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Expression and Purification of Exposed Outer Domain of *Pseudomonas aeruginosa* Opr86 protein

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ABSTRACT

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen that can form a biofilm that provides crucial drug resistance components. The bacteria have many immunogenic outer membrane proteins (OMP) including, Opr86. The Opr86 is essential for the viability of bacterium, as it has a significant role in OMP assembly and its depletion leads to alteration of cellular morphology. Therefore, Opr86 may serve as a vaccine candidate. Moreover, Opr86 may be used for raising hyperimmune serum, which may be a potential therapeutic candidate in *P. aeruginosa* infection. In the current study, gene fragment encoded exposed outer domain toward extracellular side of Opr86 (E-Opr86) was amplified by polymerase chain reaction (PCR), cloned into pJET1.2 vector, and sequenced. The gene was inserted into pET302/NT-His vector and was expressed in Rosetta-gami 2(DE3) pLysS cells. The recombinant E-Opr86 protein expression was confirmed by the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and subjected to purification using His-tag affinity chromatography. The purified protein (~18kDa) was confirmed by SDS–PAGE and by the Western blotting. This study successfully cloned and characterized Opr86 protein of *P. aeruginosa*.

HIGHLIGHTS

- Gene encoding exposed outer domain of *Pseudomonas aeruginosa* Opr86 (E-Opr86) protein was cloned and sequenced.
- Recombinant E-Opr86 protein of approximetlt 18kDa size was expressed and purifesd using bacterial expression system.

Keywords*:* *Pseudomonas aeruginosa*, biofilm, E-Opr86 protein

The *Pseudomonas aeruginosa* is a Gram-negative rod-shaped bacteria (Furchtgott *et al*., 2011) belonging to *Pseudomonas* genus, *Pseudomonadaceae* family (Wu and Li, 2014). It is an opportunistic organism known for causing nosocomial infections like pneumonia, bacteremia and cystic fibrosis in immune-compromised individuals (Zhang *et al*., 2018). The therapy of this organism is tough due to antibiotic resistance (Pang *et al*., 2019) and low evident to the immune system (Alhede *et al*., 2014) provided by biofilm state of organism exists in the body. The biofilm is an organized community of cells embedded in an extracellular polysaccharide matrix and attached to a surface (Tashiro *et al*., 2008).

As *P. aeruginosa* is a Gram-negative bacterium, it has a cytoplasmic membrane and an outer membrane. The outer membrane has numerous outer membrane proteins (OMPs) like OprD, OprE, OprF, OprH, Opr86 (Hemamalini and Khare 2014). In *P. aeruginosa*, many OMPs have been investigated as possible targets for developing vaccine candidates (Solanki *et al*., 2019). Opr86 is essential for the viability of *P. aeruginosa*, as it has a significant role in OMP assembly and its depletion leads to alteration of cellular morphology of bacterium. In addition, the antibody against Opr86 inhibits biofilm formation of *P.*

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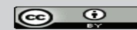

aeruginosa (Tashiro *et al*., 2008) indicated Opr86 would be a good target for the design of new antibiotics vaccine.

The Opr86 has a β-barrel structure with 14 transmembrane domains. The region between 630 and 752 amino acid residues of Opr86 formed exposed outer domain (E-Opr86) toward the extracellular side of *P. aeruginosa* (Tashiro *et al*., 2008). Therefore in the current study, E-Opr86 domain protein was expressed using a prokaryotic expression system.

MATERIALS AND METHODS

Bacterial culture revival and genomic DNA isolation

The freeze-dried bacterial culture of *P. aeruginosa*, microbial type culture collection and the gene bank (MTCC) number 3542 were procured from the Institute of Microbial Technology (IMTECH), Chandigarh. Freeze-dried bacterial culture was revived by overnight inoculation in Luria-Bertani (LB) broth at 37°C. Revived bacterial were sub-cultured overnight in 5 ml LB broth. The genomic DNA from *P. aeruginosa* (strain MTCC-3542) culture was isolated using the protocol described earlier (Sambrook and Russell, 2001). The purity and concentration of isolated DNA were checked by a spectrophotometer (NanoDrop, ThermoFisher, CA, USA) machine.

Polymerase chain reaction (PCR) amplification of E-Opr86

The partial Opr86 (E-Opr86) coding gene was amplified using a forward primer having *Eco*RI restriction enzyme (RE) site (OPR_F: 5'- ACGTGAATTCATTCCACACC GAGCTGGGCTA-3') and reverse primer having *Xho*I RE site (OPR_R: 5'- CGATCTCGAGCTTGATCCAG GTCAGGCCGA-3'). The reaction mixture (50 µl) for E-Opr86 amplification was prepared to have 10 µl of 5X Phusion HF buffer, 1 µl (10 mM) dNTPs, 1 µl (10 pmole) of each primers, 1 µl (1.84 ng) of genomic DNA, 1 µl of 2U Phusion Hot start II DNA Polymerase (MBI-Thermo Scientific, USA) and 35 µl nuclease-free water. PCR cycling condition followed initial denaturation at 94°C for 5 min followed by 30 cycles each of denaturation (94°C for 1 min), annealing (57°C for 20 s) and extension (68°C for 1 min) and a final extension at 68°C for 10 min. The PCR product was confirmed by running on 1.0 % agarose gel prepared in 1.0X TAE buffer containing 1.0 µg/ml ethidium bromide. The sample was run at 100 volts for 45 min and visualized on ChemiDoc XRS gel documentation system (BioRad, Hercules, USA). A 100 bp plus ladder marker (Fermentas) was used as a standard for determining the tentative base pair (bp) size of the PCR product.

Cloning and sequencing of E-Opr86 gene

The PCR product of E-Opr86 was purified by a PCR cleanup kit (Qiagen). The purified PCR product was ligated into pJET1.2/blunt cloning vector (Fermentas, MA, USA) using T4 Ligase. The ligated product was transformed into DH5α *E. coli* cells. Transformed cells were spread on an LB-agar plate with ampicillin (100 µg/ml). The LB-agar plate was incubated overnight at 37°C. After growing of bacterial colonies, few colonies were selected and inoculated in LB broth (with ampicillin @ 100 µg/ml) and kept overnight on a shaker incubator at 37°C. Plasmid from overnight incubated bacterial culture was isolated (Sambrook and Russell, 2001). The recombinant cloning vectors (E-Opr86- pJET1.2/blunt) were characterized by *Eco*RI and *Xho*I restriction enzyme double digestion. Subsequently, the E-Opr86 gene was cloned, confirmed by sequencing, and submitted to GenBank, NCBI database. Further, gene sequence obtained in current study and other corresponding sequences of various *P. aeruginosa* isolates obtained fron GenBank, NCBI were used for estimation of percentage similarity/divergence in E-Opr86 gene of vaious isolates and for construction of phylogenetic tree using the Lasergene (version 11.2.1) software.

Sub-cloning of E-Opr86 gene in a bacterial expression vector

The E-Opr86 gene was sub-cloned into pET302/NT-His (Thermo Fisher Scientific) bacterial expression vector. For the sub-cloning of the E-Opr86 gene, the insert from recombinant E-Opr86- pJET1.2/blunt plasmid was released using enzymes *Eco*RI and *Xho*I restriction enzyme. At the same time, expression vector pET302/NT-His was also digested with the same set of enzymes. A Double digested insert and expression vector was ligated using T4 Ligase, and the ligated product was cloned into *DH5α E. coli* cells. Confirmation of the presence and orientation of the insert in the recombinant expression

vector (E-Opr86- pET302/NT-His) was done by colony PCR with a forward T7 promoter primer and a reversed gene-specific primer. Further, confirmation was carried out by *Eco*RI and *Xho*I double digestion of recombinant expression vector.

Expression of recombinant E-Opr86 protein

The recombinant E-Opr86- pET302/NT-His vector was transformed into *E. coli* Rosetta-gami 2(DE3) pLysS cells (Sigma-Aldrich, Novagen). One colony of Rosetta-gami 2(DE3) pLysS having recombinant expression vector was grown in shaker flasks at 37ºC, in LB broth medium containing 50 µg/ml ampicillin 34 µg/ml chloramphenicol until OD_{600} reached 0.6. Then 0.1 mM of isopropyl B-D-thiogalactopyranoside (IPTG) was added to the medium to induce E-Opr86 expression. At 0, 1, 2, 3, 4, 6 hrs and overnight after induction, 1 ml cells were collected and centrifuged at 8,000 g for 10 min, and cell pellets were harvested. Each cell pellets were lysed by resuspension of pellet in 30 µl sample buffer (Laemmli, Sigma, Germany) followed by boiling for 5 minutes and ice cooling. Cell lysates were centrifuged at 12,000 g for 10 min for the removal of insoluble cell debris. Supernatant fractions obtained were run on 10 % SDS-PAGE gel along with prestained protein molecular weight marker (Fermentas) and stained with Coomassie Brilliant Blue dye.

Purification of recombinant protein

Purification of recombinant E-Opr86 protein was carried out by Ni-NTA affinity chromatography. The IPTG induced 50 ml bacterial culture after 6 hrs of induction was centrifuged at 8,000 g for 10 min, the supernatant was decanted, and bacterial pellet was further used for protein purification. The cell pellet was re-suspended in Ni-NTA lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, and pH 8.0) and subjected to sonication at ice condition on a 20 s burst cycle for 2 min, followed by centrifugation to clarify the cell lysate. The supernatant was collected and mixed gently to Ni-NTA slurry for one hr at 4°C to bind His-Tag containing recombinant E-Opr86 protein. The mixture was poured to a chromatography column and allowed to pack. The unbounded protein was allowed to come out along with lysis buffer. The packed Ni-NTA in the column was washed by 10 ml of wash buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, and pH 6.3). The recombinant protein was eluted using 2 ml of elution buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, and pH 4.5). The eluted solution was kept in a dialysis bag and dialyzed in Phosphate-buffered saline (PBS) to remove urea and other salts from the protein solution. Further, SDS-PAGE analysis and western blotting were done for reconfirmation of purified protein.

RESULTS

Genomic DNA isolation

Genomic DNA from *P. aeruginosa* culture was isolated. Subsequently, purity and concentration of isolated genomic DNA were estimated NanoDrop. The ratio of optical density (OD) values at 260 nanometer (nm) and 260 ($OD_{260/280}$) of genomic DNA was 1.84. The concentration of genomic DNA was 205 ng/µl.

PCR amplification of E-Opr86 gene

The E-Opr86 coding gene was amplified by PCR using forward and reverse primers with *EcoRI* and *XhoI* RE sites. For PCR reaction, high fidelity Phusion Hot start II DNA Polymerase kit of MBI-Thermo Scientific, USA was used. The amplified product was confirmed by 1.0 % agarose gel electrophoresis. In addition, a specific single band of approximately 460 bp was observed in agarose gel (Fig. 1).

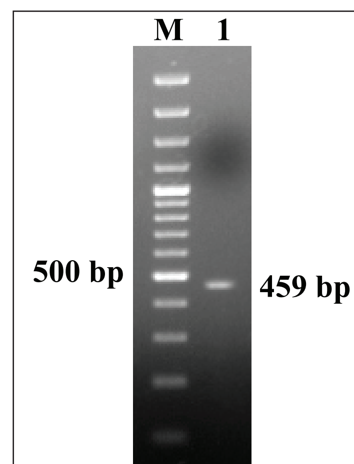

Fig. 1: PCR amplification of partial Opr86 (E-Opr86) gC gene of *P. aeruginosa*. Lane M: 100 bp DNA Ladder plus marker, lane 1: amplified *E-Opr86* gene (459 bp)

Cloning, sequencing and phylogenetic analysis of E-Opr86 gene

The purified PCR product of E-Opr86 was cloned into pJET1.2/blunt cloning vector. Recombinant cloning vectors (E-Opr86- pJET1.2/blunt) were characterized by *Eco*RI and *Xho*I double digestion. Double digestion produced two fragments of approximately 2974bp bp, and 459 bp (Fig. 2) confirmed the presence of E-Opr86 in the recombinant cloning vector. Subsequently, cloned E-Opr86 gene in recombinant cloning vector was sequenced, and the sequence was submitted to GenBank, NCBI (accession number: KF646784). Divergence analysis of E-Opr86 gene of various *P. aeruginosa* isolates indicated maximum 5.5% divergence among various isolates (Table 1).

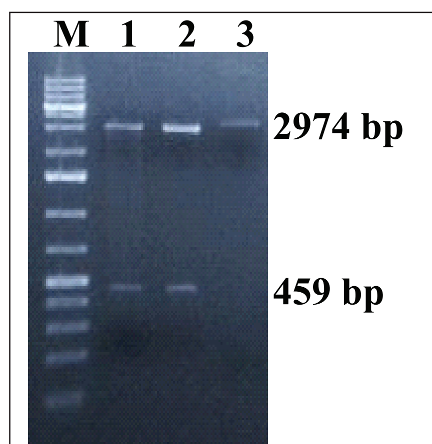

Fig. 2: Confirmation of E-Opr86 gene inserts in cloning vector by restriction enzyme digestion of recombinant plasmid. Lane M: O'GeneRuler 1 kb DNA Ladder, Lane 1 and 2: *Eco*RI/*Xho*I double digested plasmid, Lane 3: Uncut Plasmid

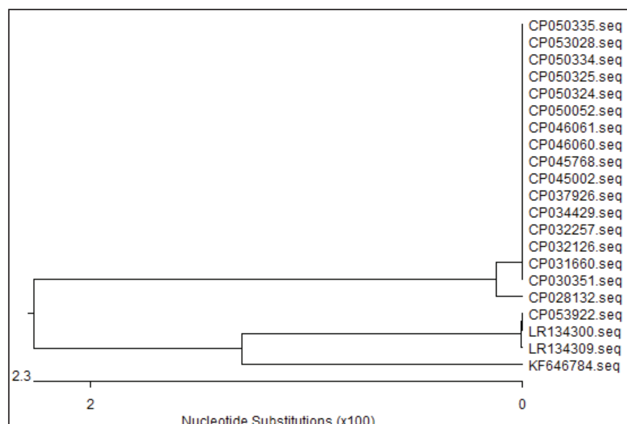

Fig. 3: Phylogentic tree based on E-Opr86 gene of various *P. auriginosa* isolates

Moreover, phylogentic analysis indicated gene used in this study was evolutionary closer to corresponding gene of isolates with accession number LR134300 and LR134309 (Fig. 3).

Sub-cloning of E-Opr86 in a bacterial expression vector

The E-Opr86 gene was sub-cloned into pET302/NT-His bacterial expression vector using *DH5α E. coli* cells. Confirmation of the presence and orientation of the insert in the recombinant expression vector (E-Opr86- pET302/NT-His) was done by colony PCR with forwarding T7 promoter primer and reversed gene-specific primer. Colony PCR gave an amplified product of approximately 500 bp (Fig. 4A). Further, confirmation was carried out by *Eco*RI and *Xho*I double digestion of recombinant expression vector. Double digestion produced two fragments of approximately 5700 bp, and 459 bp (Fig. 4B) confirmed the presence and correct orientation of insert in the recombinant expression vector.

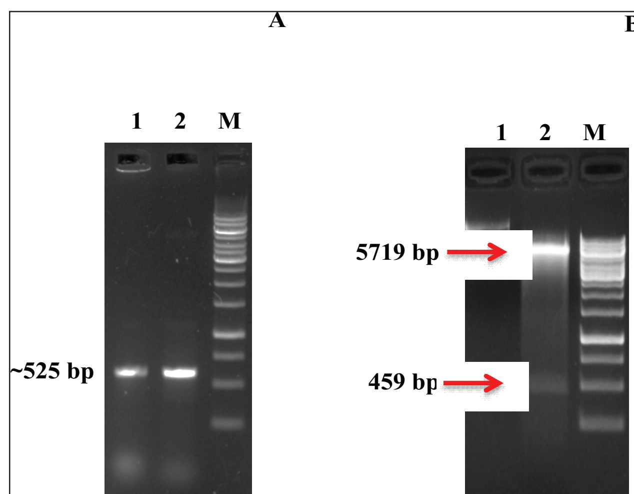

Fig. 4: Confirmation of E-Opr86 gene inserts in expression vector by **(A)** colony PCR of recombinant clone. Lane M: O'GeneRuler 1 kb DNA Ladder marker, Lane 1, 2: positive ampicillin-resistant E-Opr86- pET302/NT-His clone **(B)** restriction enzyme digestion of recombinant plasmid. Lane M: O'GeneRuler 1 kb DNA Ladder marker, Lane 1: Uncut Plasmid, Lane 2: *Eco*RI/*Xho*I double digested plasmid

Expression of recombinant E-Opr86 protein

The recombinant expression vector was isolated and transformed into *E. coli* Rosetta-gami 2(DE3) pLysS cells.

Table 1: Percentage similarity/divergence in E-Opr86 gene of vaious *P. auriginosa* isolates

Percent Identity (upper triangle); Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
1		99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	93.6	96.6	93.6	93.6	1	CP028132.seq
2	0.2		99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	93.2	96.1	93.2	93.2	2	CP030351.seq
3	0.2	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	93.4	96.3	93.4	93.4	3	CP031660.seq
4	0.2	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	93.4	96.3	93.4	93.4	4	CP032126.seq
5	0.2	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	93.4	96.3	93.4	93.4	5	CP032257.seq
6	0.2	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	93.4	96.3	93.4	93.4	6	CP034429.seq
7	0.2	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	93.4	96.3	93.4	93.4	7	CP037926.seq
8	0.2	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	93.4	96.3	93.4	93.4	8	CP045002.seq
9	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	93.4	96.3	93.4	93.4	9	CP045768.seq
10	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	93.4	96.3	93.4	93.4	10	CP046060.seq
11	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	93.4	96.3	93.4	93.4	11	CP046061.seq
12	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	93.4	96.3	93.4	93.4	12	CP050052.seq
13	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	93.4	96.3	93.4	93.4	13	CP050324.seq
14	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	93.4	96.3	93.4	93.4	14	CP050325.seq
15	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	93.4	96.3	93.4	93.4	15	CP050334.seq
16	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	93.4	96.3	93.4	93.4	16	CP050335.seq
17	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		93.4	96.3	93.4	93.4	17	CP053028.seq
18	5.3	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5		97.2	100.0	100.0	18	CP053922.seq
19	3.5	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	2.6		96.1	96.1	19	KF646784.seq
20	5.3	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	0.0	2.6		100.0	20	LR134300.seq
21	5.3	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	0.0	2.6	0.0		21	LR134309.seq
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		

One colony of transformed cells was selected, sub-cultured in LB broth with ampicillin (100mg/ml), and allowed to reach the OD of approximately 0.6 under shaking.

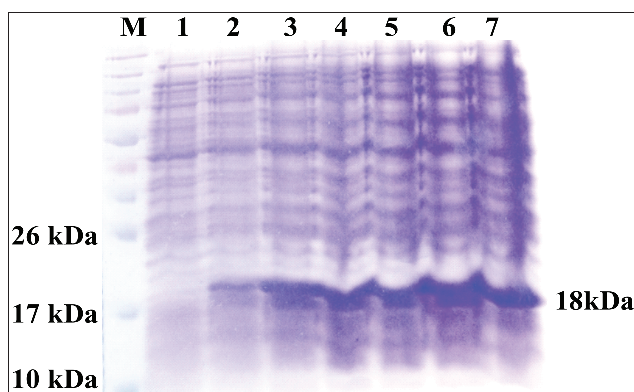

Fig. 5: SDS-PAGE analysis of expression level of recombinant protein. Lane 1: Protein molecular weight marker; Lane 2: Uninduced cell lysates; Lane 3, 4, 5, 6, and 7: induced cell lysates at various incubation time (1, 2, 3, 4, 6 h and overnight), respectively showing expression of E-Opr86 protein

Induction was done with 0.1 mM IPTG, and samples were collected at hourly intervals up to 6 hrs and after overnight shaking. Cells were pelleted out. SDS-PAGE detected the expressed protein, and an approximately 18 kDa size band of expressed protein was detected after staining with coomassie brilliant blue. Expression kinetics studies indicated that the highest expression level was available at sixth hr post-induction (Fig. 5).

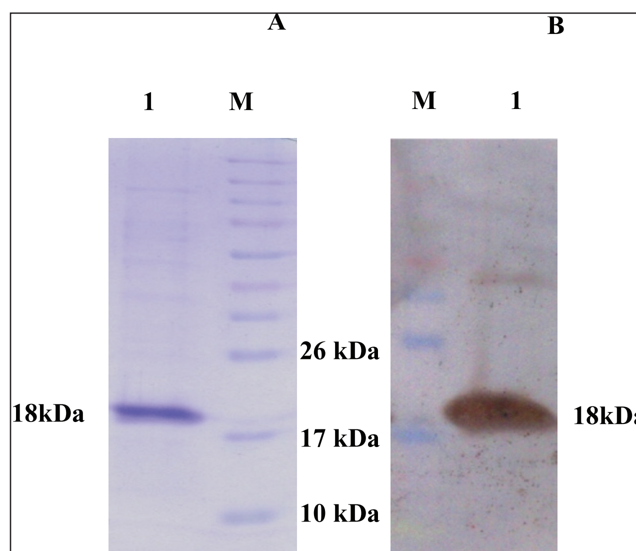

Fig. 6: Confirmation of purified recombinant protein by **(A)** SDS-PAGE analysis (Lane 1: molecular weight marker; Lane 2: purified protein) **(B)** Western blotting (Lane 1: purified protein; Lane 2: prestained protein molecular weight marker)

Purification of recombinant protein

The purification of expressed recombinant protein was carried out by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA affinity resin. The induced culture was collected and platted after 6 hrs, where the expression level was good. Then, the recombinant protein was purified using Ni-NTA affinity chromatography. SDS-PAGE and western blotting analysis of purified protein had a specific single band of approximately 18 kDa (Fig. 6).

DISCUSSION

Despite significant developments in antimicrobial therapy, there are persistent problems for effective control and treatment of *P. aeruginosa* infections. As many studies showed intravenous injections of *P. aeruginosa* specific hyperimmune globulins having protective therapeutic intervention for *P. aeruginosa* infection (Sharma *et al*., 2011; van Wye *et al*., 1990), E-Opr86 protein can be used to raise of hyperimmune serum for passive immunization against *P. aeruginosa* infections. Moreover, Opr86 is an important protein of bacterium biofilm, antibody against Opr86 inhibits biofilm formation and E-Opr86 forms exposed outer domain of Opr86 (Tashiro *et al*., 2008). Therefore, recombinant E-Opr86 can be used in the future as a potential vaccine to prevent *P. aeruginosa* infection.

For expression study, the expression vector is the most critical factor to determine the expression level. pET series vectors are routinely used to express the heterologous protein in the prokaryotic system under the control of the T7 promoter, under which much integral protein has been expressed (Korepanova *et al*., 2009). In this study, pET302/NT-His expression vector was chosen to express recombinant protein E-Opr86 under control of T7 promoter and transformed into *E. coli* BL21 (DE3) pLysS Rosseta-gami 2 cells for a high level of expression. The pET302/NT-His Vector is used for high-level expression of recombinant protein with N- terminal 6xHis-tagged protein (Singh *et al*., 2015). The *E. coli* BL21 (DE3) pLysS Rosseta-gami 2 ease codon bias and enhance disulfide bond formation in the cytoplasm when heterologous proteins are expressed in *E. coli* (Chang *et al*., 2010).

In the current study maximum level of recombinant E-Opr86 expression in *E. coli* BL21 (DE3) pLysS Rosseta-gami 2 cells were detected at 6 hrs post IPTG induction. A similar result has been reported for the maximum level of expression of OprL protein of *P. aeruginosa* at ~6 hrs post-induction (Young *et al*., 2012).

In this study, Ni-NTA affinity resin was used for the purification of the recombinant protein. The recombinant fused with Histidine tag (His-Tag) under denaturing condition with immobilized metal ion affinity chromatography (IMAC) using Ni-NTA affinity resin (Chang *et al*., 2010). The His-Tag is very useful as a fusion partner for protein purification as His-Tag has low immunogenicity and small size (0.84 kDa). His-Tag fusion proteins can be affinity purified under denaturing conditions and native conditions. Ni-NTA exhibits a high affinity for histidine residues and allows elution of bound recombinant proteins by different ways such as an imidazole gradient (20 to 250 mM), changes in pH, or metal chelation(Young *et al*., 2012). In the present study, the protein was purified under denaturing conditions and eluted at a pH of 4.5. On SDS-PAGE and western blotting, recombinant protein resulted in an intense color reaction at approximately 18 kDa, indicating the correct size of the expressed recombinant E-Opr86.

As pET302/NT-His Vector was used for expression purpose, the recombinant E-Opr86 protein obtained in this study have approximetly1.0 kDa of vector encoded His-Tag and 17 kDa of wild Opr86 protein.

This study confirmed that the gene encoding E-Opr86 protein is relatively conserved in various isolate of *P. aeruginosa.* High-level expression of recombinant E-Opr86 protein with minimum size vector encoded tag (approximetly 1 kDa) can be possible using pET302/NT-His expression vector and *E. coli* BL21 (DE3) pLysS Rosseta-gami 2 cells. As Opr86 is essential for the viability of *P. aeruginosa* and antibody against it inhibits biofilm formation (Tashiro *et al*., 2008), the current study of E-Opr86 protein expression can pave the way to development subunit vaccine against *P. aeruginosa*.

CONCLUSION

P. aeruginosa E-Opr86 protein was successfully expressed in *E. coli* Rosetta-gami 2(DE3) pLysS cells using pET302/NT-His expression vector. Purified recombinant E-Opr86 protein of approximately molecular weight 18 kDa was

confirmed by SDS-PAGE analysis and western blotting. In the future, recombinant E-Opr86 protein can be used as a vaccine candidate against *P. aeruginosa* infection.

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