CIBTech Journal of Biotechnology ISSN: 2319–3859 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/cjb.htm
2016 Vol. 5 (1) January-March, pp.6-15/Noi et al.
Research Article

THE STEPS CULTURE FOR PRODUCTION OF RECOMBINANT S1 DOMAIN OF THE PORCINE EPIDEMIC DIARRHEA VIRUS SPIKE (PEDV-S1) PROTEIN IN IMMOBILIZED *SPODOPTERA FRUGIPERDA* (SF9) CELLS

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ABSTRACT

Porcine epidemic diarrhea (PED) is an enteric disease in swine caused by an alphacoronavirus. It affects swine of all ages causing acute diarrhea and can lead to severe dehydration and death in suckling piglets. The present study was to develop a two-step culture to express the recombinant PEDV-S1 protein using the immobilized *Spodoptera frugiperda* (Sf9) cells. The first step, Sf9 cells were cultured and harvested in eutrophic medium containing 10% fetal bovine serum. The second step, Sf9 cells were immobilized using silk fibroin hydrogel and cultured in a stirred-tank bioreactor after infection by recombinant baculovirus expressing the full length PEDV-S1 gene. The data shows that the maximum yield of recombinant PEDV-S1 protein reached the concentration of 145 mg/L. The results reveal that the immobilized cells and two step culture could extend the expression period and significantly raise the production of the recombinant protein.

Keywords: *Spodoptera Frugiperda Cells, Baculovirus, Immobilization, PEDV-S1 Protein*

INTRODUCTION

Porcine epidemic diarrhoea (PED) is a highly contagious infectious disease caused by a coronavirus, porcine epidemic diarrhoea virus (PEDV). It causes acute and watery diarrhoea in pigs of all ages although the most severe signs are reported in piglets less than two weeks old, in which diarrhoea leads to severe dehydration and is associated with mortalities which can reach up to 100 % in affected litters (Pensaert & De Bouck, 1978). The PEDV genome is composed of the 50 untranslated region (UTR), at least seven open reading frames (ORF1a, ORF1b, and ORF2 through 6), and the 30 UTR (Kocherhans *et al.*, 2001). The two large ORFs 1a and 1b make up the 50 two-thirds of the genome and encode the non-structural replicase genes. The remaining ORFs in the 30 terminal region code for four major structural

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proteins: the 150–220-kDa glycosylated spike (S) protein, the 20–30-kDa membrane (M) protein, the 7-kDa envelope (E) protein, and the 58-kDa nucleocapsid (N) protein (Duarte *et al.,* 1994; Lee & Lee, 2014).

The S protein of PEDV is a type I membrane glycoprotein composed of 1,383 to 1,386 amino acids (Bosch *et al.,* 2003), depending on the strain. It contains a putative signal peptide (aa 1–24), a large extracellular region, a single transmembrane domain (aa 1,334–1,356), and a short cytoplasmic tail. Although PEDV has an uncleaved S protein because it lacks a furin cleavage site, the S protein can be divided into S1 (aa 1–735) and S2 (736 - the last aa) domains based on homology with S proteins of other cor- onaviruses (Duarte & Laude, 1994; Jackwood *et al.,* 2001; Lee *et al.,* 2010a; Sturman & Holmes, 1984). Like other coronavirus S proteins, the PEDV S protein is known to play a pivotal role, interacting with the cellular receptor to mediate viral entry and inducing neutralizing antibodies in the natural host (Bosch *et al.,* 2003; Chang *et al.,* 2002). More precisely, previous studies have shown that the S1domain includes the main neutralizing epitopes and the receptor-binding region (Lee *et al.,* 2011; Sun *et al.,* 2007). Furthermore, along with the full-length S gene, the S1 portion is known to be a suitable region for determining genetic relatedness among the different PEDV isolates and for developing differential diagnostic assays (Chen *et al.,* 2014; Lee *et al.,* 2010b). Considering these molecular and biological features of the S1 domain, it would be an appropriate target for developing effective vaccines against PEDV.

The baculovirus-insect cell system has been extensively used for the expression of recombinant protein for basic research applications, vaccines and diagnostic and therapeutic proteins. It has a disadvantage for the expression system that the specific productivity is decreased as the cell density for infection by baculoviruses is increased (Reuveny *et al.,* 1993). Previous studies have shown that serum in the medium played a promoting role on recombinant protein production up to post-infection day 1, but is not essential thereafter (Jardin *et al.,* 2007). Therefore, it is necessary to develop a novel culture method to separate the cell amplification and infection expression. Silk fibroin is an excellent biomaterial for the formation hydrogel for encapsulating cells since it has good mechanical properties, biocompatibility, and structure network.

Silk fibroin has been applied in drug release, tissue engineering and cell immobilization. In this study, Sf9 insect cells were immobilized using silk fibroin to produce recombinant PEDV-S1 protein successfully. In addition, a two step culture protocol was investigated. The first step, the insect cells were abundantly proliferated in Grace's insect medium containing serum and glucose. The second step, the insect cells were immobilized and infected with recombinant virus at the proper time in modified Grace's medium (MGM; Grace's medium containing 0.33% lactoalbumin hydrolysate and 0.33% yeastolate) without serum. The two step culture could facilitate downsteam processing and purification of target products and thereby reduces production costs.

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MATERIALS AND METHODS

Cell Lines, Baculovirus and Medium

The Bac-to-Bac expression system and Sf9 cells were purchased from Invitrogen Company (USA). The Sf9 cells were cultured in Grace's insect medium supplemented with 10% (v/v) fetal bovine serum and glucose (Gibco, USA). The immobilized Sf9 cells were grown in serum-free melanocyte growth medium (MGM). All the cell culture processes were carried out at 27°C. All medium contains 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 g/mL amphotericin. The recombinant baculovirus expressing the full length PEDV-S1 gene of high titer virus (10^8 pfu/mL as determined by plaque assay), was obtained and sustained by Vaccine and Adjuvant lab. The pFastBacHTb_PEDV-S1 was propagated by infecting Sf9 cells and harvested from supernatant centrifugated at 1,000 rpm for 5 min to remove cell debris. All virus stocks were stored at 4°C and protected from light to ensure maintenance of titer for PEDV-S1 expression. Cell density was determined by microscopically counting with a Burker-Turk hemocytometer, while cell viability was examined by trypan blue exclusion test.

Shake Flask Culture of the Sf9 Insect Cells

Corning 500 mL disposable Erlenmeyer flasks were used with a working volume of 80 mL. After suspension in fresh Grace's insect medium, Sf9 cells were grown for a few days at 27°C in a spinner flask with constant stirring at around 120 rpm. Cells in the exponential growth phase were collected by centrifugation and resuspended in fresh serum-containing medium. The cells were incubated for two days at 27°C on a reciprocal shaker for inoculation. Following removal of the cell suspension, 80 mL of fresh serum-supplemented medium was added to each flask and inoculation on a reciprocal shaker was resumed. The culture medium was replaced completely with fresh serum-supplemented medium every two days.

Immobilized Cells Culture in Bioreactor

The glass bioreactor vessel consisted of a cylindrical body and a rounded bottom. Mixing was performed by an impeller with three blades rotating at the rate of ranging from 0 to 150 rpm in the reactor. A mixed gas of oxygen and nitrogen was introduced from a nozzle at a constant rate set manually according to oxygen demanded. The dissolved oxygen (DO) concentration in the culture medium was controlled at 30% oxygen saturation by automatically changing the composition of the supplied gas based on the reading of a DO sensor. The temperature was kept constant at 27°C by recycling water from a water bath through the tank. The pH was monitored using a pH sensor. But, even without active adjustment, the pH did not change substantially in the course of the cultures. Antifoam agent (Antifoam C Emulsion, Sigma) was added into the bioreactor at the concentration of 0.1 mL/L if necessary.

The process of cells immobilization using silk fibroin was described as the following. Silk cocoons were shattered and then degelatinized twice in boiling water containing 0.5% (w/v) Na2CO3 for 1 h. The degelatinized silk fibroin was washed with the double distilled water. The clean silk fibroin was dissolved

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and boiled in 50% (w/v) CaCl2 at 100°C for 10 min. The cooled silk fibroin solution was dialyzed for 72 h to remove salt using 8 kDa dialysis-membrane. Finally, the silk fibroin was filtered through film (Φ 5 μm, Pharmacia) and subsequently became achroic transparent liquor. For making silk fibroin hydrogel the sterilized silk fibroin remained at 30°C and was mixed with Sf9 cells. The mixture gel subsequently was added into the serum-free MGM medium and stored under 8°C for 30 min. Further, the silk fibroin hydrogel was made into hydrogel beads of about 0.5 mm in diameter (Kim *et al.,* 2004; Matsumoto *et al.,* 2006; Wang *et al.,* 2008).

The time of Bac-PEDV-S1 addition was designated as post-infection time zero. The hydrogel beads and the culture broth were sampled every day to measure the immobilized cell density and the PEDV-S1 protein yield.

Quantitative Assay of Recombinant PEDV-S1

The recombinant baculovirus Bac-PEDV-S1 infected Sf9 cells when the cell culture reached the later of log phase according to the growth curve (data not shown for recombinant PEDV-S1 expression). The immobilized cells were harvested at 48 to 72 h post-infection when cells were still mostly viable (between 60 and ~ 90%) and sonicated five times for 10 s by 3 min intervals on ice, using ultrasonicator at 50% power. The lysate was cleared by centrifugation at 10,000 rpm for 30 min at 4°C to remove cell debris. The supernatant was stored at -80°C until protein purification.

The recombinant PEDV-S1 protein was determined by enzyme-linked immunosorbent assay (ELISA) (Ausubel *et al.,* 1997). The ELISA standard curve was generated using a series of PEDV-S1 standards VTI540 (Meridian life science, USA) from 1.6 to 100 ng/mL (Wang & Fan, 2000). Appropriate wells of 96-well medium binding polystyrene plates (IWAKI, Japan) were coated with serially diluted cell culture samples (100 μl/well).

The plates were sealed with a parafilm sheet and incubated in a humidified chamber at 4°C overnight. The plates were allowed to equilibrate to room temperature and were washed three times with freshly prepared phosphate buffered solution containing 0.05% Tween 20 (PBST) using a ELX405 auto plate washer (Bio-Tek Instruments Inc., USA). After washing, the plates were blocked with 1% BSA-PBS at room temperature for 2 h. After the plates were washed with PBST three times, mouse anti-Histag monoclonal antibody C65019M (Meridian life science, USA) were diluted 1:700 in PBST immediately before dispensing into the antigen-coated wells.

Hundred microlitres of diluted mouse anti-Histag monoclonal antibody was then incubated in the cell culture samples coated polystyrene plates at 37°C for 1 h. All incubations in future steps were also performed at 37°C. After incubation, the wells were washed three times. Each well was then incubated with 100 μl of a 1: 2000 (v/v) dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Roche, USA) for 45 min. The plates were washed three times. The wells were incubated in the dark with 100 μl of developing solution prepared immediately before use at room temperature for 20 min. The

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developing reaction in each well was stopped with 40 µl of 2M H2SO4. The optical density at 490 nm was determined for each well with a ELX800 Universal Microplate Reader (Bio-Tek instruments Inc., USA).

The amounts of recombinant PEDV-S1 were calculated from the OD490 values that were in the descending portion of the ELISA standard curve for the PEDV-S1 standards.

RESULTS AND DISCUSSION

The First Step for Cell Amplification in Shake Flask

The Sf9 insect cells were cultured in Grace's medium containing 10% fetal bovine serum (FBS) supplemented with limited concentration glucose to obtain the maximum cell density. In the test, the glucose feeding was started at 50 h when the initial quantity of glucose was consumed. The controlled level of glucose was determined depending on the cell density.

The results indicate that when the residual concentration of glucose was about 8 mM, the cell density reached 6.5×10^6 cells/mL. A higher concentration of glucose did not give the markable increase of cell density (Figure 1).

The Second Step for Recombinant PEDV-S1 Protein Expression

In this step, the large amount of Sf9 cells obtained at the optimal condition of shake flask culture were immobilized at a density of over 10^6cells/mL using silk fibroin hydrogel and further made into beads according to the method described in the materials and methods.

The immobilized cells were suspended and cultivated using serum-free MGM medium in the 5 L stirred-tank bioreactor. As for the immobilization cell culture, the stirring speed was an important factor to influence the cell growth and oxygen transfer. Therefore, the optimal stirring speed was determined in the 5 L bioreactor.

The figures showed that the cell density reached the maximum over 8×10^6 cells/mL beads at the stirring speed of 100 rpm. If the stirring speed exceeded 120 rpm, the hydrogel beads were damaged greatly, resulting in decreased viability (Figure 2).

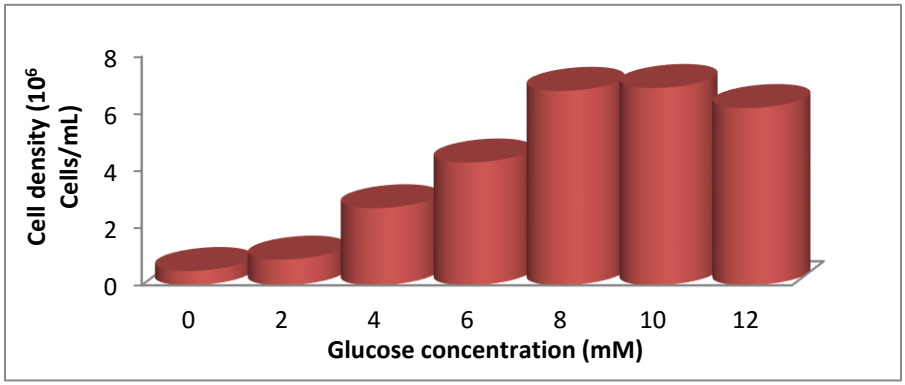

Figure 1: The Effect of Glucose on Sf9 Cell Density

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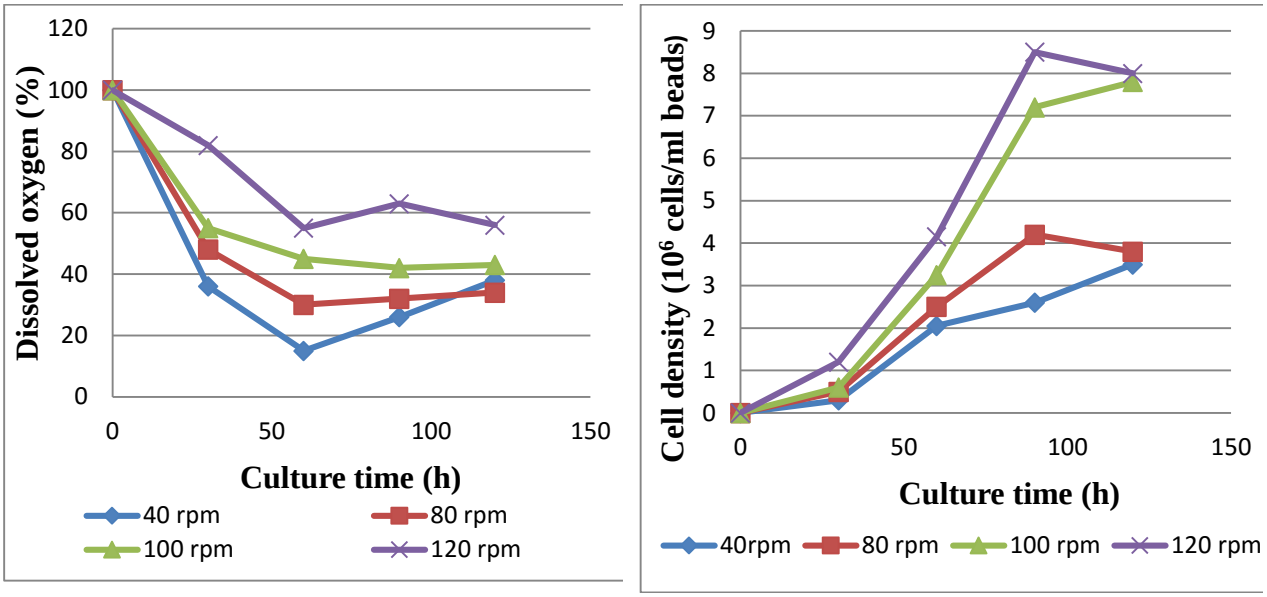

Figure 2: The Effect of Stirring Speed on the DO and Sf9 Cell Density

Effect of Multiplicity of Infection (MOI) on the Production of PEDV-S1 Protein

The effect of multiplicity of infection (MOI) was examined on recombinant PEDV-S1 production ranging from 1 to 10. Fed-batch cultures were infected by Bac-PEDV-S1 after four days when the cell density reached about 6×10^6 cells/mL. The highest yield of recombinant PEDV-S1 was obtained at MOI of about 5 with 145 mg/L (Figure 3).

Determination of Infection time

Following growth of the immobilized cells to a density of over 10^6 cells/mL beads, a high-titer Bac-PEDV-S1 was added at MOI of 5. The time of Bac-PEDV-S1 addition was designated as post-infection time zero. Time of infection is an important factor for obtaining high yield recombinant protein. Previous studies had shown that it was the proper moment of infection when the cell density was going to reach the maximum.

In this present study, the non-immobilized cells were cultured in the 5 L bioreactor as the contrast to the culture of immobilized cells. The results show that the immobilized cells grew more slowly than the non-immobilized cells. The optimal infection time should be about 120 to 130 h (Figure 4).

Infection with PEDV-S1 remains a prevalent and potentially serious health problem of animal. The PEDV-S1 is a primary vaccine candidate for the application in swine. An efficient and economical way of producing PEDV-S1 must be established. The baculovirus expression system has a number of potential benefits, such as high expression level, correct folding and post-translational modification, and production of biologically active proteins for analysis. Compared with the traditional suspended-cell culture immobilization of insect cells has some attractive characteristics, such as increasing the biomass protecting cells from shear force and facilitating the purification of the recombinant protein.

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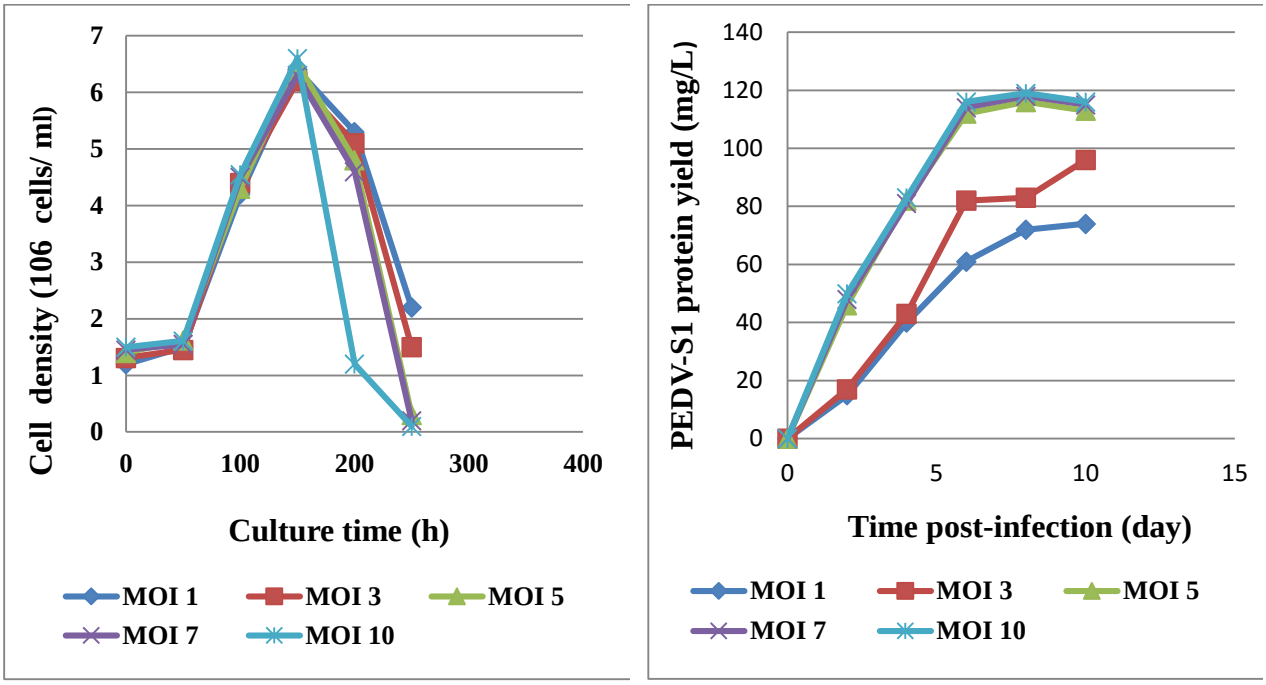

Figure 3: Optimal MOI for Sf9 Cells Growth and the Recombinant PEDV-S1 Protein Production

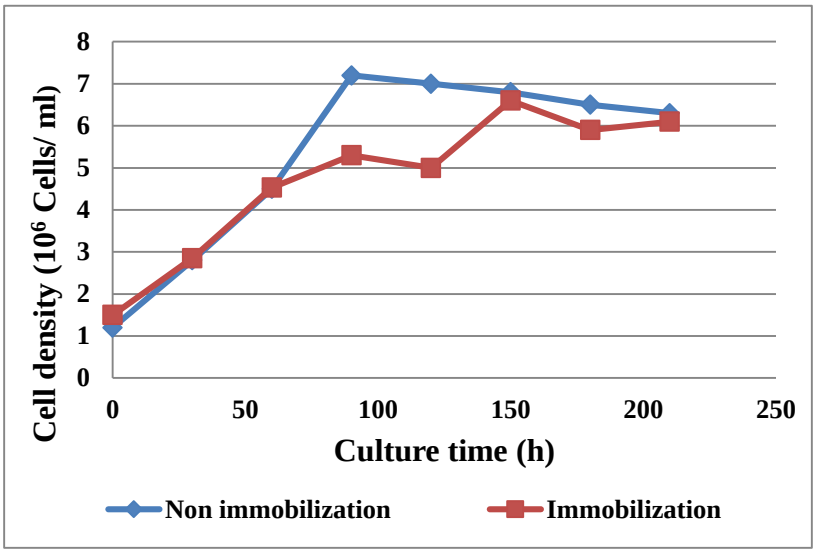

Figure 4: The Cells Growth Process of the Immobilized and Non-Immobilized Sf9 Cells

One of the crucial problem in baculovirus-insect cell expression system was that the interesting protein production was decreased while the cell density for infection was increased. In most cases, the productivity could be improved by supplying the fresh growth medium for cell culture during virus infection. However, the above procedure is impractical on a large scale. In order to obtain higher protein production under the condition of corresponding higher cell density, a combination of cell immobilization

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and two step culture procedure was developed. In the present study, a two-step culture of Sf9 cells immobilized within silk fibroin hydrogel beads was carried out in 500 mL shake flasks and a 5 L stirred-tank bioreactor. The first step was to culture cells and the second was to infect cells by Bac-PEDV-S1 for protein expression. Silk fibroin was a suitable bio-material to be used as immobilization vector (Matsumoto *et al.*, 2006).

In the first step of shake flask culture, Sf9 cells were amplified in Grace's insect medium supplemented with fetal bovine serum and glucose, and which could increase the cell density to 10^6 cells/mL. During the baculovirus infection step, the infection time was determined at the logarithmic growth phase of immobilized Sf9 cells. Recombinant protein production by densely immobilized cells in serum-free MGM medium could simplify separation and purification processes and reduce the manufacturing costs of cellular products. In this research, the dissolved oxygen concentration was maintained at 30% oxygen saturation in the bioreactor.

Maybe, excessive DO conditions can bring the formation of nascent oxygen, superoxide and peroxide which destroy cellular components and then destroy the cell growth. In our previous studies, the protein production by immobilized Sf9 insect cells was significantly depressed when the DO was below than 30%. The DO level was higher than the culture of non-immobilized cells since the immobilization limited the transfer of oxygen. Specially, the Bac-to-Bac expression system was chosen since it is a valid and multipurpose system for high production of recombinant protein. The specific PEDV-S1 protein productivity obtained by immobilized cell in bioreactor culture was higher than that by non-immobilized cell culture.

Due to the fact that glucose, stirring speed, MOI and infection time are the most essential factors affecting Sf9 cells growth, these factors have been investigated with the aim of increasing the final cell density for high protein production. In this study, the concentrations of glucose, stirring speed, MOI and infection time were optimized in cell culture. Higher cell density could be achieved at the conditions of 8 mM glucose. In contrast, lower concentrations of glucose could not provide enough carbohydrate for cell metabolism and propagation, and higher concentrations of glucose were not beneficial to cell growth due to the production of extra lactic acid or ammonia. Although the higher stirring speed was a crucial factor for increasing the concentration of the dissolved oxygen and nutrition transfer in the immobilized cell culture, the shear force of agitator at high speed would damage the gel beads and cells. Higher cell density could be achieved at the stirring speed of 100 rpm, infection time of 120 to 130 h and MOI of 5.

At the optimal conditions, the yield of recombinant PEDV-S1 protein was up to 145 mg/L cell culture, to our knowledge this is the first report of the combination of immobilization and two-step culture for the recombinant PEDV-S1 protein expression in bioreactor. In the further research, we will optimize the culture conditions of the two step culture to separate the medium from the bioreactor and replace with other medium in order to obtain higher protein productivity.

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ACKNOWLEDGMENTS

The authors would like to thank Dr. Li-Ting Cheng, Graduate Institute of Animal Vaccine Technology, College of Veterinary Medicine for his editorial comments on this letter.

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