OS Number: OS04

EXPRESSION OF RECOMBINANT PROTEIN ORF2 FROM PORCINE CIRCOVIRUS IN *ESCHERICHIA COLI* WITHOUT ISOPROPYL-β-THIOGALACTOSIDE INDUCER

Truong Quoc Phong

Hanoi University of Science and Technology, Vietnam

ABSTRACT

.

Inducible expression system *Escherichia coli* BL21 (DE3) harboring expression vector pETs is considered as the efficient system for expressing several target proteins. In this expression system, isopropyl-β-D-thiogalactoside (IPTG) is used to be an inducer for expression of target proteins. However, IPTG is quite expensive and therefore it is not suitable for the large-scale production of recombinant proteins. Gene *orf2* from porcine circovirus type 2 (PCV2) was cloned in the expression vector pET22b(+) and transformed into *E. coli* BL21(DE3) for production of rORF2 protein that will be applied to develop the diagnostic kit and vaccine for porcine. In the present study, lactose was used to be an inducer instead of IPTG. The optimal medium components for expression of recombinant protein was investigated including 0.8% peptone, 0.4% yeast extract, 0.3% glycerol, 50 mM phosphate buffer, 2 mM MgSO4, 25 mM (NH4)2SO4 and 0.03% lactose. Optimal temperature for expression of ORF2 protein was 37^{o}C. The dynamics of the growth and recombinant protein expression indicated that rORF2 was started to synthesize after 8 hours (OD_{600} ~ 2.2), increased afterward and reached the highest level after 11 hours fermentation (OD_{600} ~ 3.5). Optimal conditions established in this research are suitable for the large-scale fermentation producing recombinant ORF2 protein for the next researches.

Keywords: Escherichia coli (E. coli), isopropyl-β-D-thiogalactoside (IPTG), lactose, porcine circovirus (PCV2), expression vector pET

1. INTRODUCTION

The strong development of recombinant DNA technology has brought significant results in the development and production of recombinant proteins applying in many different fields such as agriculture, industry, medicine, food,... The expression system pET/*E. coli* BL21 (DE3) was considered as the high expression system and widely used to produce recombinant proteins (Chen, 2012). For pETs vector system, the *lac* operator sequence is positioned immediately behind the T7 promoter, forming a promoter *T7lac* (Dubendorff and Studier, 1991). With this system the regulation of target gene expression is controlled directly by the promoter *T7lac* and indirectly by *L8-UV5 lac* promoter. After adding to the culture inducer IPTG will bind to LacI, leading to release *lac* operator position, the synthesis of T7 RNA polymerase will be induced and the transcription will be occurred from the promoter *T7lac* position of a target gene. However, IPTG is an expensive substance and should not suitable for the large-scale production. Lactose is a natural inducer of the *lac* operon. In the process of metabolism in bacteria, lactose will be metabolized into allolactose after uptake into the cytoplasm. This compound binds to LacI protein to prevent the attachment of LacI to the operator position and thus it acts as an inducer to open the expression structure under the control of the L8 - UV5 and lac T7lac promoter. However, allolactose rapidly hydrolyzed by β-galactosidase resulting in the closure of the expression structure (Brooker *et al*., 2008; Tian *et al*., 2011). Unlike IPTG, lactose also serves as a carbon source for growth, therefore the synthesis of β-galactosidase will be depended on several factors, including nutritional components in the medium, carbon source, growth phase,... In order to use lactose as an effective inducer for expression system of *E. coli* BL21 (DE3) and promoter T7*lac*, the study of these factors are really necessary. *Orf2* gene of porcine circovirus (PCV2) has been genetically optimized and cloned into the pET22b(+) vector to express ORF2 protein applying in diagnosis and development of vaccine for porcine circovirus. In this study, carbon and energy source (glucose, glycerol, lactose, peptone, yeast extract), fermentation conditions (temperature, time) were studied to establish suitable conditions for production of ORF2 protein in *E. coli* BL21 (DE3) using lactose as an inducer.

2. MATERIALS AND METHODS

2.1 Materials

Recombinant strain *E. coli* BL21(DE3) harboring pET22*::orf2* was developed by our group. Culture chemicals such as lactose, glucose, glycerol, peptone, yeast extract, mineral,... were purchased from Merck (Germany), Sigma (USA), Himedia (India). Chemicals for electrophoresis of proteins: Acrylamide , Tris - HCl , sodium dodecyl sulfate, mercaptoethanol,... were purchased from Merck (Germany), Bio-Rad (USA). The standard protein ladder (# 26612) was from Thermo Scientific (USA). Components of nutrient medium: peptone; yeast extract; 50 mM phosphate buffer pH 6.8; 25 mM $(NH_4)_2SO_4$; 2 mM $MgSO_4$, glycerol; glucose; lactose.

2.2 Methods

2.2.1 Fermentation

Two milliliters of sterile culture medium containing 100 µg/ml ampicillin was transferred into the 10 ml volumetric flask. Bacteria were inoculated at initial OD_{600} of 0.03. Fermentation was carried out at the temperature of 30^oC with shaking 110 rpm for 14-16 hours.

2.2.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel contained two layers of the stacking gel of 5% and separating gel of 12%. Protein sample for electrophoresis analysis was prepared by dissolving bacterial biomass in the sample buffer (50 mM Tris-HCl pH6,8, 2% SDS, 10% Glycerol, 1% mercaptoethanol) and heating at 95^oC for 10 min. After centrifugation 12000 rpm for 10 min, the supernatant was collected and loaded on the polyacrylamide gel. The electrophoresis was carried out at the constant voltage of 110 V until the dye front reaches the bottom of the gel (Laemmli, 1970).

2.2.3 Quick gel stain

After electrophoresis, the gel was soaked in the fixing solution containing 50% ethanol and 10% acetic acid and heated to nearly boiling (using microwaves). The gel was transferred to a stain solution (5% ethanol, 8% acetic acid, 0.001% coommassie brilliant blue) and heated to nearly boiling. The protein band will gradually appear on the gel after 20 min.

2.2.4 Protein pattern analysis

Intensity of protein bands on the gel pattern were analyzed by Quantity One 4.6.9 (Bio-rad, USA). % rORF2 = C_{rORF2}/C_{ts}*100; in which C_{rORF2} – intensity of target ORF2 protein band, Cts – total intensity of all bands in one lane.

3. RESULTS AND DISCUSSIONS

3.1 Carbon and energy source for auto-induction of recombinant protein expression in *E. coli*

Auto-induction of protein expression is induction process for expression of target gene depending on the nutritional and energy demand of the body in the growth and development. For expression system containing promoter *T7lac* and using lactose as an inducer and carbon source, *T7lac* promoter is only activated whenever the host body uses lactose as a carbon source (Inada *et al.*, 1996; Pei *et al.*, 2011). The time point of induction depends on the control of the host body; hence this process is called auto-induction. Auto-induction is affected by several factors such as nutritional components, growth temperature, the cell state,... This process is completely different from the expression process using IPTG as inducer. In the presence of glucose, the metabolism of other carbon and energy sources will be inhibited by the system of phosphoenolpyruvate/carbohydrate phosphotransferase (Inada *et al.* 1996; Meadow *et al.*, 1990). In the absence of glucose, glycerol can be used as carbon source, however inhibition of use of other carbon sources is much less than glucose; the metabolism of lactose will be induced in this case and leads to induction of expression of target protein in the T7lac expression system. The appropriate concentration of nutrient components needs to be studied to achieve the highest production of recombinant target protein.

3.1.1 Glucose

Glucose, an important nutrient composition affects the growth of *E. coli* BL21 and also greatly affects the expression of recombinant proteins using lactose as an inducer. In this study, a glucose concentration range (from 0.00 to 0.05 %) was investigated to determine appropriate level for effective expression of recombinant rORF2. The result showed that the expression level of recombinant rORF2 was the same in the different glucose concentrations (Fig. 1). No addition of glucose, protein expression levels are similar in the presence of glucose.

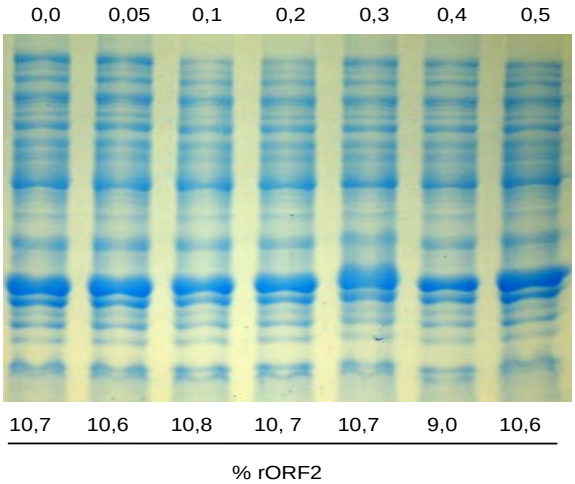

Fig. 2 Protein pattern of total extract from biomass grown in medium with different glucose concentrations.

3.1.2 Glycerol

In this study, glycerol was alternative carbon source when glucose was exhausted from medium because glycerol was good nutrition for the growth of bacteria. However, this carbon source did not strongly inhibit induction for the metabolism of lactose; *lac* promoter will therefore be activated at certain level. During growth glycerol will be decreased and simultaneously increasing the use of lactose led to the induction of recombinant protein expression under the control by the promoter *T7lac* (Eppler *et al.*, 2002). Furthermore, excess of glycerol will create more acid leading to stop the growth of bacteria (Studier, 2005). Therefore, the different concentrations of glycerol from 0.2 to 0.7% were studied to choose appropriate concentration consistent with the objective of good growth of bacteria and not inhibit induction of the metabolism of lactose. The result showed that in the range of investigated concentrations, the produced recombinant protein was 5.2 to 5.6% (Fig. 2), with no significant difference between glycerol concentrations examined. Therefore, 0.3% glycerol concentration was selected for next experiments.

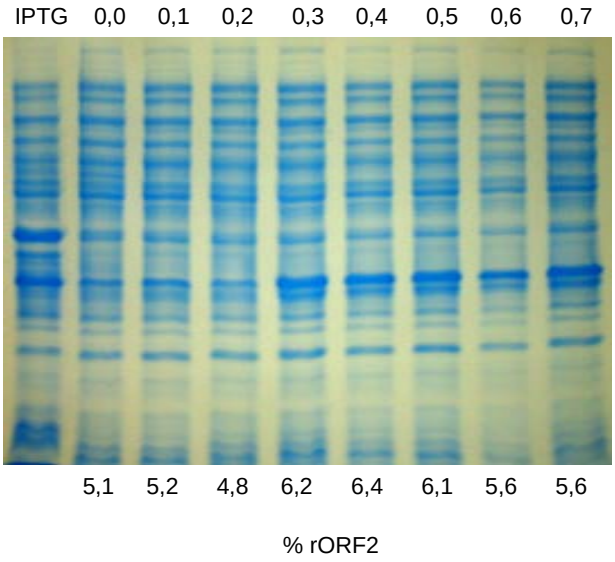

Fig. 2 Protein pattern of total extract from biomass grown in medium with different glycerol concentrations.

3.1.3 Lactose

In this study, lactose was used as an inducer to substitute IPTG, but lactose was also nutrient source used by bacteria for growth. Therefore it is necessary to determine appropriate level of lactose for induction of the strongest rORF2 expression. The result showed that the expression levels of rORF2 protein were similar with lactose concentrations vary from 0.01 to 0.1 % (Fig. 3). This result was consistent with previous study (Studier, 2005). Based on the amount of lactose used and the efficiency of rORF2 protein synthesis, lactose concentration of 0.03 % was selected as substitute IPTG inducer.

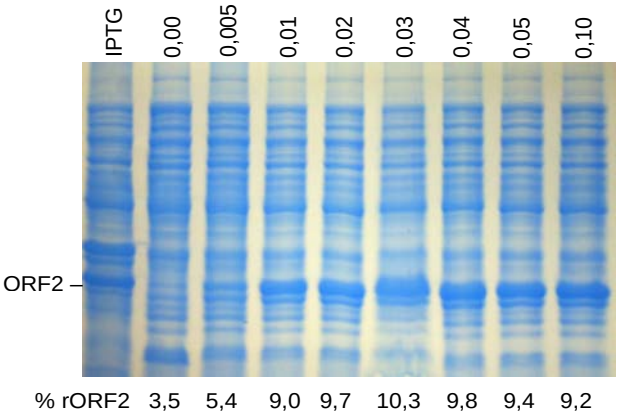

Fig. 3 Protein pattern of total extract from biomass grown in medium with different lactose concentrations.

3.1.4 Peptone

Studier (2005) had used NZ amine as a source of nitrogen in study of recombinant protein expression using lactose as inducer (Studier, 2005). However, the NZ amine was expensive nutrient source; therefore peptone was used as alternative nitrogen source in this study. A range of peptone concentrations (0 to 1.4%) was used to assess its influence to the ability of recombinant protein expression. The result showed that the level of the rORF2 protein was increased with the increase of peptone concentration (Fig. 4). Similarly, based on the amount of peptone used and the efficiency of rORF2 protein synthesis, peptone concentration of 0.8 % was selected for further experiments.

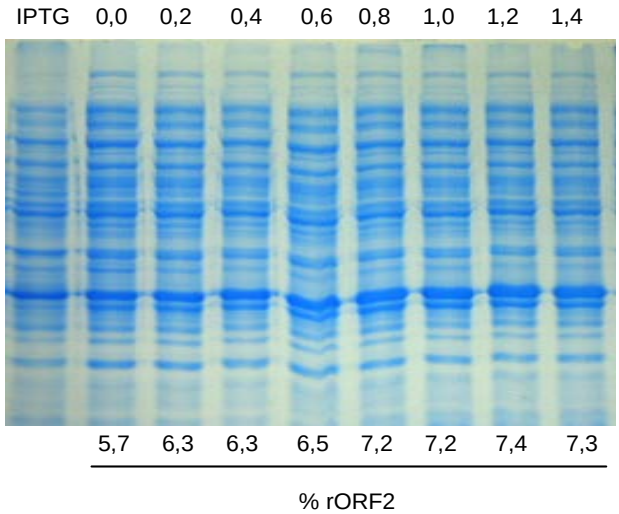

Fig. 4 Protein pattern of total extract from biomass grown in medium with different peptone concentrations.

3.1.4 Yeast extract

Yeast extract is a source of nitrogen, vitamin B, sugar and minerals for bacterial growth and development. According to previous studies, yeast extract was a very good source of nutrition to increase the level of bacterial growth (Barrette *et al.*, 2001; Li *et al.*, 2011). However, strong growth and high biomass density sometimes

decreases the level of recombinant protein expression due to the presence of bacterial populations not carry plasmids (Studier, 2005). Usually the concentration of yeast extract in nutrient medium was 0.5% (Sambrook and Russell, 2001). In this study, a range of yeast extract concentrations (0.1 - 0.6%) was investigated in order to assess its impact to rORF2 protein expression level. Results showed that the target protein was obtained the lowest (6.8%) at a concentration of 0.1% and increased when the concentration of yeast extract risen. However, the target protein was not significantly increased between 0.4 and 0.6% yeast extract used (Fig. 5). Thus, the concentration of yeast extract selected for further studies was 0.4%.

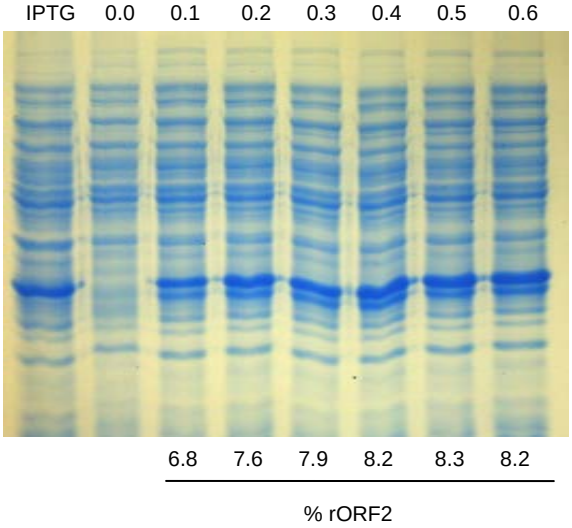

Fig. 5 Protein pattern of total extract from biomass grown in medium with different yeast extract concentrations.

3.2 Culture conditions for auto-induction of recombinant protein expression in *E. coli*

3.2.1. Temperature

Temperature is a factor affecting the growth rate of bacteria and simultaneously it will affect the expression level of recombinant target proteins.

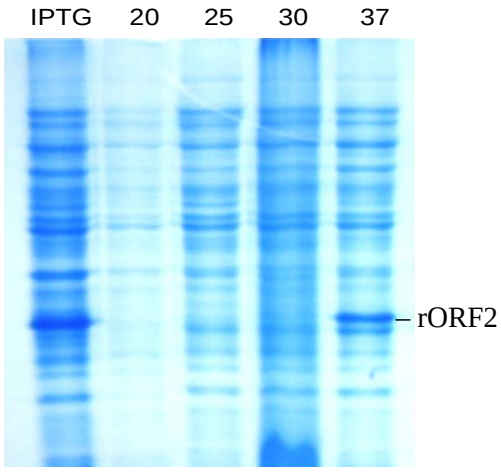

Fig. 6 Protein pattern of total extract from biomass grown at different temperatures.

Results at four different temperatures of 20, 25, 30 and 37°C showed that recombinant protein ORF2 was only expressed at 37^{o}C (Fig. 6). Therefore, temperature 37 °C was selected for fermentation of recombinant synthesis rORF2 in *E. coli*.

3.2.2. The kinetics of growth and biosynthesis of recombinant protein ORF2

As mentioned above, the expression of recombinant proteins using lactose as inducer is auto-induction process. Therefore, the determination of the kinetics of growth and protein synthesis is necessary to determine the appropriate stop point of fermentation. The monitoring result showed that the growth process entered the log phase after 5 hours, reached maximum growth after 14 hours and then moved to stationary phase (Fig. 7A).

Protein electrophoresis result showed that the recombinant protein ORF2 started to express after 8 hours and the expression gradually increased to 14 hours of fermentation; however protein synthesis was slightly increased when bacteria come into the stationary phase (Fig. 7B). The strong protein expression was determined at the end of the log phase when biomass density reached OD600 = 3.5. This result was completely consistent with previous study (Studier, 2005).

CONCLUSION

We determined the appropriate nutrient composition and concentration using lactose as an alternative inducer for high expression of recombinant ORF2 protein in *E. coli*. The appropriate temperature for culture of bacteria expressing rORF2 was 37^{o}C. Synthesis of recombinant protein ORF2 was started after 8 hours culture and reached the maximum level when entering the stationary phase. Optimal conditions established in this research are suitable for the large-scale fermentation producing recombinant ORF2 protein for the next researches.

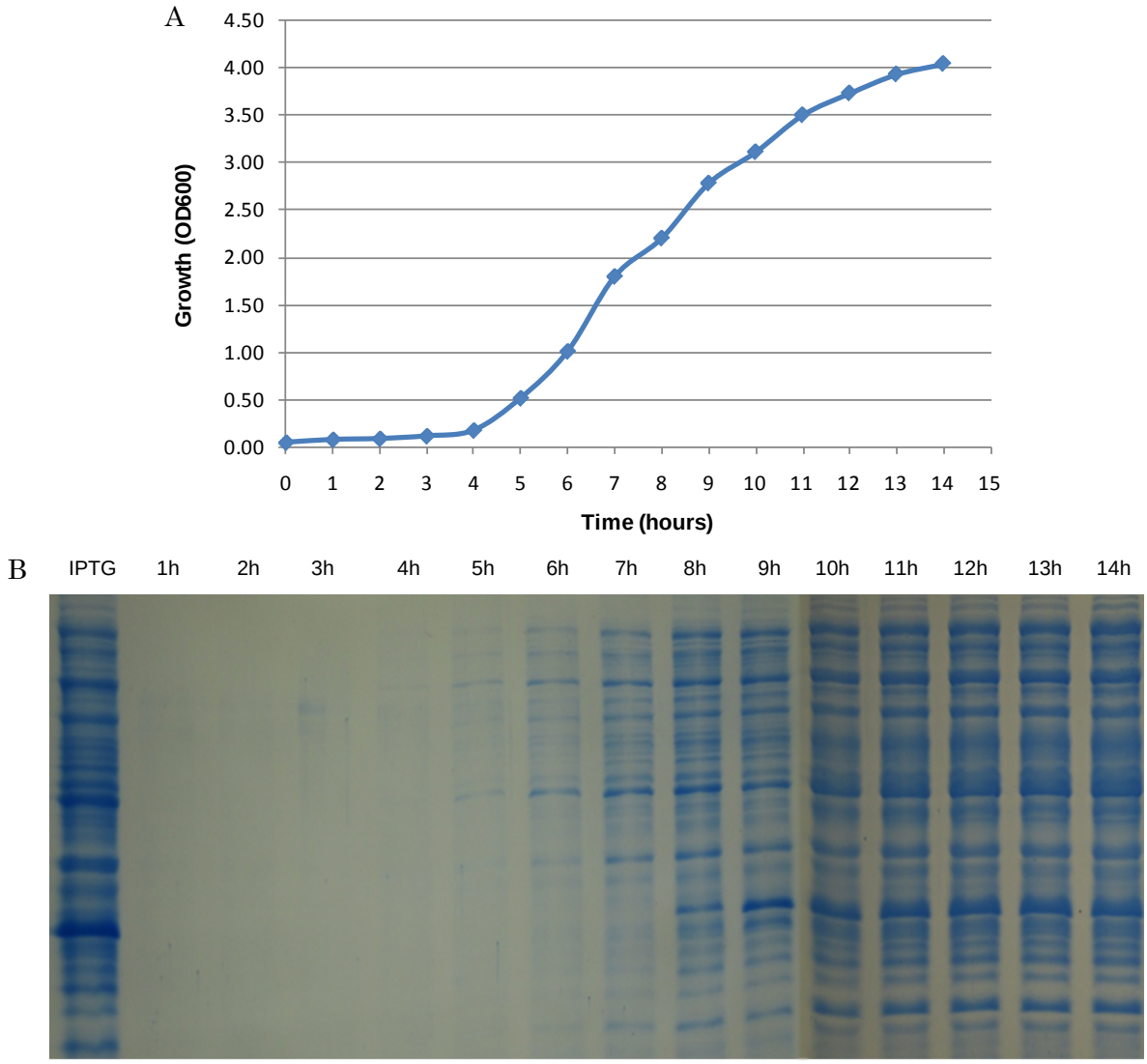

Fig. 7 (A) Growth and (B) protein pattern of total extract from biomass grown at different time points (1-14 hours).

REFERENCES

Barrette J, Champagne CP, Goulet J (2001). Growth-promoting properties of yeast extracts produced at different pH values, with different autolysis promoters and bacterial populations. *J Chem Tech Biotechnol* 76(2): 203-209.

Brooker RJ, Widmaier EP, Graham LE, Stiling PD (2008). Biology. *McGraw-Hill, Vol 1.*

Chen R (2012). Bacterial expression systems for recombinant protein production: *E. coli* and beyond. *Biotechnol Adv*. 30(5): 1102-1107.

Dubendorff JW, Studier FW (1991). Controlling basal expression on an inducible T7 expression system by blocking the target T7 promoter with lac repressor. *J. Moll. Biol.* 219: pp 45-59.

Eppler T, Postma P, Schutz A, Volker U, Boos W (2002). Glycerol-3-phosphate-induced catabolite repression in *Escherichia coli*. *J. Bacteriol.* 184: 3044-3052.

Inada K, Kimata K, Aiba H (1996). Mechanism responsible for glucose-lactose diauxie in *Escherichia coli*: Challenge to the cAMP model. *Genes Cells* 1: 293-301.

Laemmli, U.K., 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685

Li M, Liao X, Zhang D, Du G, Chen J (2011). Yeast extract promotes cell growth and induces production of polyvinyl alcohol-degrading enzymes. *Enzyme Res.* doi: 10.4061/2011/179819

Meadow ND, Fox DK, Roseman S (1990). The bacterial phosphoenolpyruvate: glycose phosphoenol transferase system. *Annua. Rev. Biochem*. 59: 497-542.

Pei, X.L., Wang, Q.Y., Li, C.L., Qiu, X.F., Xie, K.L., Huang, L.F., Wang, A.M., Zeng, Z.W., Xie, T., 2011, Efficient production of a thermophilic 2-deoxyribose-5-phosphate aldolase in glucose-limited fed-batch cultivations of Escherichia coli by continuous lactose induction strategy. Appl Biochem Biotechnol 165, 416-425.

Sambrook J, Russell DW (2001). Molecular cloning. A Laboratory Manual, 3rd ed. *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.*

Studier FW (2005). Protein production by auto-induction in high-density shaking cultures. *Protein expression and purification* 41: 207-234.

Tian H, Tang L, Wang Y, Wang X, Guan L, Zhang J, Wu X, Li X (2011). Lactose induction increase production of recombinant keratinocyte growth factor-2 in *Escherichia coli. International Journal of peptide research and therapeutics* 17(2): 123-129.

Truong Quoc Phong received the B.S. (2001), M.S. (2004), degrees in Biochemistry from University of Science, Hanoi National University. He received the Doctor degree (2008) in Molecular Biology from Greifswald University, Germnay. He is a Head of Laboratory for Proteomics, Hanoi University of Science and Technology. His current interests include: Production of recombinant proteins/enzymes, Establishment of new methods for rapid test and detection of target organisms, Application of proteomics in biomarker screening and early diagnosis of dangerous human disease