PURIFICATION OF NUCLEOCAPSID PROTEIN OF NEWCASTLE DISEASE VIRUS FOR IMMUNODIAGNOSTIC KIT

R.AHMAD-RAUS[1], R.A. AWANG[1], M. MEL[1] AND K. YUSOFF[2]

[1]*Department of Biotechnology Engineering, Kullliyyah of Engineering, International Islamic University of Malaysia, Jalan Gombak, 51100 Kuala Kumpur.*

[2] *Ministry of Science and Technology, Putrajaya, Selangor, Malaysia.*

rahaar@iium.edu.my

ABSTRACT: The objectives of the present study are to purify recombinant nucleocapsid (NP) protein of Newcastle disease virus (NDV) using microfitration system and to improve the processing conditions as well. A microporous membrane screening using two different sizes of membrane which are 0.1 µm and 0.45 µm was performed before further improvement on NP protein filtration was carried out. Several conditions of the independent variables that affect the microfiltration process such as the temperature, transmembrane pressure (TMP), and viscosity were observed. It turned out to be that the 0.45 µm membrane gave higher yield of NP protein than the 0.1 µm membrane. Thus, the 0.45 µm membrane was used to improve the filtration process for NP protein. Based on the Full Factorial Design (STATISTICA 8.0, Statsoft, Inc.), eight sets of experiment were designed to identify the best conditions for the NP protein filtration. From these experiments, the optimal conditions that gave the highest yield of NP protein are; TMP 4.5 psi, viscosity 2.39 cP and temperature 4 ºC. Based on ANOVA (analysis of variance), all the independent variables studied do not significantly affect the purification of NP protein. However, based on the lowest p value for each variable corresponding to the response, temperature has the largest effect for the NP protein yield.

ABSTRAK: Objektif kajian yang dikendalikan adalah untuk purifikasi protein rekombinan nuleokapsid (NP) virus penyakit Newcastle (NDV) menggunakan system mikrofiltrasi dan memperoleh keadaan pemprosesan yang terbaik. Dua jenis membran mikroporus disaring iaitu membrane yang mempunyai saiz liang 0.1 dan 0.45 µm. Beberapa parameter yang mempengaruhi mikrofiltrasi iaitu suhu, tekanan transmembran (TMP) dan kelikatan sampel juga dikaji. Hasil menunjukkan bahawa membran yang mempunyai saiz liang 0.45 µm memberi hasil NP protein lebih baik berbanding membran yang mempunyai saiz liang 0.1 µm. Maka, membran yang mempunyai saiz liang 0.45 µm digunakan dalam eksperimen untuk memperoleh keadan proses purifikasi terbaik. Berdasarkan Full Factorial Design (STATISTICA 8.0, Statsoft, Inc.), lapan ujikaji direka untuk menentukan keadaan proses tersebut. Hasil menunjukkan keadaan optima yang memberikan hasil protein NP tertinggi adalah TMP 4.5 psi, kelikatan sampel 2.39 cP dan suhu 4 ºC. Berdasarkan ANOVA (analysis of variance), semua variabel independen yang dikaji tidak memberi kesan secara signifikan terhadap purifikasi protein NP. Walau bagaimanapun, berdasarkan nilai p untuk setiap variabel; suhu memberi kesan terbesar dalam purifikasi protein NP.

KEYWORDS*: Microfiltration, transmembrane pressure, full factorial design, nucleocapsid protein*

1. INTRODUCTION

Purification of recombinant protein normally adopts chromatography system as it yielded somewhat pure protein. However, the method is time consuming and laborious as well as requires each of the resulting chromatography fractions to be analyzed whether they contain the target purified protein or not. On the other hand, filtration method is rapid, does not require preparations of many chemical solutions and detection of one permeates for the target purified protein. It is used to separate particulate or solute components in a fluid suspension or solution according to their size by flowing under a differential pressure through a porous membrane [1]. Filtration can be divided into two categories which are microfiltration and ultrafiltration based on the pore size of the membrane.

Microfiltration is capable of separating bigger compounds from fluid suspension while ultrafiltration is the reverse. To be exact, microfiltration removes contaminants from a fluid by passage through a microporous membrane with the pore size range 0.1 to 10 μm [2]. In this study, microfiltration was used to purify the nucleocapsid (NP) protein of Newcastle disease virus (NDV) that was expressed by recombinant bacteria *Escherichia coli*. The optimized operating conditions to remove NP proteins from other bacterial lysate contaminants were determined in this study as NP protein can be easily degraded [3].

NP protein is one of the six structural proteins of NDV [4]. It is a major protein in the virus and forms a herringbone-like structure inside the virus and bacteria. Apart from herringbone-like structures, it can form ring-like particles in E. coli [3]. NP protein is highly immunogenic in nature and induces significant antibody response in rabbits, mice and chickens although it fails to confer any immunoprotection [5]. Due to its relative abundance and immunogenic property, NP protein can be used as an antigen in immunodiagnostic kit. The ultimate aim of this study is to purify this protein using microfiltration so as to obtain NP protein to be used in the immunodiagnostic kit.

2. METHODOLOGY

2.1 Microorganism

E. coli TOP10 (Invitrogen, USA) transformed with pTrc His2 TOPO (Novagen, USA) harboring NP gene of NDV AF2240 was obtained from Virology Laboratory, Department of Microbiology, Universiti Putra Malaysia.

2.2 Inoculum Preparation

Overnight culture of recombinant *E. coli* was incubated with Luria-Bertani (LB) broth for 4 hr at 37 ^{o}C and shaken at 250 rpm to produce 50 ml starter culture. This starter

culture was then used for preparing larger culture volume by growing it in 500 ml LB broth at the same conditions. Subsequently, this large culture is used as the inoculums for 5 l culture.

2.3 Cell Cultivation in Stirred-tank Bioreactor

The inoculums was transferred aseptically into 5-litre B-Braun fermenter and was cultured at 37 ^{o}C, pH 7.2, agitation at 250 rpm and 80% pO_2 for 8 hr. Induction with 1 mM IPTG was carried out at 3 hr to stimulate high level expression of recombinant NP protein. At the end of the incubation time, the cell was harvested and the resulting pellet diluted with sterile distilled water before it was homogenized into HPH.

2.4 High Pressure Homogenizer (HPH)

The diluted pellet was homogenized using HPH at pressure 800 bars and pump speed 7 psi [6]. Homogenization was carried out for three cycles before the supernatant of the homogenized *E. coli* was harvested using centrifugation.

2.5 Microfiltration

After the centrifugation of homogenized cells, the resulting supernatant was used for microfiltration to purify NP protein. Microfiltration was carried out by forcing the supernantant through the crossflow membrane filter. To determine the best membrane filter to be used to separate NP protein from other contaminants, screening experiments which used two different membrane pore sizes were conducted. Two membrane pore sizes screened were 0.45 µm and 0.1 µm. The best membrane pore size was used in the optimization experiments. To design optimization experiments for determining optimal process conditions to purify NP protein, a two-level full factorial design (STATISTICA 8.0, Statsoft, Inc.) was employed. Three experimental parameters (factors) i.e. transmembrane pressure (TMP), operating temperature and viscosity of samples used were studied to achieve the optimal conditions. The levels of parameters for experimental design are shown in Table 1. A total of 8 set experiments are summarized in Table 2. The NP protein yield was taken as the response (dependent variable, Y) in the experimental design.

Table 1 – The parameters and their respective levels

Parameters	**Low level**	**High level**
Transmembrane pressure (psi)	3.5	4.5
Temperature (^{o}C)	4	28
Viscosity (cP)	2.13*	2.39**

* and **: Supernatant at 7.5 and 9.9 µg/ml protein are used for the experiments, respectively. The viscosities of both supernatants are determined using viscometer.

Once the experiments are performed, the response (NP protein) was fitted to the factorial equation which includes the effect of linear and interaction. The equation is as follows:

$$Y = \beta_o + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \qquad \text{(Eq. 1)}$$

where, Y is the dependent variable (NP protein yield); A, B and C are the independent variable (TMP, operating temperature and viscosity of sample); β_0 is the regression coefficient at center point; β_1, β_2 and β_3 are the linear coefficients; and β_{12}, β_{13} and β_{23} are the second order interaction coefficient.

2.6 Statistical Analysis

Statistical software, STATISTICA 8.0 was used to analyze and develop a regression model of the experimental data, coefficients of all effects and analysis of variance (ANOVA) [7]. The developed regression equation was used to predict the optimum combinations considering the effects of linear and interaction on NP protein yield.

2.7 SDS-PAGE and Western Blot

Permeates were detected for NP protein by running them on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmeli [8]. The fractionated proteins were blotted onto a nitrocellulose membrane (Osmonics, USA) and Western blotting was performed according to Harlow and Lane [9] with some modifications. The blotted membranes were incubated with anti-myc antibody conjugated to alkaline phosphatase (Invitrogen, USA, 1:5000 dilution) before developing it with substrate. Nitro-blue tetrazolium chloride (0.396 mM, Amresco, USA) and bromochloroindolyl phosphate (0.429 mM, Amresco, USA) were used as a substrate solution. Amresco, USA) and bromochloroindolyl phosphate (0.429 mM, Amresco, USA) were used as a substrate solution.

3. RESULTS AND DISCUSSION

3.1 Microfiltration Membrane Screening

In order to optimize the purification of NP protein using microfiltration, two different pore sizes of microfiltration membrane which are 0.1 µm and 0.45 µm were screened to determine which pore size of microfiltration membrane would yield higher NP. The lysed culture was filtrated using these two membranes and later the permeates were analyzed using SDS-PAGE and Western Blot (Fig. 1). Figure 1 shows that the membrane size of 0.45 µm produced more NP protein compared to that of 0.1 µm.

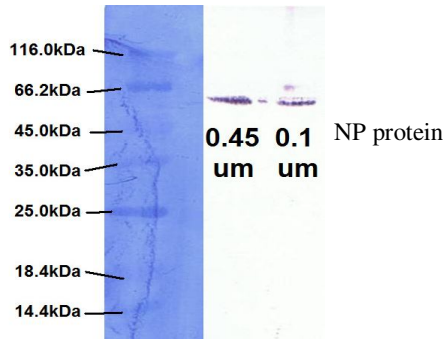

Fig. 1: NP protein after microfiltration using 0.45 µm and 0.1 µm microfiltration membrane. NP protein (53 kDa) was identified using Western Blot Technique.

3.2 Optimization of Operating Conditions of the Microfiltration to Purify NP Protein

In this study, 8 experiments (Table 2) as indicated by full factorial design (FFD) were carried out to obtain the optimized operating conditions of microfiltration to purify NP protein from recombinant *E. coli* lysate. The purified NP proteins of all 8 runs that were analyzed using SDS-PAGE and Western Blot were quantified by AlphaImager System and the protein yield of each run was summarized in Table 2.

The results obtained from the FFD were analyzed to develop the regression equation which shows the response or dependent variable of NP protein (y) as a function of TMP (A), operating temperature (B) and viscosity of sample (C). The equation is as follows:

$$Y = -2.83362 + 0.53704(A) + 0.04448(B) + 1.21250(C) - 0.00452(A)*(B) - 0.19423(A)*(C) - 0.01466(B)*(C) \quad \text{(Eq. 2)}$$

Table 2: NP protein yield after microfiltration and average flow rate of microfiltration

Run	Transmembrane pressure, TMP (psi)	Temperature (^{o}C)	Viscosity (cP)	NP protein (U/µl)	
				Observed	Predicted
1	3.5	4	2.13	0.186	0.170
2	4.5	4	2.13	0.260	0.276
3	3.5	4	2.39	0.278	0.294
4	4.5	4	2.39	0.364	0.348
5	3.5	28	2.13	0.093	0.109
6	4.5	28	2.13	0.121	0.105
7	3.5	28	2.39	0.156	0.140
8	4.5	28	2.39	0.071	0.087

The predicted yields of NP protein for each run using Eq. 2 are summarized in Table 2. The FFD results showed that the best combination of TMP, operating temperature and viscosity of sample were 4.5 psi, 4 ^{o}C and 2.39 cP, respectively, which gave a maximum NP protein of 0.364 U/µl, which is slightly lower than the experimental results. In this experiment, the value of R squared is 0.97 for the NP protein yield. This value indicates a high degree of correlation between the experimental and the predicted values. The value of R squared indicates that 97% of the variables: TMP, operating temperature and viscosity of sample contribute very positively to the response. The value of R squared is also a measure of fit of the model [10] and it can be mentioned that only about 3% of the total variations were not explained by the NP protein yield.

The probability value (p-value) is a tool for evaluating the significance and contribution of each of the parameters to the statistical factorial model equation. The pattern of interactions between the variables is indicated by these coefficients. The variables with low P values contribute to the model, whereas those with high P values can be neglected and eliminated from the model [11]. The p-values for the linear and interactive terms are presented in Table 3. It can be seen that the linear operating temperature term (B) had the largest effect ($p<0.325$), followed by, the interactive TMP and operating temperature term (AB) (0.332), the interactive operating temperature by viscosity of sample term (A) ($P<0.382$), and others. However, all parameters do not significantly affect the yield of NP protein. Due to this the developed model regression equation is not adequately valid to predict optimum microfiltration processing conditions. Further experiments have to be carried out in the future with different range of independent variables in order to develop regression equation that can be used to predict optimized processing conditions.

Table 3: p-value for NP protein yield

Parameter	**p value**
TMP (A)	0.505124
Operating Temperature (B)	0.325438
Viscosity of Sample (C)	0.433435
A by B	0.332706
A by C	0.567353
B by C	0.381505

3.3 Flow Rate

Generally, flow rate of microfiltration process can be divided into three different zones namely, pore blocking zone, cake formation zone and constant rate zone [12] (Fig. 2).

It is apparent from the current study that the flow rate (Fig. 3 and 4) obtained from microfiltration optimization experiments shows decreasing pattern, almost similar with Fig. 2. Closer observations of all the flow rate patterns obtained in this study showed that there is a slight difference in flow rate pattern operated at cold temperature (Fig. 3) and room temperature (Fig. 4). For microfiltration operated at cold temperature (run 1-4), it

was observed that the flow rate has sharp decrease after approximately 2-5 minutes of microfiltration while for those runs operated at room temperature (run 5-8), there was a slight decrease. It seems that at cold temperature, there is probably more formation of cake compared to that of at room temperature and causes the sharp decrease in the flow rate. According to Qin and Chung [13] who observed similar pattern, the flow of warmer fluid through the membrane causes the membrane pore to open up and thus has less effect on the flow rate. Whether there is more cake formed when operating at low temperature was not investigated by them.

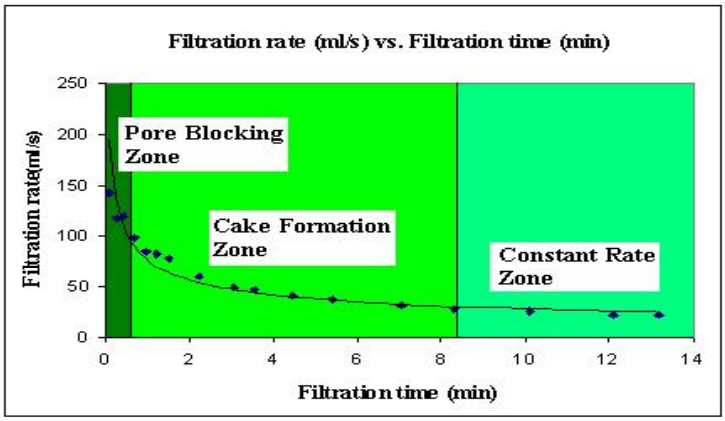

Fig. 2: Typically graph filtration rate versus filtration time
(Source: Qin and Chung, 1999)

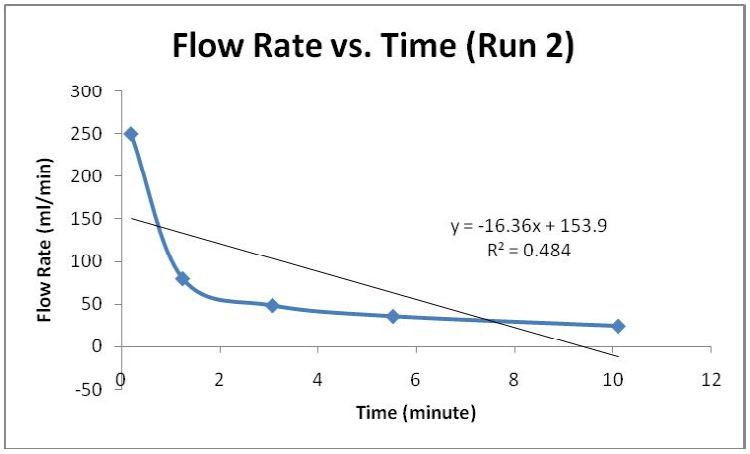

Figure 3: Graphs for flow rate vs. time under cold temperature.
The microfiltration was operated at 4 ^{0}C; TMP, 4.5 psi and viscosity of sample used was 2.13 cP. An identical pattern of flow rate was observed with runs 1, 3 and 4.

The highest average flow rate obtained when microfiltration is operated at room temperature; TMP, 3.5 psi and low viscosity of the sample was used (136 ml/min, run 5, Table 2). However, the highest NP protein purified produced from run 4 of which the

average flow rate is 48 ml/min, almost 3 times lower than that of run 5. Although lower flow rate means the entire process of filtration takes more time to produce product, run 4 is still the best operating conditions to purify NP protein as it produces more than 3 times NP protein compared to that of run 5. Furthermore, the average flow rate of run 4 was not as low as other runs such as run 3, 7 and 8 (21, 33, 29 ml/min, respectively).

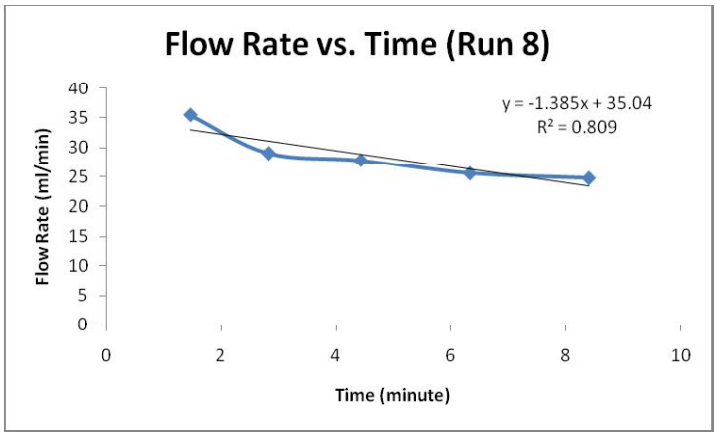

Fig. 4: Graphs for flow rate vs. time under room temperature.
The microfiltration was operated at 28 ^{0}C; TMP, 4.5 psi and viscosity of sample used was 2.39 cP. An identical pattern of flow rate was observed with runs 1, 3 and 4.

4.0 CONCLUSIONS

Results obtained from this study show that a TMP of 4.5 psi, operating temperature of 4 ^{0}C and viscosity of sample of 2.39 cP gave the highest NP protein of 0.364 U/µl. From regression analysis, temperature has the largest effect for the NP protein yield ($p<0.325$). However, the regression model developed from this study is inadequate to identify optimized operating conditions for filtration of NP protein. Future studies with at least three levels for each parameter need to be conducted to obtain the optimized conditions. It is hoped that the optimized operating conditions for filtration of NP protein will effectively purify the protein at shorter period of time with less operating cost.

5.0 ACKNOWLEDGEMENT

The authors are grateful to the International Islamic University of Malaysia (IIUM) for their support in funding this research and also to the Department of Biotechnology Engineering, IIUM for providing lab facilities to complete this work.

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