

*Full Length Research Paper*

## **Optimization of Newcastle disease virus production in T-flask**

**Mohd Azmir Arifin<sup>1</sup>, Maizirwan Mel<sup>1\*</sup>, Siti Hajar Salim<sup>1</sup>, Mohamed Ismail Abdul Karim<sup>1</sup> and Sharifah Syed Hassan<sup>2</sup>**

<sup>1</sup>Department of Biotechnology Engineering, Kulliyyah of Engineering, International Islamic University Malaysia, Gombak, P.O. Box 10, 50728 Kuala Lumpur, Malaysia.

<sup>2</sup>School of Medicine and Health Sciences, Monash University, Sunway Campus, Jalan Lagoon Selatan, Bandar Sunway, 46150 Selangor, Malaysia.

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**Newcastle disease (ND) is regarded as one of the most important diseases in the poultry industry. Currently, vaccines for ND are produced by using embryonated chicken eggs, a method which has the disadvantages of being labour-intensive, time consuming and requires large area for the incubation of eggs. In the present study, the production of lentogenic Asplin F strain of Newcastle disease virus by using cell culture method was studied. Experiments were carried out in T-flasks to investigate the effects of serum concentration in the culture medium during virus replication phase and multiplicity of infection (MOI) on ND virus propagation in DF-1 cells. Results show that virus infectivity titre of  $6.62 \times 10^7$  TCID<sub>50</sub>/ml was achieved when serum concentration of 0.5% and MOI of 20 was used.**

**Key words:** Newcastle disease virus, Asplin F strain, DF-1 cell, T-flask.

### **INTRODUCTION**

Newcastle disease (ND) is regarded as one of the most important disease in the poultry industry (Adene, 1990; Spradbrow, 1987). The disease which had its first outbreaks in 1926, in Java, Indonesia (Kraneveld, 1926), and in Newcastle-upon-Tyne, England (Doyle, 1927) is highly contagious, affecting 27 of the 50 orders of birds (Yusoff and Tan, 2001). Chickens are the most susceptible host, in which the severity of the disease may vary from mild infection with no apparent clinical signs to a severe form causing 100% mortality. The causative agent responsible for the disease is the Newcastle disease virus (NDV) (Alexander, 1997). NDV also known as avian paramyxovirus 1 is a member of the genus Avulavirus of the family Paramyxoviridae, in the order Mononegavirales (Mayo, 2002). This family also includes other important pathogens such as the mumps virus, human parainfluenza virus, sendai virus, simian virus 5 and recently emerging nipah and hendra viruses (Lamb et al., 2005).

NDV strains are classified into three main pathotypes: Lentogenic, mesogenic and velogenic, based on the clinical signs and severity of the disease. Lentogenic strains cause mild or avirulent infections that are largely limited to the respiratory system. Mesogenic strains are of intermediate virulence causing respiratory infection with moderate mortality, while velogenic strains are highly virulent causing 100% mortality in chickens. Velogenic strains can be further categorized into two types: Viscerotropic and neurotropic. Viscerotropic velogenic strains produce lethal hemorrhagic lesions in the digestive tract, whereas neurotropic velogenic strains produce neurological and respiratory disorders (Alexander, 1997; Spradbrow, 1987).

The disease caused by NDV remains a potential threat to commercial or backyard production until today. In 2002, an outbreak caused by a virulent strain of NDV occurred in California, USA. The outbreak has caused \$200 million worth of losses from the depopulation of birds (Kapczynski and King, 2005). Although, the disease is not yet eradicated, it can be controlled either by importing birds from disease free flocks or through vaccination that must continue throughout the life of the bird

\*Corresponding author. E-mail: [maizirwan@iium.edu.my](mailto:maizirwan@iium.edu.my). Tel: +6-03-61964566. Fax: +6-03-61964442.

**Table 1.** 3<sup>\*\*</sup>(2-0) Full factorial design for NDV propagation in T-flask.

Parameter	Level		
	-1	0	1
Serum concentration (%), A	0.5	1.5	2.5
MOI, B	0.2	2	20

(Mayo, 2002).

Currently, vaccines for ND are produced by using embryonated chicken eggs, a technology that has remained almost unchanged since the late 1930s. This traditional method however poses some drawbacks, such as the need for high amounts of specific pathogen-free eggs, high labor-intensity, time consuming and requires large area for the incubation of eggs. Besides, the process is slow and difficult to scale-up, so large strategic stocks must be kept to respond in cases of epidemics (Souza et al., 2009). An alternative to this method is the propagation of NDV in cell culture systems. It has been reported that Newcastle disease virus strains are able to replicate in an enormous range of cells (Lancaster, 1966). Among cell substrate systems that have been identified are Vero, CEF and DF-1 cells (de Leeuw and Peeters, 1999; DiNapoli et al., 2007; Ravindraa, 2008).

In the present study, the production of Newcastle disease virus (lentogenic Asplin F strain) in T-flasks using DF-1 cells as hosts was studied. The influence of factors such as serum concentration during virus replication phase and multiplicity of infection, MOI on virus yield was investigated. This study serves as the foundation for future live Newcastle disease vaccine production in larger cell culture systems such as the bioreactor.

## MATERIALS AND METHODS

### Cell line and virus strain

Established DF-1 cell line (ATCC-CRL-12203<sup>TM</sup>) was purchased from the American Type Culture Collection (ATCC). Lentogenic Asplin F strain of NDV was obtained from Malaysia Vaccine Pharmaceuticals (MVP) Sdn. Bhd.

### Culture medium and chemicals

Dulbecco's modification of Eagle's medium, DMEM (with glucose and L-glutamine) in powder form and fetal bovine serum (FBS) were supplied by Gibco<sup>®</sup>.

### Cell infection with NDV in T-flask

Confluent monolayers of cells were used for infection with NDV as described by Hussain and Rasool (2005). Spent medium was removed and cell monolayer was washed with 5 ml of PBS. Later, cells were infected with NDV according to the designed levels by inoculation of virus in DMEM supplemented with trypsin. The virus inoculum was spread uniformly and incubated in humidified CO<sub>2</sub> incubator for 1 h with intermittent rotation to allow adsorption. Five

milliliters of maintenance medium with designed concentration of serum was added to the flask. The flask was later returned to humidified CO<sub>2</sub> incubator and monolayers were examined daily under inverted microscope for evidence of CPE.

### Virus harvesting

The virus was harvested by the combination of freeze thawing, sonication and centrifugation process. Four days post inoculation flasks were transferred to -80°C freezer for 1 h and later thawed at room temperature. This process was then resumed with sonication at 15°C for 1.5 min. Subsequently, virus suspensions were poured into centrifuge tubes and centrifuged at 300 g for 10 min at 4°C to pellet the cell debris. The clear supernatant fluid containing virus was collected carefully, labeled and kept in -80°C freezer.

### TCID<sub>50</sub> assay

Fifty percent tissue culture infectious dose (TCID<sub>50</sub>) assay was carried out in 96 well flat shaped microtiter plates. Plates were seeded with DF-1 cells such that confluence was reached in 2 to 3 days. DF-1 cells were grown in DMEM media containing 10% FBS and grown at 37°C, 5% CO<sub>2</sub>. Appropriate dilutions of each virus were prepared in DMEM medium and 100 µl of each dilution was added to each well. Plates were incubated at 37°C for 30 to 60 min before 100 µl of DMEM was added to each well. The plates were then incubated for 4 days until the CPE effect appeared. The log TCID<sub>50</sub>/ml calculation was based on the Reed and Muench (1932) formula.

### Experimental design

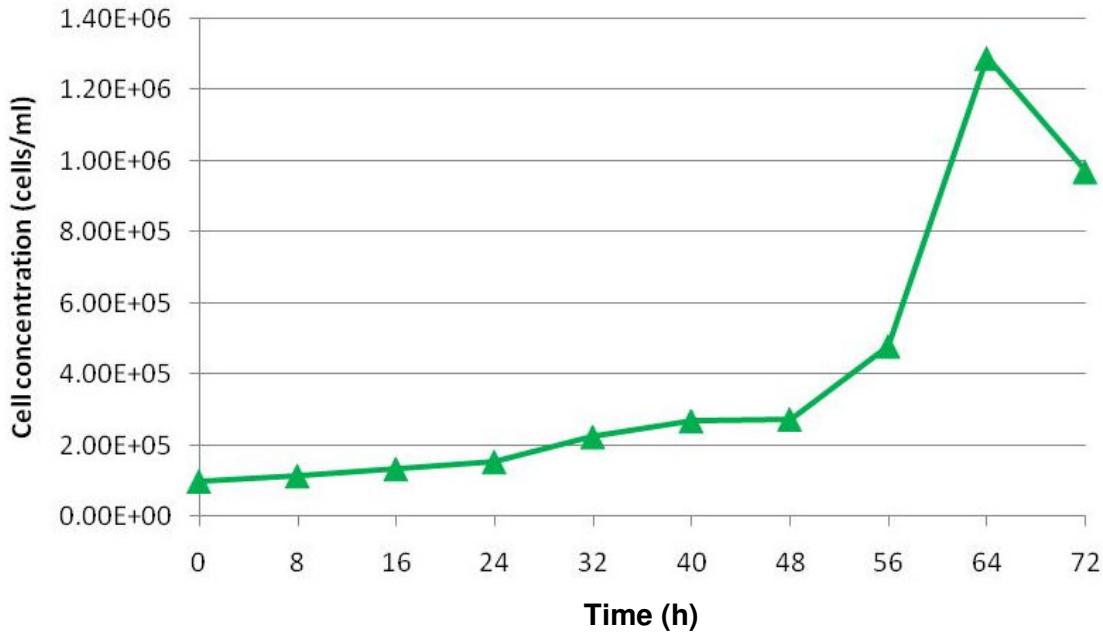
Optimization of NDV propagation in T-flask was carried out according to 3<sup>\*\*</sup>(2-0) full factorial design (FD) generated by STATISTICA<sup>®</sup>. Two selected parameters: Serum concentration and MOI were varied in this experiment to investigate their optimum value in response to virus infectivity titre produced. The design consists of 9 main runs, 4 replicates at the centre point and each parameter had 3 levels. The layout of the design is shown in Table 1.

## RESULTS AND DISCUSSION

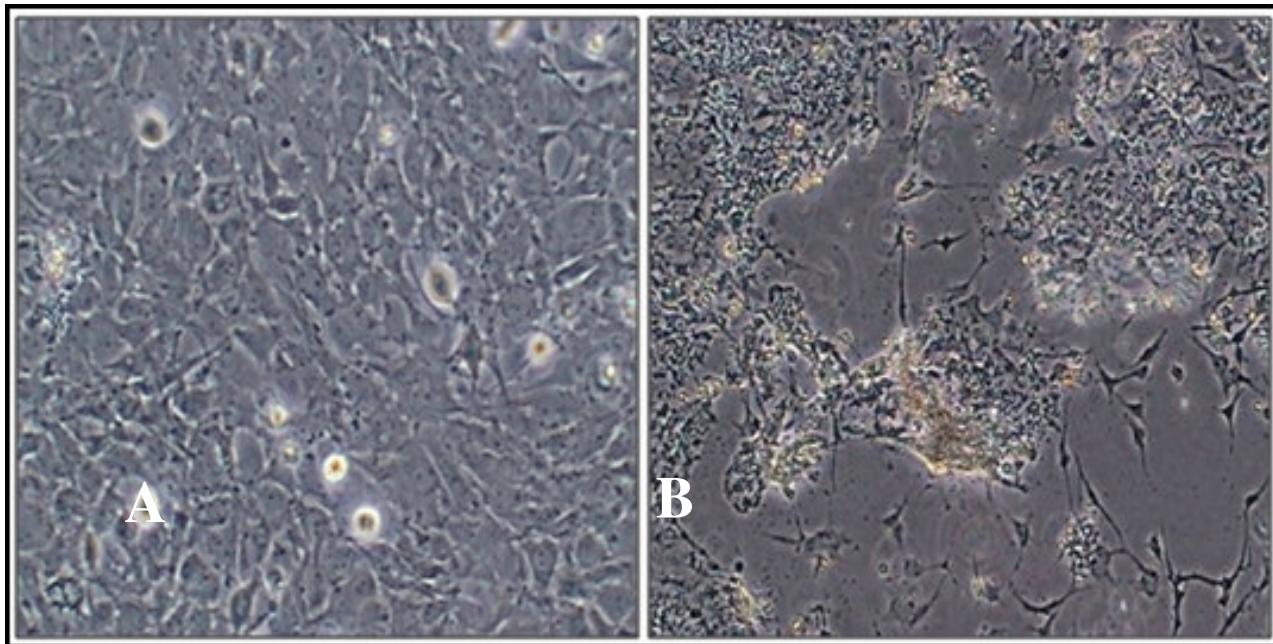
### Determination of time of infection (TOI)

To propagate viruses in cell culture, a suitable time of infection (TOI) is required. To this regard, the growth profile of DF-1 cell in the T-flask was studied and the appropriate time to infect with the virus was thereof determined.

As shown in Figure 1, culture of DF-1 was started by inoculation of 1.00 x 10<sup>5</sup> cells/ml into the flask. Growth of DF-1 cells was in lag phase from 0 h until 48 h. Starting



**Figure 1.** Growth profile of DF-1 cell in T-flask.



**Figure 2.** Morphology of DF-1 cell in T-flask: (A) Culture reached confluent state 64 h after cell inoculation, (B) CPE showed by the culture 96 h after the infection by NDV.

from 48 h, the culture entered exponential phase and reached the maximum cell concentration of  $1.29 \times 10^6$  cells/ml at 64 h. Once the maximum was reached, cell concentration dropped and entered the death phase. Based on this, TOI was determined to be between 56 and 64 h, where the culture will be in the exponential phase.

During this period, cells which will serve as the virus replication hosts are highly available and this will contribute to higher virus production.

Figure 2 shows the morphology of DF-1 cells before and after the infection by NDV. As can be observed, characteristic changes in the appearances of DF-1 cells

**Table 2.** Experimental design using 3<sup>\*\*</sup>(2-0) full factorial design (FD) with experimental and predicted (using model equation) values of virus infectivity titre.

Standard run	Serum concentration (%)	MOI	Virus infectivity titre (TCID <sub>50</sub> /ml)	
			Experimental	Predicted
1	2.50000	2.00000	5.76 x 10 <sup>7</sup>	5.71 x 10 <sup>7</sup>
2	1.50000	20.00000	5.89 x 10 <sup>7</sup>	6.10 x 10 <sup>7</sup>
3	0.50000	0.20000	1.11 x 10 <sup>7</sup>	3.69 x 10 <sup>7</sup>
4	0.50000	20.00000	6.62 x 10 <sup>7</sup>	6.47 x 10 <sup>7</sup>
5	2.50000	0.20000	2.75 x 10 <sup>7</sup>	2.86 x 10 <sup>7</sup>
6	1.50000	0.20000	1.48 x 10 <sup>7</sup>	1.96 x 10 <sup>7</sup>
7	1.50000	2.00000	5.13 x 10 <sup>7</sup>	4.98 x 10 <sup>7</sup>
8	0.50000	2.00000	2.95 x 10 <sup>7</sup>	3.69 x 10 <sup>7</sup>
9	1.50000	2.00000	5.14 x 10 <sup>7</sup>	4.98 x 10 <sup>7</sup>
10	1.50000	2.00000	5.11 x 10 <sup>7</sup>	4.98 x 10 <sup>7</sup>
11	2.50000	20.00000	5.26 x 10 <sup>7</sup>	5.18 x 10 <sup>7</sup>
12	1.50000	2.00000	5.12 x 10 <sup>7</sup>	4.98 x 10 <sup>7</sup>
13	1.50000	2.00000	5.13 x 10 <sup>7</sup>	4.98 x 10 <sup>7</sup>

were very obvious in which the formation of giant multi-nucleated cell or syncytia, formation of plaques and also elongation of cytoplasmic tails has occurred after the infection. These changes are known as cytopathic effect or CPE. CPE is defined as pronounced morphologic changes induced in individual cells or groups of cells by virus infection, which are easily recognizable under a light microscope. CPE are very reproducible and can precisely characterize the virus type providing significant clues to the identity of a virus (Cann and Irving, 1999).

#### Optimization of NDV production in T-flask

Virus infection parameters, serum concentration during virus replication phase and multiplicity of infection (MOI) as independent factors were optimized to yield high virus infectivity titre. Experiments were carried out as per the design matrix of the 3<sup>\*\*</sup>(2-0) full factorial design (FD) (Table 2), and the final virus titre produced by the culture was used as the response. For predicting the optimal values of virus titre obtained within the experimental constraints, a second order polynomial model was fitted to the experimental results by using the STATISTICA® software. The model developed is as follows:

$$Y \text{ (Virus infectivity titre, TCID}_{50}/\text{ml}) = -8222414 + 20301437(A) - 2781034(A^2) + 19951268(B) - 815773(B^2) + 920045(AB) \quad (1)$$

Where, the virus infectivity titre as yield (Y) is a serum concentration (A) and MOI (B).

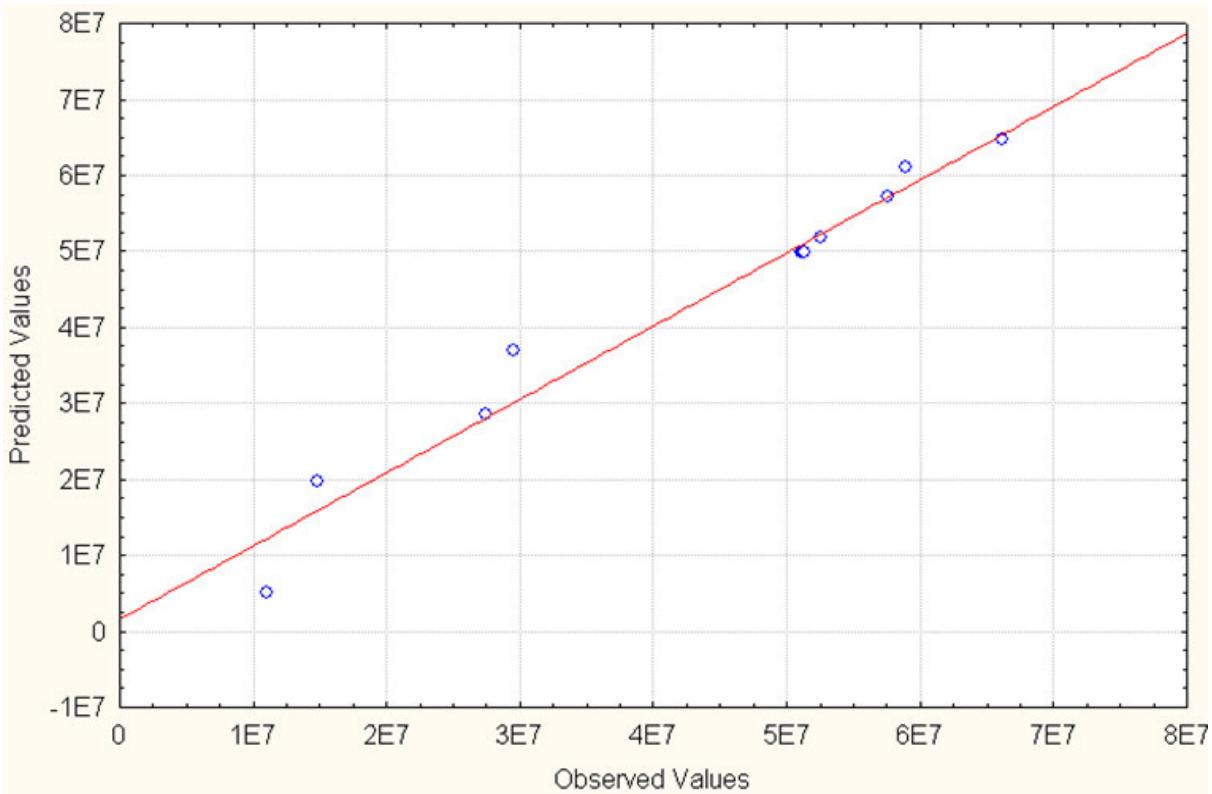
The statistical model was checked by Fischer variance ratio, the F-value and the analysis of variance (ANOVA) for the response surface quadratic model is summarized in Table 3. In Table 3, the model F-value of 36.7793

implies that the quadratic regression model was significant. F-value is a statistically valid measure of how well the factors describe the variation in the mean of data. The greater the F-value from 1, the better the factors explain the variation in the data about its mean, and the estimated factor effects are real (Mannan et al., 2007). Model was further approved to be significant by having a very low probability value ( $(P_{\text{model}} > F) = 0.0001$ ). At the model level, the correlation measures for the estimation of the regression equation are the correlation coefficient R and the determination coefficient R<sup>2</sup>. The correlation between the experimental and predicted values is better when the value of R is closer to 1 (Haaland, 1989; Wang and Lu, 2005). In this experiment, the value of R and R<sup>2</sup> were 0.98149 and 0.96333, respectively. These values indicate a high degree of correlation between the experimental and the predicted values. The value of R<sup>2</sup> indicates that 96.333% of the factors: Serum concentration and MOI contribute very positively to the response. The value of R<sup>2</sup> is also a measure of fit of the model and it can be mentioned that only 3.667% of the total variation were not explained by the virus infectivity titre. The value of the adjusted determination of coefficient was also very high (0.93714) which indicates high significance of the model.

The P values are used as tool to check the significance of each of the factors which, in turn, are necessary to understand the pattern of the mutual interactions between the factors. The lesser the P value than 0.05, the higher the significance of the corresponding factor (Liu et al., 2003). Based on this, the factors that were highly significant in the model were the MOI concentration (B), square terms of MOI (B<sup>2</sup>) and the interactive term between serum concentration and MOI (AB). Serum concentration (A) and its square term (A<sup>2</sup>) were not significant because their P values were larger than 0.05.

**Table 3.** Analysis of variance (ANOVA) for response surface quadratic model for optimization.

Source	Sum of squares	F value	p value
Model	$3.520427 \times 10^{15}$	36.7793	<0.0001
Serum concentration, A	$3.907699 \times 10^{13}$	2.0413	0.196144
MOI, B	$2.575082 \times 10^{15}$	134.5168	0.000008
A <sup>2</sup>	$2.136099 \times 10^{13}$	1.1159	0.325900
B <sup>2</sup>	$1.474961 \times 10^{15}$	77.0489	0.000050
AB	$4.059055 \times 10^{14}$	21.2036	0.002470

**Figure 3.** Plot of observed versus predicted values for virus infectivity titre.

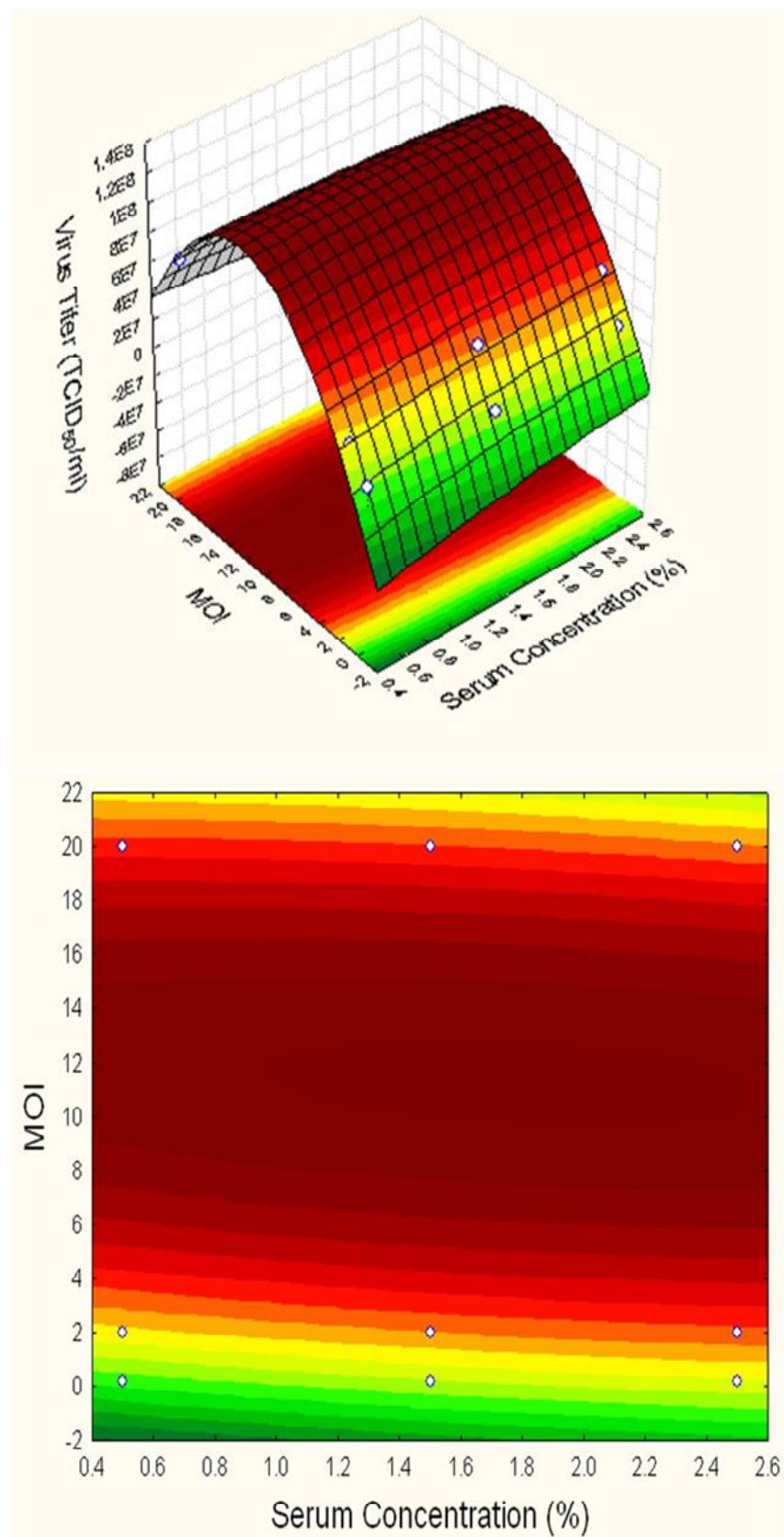
However, in this study, all terms were included due to the significance of overall model.

Figure 3 shows the virus infectivity titre predicted by the model and the real values obtained from the experiment. It can be observed that the point's distribution around the line for the response fitted to the linear model.

The 3D response surface plots described by the regression model were drawn to illustrate the effects of the independent factors and the interactive effects of each independent factor on the targeted response. The shape of the corresponding 2D contour plots indicates whether the mutual interactions between the independent variables are significant or not. An elliptical contour plots indicates that the interactions between the independent factors are significant. While a circular contour plot indicates that the interactions between related factors are

negligible (Mannan et al., 2007). By analyzing the 3D response surface plots and the corresponding 2D contour plots, the optimal values of the independent factors could be observed, and the interaction between each independent factor can be easily understood (Li and Hanson, 1989). Figure 4 shows elliptical contour plots since the interactive term between serum concentration and MOI, AB was significant (Table 3). The maximum virus infectivity titre can be obtained at the point of intersection of the major and minor axes of the ellipse or by solving the inverse matrix of Equation 1. By using STATISTICA®, the optimum values for each factor were determined: 1.79% for serum concentration and 11.22 for MOI which will yield the maximum virus infectivity titre of  $1.22 \times 10^8$  TCID<sub>50</sub>/ml.

Genzel et al. (2006) has reported that the MOI influences



**Figure 4.** 3D response surface and 2D contour plots showing the effect of serum concentration (%) and MOI on virus infectivity titre.

virus growth dynamics but not final virus yield. In the study of propagation of influenza virus in Vero and MDCK cells conducted by Audsley and Tannock (2005), it was observed that cells infected with higher MOIs maximum titers were attained earlier than in cultures infected at lower MOIs. While according to Maranga et al. (2004), in his study of production of virus-like particles with a baculovirus insect cell system, they noticed that at high MOIs, the specific productivity decreased when cells were infected at late growth phase.

Different theories have encouraged us to investigate the effects of MOI on Newcastle disease virus production. As shown in Table 2, three different MOIs: 0.2, 2.0 and 20 were used. Based on the result, the maximum virus infectivity titre of  $6.62 \times 10^7$  TCID<sub>50</sub>/ml was achieved when we used high MOI, 20 (while serum concentration used was 0.5%). High MOI probably better suited NDV production in T-flasks. While for serum concentration during viral replication phase, it has been analyzed by STATISTICA® that the factor was not significant towards virus infectivity titre. In addition, serum was suggested to be removed from the culture medium to facilitate the growth of virus in the host cells (Pattinson, 2008).

Apart from serum concentration and MOI, other factors such as type of cell, type of culture medium and time of infection also may partially contribute to the results we obtained in this experiment. Investigation on their effects on virus production is much recommended.

## Conclusion

In the present study, two factors of interest for the establishment of a new platform for the production of Newcastle disease virus were investigated. It was verified by statistical analysis using STATISTICA®, that multiplicity of infection, MOI has substantial effect on the virus infectivity titre. High virus infectivity titre of  $6.62 \times 10^7$  TCID<sub>50</sub>/ml was achieved when high MOI of 20 was used. Serum concentration during virus replication however was analyzed to have minor effect on the virus titre. Also, it has been analyzed that the optimum conditions to produce Newcastle disease virus using DF-1 cell culture are serum concentration of 1.79% and MOI of 11.22. These results constitute important information when large scale production of Newcastle disease virus is considered.

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