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Original Article

Evaluation of Pertussis Toxin Expression in B2 and THIJS Media

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ABSTRACT

Whole-cell pertussis vaccine (wP) has been imperative and highly effective in preventing childhood deaths due to pertussis. Pertussis toxin is one of the virulence factors of *Bordetella pertussis* in all available pertussis vaccines. wP production in Razi Vaccine and Serum Research Institute is according to bioreactor culture of *B. pertussis* strains in B2 medium. The aim of this study was to evaluate *B. pertussis* strain 509 PT production in B2 and Thalen-IJssel (THIJS) media by Chinese Hamster Ovary (CHO) cell and enzyme-linked immunosorbent assay methods (ELISA). In the current study, *B. pertussis* strain 509 was cultured in B2 and THIJS media. Six samples were taken during the log growth phase within 2-3 h intervals (triplicate). The growth rate was calculated using opacity and the quantification of cell-associated and released PT measured by ELISA and CHO cell assays. THIJS medium was significantly showed an increase in the bacterial growth rate. During the first 29 h, bacterial concentrations in B2 and THIJS culture medium were 19 and 29 IOU, respectively. In THIJS medium, greater amount of pertussis toxin production was cell-associated. In B2 medium, maximum cell-associated toxin by ELISA and CHO cell assays were in the ODs of 1.1 and 0.9 and for THIJS medium in the ODs of 1.6 and 1.1, respectively. *B. pertussis* strain 509 in THIJS medium produced higher cell mass and cell-associated pertussis toxin than that of B2. It can be used for the production of whole-cell vaccine with higher pertussis toxin and accordingly using lower biomass per dose leading to the reduction of vaccine toxicity.

Keywords: *Bordetella pertussis*, Pertussis Vaccine, ELISA, CHO cells, Pertussis Toxin

Évaluation de l'expression de la toxine de coqueluche dans les milieux B2 et THIJS

Résumé: Le vaccin anticoquelucheux à germes entièrs a été impératif et très efficace dans la prévention des décès d'enfants dus à la coqueluche. La toxine de la coqueluche est l'un des facteurs de virulence de *Bordetella pertussis* dans tous les vaccins anticoquelucheux disponibles. La production de wP à l'Institut de recherche sur les vaccins et les sérums de Razi est menée en conformité à la culture en bioréacteur des souches de *B. pertussis* dans un milieu B2. Le but de cette étude était d'évaluer la production de la souche 509 PT de *B. pertussis* dans les milieux B2 et Thalen-IJssel (THIJS) par dosage sur la cellule d'ovaire de hamster chinois (CHO) et par ELISA. Dans cetteétude, la souche 509 *de B. pertussis* a été cultivée dans les milieux B2 et THIJS. Six échantillons ont été prélevés au cours de la phase de croissance logarithmique dans des intervalles de 2 à 3 heures (en triple exemplaire). Le taux de croissance a été calculé en utilisant l'opacité et la quantification du PT libéré et associé aux cellules mesuré par les tests ELISA et cellulaire CHO. Le milieu THIJS a montré de manière significative une augmentation du taux de croissance bactérienne. Au cours des 29 premières heures, les concentrations bactériennes dans les milieux de culture B2 et THIJS étaient respectivement de 19 et 29 IOU.

Dans le milieu THIJS, une plus grande quantité de production de toxine de coqueluche était associée aux cellules. Dans le milieu B2, les concentrations maximales de toxines obtenues par les tests ELISA et CHO étaient dans les ODs de 1,1 et 0,9 et dans le milieu THIJS dans les ODs de 1,6 et 1,1, respectivement. La cuture de la souche 509 de *B. pertussis* dans le milieu THIJS a produit un taux de toxines de pertussis par cellule et par masse cellulaire supérieure à celui obtenu en mileieu B2. Ce dernier peut être utilisé pour la production de vaccins à germes entiers contenant une concentration plus importante de toxine anticoqueluche et, par conséquent, d'utiliser une biomasse moins importante de microorganism par dose entraînant une réduction de la toxicité du vaccin.

Mots-clés: *Bordetella pertussis*, vaccin anticoquelucheux, ELISA, cellules de CHO, toxine de coqueluche

INTRODUCTION

Pertussis is a contagious and respiratory infection disease caused by *Bordetella pertussis*. In 2008, pertussis was globally associated with an estimated 16 million cases and 195000 deaths (Black et al., 2010). In 2013, out of 3.257 million (51·8%) children who died of infectious diseases, pertussis accounted for 2% of the mortalities in this population (Liu et al., 2015). Most deaths occurred in neonates who were either unvaccinated or incompletely vaccinated. Widespread vaccination has led to lower incidence of disease and deaths caused by pertussis (Edwards, 2005); however, pertussis remains one of the top 10 causes of death in children (Crowcroft et al., 2003). Whole cell pertussis vaccines (wP) are used as a part of combined DTP vaccine. wP is still produced and used in both developed and developing countries and has successfully contributed in the control of whooping cough (Witt et al., 2013). Strategic Advisory Group of Experts on immunization at World Health Organization (WHO) has recommended that countries considering a switch from wP to acellular vaccine (aP) should await for further guidance (2014). For wP production, a high yield of virulence factors per unit biomass production is desirable. The more vaccine doses per production run obtained, the lower the costs (Thalen et al., 1999). The amount of lipopolysaccharides per dose for safety is more important than the yield of virulence factors which consequently leads to less adverse reactions. As the culture supernatant is discarded, it is important for cellular vaccines (wP) that all produced virulence factors remain cell-associated (Thalen et al., 2006a). Pertussis toxin (PT) is a member of the AB5 family of toxins, comprised of six subunits, namely S1, S2, S3, S4, and S5 in a 1:1:1:2:1 ratio (Craig-Mylius and Weiss, 2000). S1 is the A subunit of PT which causes damage to host cells. PT seems to require a secretary complex pathway for assembly and secretion from the bacterium (Coutte and Locht, 2015). The growth rate, virulence factors properties, and metabolism of *B. pertussis* depend on various parameters of cultivation (Thalen et al., 2006b; Streefland et al., 2009a). The most important factor is the selection of appropriate medium and the time of stopping culture. Fine and Clarkson showed that vaccine effectiveness of different manufacturers and batches of a vaccine manufacturer were different (Fine and Clarkson, 1987). Stephen Hoff studied differences in immunogenicity of two different wP vaccines in children and showed a significant difference regarding the immunogenicity in children (Steinhoff et al., 1995). Westdijk and Thalen indicated that the amount of free and cell associated antigens vary in culture duration (Westdijk et al., 1997; Thalen et al., 2006b). Maximum toxin production was enhanced during the logarithmic growth phase and decreased from start to the end of the stationary phase. Nevertheless, results indicated batch to batch inconsistency due to culture parameters variation and stopping times (van de Waterbeemd et al., 2009). Potency of the wP vaccine is related to the amount of antigenic determinants expressed on the surface of the

B. pertussis. Verification and determination of PT in culture duration is very critical due to the fact that the most antigenic immunogenicity of the vaccine in wP is related to PT and its expression is representative for many expressed antigenic determinants (Thalen et al., 2006b). ELISA test could not discriminate between inactive and active PT. CHO cell clustering assay which is generated by a promotor regarding B oligomer can be used for measuring of PT holotoxin activity (Gillenius et al., 1985). *B. pertussis* is a fastidious microorganism and can be challenging to grow it in different media (Thalen et al., 1999). For wP vaccine production, *B. pertussis* is typically cultured in B2 medium and stopped according to optical density. The quantitative and qualitative detection of PT during culture enhance quality and consistency of wP vaccine production. The aim of the present study was to evaluate growth yield and PT production of *B. pertussis* (vaccine) strain 509 cultured in B2 and THIJS media. Growth was monitored by measuring pH, optical density at OD590 and pertussis toxin activity and production quantitatively by CHO cell and ELISA test, respectively.

MATERIAL AND METHODS

B. pertussis vaccine strain 509, which is one of the two strains used in DTP vaccine in 10 IOU per dose, was obtained from Razi Vaccine and Serum Research Institute (RVSRI). The strain is maintained in lyophilized state at -20 ºC. A lyophilized ampoule was inoculated into 100 ml Verwey medium (Verwey et al., 1949) on a 200 revolutions per minute (rpm) rotary shaker at 35 °C for 2-3 days until it reached 20±4 IOU/ml. At the end of the culture, growth and purity were checked by gram staining and cultured on the BG and nutrient agar. 10 ml of pure culture was used to inoculate a second shake flask with 200 ml Verwey medium. After incubation for 24h at 35°C, glycerol (87% weight volume^{-1}) was added to a final concentration of 10% volume volume^{-1}, and then the suspension was frozen in aliquots of 5 ml at −70 °C. The preculture medium was inoculated with 10 ml of these −70 °C working seed lots. Pre-warmed 0.5 L bottles containing 100 ml medium were inoculated with a 5% inoculum from a −70 °C stock and incubated for 24 h to an OD of 1. This culture was used to inoculate THIJS and B2 media using a 1% inoculum. The optical density was adjusted to 0.02. Bottles were incubated at 35°C, on a rotary shaker at 200 rpm. The same procedure was carried out for both the THIJS and B2 media. All experimental cultures were performed in triplicate and all samples analyzed individually.

Preparation of media. Composition of B2 basal medium for 1 liter: Bactocasamino acid (BCA) 12g, L-glutamic acid 10g, NaCl 5g, KH_2PO_4 1g, $MgSO_4$ 0/2g, $CaCl_2$ 3g, soluble starch 3g. Supplement composition for 1 liter: Yeast extract 4g, $FeSO_4$ 20mg, $CuSO_4$ 1mg, Glutathione 20mg. The basal medium was heat-sterilized at 110 °C for 20 minutes; the supplement was filter-sterilized into bottle before inoculation. Per liter basal medium, 20 ml supplement was added. Composition of THIJS basal medium for 1 liter: NaCl 3.319g, NH_4Cl 0.107g, KH_2PO_4 0.5g, KCl 0.5g, $MgCl_2 \cdot 6H_2O$ 0.1g, TRIS 1.525g, Na-glutamate$\cdot H_2O$ 1.87g, L-(+)-lactate (40% weight volume^{-1}) 3.76 ml, 5M NaOH solution 2.071 ml. Supplement composition for 1 liter: L-cystine 4.0g, $CaCl_2 \cdot 2H_2O$ 2.6g, glutathione (reduced) 10g, $FeSO_4 \cdot 7H_2O$ 1g, nicotinic acid 0.4g, L-ascorbic acid 2g and 1M HCl 2ml. The basal medium was heat-sterilized at 110 °C for 20 minutes. Moreover, the supplement was filter-sterilized into bottle before inoculation. 20 ml supplement was added to per liter basal medium.

Cell mass determination by opacity test. Opacity Reference standard 10 IOU WHO 5th IRP was used for opacity control. The cell mass concentration during cultivation process was determined by measuring opacity using opacity standard tube. The samples were aseptically collected at different time intervals, 17, 20, 23, 25, 27, and 29 h for all the batches. The optical density of the suspensions was measured using

spectrophotometer (Pharmacia, ultraspec 2000) at 590 nm.

Total PT amount and activity determination. THIJS and B2 cultures were tested in triplicate. Samples were centrifuged at 8000g for 10 min at 4°C and supernatant were filtered by 0.22 µm. Subsequently, cell pellet resuspended in PBS to the volume of sample initiated and finally stored at -20°C. Every sample taken was assayed in duplicate, both for ELISA and CHO cell assays. PT yield of samples was calculated by dividing the average amount of PT measured by the cell mass. ANOVA was used to evaluate the significance of differences ($P<0.05$) in antigen content during bacterial growth. In addition, independent sample t-test was applied regarding the B2 and THIJS media.

CHO cell assay. Amount of toxin activity was quantified using the CHO cell assay as previously described (Gillenius et al., 1985; Halperin et al., 1990). CHO cells (Razi Cell Bank, RVSRI, Iran) were cultured in the Roswell Park Memorial Institute (RPMI) medium (Gibco) supplemented with 10% volume per volume (v/v) fetal bovine serum (Gibco) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Single cell suspensions were obtained from confluent monolayers of CHO cells by treatment with a solution of 0.1% weight per volume (w/v) trypsin and 0.02% w/v Ethylenediaminetetraacetic acid (EDTA) in Phosphate-buffered saline (PBS) with pH 7.2. The released cells were adjusted to a final concentration of $1x10^5$ cells per ml in complete RPMI. 100 microliters of the trypsinized CHO cells was placed in each well in a 96-well flat-bottom microplate (SPL, South Korea). After 18-24 h incubation for attachment, 30 µl was added from each sample and standard toxin (edqm) to the first and second wells in each row in duplicate. Subsequently, the volume in first and second wells was adjusted to 200 µl. Samples from second to eleventh wells diluted in twofold dilution steps using a multichannel pipette. The volume in all wells was adjusted to 200 µl. After incubating for 20-24 h at 37 °C, the clustering effect of test samples on the cells was observed using an inverted microscope. The highest dilution of a test sample showing the clustering effect was considered as titer, and the concentration of PT was determined using a PT standard (World Health, 2013).

ELISA assay. Pertussis toxin ELISA kit (ALPHA DIAGNOSTIC, USA) was used for the detection and measurement of pertussis toxin. This kit is a sandwich ELISA for the detection and measurement of pertussis toxin/toxoid in vaccines. PTX ELISA kit is based on binding of PT to an antibody coated on the plate and antibody-HRP conjugate. Absorbance was then measured on a microtiter well ELISA reader at 450 nm and the concentration of PT in samples and control was read off via the standard curve. All samples were tested at least in two dilutions that were within the standard range. Samples containing PT more than highest standard (100 ng/ml) were further diluted and the results obtained were multiplied by the appropriate dilution factor.

RESULTS

B. pertussis strain 509 was cultivated in triplicate. First, *B. pertussis* was grown in Verwey medium. After 24-36 h, the bacteria were adjusted to an optical density at OD590 of 1 and inoculated to B2 and THIJS media. The bacteria were allowed to grow for 30 h at 35 °C for PT production. In this study, the samples were taken in the exponential phase. After 17 h of bacterial growth, sampling was started and taken every 2-3 h (Figure 1).

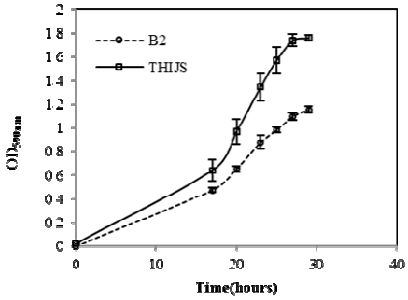

Figure 1. Cultivation of *B. pertussis* strain 509 in B2 and THIJS media. Error bars represent the standard deviation of three separate cultivations.

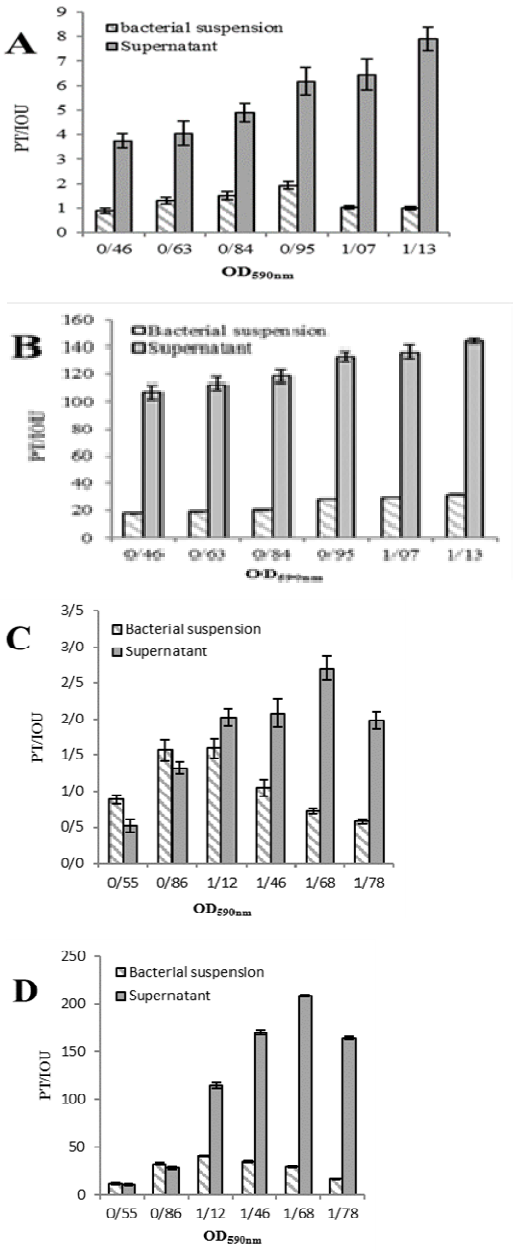

Figure 2. Free and cell-associated PT production in culture duration in B2 and THIJS media
A: CHO cell assay for B2 medium
B: ELISA assay for B2 medium
C: CHO cell assay for THIJS medium
D: ELISA assay for THIJS medium

In THIJIS medium, bacteria grew more rapidly; therefore, in 29- hour culture, maximal biomass concentrations of 29 IOU reached to compare with 19 IOU by B2 medium. In B2 medium the optical density changes in culture duration were with a gentle slope. Consequently, the time they reach the stationary phase in growth curve was unclear; however, in THIJS, the growth rate time to reach the stationary phase was short and the increase of optical density stopped after 27 h and remained constant.

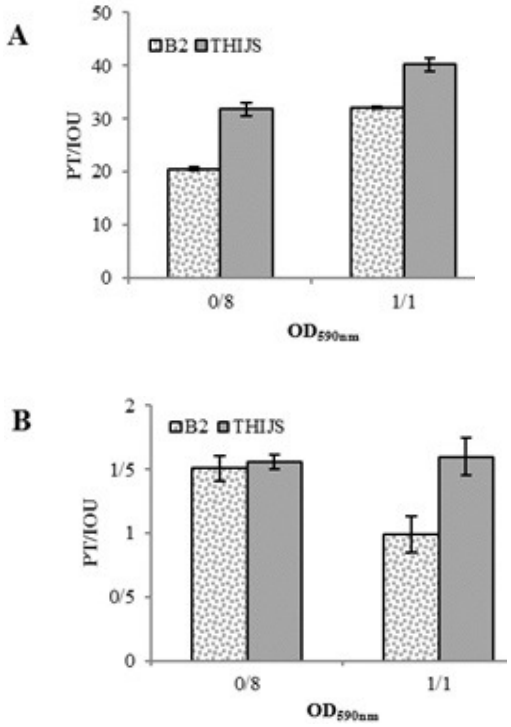

Figure 3. Amount of cell-associated PTX/IOU ratio in B2 and THIJS media measured by ELISA (A) and CHO cell (B) assays. In both, amount of cell-associated PT in THIJS medium was more than B2 medium.

PT production in B2 medium. In ELISA and especially in CHO cell assays, cell-associated PT production raised with a slope similar to bacterial growth curve; therefore, there was no significant difference within two consecutive samples in 2 hours intervals ($P>0.05$). However, the difference rate of toxin production in alternate samples was significant during cultivation ($P<0.05$). In CHO cell assay, the ratio of released and cell-associated PT during bacterial growth was different (Fig. 2A). Maximum values of

released and cell-associated active PT were 149/9 IU/ml and 31/2 IU/ml in ODs of 1/1 and 0/9, respectively. PT released in supernatant raised in culture period up to OD of 1/1, whereas cell-associated PT production was stopped in OD of 0.9 and then decreased. Approximately 24% of the toxin remained cell-associated in ODs of 0.6, 0.8, and 0.9; however, it decreased during the continuation of cultivation and reached 11% in OD of 1.1. These results showed that while total active toxin production increased by the end of the logarithmic phase, cell-associated PT production had already stopped before this stage. In ELISA assay, from starting to finishing of sampling, the concentration of cell-associated and free PT raised (Fig. 2B). Maximum values of released and cell-associated PT were 2.7 µg/ml and 0.6 µg/ml in OD of 1/1. Cell-associated PT slightly increased from OD of 0.9, where about 18% of total PT production in ODs of 0.9, 1, and 1.1 was cell-associated. These results indicated that the cell-associated PT production did not increase parallel to the optical density and more toxins were released into culture medium.

PT production in THIJS medium. In THIJS medium, such as B2, ELISA and especially CHO cell assays did not have enough sensitivity and significant difference for two consecutive cell-associated PT samples ($P>0.05$). Due to the slow rise of cell-associated PT production, the rate of toxin production showed significant difference in alternate samples determined during cultivation ($P<0.05$). In CHO cell assay, maximum released and cell-associated active PT were 74.9 IU/ml and 35/4 IU/ml in ODs of 1/6 and 1/1, respectively. In OD of 0.5, the amount of PT was greater than free toxin and about 66% of the total toxin production was cell-associated (Fig. 2C). This trend changed after OD of about 1, and amount of toxin released into the culture medium was higher than cell-associated toxin and declined to about 21% in ODs of 1.6 and 1.7. Active PT production was stopped in OD of 1.1 and then decreased. In ELISA assay, in ODs of 0.5 to 0.8, about 53% of total toxin was cell-associated, whereas it declined to 9% in OD of 1.7.Maximum released and cell-associated total PT were 5.7µg/ml and 0.8µg/ml in ODs of 1/6 and 1/1, respectively (Fig. 2D).

DISCUSSION

Whole cell pertussis vaccines are produced by *B. pertussis* selected strains under suitable conditions for the expression of the desired phenotypic phase I antigens. After the completion of the culture, bacterial suspension is concentrated and inactivated. Cultivation is typically stopped at a given time, and OD regardless of the virulence factors production leads to differences in the quality of each batch vaccine. One of the most efficient vaccine production processes is to provide suitable culture components for the growth of the bacteria. In this study, it was shown that the selection of appropriate culture medium can increase the bacterial growth rate and the production of cell-associated virulence factors. PT production is different during cultivation period and mostly near the end of the logarithmic phase. Due to the correlation of PT content with potency, the assessment of PT production in the logarithmic growth phase before reaching stationary phase is extremely critical. In the current study, pertussis toxin production in B2 and THIJS media during the log growth phase in free and cell-associated was evaluated and measured using CHO cell and ELISA assays. Although the ELISA and CHO cell assays did not show significant differences regarding the two consecutive measurements, the toxin production process during cultivation was conclusive.

However, in B2 medium, due to the gentle slope in growth curve, specifying the exact time of getting into the stationary phase was not known, whereas it was obvious and fast in THIJS medium In THIJS medium, OD of culture reached 1.1 faster than that of B2 medium. This showed that the performance of culture components in THIJS was better than that of B2 for bacterial growth. In a study performed by Westdijk et al. on *B. pertussis* strain 509, samples from three time points (logarithmic phase, start, and end of stationary phase) were taken and examined for virulence factors production (Westdijk et al., 1997). The amount of cell-

associated and free pertussis toxin in culture was inconsistent with the current study which may be due to the differences in methods of bacterial culture. However, in the study conducted by Thalen et al., the amount of PT in supernatant was more than that of cell-associated (Thalen et al., 2006b). For illustration of the difference(s) between two culture media, two identical ODs at 0.8 and 1.1 at the end of logarithmic phase were selected. According to figure 3A, PT production in both media at OD of 1.1 was higher than OD of 0.8 and the highest PT concentration was achieved by THIJS medium in OD of 1.1 ($P< 0.05$) in ELISA assay. In OD of 0.8, the rates of cell-associated PT to total toxin were 53% and 14% in THIJS and B2, respectively. In OD of 1.1, the rates of cell-associated PT to total toxin were 26% and 18% in THIJS and B2, respectively. These results showed that in OD of 0.8 and 1.1, cell-associated toxin levels in THIJS medium were 55% and 25%, respectively which were higher than those of B2 medium. Figure 3B demonstrates that in CHO cell assay, the highest amount of active cell-associated toxin was related to B2 medium in OD of 0.9; nevertheless, more of the toxin in culture duration released into the supernatant and cell-associated PT declined in OD 1/1. On the other hand, in THIJS medium, the amount of cell-associated toxin declined gradually in cultivation period. In OD of 0.8, no significant difference was found between B2 and THIJS ($P = 0.59$). In OD of 1.1, the amount of cell-associated toxin in THIJS medium was 66% which was more than that in B2 medium ($P< 0.05$). The results showed more levels of remained cell-associated toxin produced in THIJS medium. The *B. pertussis* strain 509 produced more PT (30%) at THIJS than B2 medium. In terms of quantity, the most amount of cell-associated toxin was in OD of 1.1 in THIJS medium, whereas in terms of quality, the highest cell-associated toxin was in OD of 0.9 in B2 medium. However, the current results showed that the best culture stop (harvest) time in THIJS medium was quantitatively and qualitatively in OD of 1.1. With regard to the B2 medium, the better quality and quantity of PT production were in OD of 0.9 and 1.1, respectively. Therefore, the best time for culture stopping (harvesting) differs in various media. This study revealed that the amount of cell-associated and free toxin vary during the culture and the increase of OD cannot be a suitable factor to determine the raise of toxin production. PT production before getting into stationary phase is dropped which shows the presence of inhibitory components in the medium or byproducts causing down-regulation of PT production without any observable effect on the growth of bacteria (Bogdan et al., 2001). According to the total pertussis toxin increment and active toxin reduction at the end of logarithmic phase, it can be stated that measured PT is the combination of active and inactive or degraded toxin. Some of the PT molecules are degraded during the cultivation at logarithmic phase that show rising of proteolytic activity during the growth phase of the culture (Thalen et al., 2006b). Therefore, to improve the quality of wP production, an online monitoring and control system that can measure the critical parameters during culture is required in order to find the best harvesting time (Streefland et al., 2007; Streefland et al., 2009b).

In conclusion, a low bacterial mass with high virulence factors is highly desired in pertussis vaccine production. Accordingly, THIJS medium can be a good alternative for B2 medium. These findings may be used to produce a whole-cell vaccine with high potency, less biomass per dose. Consequently, it reduces toxicity compared to conventional vaccine.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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