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Research Article

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Cloning and Expression of Outer Membrane Protein Omp38 Derived from *Aeromonas hydrophila* in *Escherichia coli*

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ABSTRACT

Background: *Aromonas hydrophila* is an aquatic bacterium involved in various diseases in fish, resulting in serious economic losses every year. In previous studies, the outer membrane protein Omp38 was demonstrated to have high immunoprotection capacity, suggesting the use of this protein as a vaccine candidate to protect fish against *A. hydrophila* in fish aquacultures.

Methods: The gene coding for Omp38 was amplified from *A. hydrophila* genome and inserted into *Bam*HI/XhoI sites of plasmid pET-28a(+). The recombinant plasmid was then introduced into *E. coli* BL21(DE3). Transformed *E. coli* cells were treated with IPTG to induce the expression of Omp38 fused with 6xHis tag. The presence of 6xHis-Omp38 was detected by western blot analysis using an anti-Histidine antibody.

Results: We successfully constructed an *E. coli* strain that can highly express Omp38 protein (comprising ~40% of total cell proteins). The Omp38 protein was expressed as the form of inclusion bodies so that it could be easily obtained from cell lysate with high purity (≥95%) by centrifugation.

Conclusion: This study was the first step in an attempt to produced Omp38-based vaccine to prevent the outbreak of diseases caused by *A. hydrophila* in fish farming.

Key-words: *Aeromonas hydrophila, Escherichia coli*, Omp38, Outer membrane protein, Recombinant protein

INTRODUCTION

A.hydrophila is a facultative anaerobic, Gram-negative, and rod-shape bacterium that can live in a wide range of temperatures, pHs, conductivities and turbidities, but favorably grows in warm climates [1,2]. *A. hydrophila* is considered as an opportunistic pathogen responsible for numerous diseases in human such as gastroenteritis, skin infections, peritonitis, bacteremia, hemolytic uremic syndrome, and necrotizing fasciitis, etc [3, 4]. Especially, this bacterium is also a well-established fish pathogen that causes hemorrhagic septicemia and red sore disease in carp, catfish, salmone, dogfish, and goby, that results in huge global economic losses every year [1,5]. In China, outbreaks of motile *Aeromonas* septicemia were responsible for losses of 2,200 tons of dead fish per year [6]. In the summer of 2009, an outbreak of the same disease resulted in an estimated loss of more than 12 million dollars in catfish farming in the southeastern United States [7].

To prevent the infection of *A. hydrophila,* commercial antibiotics are traditionally and popularly used in fish farming [8]. However, the overuse of antibiotics has raised concerns regarding the development of antibiotic resistance that seriously affects public health [9,10]. Therefore, in previous studies, some vaccines, including heat-killed bacteria [11], S-layer recombinant protein [12],

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lipopolysaccharide [13], attenuated bacteria [14], and biofilm oral vaccine [15] were developed to control the spread of *A. hydrophila* and also to reduce the use of antibiotics. Recently, the bacterial outer membrane proteins (Omps) have been gained attention as potential vaccines due to their high immunoprotective capacities [16,17]. Importantly, among various Omps in *A. hydrophila,* the vaccination with Omp38 can effectively protect fish against this bacterium through the stimulation of both specific and non-specific immune responses as demonstrated by Wang *et al.* [18].

For the reasons mentioned above, in this study, we focused on establishing an *E. coli* strain that can express *A. hydrophila* Omp38 for the future application in vaccine production. The recombinant Omp38 can also be used to produce a specific antibody, which has not yet been available on market.

MATERIALS AND METHODS

Bacterial strains, Medium and Other Reagents- The *E. coli* strains DH5α (F^- Φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (r_K^-, m_K^+) phoA supE44 λ^- thi-1 gyrA96 relA1) and BL21(DE3) (F^- ompT hsdS_B (r_B^-, m_B^-) gal dcm (DE3) (Thermo Fisher Scientific, USA) were used for the cloning and expression of recombinant Omp38. The *A. hydrophila* strain used in the current study for omp38 gene amplification was isolated from diseased fish in aquaculture of the Mekong Delta, Vietnam. All bacterial strains were cultured at 37°C in LB broth (1% trypton, 0.5% yeast extract, and 0.5% NaCl) with reciprocal shaking (250 rpm). All enzymes for DNA cloning were purchased from Invitrogen (USA). The DNA ladder HyperLadder™ 1 kb and the Peptide Molecular Weight Marker were purchased from Bioline (USA) and GE Healthcare (USA), respectively. The other chemicals and reagents were obtained from Merck (USA).

Plasmid construction- The coding sequence of Omp38 (1008 bp) was amplified from *A. hydrophila* genome using primer pair omp38-F (5'-GGA AGA TCT GGATCC GTC ATC TAT CAG GCC GAT GAC GGC TCC AAC GTC G-3') and omp38-R (5'-CCG CTC GAG CGG ATG GTG ATG GTG ATG GTG GAA CTC GTA GCG CAG GCC GAG GTG GTA-3'). The plasmid pET-28a(+) (Novagen, USA) was isolated from DH5α/pET-28a(+) using EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio Basic Inc., Canada). The PCR product was cloned into BamHI/XhoI sites of plasmid pET-28a(+) and in-frame with polyhistidine tag (6xHis) sequence to construct pET-omp38 [19]. The ligation mixture was then transformed into DH5α cells and the transformed clones were screened on 100 µg/ml ampicillin containing agar plates [20]. The recombinant plasmids containing omp38 gene were selected using PCR and further verified by Sanger sequencing.

Expression of recombinant Omp38- The plasmid pET-omp38 was transformed into BL21(DE3) cells to establish the strain BL21(DE3)/pET-omp38 that can express the recombinant protein fused with 6xHis tag (6xHis-Omp38). A colony of transformed cells was cultured in 100 ml LB medium containing 100 µg/ml ampicillin at 37˚C with shaking (250 rpm) in an Erlenmeyer flask (300 ml). When the culture reached OD ~ 0.8, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of 6xHis-Omp38. Cells were further cultured for 4 hours and then 1 ml of the culture was centrifuged at 6,000 g for 10 minutes to harvest cells. Cells were washing and re-suspended in 1 ml of 0.1 mM phosphate buffer pH 7. After that, cells were disrupted by sonication and 500 µl of cell lysate was centrifuged at 10,000 g for 5 minutes to separate the supernatant and pellet fractions. The pellet fraction was then suspended into 500 µl of 0.1 mM phosphate buffer pH 7. Fifty microlitter of total cell lysate, supernatant and pellet fractions were mixed with 10 µl sample buffer 6X (0.35 M Tris-HCl, 10% SDS, 36% glycerol, 0.6 M dithiothreitol, 0.012% bromophenol blue, pH 6.8) and heated at 100˚C for 10 minutes for protein denaturation. After that, 10 µl of each sample was applied to each lane of a polyacrylamide gel (12.5%) for SDS-PAGE analysis [21]. An His-probe antibody (H-3): sc-8036 (Santa Cruz Biotechnology Inc., USA) was used to detect 6xHis-Omp38. We used Anti-mouse IgG (whole molecule)-Peroxidase antibody (A-9044, Sigma-Aldrich, USA) as the second antibody, and the signal was detected by ECL™ Prime Western Blotting Detection Reagent (GE Healthcare Amersham™, UK). The purity of Omp38 protein in pellet fraction was determined using ImageJ software [22].

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RESULTS

Amplification of omp38 gene and construction of plasmid pET-omp38- For the high expression of Omp38 protein, pET-28a(+) was chosen as a vector to carry the target gene into *E. coli* cells. The omp38 gene was inserted into BamHI/XhoI site of pET-28a(+) so that this gene can be effectively expressed under the control of the very strong *T7* promoter.

With the purpose mentioned above, the omp38 gene was amplified from *A. hydrophila* by PCR, with a BamHI and an XhoI at the 5'- and 3'-end, respectively. Electrophoresis of PCR products showed that the length of amplicons was approximately 1,000 bp that was in accordance with the theoretical length of omp38 gene (Fig. 1A). The PCR products were then digested with restriction enzymes BamHI and XhoI, yielding a DNA fragment with two sticky-ends for DNA cloning.

The plasmid pET-28a(+) was also digested with the same restriction enzymes, that generated a single DNA band between 5,000 and 6,000 bp in size (Fig. 1B). After digested, PCR products and plasmids were ligated using T4 DNA ligase and the ligation mixture was then transformed into DH5α cells to select recombinant plasmids.

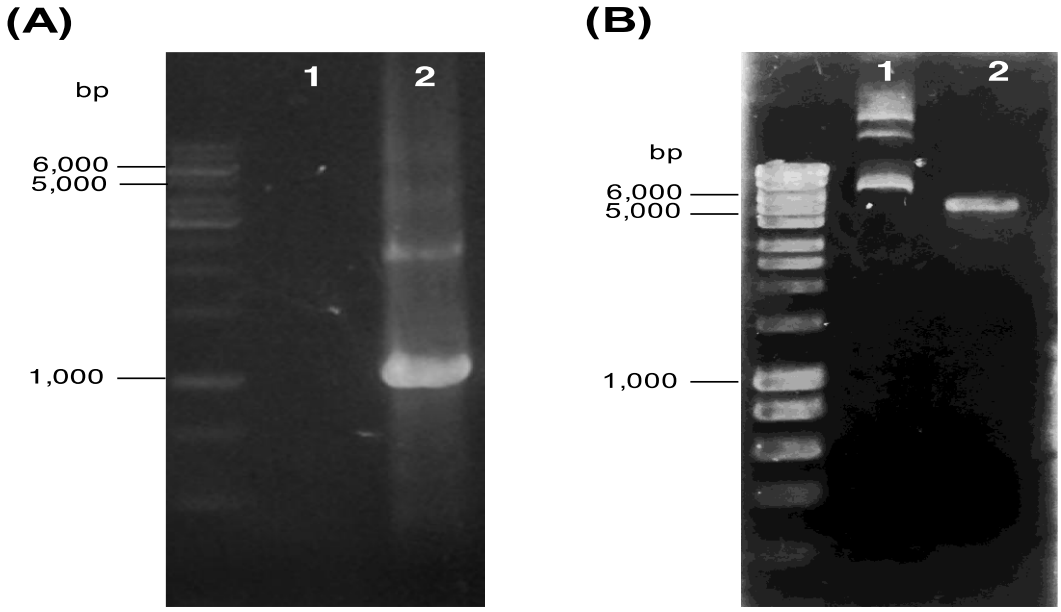

Fig. 1: Gene and plasmid preparation

(A) ORF sequence of omp38 gene was amplified from *A. hydrophila* genome by PCR. Samples were as follows: 1- No template control; 2- PCR products

(B) Plasmid pET-28a(+)

Samples were as follows: **1-** Purified plasmid**; 2-** Plasmid digested with BamHI/ XhoI

The transformed cells were then spread on an LB agar plate containing 100 µg/ml ampicillin. Ten colonies grown on this plate were randomly chosen and subjected to colony-PCR with primers omp38-F/R (Fig. 2A). The electrophoresis result showed that DNA bands representing omp38 gene were present in 6/10 samples, suggesting that these colonies were successfully transformed with pET-omp38.

We next isolated plasmids from the above six omp38-positive clones and used them as templates for PCR analysis with omp38-F primer and the T7-terminator primer (5'-GCT AGT TAT TGC TCA GCG G-3') to verify the insertion of omp38 gene into plasmid pET-28a(+). We found that all reactions from these six plasmids produced a DNA band around 1,100 bp (Fig. 2B), that was equal to the size of omp38 ORF plus the length from XhoI position to the T7-terminator primer binding site. This result indicated that all these plasmids contained omp38 gene with proper direction.

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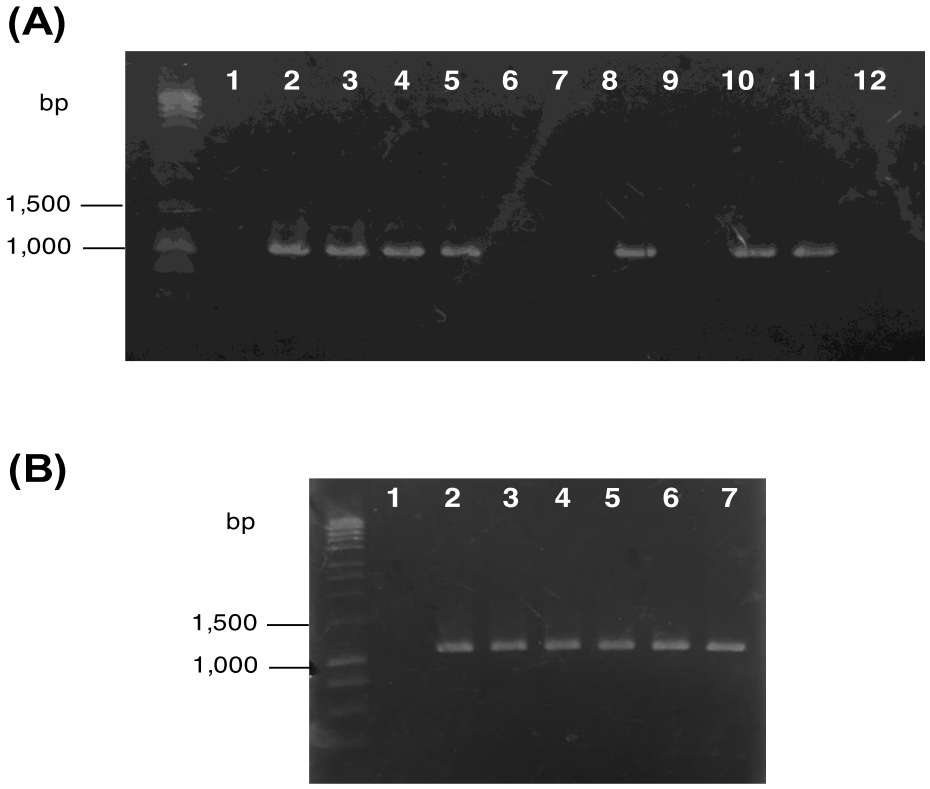

Fig. 2: Selection of recombinant plasmids

(A) Obtained colonies were analyzed by PCR with primers omp38-F/R to select the omp38-positive colonies. Samples were as follows: **1-** No template control; **2-** PCR product from *A. hydrophila* genome (positive control), **3 to 12-** PCR products from ten randomly chosen colonies

(B) Candidate plasmids were verified by PCR with omp38-F and T7 terminator primers. Samples were as follows: **1-** No template control; **2 to 7-** PCR products from six plasmids

DNA sequencing and Database search- The cloned omp38 gene was sequenced using the Sanger method and the result (Fig. 3) was submitted to a BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) to check the sequence accuracy. The interpreting BLAST output showed that there are some variety of Omp38 proteins among *A. hydrophila* strains and the omp38 sequence obtained in this study was the same as those found in nine *A. hydrophila* strains i.e. ZYAH72, GYK1, D4, JBN2301, NJ-35, J-1, pc104A, AL09-71, and ML09-119 with 100% identity with 100% coverage. These data demonstrated that we successfully and correctly inserted omp38 gene into pET-28a(+).

Expression of Omp38 protein- The plasmid pET-omp38 was transformed into BL21 (DE3) cells to produce Omp38. The BL21 (DE3) strain carries the gene coding for T7 RNA polymerase under the control of lacUV5 promoter. The adding of IPTG induces the expression of T7 RNA polymerase and subsequently stimulates the expression of omp38 gene downstream of the T7 promoter in pET-omp38 plasmid.

Therefore, a colony of transformed cells was cultured in LB medium containing 100 µg/ml ampicillin and 1 mM IPTG was added to induce the expression of recombinant protein. Cells were disrupted and then soluble proteins and aggregates were separated and analyzed by SDS-PAGE method. Lysates of BL21(DE3) cells and BL21(DE3)/pET-omp38 cells untreated with IPTG were used as the negative controls. A clear protein band around 38 kDa appeared in induced BL21(DE3)/pET-omp38 sample and was absent in all negative controls (Fig. 4A). Since omp38 protein is fused with 6xHis tag, this band could be detected by western blot analysis with anti-Histidine antibody (Fig. 4B). These results indicated that the addition of IPTG induced the expression of 6xHis-Omp38 protein and that we successfully established an *E. coli* strain that can produce Omp38 protein.

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1   GTC ATC TAT CAG GCC GAT GAC GGC TCC AAC GTC GAT CTC TAC GGT CGC CTC GGC TTC AAC
61  ATC TCG GAC AAG AAG AGC GGC AAC GAT CAG GGC GAC TTC GAC GGT CGC ATC GGC TTC ACC
121 GCC CGC CAG ACC GTC AAC GAG CAG GTA GCC GTG ATC GGC ATG GCT CAG TAC CAG GTC AAC
181 GCC GCC GAA TAC GCC AAC AAC GTG CAG AAG AAC AGC AGC GAT CTC ACC GCC CGT TAC GTC
241 TGG GCC GGG CTC GAT GGC GGC CAG TGG GGC AAG CTG ACC GGT GGC CGG GTC TCC TCC GGG
301 CTC ATC ATG TTC ACC GAC ATC GGC GAC GTG TTT GCC GCC TCC GAT GTC TCC ATG GCG CGC
361 CAG GCC AGC AAA GTG GAC TCC ACC GCG GTA CAG GTG TTC CGT CAG GAC GGC ACC CTG CAA
421 TAC CAG AAC ACC CTC GGC AAT CTG GAC TTG TCG CTG GCC TAC ATC CTC GGC AAC GGC ACC
481 TCG GAT CTC AAC TAC GGC TAC AAC GGC GCC CTG CGC TAC ACC TTC GAT CTC GGG GCG GCG
541 GGC AAG CTG GCG CCG GTC ATC GCC TAC CAG CAG AGC AAG GCG GCC CAG ACC GGT GCC AGC
601 AAA GTC GAC CCC AAT GCC GAC GAG TAC CGC TTC AGC GGC ATC GGC ACC CGC TAC ACC CTG
661 GGA CCG CTG ATG CTG GGC GCC CTC TAT GCC AAG GAT CAG GTG AGC TAT CGC AAC GGC CAA
721 GCG GAC AGC GAA GAC AAG GTG TGG GAG CTG ACC GCC GTC TAC GAC ATC CAC CAG AAG TGG
781 GCC GCC CGC GCC GGC TAC CGC CAC CTC GAC AAC GAC GGG GGA GAC GGC ATG AGC CTG CGC
841 GAC ACT ACC CTG GAG CTG CAG TAC AAG CTC ACT CCG CGC TCC TCC CTC TAT AGC GCC TAC
901 GTG TTC CGG GGC GGT GAA GAC GGG CTG GCC GGC AGC ACG GTC ACC AGT TTT GGC GGC AGC
961 AGC AAC AGC GAG GAT TTC TAC CAC CTC GGC CTG CGC TAC GAG TTC TGA
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Fig. 3: DNA sequence of the omp38 gene that was cloned in this study

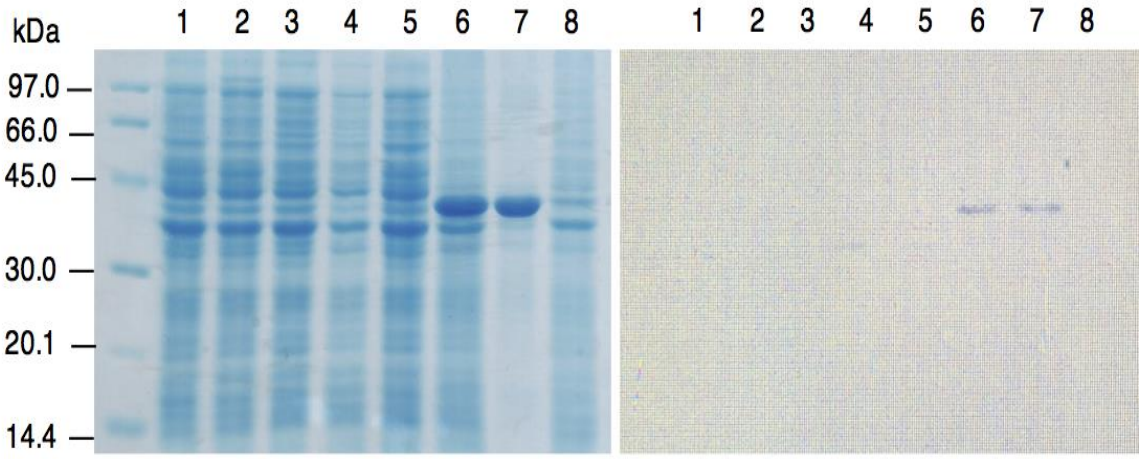

Fig. 4: The expression of 6xHis-Omp38 protein was analyzed by SDS-PAGE **(A)** Western Blot analysis **(B)** Samples were as follows: **1-** Un-induced BL21(DE3); **2-** IPTG-induced BL21(DE3); **3-** Un-induced BL21(DE3)/pET-28a(+); **4-** IPTG-induced BL21(DE3)/pET-28a(+); **5-** Un-induced BL21(DE3)/pET-omp38; **6-** IPTG-induced BL21(DE3)/pET-omp38, total proteins; **7-** IPTG-induced BL21(DE3)/pET-omp38, insoluble fraction, **8-** IPTG-induced BL21(DE3)/pET-omp38, soluble fraction

Additionally, we also found that this protein was mostly expressed in inclusion body form since it was not observed in the soluble fraction of cell lysate (Fig. 4A). The formation of such aggregates is considered to be a benefit for protein purification, as almost bacterial native proteins are soluble. Accordingly, the ImageJ analysis of SDS-PAGE picture (Fig. 4A) showed that the Omp38 protein comprised approximately 40% of total proteins in cell lysate (well 6) and was present in insoluble fraction with high purity (~95%) (well 7).

DISCUSSION

As stated in pET system manual (Novagen, USA), the pET plasmid system is a very powerful tool for recombinant protein expression, which allowed the high expression level of target protein, up to 50% of the total cell protein amount [19]. Therefore, in this study, we chose pET-28a(+) plasmid for the expression of our target protein Omp38 and succeeded in creating the *E. coli* strain BL21(DE3) expressing 6xHis tag-fused Omp38 protein, which represented ~40% of total cell proteins (Fig. 4A). This result was achieved under the current unoptimized

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conditions in a 100 ml-flask scale and might be improved by the further optimization and tighter control of the medium, pH, oxygen concentration, and other culture conditions using a fermenter.

In two previous studies, in order to evaluate the Omp38 protein as a vaccine antigen against *A. hydrophila*, Wang *et al.* [18]; Ni *et al.* [23] also used the same system for Omp38 expression, and after purification using HisTrap™ HP, the protein purities obtained were about 80% and 90%, respectively. We here found that the target protein 6xHis-Omp38 was expressed mostly in inclusion body form and comprised about 95% of the aggregated protein in *E. coli* cells (Fig. 4A, well 7). Therefore, compared to the above studies, our target 6xHis-Omp38 protein can be easily obtained with higher purity using a centrifuge. However, if a very high purity is required (>95%), the recombinant Omp38 can be simply purified using Ni-NTA affinity chromatography [24].

Additionally, Kasik *et al.* [25] found that inclusion bodies carrying the E2 antigen of classical swine fever virus induced both systemic and mucosal responses when being fed to mice without any adjuvant. Therefore, it seems that the obtained Omp38 inclusion bodies can be directly used as an oral vaccine without any further step. On the other hand, according to Ni *et al.* [23] the omp38 gene could be detected in 75% of the tested *A. hydrophila* strains, suggesting that although Omp38 protein showed high immunoprotective activity, the vaccination with Omp38 protein could protect fish against most, but not all, *A. hydrophila* strains. Therefore, an approach based on the combination of Omp38 and some other outer membrane proteins should be considered to widen the range of protected strains.

CONCLUSIONS

In this study, we successfully established an *E. coli* strain that could highly express Omp38 protein (making up ~40% of total proteins) when being induced by IPTG. The Omp38 protein was expressed as inclusion bodies so that it could be easily obtained with high purity (~95%) from cell lysate by centrifugation.

The inclusion body obtained in this study can be directly used as a potential vaccine candidate for fish to control the spread of *A. hydrophila* or to produce a specific antibody by immunization of animals. Since inclusion bodies are relatively stable, the mixing of Omp38 aggregates and food for oral administration might be an effective approach for labor-effective fish vaccination. Additionally, the simple process to obtain high-purity Omp38, as shown in this study, might also enable us to produce a cost-effective vaccine that can replace antibiotics in fish farming.

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Research design: Dang Thi Phuong Thao
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Literature search: Le Thi Kim Phuong, Nguyen Thi My Trinh
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Critical review: Nguyen Thi My Trinh, Dang Thi Phuong Thao
Article editing: Nguyen Thi My Trinh
Final approval: Dang Thi Phuong Thao

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