAGGTCGAAATCACTTATGG
TCAAGGCACTGCAGAAGGTAAAATTTCTAAG
CTGATCACTGGTAAAAAATTGGTTTTAGATC
AAATGGTTAATGGTTCATTTATTCAAGGTGA
AGGTACTGACTTAGCAGATAAATTTATCCCG
AATGCAGTAAAAAAGAAATCTAAATAGTATC
TAGTGTAAACATTAGCTTACTTAAAAGCCTC
TCTCTTGAGAGG

>Nucleotide sequence of *fimA* gene (serogroup E) of clone FimE was found to be 591 bp and its nucleotide sequence is given as;

GCGTCAGGCAACTGACTCTAAACAAGATG
ATATTTAATGTTCACATTCTTAATAGGAGAA
TATGATGAAAAGTTTACAAAAAGGTTTCACC
TTAATCGAACTCATGATTGTAGTTGCAATTA
TCGGTATCTTAGCTGCTTTCGCTATCCCTGCA
TATAACG ACTACATCGCTCGTTCACAAGCAGCTGAAGG
TTTATCATTAGCTGATGGCTTAAAAATCCGTATCGCT
GATCACTTAGAAAATGGCTCTTGTACAGAGGA
TGCTAATGCTGGTGAAAAAGGCAACCAAG
ACACAGGCAAGTACGCTTTAGCAGAAATT
GGGGGTACTTATGCTCAAGATGCTACCAAC
CTCAAACCAGAAGATAAGAATGGTTGTACAGTTACCAT
CACTTATGGTAAAGGCACTGCGGGGGCAA
AGATCTCCAAGTTGATCGATACCAAAGTG
TTAGAACTTGAACAATTGGTTAATGGTTCT
TACACCCAAGGCGGCGCCACTACTTTGGAT
GCTAAATTCATTCCAAATG CAGTGAAACCAACCAAATAGT
ATCTAGTGTAAACATTAGCTTACTTAAA
AGCCTCTCTCTTGAGAGG.

>Nucleotide sequence of cloned *D. nodosus* serogroup B cdsfimA gene (forward strand), found to be 522 bp, is diven as;

G^GATCCTTCACCTTAATCGAACTCATG
ATTGTAGTTGCAATTATCGGTATCTTAGCT
GCTTTCGCTATCCCTGCATACAACGACTACAT
CGCTCGTTCACAAGCTGCTGAAGGCGTTAGTT
TGGCTGATGGTTTAAAAATCCGCATCGCTGAGAA
CTTGCAAGACGGCGAATGTAAAGGACCG
GATGCAGATCCAGCATCTGGTGCTGTTGGCAA
CCGAGACAAAGGTAAGTATGCCTTAGCAGAA
ATTAAGGGTGACTATGATGCATCAAAGACAGAC
GCTGGCGATCCGAATGGTTGTAAGGTCGAA
AATCACTTATGGTCAAGGCACTGCAGAAGGTA
AAATTTCTAAGCTGATCACTGGTAAAAAATTG
GTTTTAGATCAAATGGTTAATGGTTCATTTATT
CAAGGTGAAGGTACTGACTTAGCAGATAA
ATTTATCCCGAATGCAGTAAAAAAGAAAT CTAAATAGTAT
CTAGTGTAAACATTAGCTTACTT^AAAAGCCTCTCTCTTGA
GAGGAAGCT.

>Sequence of *cdsfimA* of *D. nodosus* serogroup B in pET32a(+)

ATGCACCATCATCATCATCATG^GATCCTTCACCT
TAATCGAACTCATGATTGTAGTTGCAATTATCGGTATCTT
AGCTGCTTTCGCTATCCCTGCATACAACGACTACA
TCGCTCGTTCACAAGCTGCTGAAGGCGTTAG
TTTGGCTGATGGTTTAAAAATCCGCATCGCTG
AGAACTTGCAAGACGGCGAATGTAAAGGACC
GGATGCAGATCCAGCATCTGGTGCTGTTGGCA
ACCGAGACAAAGGTAAGTATGCCTTAGCAGAAATT
AAGGGTGACTATGATGCATCAAAGACAGACG
CTGGCGATCCGAATGGTTGTAAGGTCGAAATC
ACTTATGGTCAAGGCACTGCAGAAGGTAAAAT
TTCTAAGCTGATCACTGGTAAAAAATTGGTTTT AGA T C A
AATGGTTAATGGTTCATTTATTCAAGGTGAA
GGTACTGACTTAGCAGATAAATTTATCCCGA
ATGCAGTAAAAAAGAAATCTAAATAGTATCT
AGTGTAAACATTAGCTTACTTAAAAGCCTCT
CTCTTGAGAGGAAGCTTGCGGCCGCACTCGA
GCACCACCACCACCACCACTG.

The sequence of insert is shown in upper case letters and that of vector in lower case letters. TAG in red is the endogenous stop codon retained in the insert.

Discussion

The conventional vaccines prove relatively costly due to the fastidious requirement and sparse growth of *D. nodosus* in culture, and the difficulty of obtaining stable fimbrial production, especially under liquid-fermentation conditions required for commercial production. These difficulties could be circumvented by use of recombinant DNA technology to direct the production of appropriate antigens by an alternative host cell. It has been established that the prominent serological and immune-protective antigens of *D. nodosus* are the fimbriae, the genes encoding the structural subunit of fimbriae can be expressed in a less fastidious surrogate host having good growth characteristics. Following the realization that protective vaccines only needed to contain fimbrial proteins, the next step in the development is to enhance the efficiency of production of the fimbrial antigens. However, the fimbriae are also attributed with antigenic variation, so it is expected that area specific (mono or bivalent) fimbrial vaccines will remain the best option, until an antigen covering all serogroups is found [4]. The serological diversity studies of *D. nodosus* in Jammu & Kashmir so far indicate that serogroup B is predominantly prevalent followed by serogroup E [2,13-15]. Keeping this in view, the fimbrial subunit gene (*fimA*) of two predominant *D. nodosus* serogroups in J&K i.e., serogroup 'B' and serogroup 'E' were undertaken for cloning and expression in *E. coli*.

Fimbriae of *D. nodosus* are constructed from a single repeated

polypeptide subunit, whose length varies from 151 to 154 amino acid residues [16,17]. Nucleotide sequence of *fimA* gene serogroup B was 598 bp, while that of serogroup E was 591 bp. The cloned full length *fimA* gene of serogroup B had 99.67% homology with fimbrial subunit gene of *Dichelobacter nodosus* strain JKS-01B, (Accession no. GU362417). The *fimA* gene of serogroup E showed maximum homology, 99.83%, with *Dichelobacter nodosus* strain JKS-08E (Accession no. HM190159). The nucleotide sequence of cloned *D. nodosus* serogroup B *cdsfimA* gene was 522 bp, with an endogenous stop codon. The gene encoded a 155 amino acid fimbrial subunit protein. Mattick et al., [17] stated that the length of the fimbrial subunit varies from 145 to 160 amino acid residues.

The strategy was aimed at expression of fimbrial subunit protein tagged with (Trx- 6x His tag) in *E. coli* BL21 (DE3), using pET System expression vector (pET32a). The pET vectors are the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli*, which drive expression of a recombinant gene under the control of the T7 RNA polymerase promoter and lac operator [18,19]. The vectors are designed for use in λDE3 lysogen strains of *E. coli*, which besides other strains include BL21(DE3), harboring a genomic copy of the gene for T7 RNA polymerase under the control of the lac repressor [20,21]. Besides, the selective markers bla (-lactamase, or ampicillin resistance) are available with the pET32a vectors. The vector contain T7 lac promoters and restriction sites for *BamHI* and *HindIII* [22].

Then *cdsfimA* was attempted for directional cloning into expression vector pET32a(+) and recombinant vector was transformed into competent BL21(DE3) cells. Initially there was absence of transformed colonies on the LB/Amp plates. The reason for absence of recombinant colonies on cultural medium could be due to toxicity of the recombinant *fimA* target protein expressed from the correctly oriented insert after transformation into host bacterial cells, thereby killing the host cells and thus prevented the growth of any recombinant colonies on LB/Amp agar plates. Low expression levels or no expression at all can be caused by toxicity of the target protein. The lac promoters are not very tightly regulated and show some degree of leaky expression before the addition of the inducer. This leakiness often leads to plasmid instability and/or loss of plasmid. As a consequence, the culture will be overgrown by cells that have lost the plasmid (especially when ampicillin is used as a selectable marker) or will not grow at all. This difficulty was overcome by the addition of 1% glucose to LB/Amp culture medium. Addition of 1% glucose to the culture medium represses the induction of the lac promoter by lactose, which is present in LB media (https://www.embl.de/pepcore). Yet a very low number of recombinant colonies were obtained on LB/Amp/Glu agar plates. However, it is established that when using recombinant pET vectors, BL21 (DE3) and its derivatives are transformed at about one tenth (1/10), the efficiency of the other strains [22]. Upon sequencing of the positive recombinant clone (BL21 carrying *cdsfimA* of *D. nodosus* serogroup B in pET32a), the sequence of *cdsfimA* insert was found to be in correct orientation inside pET32a, with the ATG start codon, thiredoin (trx) and histidine(6x) tags present upstream of the insert.

After induction of expression (using IPTG), the toxic nature of fimbrial protein was confirmed by marked decline in viability and division of recombinant BL21 cells. There was a clear decline in turbidity of broth culture induced by IPTG as compared to un-induced control. The synthesis of target proteins in most cases, driven by T7 RNA polymerase, compromises the cell's ability to carry out normal functions, thereby slowing its growth [22]. Bhat, M. A [10], thesis submitted to SKUAST-Kashmir, India), observed a similar sizeable decline in viability and division of cells concomitant with the full induction of fimbrial synthesis, as judged by decline in O.D-600 value as compared to the control. Further, Elleman et al., [23], observed the inability of induced cells to form colonies on solid media, due to cell lysis in liquid cultures post induction. Infact, recombinant proteins with hydrophobic regions often have a toxic effect on host cells, most likely due to the association of the protein with or incorporation into vital membrane systems. Our study reconfirmed that the recombinant fimbrial proteins did not assemble to mature fimbriae in E. coli, after observing that the expressed protein could not be released from the host without the use of detergent (SDS). There are sufficient evidences that the fimbrial subunit genes of *D. nodosus* expressed in *E. coli*, remain embedded in bacterial cell envelope (primarily in the inner membrane) and not assembled into mature fimbriae [23]. The possible explanations for lack of formation of mature fimbriae in recombinant *E. coli* BL21(DE3) cells are; that other genes are required for fimbrial biosynthesis which were absent from the cloned fimbrial gene of *D. nodosus*, or that these genes or their products were not properly functional in *E. coli*.

The molecular mass of recombinant fimbrial subunit protein (tagged with Trx-6x His tag), obtained by separation of different bacterial proteins from cell lysates through 12% SDS-PAGE technique was estimated to be approximately 35 kDa, which is very close to the expected molecular weight. However, host cells expressing serogroup 'E' recombinant fimbrial subunit protein showed more expression on SDS-PAGE analysis. It is because after induction of expression, the recombinant host cells (carrying Serogroup 'E' *cdsfimA* insert) were subjected to overnight incubation(~20 hours) at 37˚c and the cell lysate after SDS treatment was freeze thawed several times, which lead to greater yield of serogroup E recombinant fimbrial protein (Plate-7). The intensity of the bands in the gel are usually proportional to the amount of protein [24]. In concurrence to this finding, Johnson and Hecht et al., [25] verified that repeated cycles of freezing and thawing are sufficient to separate highly expressed recombinant proteins away from the cellular milieu of E. coli. Freezing and thawing liberates recombinant proteins from the bacterial cytoplasm, but does not release the bulk of endogenous *E. coli* proteins. Also, Fazaeli et al., [26], reported a marked increase in the yield of recombinant cholesterol oxidase (a bacterial flavoprotein) production, when cultures were incubated at 30˚C for 16 hours.

The exact identity of the expressed protein was established by subjecting the recombinant *E. coli* BL21 (DE3) cells expressing the target protein to Western transfer analysis. The expression of Trx-polyhistidine tagged *fimA* fusion protein was identified by Ni-NTA HRP conjugate. The His Tag could be used for identification of fusion protein after purification via Western blotting [27]. These antibodies picked up band of approximately 35 kDa in the induced cell lysate regions. However, no such signal was produced in the lane from non induced cell lysate.

Batch purification of 6xhis tagged recombinant fimbrial protein from *E. coli* BL21 (DE3) was done under native conditions using Ni-NTA affinity chromatography. Upon purification of the target recombinant protein, the purified fraction was obtained in the second and third elution as confirmed by SDS-PAGE analysis of different fractions. The fusion protein expressed by recombinant pET32a(+)

vector besides thioredoxin also contains a His Tag which is often used for affinity purification [27]. The His•Tag sequence binds to divalent cations like Ni^{2+} immobilized on the His•Bind metal chelation resin. After unbound proteins are washed away, the target protein is recovered by elution with imidazole [22].

Conclusion

The *fimA* gene of *D. nodosus* serogroup B and E were successfully expressed in *E. coli* BL 21 using expression vector pET32a(+). As a widely used biotechnique, construction of pET 32 vector for protein expression and purification in *E. coli* is fast, inexpensive and scaleable. The *D. nodosus* fimbrial subunit proteins expressed in *E. coli* remained embedded in bacterial cell membranes and could not assemble into mature fimbriae. The expressed fimbrial subunit protein of *D. nodosus* proved toxic to the host cells. Increasing the incubation time post induction of expression and repeated freeze thawing of cell lysate increases the yield of expressed recombinant protein. Ni-NTA affinity chromatography proved to be a rapid and excellent technique to purify in bulk (under native conditions) the his-tagged recombinant fimbrial protein of *D. nodosus*.

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References

1. Farooq S, Wani SA, Hussain I, Bhat MA. Prevalence of ovine footrot in Kashmir, India and molecular characterization of Dichelobacter nodosus by PCR. Indian Journal of Animal Sciences. 2010; 80: 826-830.
2. Rather MA, Wani SA, Hussain I, Bhat MA, Kabli ZA, Magray SN. Determination of prevalence and economic impact of ovine footrot in central Kashmir India with isolation and molecular characterization of Dichelobacter nodosus. Anaerobe. 2011; 17: 73-77.
3. Liardet DM, Chetwin DH, McNerney DM, Hindmarsh FH. Reduction of the prevalence of footrot on New Zealand farms by vaccination. N Z Vet J. 1989; 37: 129-130.
4. Dhungyel O, Hunter J, Whittington R. Footrot vaccines and vaccination. Vaccine. 2014; 32: 3139-3146.
5. Egerton JR, Ghimire SC, Dhungyel OP, Shrestha HK, Joshi HD, Joshi BR, et al. Eradication of virulent footrot from sheep and goats in an endemic area of Nepal and an evaluation of specific vaccination. Vet Rec. 2002; 151: 290-295.
6. Gurung RB, Dhungyel OP, Tshering P, Egerton JR. The use of an autogenous Dichelobacter nodosus vaccine to eliminate clinical signs of virulent footrot in a sheep flock in Bhutan.Vet J. 2006; 172: 356-363.
7. Every D, Skerman TM. Protection of sheep against experimental footrot by vaccination with pili purified from Bacteroides nodosus. N Z Vet J. 1982; 30: 156-158.
8. Egerton JR, Cox PT, Anderson BJ, Kristo C, Norman M, Mattick JS. Protection of sheep against footrot with a recombinant DNA-based fimbrial vaccine. Veterinary Microbiology. 1987; 14: 393-409.
9. Stewart DJ, Clark BL, Peterson JE, Griffiths DA, Smith EF, O'Donnell IJ. Effect of pilus dose and type of Freund's adjuvant on the antibody and protective responses of vaccinated sheep to Bacteroides nodosus. Research in Veterinary Sciences. 1983; 35: 130-137.
10. Bhat MA, Wani SA, Hussain I, Magray SN, Muzafar M. Identification of two new serotypes within serogroup B of Dichelobacter nodosus. Anaerobe. 2012; 18: 91-95.
11. La Fontaine S, Egerton JR, Rood JI. Detection of Dichelobacter nodosus using species specific oligonucleotides as PCR primers. Veterinary Microbiology. 1993; 35: 101-117.
12. Laemmli U. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature. 1970; 227: 680-685.
13. Taku A, Chhabra R, War BA. Footrot on a sheep breeding farm in the Himalayan state of Jammu and Kashmir. Rev Sci Tech. 2010; 29: 671-675.
14. Badroo GA, Taku A, Mir IA, Bhat MA, Kumar B, Gazal S. Virulence Characterization of Dichelobacter nododsus from Clinical Cases of Ovine Footrot. Israel Journal of Veterinary medicine. 2017; 72: 17-21.
15. Wani SA, Farooq S, Kashoo ZA, Hussain I, Bhat MA, Rather MA, Aalamgeer S. Determination of prevalence, serological diversity, and virulence of Dichelobacter nodosus in ovine footrot with identification of its predominant serotype as a potential vaccine candidate in J&K, India. Trop Anim Health Prod. 2019; 51: 1089-1095.
16. McKern NM, O'Donnell IJ, Inglis AS, Stewart DJ, Clark BL. Amino acid sequence of pilin from Bacteroides nodosus (strain 198), the causative organism of ovine footrot. FEBS Lett. 1983; 164: 149-153.
17. Mattick JS, Anderson BJ, Mott MR, Egerton JR. Isolation and characterisation of Bacteroides nodosus fimbriae: structural subunit and basal protein antigens. Bacteriol. 1984; 160: 740-747.
18. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 1990; 185: 60-89.
19. Dubendorff JW, Studier FW. Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor. J Mol Biol. 1991; 219: 45-59.
20. Studier FW, Moffatt BA. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol. 1986; 189: 113-130.
21. Rosenberg AH, Lade BN, Chui D, Lin S, Dunn JJ, Studier FW. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene. 1987; 56: 125-135.
22. Mierendorf RC, Morris BB, Hammer B, Novy RE. Expression and Purification of Recombinant Proteins Using the pET System. Springer. 2000; 13: 257-292.
23. Elleman TC, Hoyne PA, Stewart DJ, McKern NM, Peterson JE. Expression of pili from Bacteroides nodosus in Pseudomonas aeruginosa. J Bacteriol. 1986; 168: 574-580.
24. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248-254.
25. Johnson BH, Hecht MH. Recombinant Proteins Can Be Isolated from E. coli Cells by Repeated Cycles of Freezing and Thawing. Nature Biotechnology. 1994; 12: 1357-1360.
26. Fazaeli A, Golestani A, Lakzaei M, Rasi Varaei SS, Aminian M. Expression optimization, purification, and functional characterization of cholesterol oxidase from Chromobacterium sp.DS1. Plos One. 2019; 14: e0212217.
27. Liu ZQ, Yang PC. Construction of pET-32 α (+) vector for protein expression and purification. N Am J Med Sci. 2012; 4: 651-655.