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The Improvement of Recombinant PirA Antigen Expression in *Escherichia coli* M15

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Abstract: Acute Hepatopancreatic Necrosis Disease (AHPND), previously name Early Mortality Syndrome (EMS), is a disease in shrimps which is caused by *Vibrio* spp. This disease has led to serious shrimp mortality (up to 100%) and caused significant economic losses to shrimp industry. Many studies have shown that AHPND was caused by *Vibrio* spp. carrying *PirA* and *PirB* toxin genes located on the same large plasmid (63-70 kb); they encoded for a binary protein PirAB, which was proved to be the virulence factor of this disease. This research was carried out to evaluate the effect of different environmental conditions during induction on growth of recombinant *Escherichia coli* M15 and the expression of recombinant PirA antigens. The recombinant *E. coli* M15 strain carrying the *pirA* gene derived from our previous research. The gene encoding the pirA antigen was expressed as a fusion protein containing histidine tag at the N-terminal end of the peptide using pQE30 (pQE30-6xHis-PirA) cloned in *E. coli* M15 cells. Isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration, induction time, and cell density before induction, were selected as three important factors to optimize. The expression levels of 6xHis-PirA fusion protein under the different growth conditions were analyzed. SDS-PAGE analyses was carried out for further confirmation of interest-gene expression. The highest level of 6xHis-pirA fusion protein in recombinant *E. coli* 15 was determined after induction by 1.00 mM IPTG, for 4 hours after induction, when cell density (OD_{600nm}) reached a value of 1.0 (at 37°C, 200 rpm). From the present outcomes, we conclude that optimizing the three important factors of IPTG concentration, induction time, and cell density before induction can improve 6xHis-pirA fusion protein yield in the *E. coli* M15 expression system.
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1. Introduction

Acute Hepatopancreatic Necrosis Disease (AHPND), previously named Early Mortality Syndrome (EMS), is a disease in shrimps which is caused by *Vibrio* spp. The disease usually appears in tiger shrimp (*Penaeus monodon*) and Pacific whiteleg shrimp (*Litopenaeus vannamei)* within 20-30 days of stocking pond, with postlarvae. In severely affected ponds, the disease has led to serious shrimp mortality (up to 100%) and caused significant economic losses to shrimp industry (Lightner et al., 2012; De Schryver et al., 2014; Zorriehzahra et al., 2015; Lua et al., 2016; Li et al., 2017). By 2011, the disease was confirmed in Vietnam (Lightner et al., 2012; Tran et al., 2013; Zorriehzahra et al., 2015; Dang et al., 2018) and then the disease has spread to many provinces throughout the country such as Thua Thien Hue (Dao et al., 2014; Khanh et al., 2019); Bac Lieu (Nghia et al., 2015); Soc Trang (Dung et al., 2017); Tra Vinh, Ca Mau (Dang et al., 2018); etc. This is the most severe disease currently affecting shrimp aquaculture in Vietnam (Dang et al., 2018).

Numerous studies have shown that AHPND was caused by *Vibrio* spp. carrying *PirA* and *PirB* toxin genes located on the same large plasmid (63-70 kb); they encoded for a binary protein PirAB, which was proved to be the virulence factor of this disease (Han et al., 2015; Lee et al., 2015; Sirikharin et al., 2015). In previous report, we isolated two strains of *Vibrio parahaemolyticus* K5 and *Vibrio parahaemolyticus* K21 from whiteleg shrimp (*L. vannamei)* cultured in Phong Dien District, Thua Thien Hue Province,

Vietnam. Both the strains carried *PirA* and *PirB* toxin genes, have predicted sizes as 336 bp and 1,317 bp, respectively (Khanh et al., 2019). Application of recombinant DNA technology to produce recombinant PirA and PirB antigens is not only raw materials for research to development antibody preparations that can prevent and treat AHPND but also for diagnostic in shrimps.

Recombinant proteins can be expressed using eukaryotic and prokaryotic expression systems, including yeasts, insect cells, mammalian cells, *Bacillus subtilis*, and *Escherichia coli*. The *E. coli* expression system is the first-choice host for recombinant protein expression, because these bacteria cells can be readily manipulated, are cultured inexpensively and grow rapidly (Ghosh et al., 2004; Francis et al., 2010; Vaz et al., 2011; Rosano et al., 2014; Joseph et al., 2015; Baeshen et al., 2015; Jia et al., 2016; Gomes et al., 2020), with the expressed protein comprising up to 50 percent of the total cellular protein (Francis et al., 2010). On the other hand, it is well suited for the production of antigens for antibody generation (Rancour et al., 2010). Many of studies investigated the optimum expression conditions to improve the yield of recombinant antigents in *E. coli* (Abath et al., 1997; Hu et al., 2004; Vaz et al., 2011; Zhao et al., 2011; Loc et al., 2014; Arévalo-Herrera et al., 2015; Lan et al., 2014, 2016, 2018).

Culture conditions play important roles in recombinant protein expression (Mohajeri et al., 2016; Papaneophytou et al., 2012). Therefore, it is important to balance the induction capacity as well as protein production based on the environmental conditions employed during the expression of target protein (Azaman et al., 2010). In previous research, we successful expressed the *pirA* and *pirB* genes encoding PirAB toxin protein in recombinant *E. coli* M15 in laboratory-scale. The objective of current study is to evaluate the effect of different environmental conditions during induction such as inducer concentration, induction time, and cell density before induction (at the time of adding inducer) on growth of recombinant *E. coli* M15 and the expression of recombinant PirA antigens.

2. Material and Methods

Bacterial strain

The recombinant *E. coli* M15 strain carrying the *pirA* gene derived from our previous research was used in the present study. The gene encoding the pirA antigen was expressed as a fusion protein containing histidine tag at the N-terminal end of the peptide using pQE30 (pQE30-6xHis-PirA) cloned in *E. coli* M15 cells (Qiagen, Germany).

Chemicals and reagents

Tryptone, peptone, yeast extract, ampicillin, and kanamycin were purchased from Biobasic (USA). NaCl; KCl; K_2HPO_4; KH_2PO_4; HCl; glycerol; Triton X-100; glacial acetic acid; Tris-HCl; tris base were purchased from Merck (Germany). Imidazole; acrylamide; bis-acrylamide; methanol were purchased from Sigma-Aldrich (USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG); sodium dodecyl sulfate (SDS); ammonium persulfate (APS); N, N, N', N'-tetramethylethylenediamine (TEMED); Tris; 2-Mercaptoethanol; bromophenol blue; glycine; coomassie brilliant blue R-250 were purchased from Bio-Rad (USA). PageRuler™ prestained protein ladder (10-170 kDa) was purchased from Thermoscientific (USA).

Cell cultivation

The recombinant *E. coli* M15 hosts harboring pQE30-6His-PirA construct were cultured in test-tubes containing 5 mL of Luria-Bertani (LB) broth containing ampicillin (100 µg/mL) and kanamycin (50 µg/mL). The culture was shaken at 37°C, 200 rpm. The test-tubes were seeded with 4% (v/v). Once the optical density (OD) of cells at 600 nm reached to 0.5-1.0, the expression of recombinant PirA antigens was induced by adding IPTG.

Determination of the optimized expression conditions

For optimization of recombinant PirA antigen expression, cultivations were performed under different IPTG concentrations; different induction time; and different cell densities before induction.

In order to optimize IPTG concentration for recombinant PirA antigen expression in *E. coli* M15, different IPTG concentrations (0.1, 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 mM) were added and cultured after reaching the desired OD_{600nm} of 0.5-1,0 at 37 °C, 200 rpm. Finally, the samples were collected after 4 hours of the induction. To evaluate maximum yield of recombinant PirA antigens, incubation time after induction was studied. The cells were grown at 37 °C, 200 rpm to reach OD_{600nm} of 0.5-1,0. Then, IPTG with optimal concentration was added to induce expression, and the samples were collected at different time intervals (2, 4, 6, 8, and 10 hours) after induction. In order to determine the effect of cell density before induction on recombinant PirA antigen expression, the cells were grown at 37 °C, 200 rpm. When OD_{600nm} of the cultures reached 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6, induction was made by IPTG with optimal concentration and the samples were collected at 2, 4, 6, 8, and 10 hours before induction.

SDS-PAGE

After the induction time, the cell pellets were harvested by centrifugation at 12,000 rpm for 2 minutes. Next, the total cell pellets were lysed (lysis buffer=50 mM potassium phosphate (pH=7.8), 400

mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole), followed by sonication 5 times with a burst duration of 60 seconds each. The sonicated lysates were centrifuged at 12,000 rpm for 10 minutes to separate soluble and insoluble proteins, and the supernatants containing the soluble proteins were collected into fresh tubes. The proteins were then electrophoresed in a 15% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). In the parallel experiment, uninduced culture was grown, harvested and sample analyzed on 15% SDS-PAGE. In the negative control experiment, untransformed *E. coli* M15 pQE30/PirA strain was cultivated in the identical conditions. Equal number of cells of transformed and untransformed culture with and without IPTG induction were loaded on 15% SDS-PAGE. The gel was stained with coomassie brilliant blue for 30 minutes. Then, the gel was washed with a washing solution (30% (v/v) methanol, 10% (v/v) acetic acid) until the gel became clear and protein bands appeared. The PageRuler™ prestained protein ladder was used for assessment of protein size.

Analytical Procedure

Optical density of each sample was measured at the final expression time using a spectrophotometer (Hitachi, Japan). Data were statistically analysed using Minitab software version 16.2.0 and Microsoft Excel 2013 to calculate the mean and standard deviation. One-way ANOVA was used to identify statistically significantly different means compared between treatments with Tukey's test at a probability level of $p<0.05$.

3. Results

To optimize the expression of recombinant proteins, many expression systems have been developed. One of the most important characteristics of promoters in these systems is the simple and highly economical induction method. Therefore, many different inducers can be used depending on the vector system carrying the target gene. IPTG is a strong inducer of *lac*, *tac* and *trc* promoters so it is commonly used to induce recombinant protein expression in *E. coli* (Menzella et al., 2003). Depending on the type of protein being synthesized, the concentration of the optimal IPTG inducer is different.

The recombinant *E. coli* M15 cell growth rates at different IPTG concentrations was shown in Table 1. Results presented in Table 1 indicated differences between the cell densities (OD_{600nm}) at different IPTG concentrations (from 0-1.5 mM) after 4 hours induction were no statistically significant. Study on SefA protein expression of *Salmonella enterica* Serovar Enteritidis in *E. coli* BL21 showed that IPTG concentration did not affect the cell growth (Huyen et al., 2008).

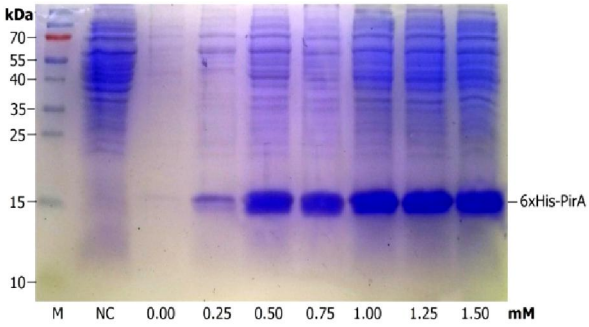
Table 1. Effect of IPTG concentration on the growth of recombinant *E. coli* M15 cell expressing 6xHis-pirA fusion protein (OD_{600nm})

IPTG concentration (mM)	NC	0.00	0.25	0.50	0.75	1.00	1.25	1.50
Before induction	0.92^{a} ±0.08	0.93^{a} ±0.08	0.89^{a} ±0.04	0.95^{a} ±0.06	0.96^{a} ±0.06	0.87^{a} ±0.03	0.97^{a} ±0.21	0.84^{a} ±0.06
After 4 hours of induction	2.82^{a} ±0.32	2.99^{a} ±0.30	2.77^{a} ±0.19	2.74^{a} ±0.15	2.88^{a} ±0.30	2.77^{a} ±0.19	2.80^{a} ±0.31	2.72^{a} ±0.06

$^{a-d}$: Values with different letters on the same row showed significant differences ($p<0.05$).

The effect of IPTG concentrations on the soluble fractions of 6xHis-PirA was compared on 15% SDS-PAGE gel (Figure 1). Results showed that all IPTG concentrations produced the soluble 6xHis-PirA extracts (prominent bands at roughly 15 kDa). It was found that a low 0.25 mM IPTG concentration was enough to induce the 6xHis-PirA fusion protein expression. In the absence of IPTG induction, prominent band migrating at approximate 15 kDa were not observed. The level of recombinant 6xHis-PirA expression in soluble fraction of *E. coli* reached the highest value at IPTG concentration from 1.00-1.50 mM. These values were similar at IPTG concentrations between 1.00 and 1.50 mM. The result indicated that a higher IPTG concentration had no significant effect on the yield of 6xHis-PirA protein. On the other hand, 1 mM IPTG was widely used (Donovan et al., 1996). Therefore, for further optimization experiments 1.00 mM IPTG concentration was used.

The present results were appropriate to the previous reports. The findings of Ribeiro et al. (2019) showed that the optimal IPTG concentration for 503 antigen expression of *Leishmania i. chagasi* in *E. coli* M15 was 1.00 mM with the maximum antigen concentration obtained being 0.19 g/L. According to Lan et al. (2016), the 3-1E recombinant antigen of *Eimeria* began to be synthesized in *E. coli* BL21 (DE3) cells when induced by 0.2 mM IPTG and reached the highest value at IPTG concentration from 0.8 to 1.0. Results of research on *Interleukin-2* (*IL-2*) gene expression of human in *E. coli* cells showed that IL-2 recombinant product were synthesized at 37°C, 1.0 mM IPTG and cell density at the time of induction OD_{600nm}=0.9-1.2 (Tan et al., 2009).



Effect of induction time

Another induction factor that influence the expresstion of recombinant protein in *E. coli* is the induction time. The induction time has a great influence on the expression performance of 6xHis-PirA fusion protein. If the cell biomass is collected early, the protein has not been synthesized to the maximum, while the cell biomass is collected late, a number of recombinant protein inhibitors and degraders will be formed. When the culture medium lacks nutrients, the cells will secrete protease to hydrolyze, convert dead cells and by-products into a source of nutrients for the cells, so foreign protein products are often hydrolyzed by protease (Shojaosadati et al., 2008).

The growth of recombinant *E. coli* M15 cells at different intervals of after induction incubation (OD_{600nm}) was presented in Table 2. Results presented in Table 2 showed after 4 hours of induction, the highest cell density reached OD_{600nm}=5.19.

ecombinant *E. coli* M15 cell expressing 6xHis-pirA fusion

	8	**10**	
	4.81^{a}±0.56	3.24^{b}±0.59	3.43^{b}±0.26

d significant differences ($p<0.05$).

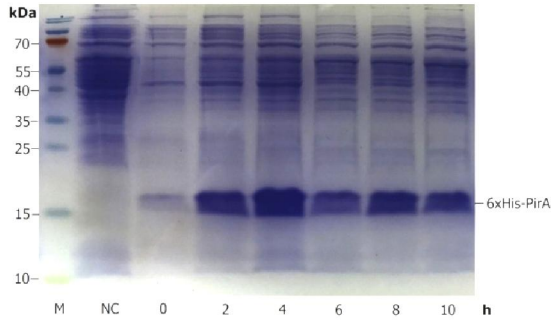


Figure 2. Expression of 6xHis-PirA fusion protein at different induction time. M: PageRuler ™ prestained protein ladder (10-170 kDa, Thermoscientific). NC: *E. coli* M15 cells without the pQE30-6xHis-PirA construct; 0: Recombinant *E. coli* M15 cell density before induction of IPTG; 2 h, 4 h, 6 h, 8 h, and 10 h: induction time.

Duong et al. (2017) expressed successfully *Cry2A* gene from *Bacillus thuringiensis* in *E. coli* BL21 host cell. *E. coli* BL21 cells carrying recombinant plasmid *pET22b (+)/Cry2A* were cultured in LB medium, induced by 1.00 mM IPTG, induction time of 4 hours, at 30°C, Cry2A recombinant protein was expressed in the fusion form with size of about 70 kD. Vaz et al. (2011) also found that eIF antigen production was associated to growth, with maximum expression 4 hours after culture induction in 2xTY medium. Loc et al. (2014) researched on enhancing expression of recombinant fimbrial adhesion K88-1NT isolated of enterotoxigenic *E. coli* from piglet. The results indicated highest level of K88-1NT in recombinant *E. coli* BL21 (DE3) was determined after induction by 0.1% lactose for 6 hours at 35ºC when cell density (OD600nm) reached 0.5. In other research, it showed that highest anti-HER2 scFv expression in recombinant Origami strain was achieved 24 hours after IPTG induction (1.00 mM), at 37 °C (Farshdari et al., 2020).

Effect of cell density before induction

Numerous studies showed that the growth stage of the cell at which protein is induced to express has a large effect on protein synthesis and activity.

Therefore, it is necessary to optimize cell density before adding inducers to improve the efficiency of recombinant protein expression (Byun et al., 2007).

Effect of recombinant *E. coli* M15 cell density was investigated at different growth phases (OD_{600nm} from 0.4 to 1.6) with 1.00 mM IPTG, at 37ºC, after 4 hours of induction. The results were presented in Table 3 and Figure 3.

Table 3. Effect of recombinant *E. coli* M15 cell density at the time of adding the IPTG (OD_{600nm})

At the time of induction	**0.4**	**0.6**	**0.8**	**1.0**	**1.2**	**1.4**	**1.6**
After 4 hours of induction	1.84^{c} ±0.08	2.29^{bc} ±0.31	2.70^{b} ±0.53	3.37^{a} ±0.15	2.52^{b} ±0.41	2.44^{b} ±0.34	2.35^{b} ±0.28

$^{a-d}$: Values with different letters on the same row showed significant differences ($p<0.05$).

The results presented in Table 3 showed the time of adding the IPTG at the cell density OD_{600nm}=1.0 produced the highest cell density of OD_{600nm}=3.37 after 4 hours of induction.

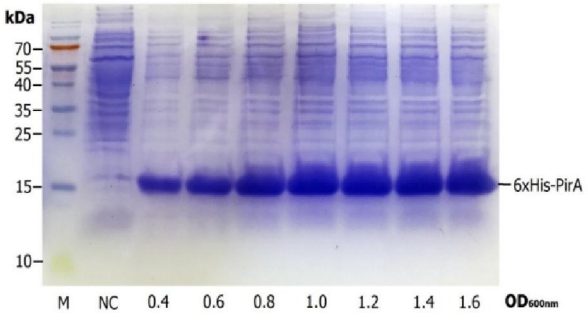


Figure 3. Expression of 6xHis-PirA fusion protein with different cell densities at the time of adding the IPTG. M: PageRuler ™ prestained protein ladder (10-170 kDa, Thermoscientific). NC: *E. coli* M15 cells without the pQE30-6xHis-PirA construct; 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6: Recombinant *E. coli* M15 cell density at the time of adding the IPTG.

The results in Figure 3 indicated that the cell density at before induction clearly affected the ability of 6xHis-PirA fusion protein synthesis. 6xHis-PirA fusion protein has begun to be synthesized when IPTG inducer was added to the cultures at cell density (OD_{600nm}) reached from 0.4-1.6. The highest amount of 6xHis-PirA fusion protein was produced at OD_{600nm}=1.0. However, when IPTG induction at higher cell densities, this fusion protein almost was not synthesized. Thus, the recombinant *E. coli* M15 cell density (OD_{600nm}) at before induction (the time of adding IPTG inducer) for the best 6xHis-PirA fusion protein expression was 1.0.

For the expression of different recombinant proteins in *E. coli*, the appropriate cell density at the induction time was different (Loc et al., 2014). The outcomes of Juhasz et al. (2003) showed that the cell density ($OD6_{00nm}$) from 0.8-1.0 was the most appropriate time to induce recombinant thermostable endoglucanase expression from *Clostridium thermocellum* in *E. coli*. Huong et al. (2016) optimized the conditions for the expression of interleukin-3 (IL-3), which included *E. coli* host strain JM109, LB cultivation medium, induction temperature was 25ºC; induction with 0.05 mM IPTG at OD_{600nm}=1.0. The cell density of OD_{600nm}=0.5 was the most suitable time to supplement IPTG to induce the expression of amino peptidase N of pigs in *E. coli* BL21 (DE3) (Liu et al., 2009). Xu et al. (2006) studied the expression of bioactive human beta-defensin-4 in *E. coli* and found that the recombinant protein was produced strongly if induced at a cell density of OD_{600nm}=15. Besides, producing the same recombinant protein at different production scales and different culture methods, the optimal cell density at the before induction was not similar. The outcomes of study on streptokinase production in *E. coli* BL21 (DE3) revealed the induction was performed at the culture scale in a flask at the cell density (OD_{600nm}) reached from 1.0-1.2, while at the scale of fermentation system, OD_{600nm} from 3.8-4.2 (intermittent culture) or OD_{600nm}=30 (intermittent culture with nutritional supplement) produced the best results (Goyal et al., 2009).

Our results showed that the highest level of 6xHis-pirA fusion protein in recombinant *E. coli* 15 was determined after induction by 1.00 mM IPTG, for 4 hours after induction, when cell density (OD_{600nm}) reached a value of 1.0 (at 37°C, 200 rpm). The information gathered may be used for optimization of the fermentation process, which aims at improving the production of 6xHis-pirA fusion protein in *E. coli* M15 from *Vibrio*. From these outcomes, we concluded that optimizing the three important factors of IPTG concentration, induction time, and cell density before induction can improve 6xHis-pirA fusion protein yield in the *E. coli* M15 expression system.

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References

1. Abath FGC, Xavier EM, Silva JBAB, Junior MAM, Montenegro SML. Expression of recombinant antigens in *Escherichia coli*: Application on immunochemical studies of *Schistosoma mansoni* tegumental antigens. Mem. Inst. Oswaldo Cruz., Rio de Janeiro 1997;92(5): 637-641.
2. Arévalo-Herrera M, Vallejo AF, Rubiano K, Solarte Y, Marin C, Castellanos A, Céspedes N, Herrera S. Recombinant Pvs48/45 antigen expressed in *E. coli* generates antibodies that block malaria transmission in *Anopheles albimanus* mosquitoes. PLoS ONE 2015;10(3):e0119335.
3. Azaman SNA, Ramanan RN, Tan JS, Rahim RA, Abdullah MP, Ariff AB. Screening for the optimal induction parameters for periplasmic producing interferon-α2b in *Escherichia coli*. African Journal of Biotechnology 2010;9(38):6345-6354.
4. Baeshen MN, Al-Hejin AM, Bora RS, Ahmed MMM, Ramadan HAI, Saini KS, Baeshen NA, Redwan EM. Production of biopharmaceuticals in *E. coli*: Current scenario and future perspectives. J. Microbiol. Biotechnol. 2015;25(7):953-962.
5. Byun SG, Kim MD, Lee WH, Lee KJ, Han NS, Seo JH. Production of GDP-L-fucose, L-fucose donor for fucosyloligosaccharide synthesis, in recombinant *Escherichia coli*. Applied Microbiology and Biotechnology 2007;74(4):768-775.
6. Dang TL, Pham AT, Phan TV. Acute Hepatopancreatic Necrosis Disease (AHPND) in Vietnam. The Journal of Asian Fisheries Society 2018;31S (2018):274-82.
7. Dao NTB, Linh NQ, Khanh NV. Research some characteristics of *Vibrio parahaemolyticus* which causes EMS disease on juvenile shirmp at Dien Huong community, Phong Dien district, Thua Thien Hue province. Journal of Science, Hue University 2014;98(10):13-22.
8. De Schryver P, Defoirdt T, Sorgeloos P. Early mortality syndrome outbreaks: A microbial management issue in shrimp farming? PLOS Pathogens 2014;10(4):e1003919.
9. Donovan RS, Robinson CW, Glick BR. Review: Optimizing inducer and culture conditions for expression of foreign proteins under the control of the *lac* promoter. J. Ind. Microbiol. Biotechnol. 1996;16:145-154.
10. Dung DTT, Quang VD and Trang PTP. Isolation and selection of *Lactobacillus* spp. antagonistic to *Vibrio parahaemolyticus* causing the (Early Mortality Syndrome) shrimp disease in Soc Trang province. Science and Technology Development 2017;3(T20-2017):5-15 (in Vietnamese).
11. Duong PT, Binh NĐ, Ha TTT, Khanh LD. Expression of *Cry2A* gene acitive on house fly larval (*Musca domestica*) from a novel *Bacillus thuringiensis* serovar *kurstaky* MSS8.4. Journal of Biotechnology 2017;15(3):563-569 (in Vietnamese).
12. Farshdari F, Ahmadzadeh M, Nematollahi L, Mohit E, The improvement of anti-HER2 scFv soluble expression in *Escherichia coli*, Brazilian Journal of Pharmaceutical Sciences, Braz. J. Pharm. Sci. 2020;56:e17861, 1-8.
13. Francis DM, Page R. Strategies to optimize protein expression in *E. coli*. Current Protocols in Protein Science 2010;5.24.1-5.24.29.
14. Ghosh S, Rasheedi S, Rahim SS, Banerjee S, Choudhary RK, Chakhaiyar P, Ehtesham NZ, Mukhopadhyay S, Hasnain SE. 2004, Method for enhancing solubility of the expressed recombinant proteins in *Escherichia coli*. BioTechniques 2004;37:418-423.
15. Gomes L, Monteiro G, Mergulhão F. The impact of IPTG induction on plasmid stability and heterologous protein expression by *Escherichia coli* biofilms. International Journal of Molecular Sciences 2020;21:576.
16. Goyal D, Sahni G, Sahoo DK. Enhanced production of recombinant streptokinase in *Escherichia coli* using fed-batch culture. Bioresource Technology 2009;100(19):4468-4474.
17. Han JE, Tang KF, Tran LH, Lightner DV. Photorhabdus insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. Diseases of Aquatic Organisms 2015;113(1):33-40.
18. Hu PQ, Tuma-Warrino RJ, Bryan MA, Mitchell KG, Higgins DE, Watkins SC, Salter RD. *Escherichia coli* expressing recombinant antigen and Listeriolysin O stimulate class I-restricted

CD8-T cells following uptake by human APC. The Journal of Immunology 2004;172:1595-1601.
19. Huong DT, Quy NT, Ha DTN, Hong LTTH, Nam HT. Optimization of fermentation conditions for the expression of Interleukin-3 in fusion with PelB in *E. coli*. Journal of Biology 2016;38(2):250-256 (in Vietnamese).
20. Huyen DT, Giang LQ, Tan TN, Thanh TL, Hai TN (2008). Overexpression of protein Sefa derived from Samonella enterica serovar enteritidis in *Escherichia coli* BL21. Journal of Biotechnology 2008;6(2):175-182 (in Vietnamese).
21. Jia B, Jeon CO. High-throughput recombinant protein expression in *Escherichia coli*: current status and future perspectives. Open Biol. 2016;6:160196.
22. Joseph BC, Pichaimuthu S, Srimeenakshi S, Murthy M, Selvakumar K, Ganesan M, Manjunath SR. An overview of the parameters for recombinant protein expression in *Escherichia coli*. J. Cell Sci. Ther. 2015;6(5):221.
23. Juhasz T, Szekely E, Simándi B, Szengyel Z, Réczey K. Recovery of a recombinant thermostable endoglucanase from *E. coli* using supercritical carbon dioxide cell disruption. Chemical and Biochemical Engineering Quarterly 2003;17(2):131-134.
24. Khanh NV, Linh NQ, Lan TT, Van TQK, Co NTK, Dung TQ. Isolation and screening of *Vibrio parahaemolyticus* strains to cause acute hepatopancreatic necrosis disease in white-leg shrimps cultured in Phong Dien, Thua Thien Hue using *16S rRNA* marker. Journal of Science, Hue University 2019;128(1E):47-58 (in Vietnamese).
25. Khimmakthong U, Sukkarun P. The spread of *Vibrio parahaemolyticus* in tissues of the Pacific white shrimp Litopenaeus vannamei analyzed by PCR and histopathology. Microbial pathogenesis 2017;113:107-12.
26. Lan DTB, Long PT, Thao LD, Viet LQ, Nhung HTT, Huong DT, Thinh LC, Chuong HV, Long DT, Hien NTT, Enhancing expression of recombinant protein LTA of *Escherichia coli* in competent cells, *Escherichia coli* BL21 (DE3), Journal of Veterinary Science and Technology 2018;25(5):49-55 (in Vietnamese).
27. Lan DTB, Long PT, Chuong HV, Long ĐT, Quang HT, Thao LD, Viet LQ, Thinh LC, Huong DT, Nhung HTT. Enhancing expression level of recombinant antigen 3-1E of *Eimeria* in *Escherichia coli* BL21 (DE3). Journal of Veterinary Science and Technology 2016;28(2):55-63 (in Vietnamese).
28. Lan DTB, Long PT, Loc NH, Quang HT, Lan TT, Viet LQ, Long DT, Thinh LC, Chuong HV, Giang DTT Enhancing expression of recombinant protein *Cp23* of *Cryptosporidium parvum* in *Escherichia coli* BL21 (DE3), Journal of Veterinary Science and Technology 2014;21(6):35-42 (in Vietnamese).
29. Lai HC, Ng TH, Ando M, Lee CT, Chen IT, Chuang JC, Mi-De Y, Chiang YA, Takeyama H, Hamaguchi HO, Lo CF, Aoki T, Wang HC. Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in shrimp. Fish & Shellfish Immunology 2015;47(2):1006-1014.
30. Lee CT, Chen IT, Yang YT, Ko TP, Huang YT, Huang JY, Huang MF, Lin SJ, Chen CY, Lin SS, Lightner DV, Wang HC, Wang AHJ, Wang HC, Hor LI, Lo CF. The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. Proceedings of the National Academy of Sciences (PNAS) 2015;112(34):10798-10803.
31. Li P, Kinch LN, Ray A, Dalia AB, Cong Q, Nunan LM, Camilli A, Grishin NV, Salomon D, Orth K. Acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* strains maintain an antibacterial type VI secretion system with versatile effector repertoires. Appl. Environ. Microbiol. 2017;83:e00737-17.
32. Lightner DV, Redman R, Pantoja C, Noble B, Tran L. Early mortality syndrome affects shrimp in Asia. Global Aquaculture Advocate 2012;15(1):40.
33. Liu L, Xiao J, Zhang M, Zhu W, Xia X, Dai X, et al. A *Vibrio owensii* strain as the causative agent of AHPND in cultured shrimp, *Litopenaeus vannamei*. Journal of Invertebrate Pathology 2018;153:156-64.
34. Loc NH, Quynh NTK, Quang HT, Lan TT, Ngoc LMT, Lan DTB, Long PT. Enhancing expression of recombinant of fimbrial adhesion K88 isolated of enterotoxigenic *Escherichia coli* from piglet, Journal of Biology 2014;36(1se):120-125 (in Vietnamese).
35. Lua DT, Khue NV, Van PT. Non-Vibrio Parahaemolyticus causing Acute Hepatopancreatic Necrosis on brackish shrimps. Vietnam J. Agri. Sci. 2016;14(5):690-698 (in Vietnamese).
36. Menzella HG, Ceccarelli EA and Gramajo HC. Novel *Escherichia coli* strain allows efficient recombinant protein production using lactose as inducer. Biotechnology and Bioengineering 2003;82(7):809-817.
37. Mohajeri A, Pilehvar-Soltanahmadi Y, Abdolalizadeh J, Karimi P, Zarghami N, Effect

of Culture Condition Variables on Human Endostatin Gene Expression in *Escherichia coli* Using Response Surface Methodology, Jundishapur J. Microbiol. 2016;9(8):e34091.
38. Nghia NT, Oanh DTH, Phu TQ, Tuan PA. Isolation and determination of the ability to cause acute hepatopancreatic necrosis syndrome of *Vibrio parahaemolitycus* bacteria isolated from cultured shrimp in Bac Lieu province. Journal of Science, Can Tho University 2015;39(2015):99-107 (in Vietnamese).
39. Papaneophytou CP, Kontopidis GA. Optimization of TNF-alpha overexpression in *Escherichia coli* using response surface methodology: Purification of the protein and oligomerization studies. Protein Expr. Purif. 2012;86(1):35-44.
40. Rancour DM, Backues SK, Bednarek SY, 2010, Protein antigen expression in *Escherichia coli* for antibody production, in S.D. Schwartzbach, T. Osafune (eds.), *Immunoelectron Microscopy*, Methods in Molecular Biology 657, Springer Science+Business Media, LLC 2010.
41. Ribeiro VT, Asevedo EA, de Paiva Vasconcelos LTC, Oliveira Filho MA, de Araújo JS, de Macedo GR, de Sousa Júnior FC, dos Santos ES. Evaluation of induction conditions for plasmid pQE-30 stability and 503 antigen of *Leishmania i. chagasi* expression in *E. coli* M15. Applied Microbiology and Biotechnology 2019;103(16):6495-6504.
42. Rosano GL, Ceccarelli EA. Recombinant protein expression in *Escherichiacoli*: advances and challenges. Frontiers in Microbiology 2014;5(172):1-17.
43. Shojaosadati SA, Varedi Kolaei SM, Babaeipour V, Farnoud AM. Recent Advances in High Cell Density Cultivation for Production of Recombinant Protein. Iranian Journal of Biotechnology 2008;6(2): 63-84.
44. Sirikharin R, Taengchaiyaphum S, Sanguanrut P, Chi TD, Mavichak R, Proespraiwong P, et al. Characterization and PCR detection of binary, Pir-like toxins from *Vibrio parahaemolyticus* isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp. PloS ONE 2015;10(5):e0126987.
45. Tan TN, Duc VM, Lan Anh LT, Chi BK, Hai TN. Expression of human IL-2 gene in *Escherichia coli*. Journal of Biotechnology 2009;7(1):35-39 (in Vietnamese).
46. Tran L, Nunan L, Redman RM, Mohney LL, Pantoja CR, Fitzsimmons K, Lightner D. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Diseases of Aquatic Organisms 2013;105(1):45-55.
47. Tran L, Nunan L, Redman RM, Mohney LL, Pantoja CR, Fitzsimmons K, et al. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Diseases of Aquatic Organisms 2013;105(1):45-55.
48. Vaz MRF, França RLS, Andrade SSL, Junior FCS, Santos ES, Martins DRA, Macedo GR. Influence of culture medium on the production of eIF antigen from *Leishmania chagasi* recombinant *Escherichia coli*, Brazilian Journal of Microbiology 2011;42:1390-1396.
49. Xu Z, Zhong Z, Huang L, Peng L, Wang F and Cen P. High-level production of bioactive human beta-defensin-4 in *Escherichia coli* by soluble fusion expression. Applied Microbiology and Biotechnology 2006;72(3):471-479.
50. Zhao Y, Wang C, Lu Y, Amer S, Xu P, Wang J, Lu J, Bao Y, Deng B, He H, Qin J (2011). Prokaryotic expression and identification of 3-1E gen of merozoite surface antigen of *Eimeria acervulina*. Parasitology Research 2011;109(5):1361- 1365.
51. Zorriehzahra MJ, Banaederakhshan R. Early mortality syndrome (EMS) as new emerging threat in shrimp industry. Adv. Anim. Vet. Sci. 2015;3(2s):64-72.

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