CLONING AND EXPRESSION OF THE NUCLEOCAPSID PROTEIN OF NEWCASTLE DISEASE VIRUS IN *Pichia pastoris* (Guillierm.) Phaff

By

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Chairman : Fatemeh Jahanshiri, PhD

Faculty : Biotechnology and Biomolecular Sciences

Newcastle disease virus (NDV) is the only member of the genus *Avulavirus* of the family *Paramyxoviridae*. NDV causes a respiratory disease in birds known as Newcastle disease (ND) which may result in high mortality in susceptible hosts such as chickens leading to substantial loss in the poultry industry. Recent outbreak has been reported in many countries including Malaysia. The continuing treat of ND to the poultry industry requires routine testing through development of better diagnostic tools. Therefore, the objective of the current study was to express the immunogenic nucleocapsid (*NP*) gene in a *Pichia pastoris* expression system with a view to develop a potential and cost effective antigen for development of a diagnostic test.

In the present study, the gene encoding NP protein of Newcastle disease virus strain AF2240 was cloned into expression vector, pPICZA and placed under the control of methanol inducible alcohol oxidase (*AOX*) promoter. Then recombinant multi-copy number *Pichia* cells with Mut^+ phenotype were selected for NP protein expression. The optimization of the NP protein production in 50 ml culture was carried out for

methanol concentration and different loaded volume in identical shake flask. A time course study for NP production in 250-ml flask with the optimized conditions was performed as well. The result showed that NP protein could be detected after 12 h of methanol induction and the level of protein expression decreased over time. The recombinant NP was purified from the yeast culture using sucrose gradient ultracentrifugation. The high level and intact recombinant nucleocapsid protein expression (570 mg/l) was obtained after 24 h of induction with 1% methanol when 10% of the shake flask was loaded with MMH (minimal methanol with histidine) medium. Western blot analysis using polyclonal NP antibody confirmed the expression of NP with the molecular weight of 53 kDa indicating that NP protein retained its antigenicity. The recombinant NP protein was highly stable in *P. pastoris* system because there was no degraded product after purification. This result proved that the yeast expression system produces a high yield of recombinant NP protein. The production of recombinant NP protein in bulk as the antigen for diagnostic tools would facilitate the monitoring of NDV infection as well as allowing a more effective control of the disease.

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KLONING DAN PENGEKSPRESAN PROTEIN NUKLEOKAPSID VIRUS PENYAKIT NEWCASTLE DALAM *Pichia pastoris* (Guillierm.) Phaff

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Virus penyakit Newcastle (NDV) adalah sejenis virus yang hanya tergolong dalam genus *Avulavirus* daripada keluarga *Paramyxoviridae*. NDV menyebabkan penyakit pernafasan pada spesis burung dan dikenali sebagai penyakit Newcastle (ND). Virus ini menyebabkan kadar kematian yang tinggi pada perumah yang mudah dijangkiti seperti ayam seterusnya menyebabkan kerugian yang besar dalam industri penternakan. Terkini, penyebaran wabak ini telah dilaporkan di banyak negara termasuk Malaysia. Ancaman yang berterusan daripada penyakit Newcastle ini terhadap industri penternakan memerlukan pemeriksaan rutin melalui pembangunan alat diagnostik yang lebih baik. Oleh kerana itu, objektif kajian ini adalah bertujuan untuk mengekspresikan gen nukleokapsid (NP) yang imunogenik dalam sistem pengekspresan *Pichia pastoris* yang berpotensi untuk menghasilkan antigen secara kos efektif bagi pembangunan ujian diagnostik.

Dalam kajian ini, gen yang mengkodkan nukleokapsid protein daripada virus penyakit Newcastle (NDV) strain AF2240 telah diklonkan ke dalam vektor pengekspresan *Pichia pastoris* (pPICZA) dan ditempatkan di bawah kawalan promoter aruhan metanol alkohol oksidase (*AOX*). Rekombinan *Pichia* yang mempunyai multi-salinan dengan fenotip Mut^+ telah dipilih untuk mengekspreskan protein NP. Pengoptimuman pengeluaran protein NP dalam 50 ml kultur telah dijalankan bagi melihat kesan kepekatan metanol dan oksigen terlarut. Kajian profil masa untuk pengeluaran protein NP juga dilakukan di dalam 250-ml kelalang kon dengan keadaan yang optimum. Keputusan kajian menunjukkan bahawa pengeluaran protein NP dapat dikesan selepas 12 jam diaruhkan. Rekombinan protein NP daripada kultur yis ditulenkan melalui kaedah pengemparan sukros berperingkat. Hasil pengeluaran yang tinggi telah diperolehi iaitu sebanyak 530 mg/l daripada proses penulinan rekombinan protein NP setelah diaruhkan selama 24 jam dengan 1% kepekatan metanol dalam media MMH (Minimal Methanol dengan Histidine). Analisis blot Western menggunakan antibodi poliklonal NP mengesahkan bahawa pengekspresan protein NP adalah pada berat molekul 53 kDa, menunjukkan bahawa protein NP mengekalkan antigenisitinya. Recombinan protein NP adalah sangat stabil dalam sistem *P. pastoris* kerana tiada produk terdegradasi selepas proses penulinan. Keputusan ini membuktikan bahawa sistem pengekspresan yis dapat mengeluarkan rekombinan protein NP dengan hasil yang tinggi. Pengeluaran protein NP pada jumlah yang tinggi mampu bertindak sebagai antigen untuk alatan diagnostik seterusnya dapat memudahkan pemantauan jangkitan NDV kepada industri penternakan selain membolehkan kawalan yang lebih efektif terhadap penyakit ini.

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I certify that a Thesis Examination Committee has met on 4 November 2010 to conduct the final examination of Syazwan bin Saidin on his thesis entitled "Cloning and Expression of the Nucleocapsid Protein of Newcastle Disease Virus in *Pichia pastoris* (Guillierm.) Phaff" in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Master of Science.

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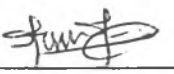
DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at other institution.



SYAZWAN BIN SAIDIN
Date: 04 November 2010

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Ω	ohm (SI unit of electrical resistance)
°C	degree centigrade
µF	microfarad (10^{-6} F)
µg	microgram (10^{-6} g)
µl	microliter (10^{-6} L)
µM	micromolar (10^{-6} M)
ATP	adenosine triphosphate
AOX	alcohol oxidase
AOX1	alcohol oxidase 1
AOX2	alcohol oxidase 2
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BCP	1-bromo-3-chloro-propane
BHK	Baby hamster kidney
bp	base pair
C	bis-acrylamide monomer
C -terminus	carboxy terminus
$CaCl_2$	calcium chloride
CAT	catalase
CHO	Chinese hamster ovary
dH_2O	distilled water
DHAS	dihydroxylacetone synthase
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides

DO	dissolved oxygen
DTT	1,4-dithiotritol
EDTA	ethylenediaminnetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F	fusion protein
FLD1	formaldehyde dehydrogenase
GAP	glyceraldehydes-3-phosphate dehydrogenase
h	hour
His	histidine
HN	haemagglutinin-neuramidase protein
kb	kilo base
KCl	potassium chloride
kDa	kilo Dalton
KH_2PO_4	potassium dihydrogen phosphate
KV	kilo volt
L	large protein
LB	Luria Bertani
LiAc	litium acetate
M	matrix protein
M	Molar
mA	miliampere
MDH	minimal dextrose with histidine
MgCl2	magnesium chloride
MGYH	minimal glycerol with histidine
min	minute

MMH	minimal methanol with histidine
MOP	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
NaCl	natrium chloride
Na_2HPO_4	disodium phosphate
NBT	nitro blue tetrazolium
ND	Newcastle disease
NDV	Newcastle disease virus
NME	N-terminal methionine excision
NP	nucleocapsid protein
NP^o	unassembled nucleocapsid
NRRL	Northern Regional Research Laboratories
nt	nucleotide
N-terminus	amino terminus
OD	optical density
OIE	World Organization for Animal Health
ORF	open reading frame
P	phosphoprotein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCI	phenol:chloroform:isopropanol
PCR	polymerase chain reaction
pH	*puissance hydrogen*
PHOI	*P. pastoris* acid phosphatase
PVDF	polyvinylidene fluoride

RNA	ribonucleic acid
rpm	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulphate
SCO	super optimal broth with catabolite repression
SCP	single cell protein
SIBIA	Salk Institute Biotechnology/Industrial Associate
T	acrylamide monomer
TAE	Tris-acetate-EDTA buffer
Taq	*Thermus aquaticus*
TBST	transfer buffer solution with Tween 20
TE	Tris-EDTA
TEMED	tetramethyl ethylenediamine
Tris-Cl	tris(hydroxymethyl)amino methane
TT	transcription termination sequence
U	unit
uv	ultraviolet
v/v	volume/volume
Vol	volume
w/v	weight/volume
xg	g-force
YNB	yeast nitrogen base
YPD	yeast peptone dextrose
YPDSZ	yeast peptone dextrose sorbitol with zeocin

CHAPTER 1

INTRODUCTION

Newcastle disease (ND) is responsible for one of the most overwhelming diseases of poultry and has a substantial economic impact on the poultry industry. This disease is caused by an avian paramyxovirus type I (APMV-1), from the genus *Avulavirus* belonging to the family *Paramyxoviridae*, known as Newcastle disease virus. There is a wide host range and different clinical severity of this virus depending on its pathogenicity (Alexander, 1998).

Vaccination of chickens particularly those raised for commercial consumption are carried out throughout the world. Although effective live or inactivated Newcastle disease vaccines are currently available, the virus remains an ongoing threat to commercial flocks. For continuation of successful international poultry trades, introduction of a systematic Newcastle disease control measure is desirable. In Malaysia, the disease appears to be endemic; therefore a constant surveillance and vaccination program is required which could be achieved through development of better diagnostic techniques.

Currently, the whole inactivated virus is used as the coating antigen for the commercially available kit such as FlockChek* Newcastle Disease Virus Antibody Test Kits (Idexx, USA). However, because of the difficulties in preparation of the antigen (whole virus) for this kind of kit, the potential of the internal viral components to function as the basis for a diagnostic system has gained a lot of

interest. Among viral proteins, the NDV nucleocapsid protein (NP), which is the most abundant viral protein, has been well defined (Nishikawa *et al.*, 1987) as a major immunogen which can be used as the antigen in serological tests. Interestingly, the highly conserved nucleocapsid protein of NDV involves not only in important biological functions in the virus life cycle but also in inducing a high level of NDV-specific antibodies in chickens.

As a platform for making recombinant proteins, *Escherichia coli* expression system is unbeatable as it grows quickly and has a simple genetic manipulation. However, *E. coli* fails to handle posttranslational modification, for example the *E. coli*-produced proteins are mostly mis-folded and insoluble. There are other expressions systems such as mammalian cells in which proteins are folded properly, however the yield is very low and commercial production using this system is costly. As an alternative, one organism that potentially combines the advantages of bacterial and mammalian expression systems is *Pichia pastoris,* a harmless species of methylotrophic yeast that uses methanol as its carbon source. This yeast is known as an efficient host for the production of recombinant proteins for several factors such as the simplicity of technique needed for the molecular genetic manipulation of *P. pastoris*, the ability of *P. pastoris* to produce foreign proteins at high levels, the presence of many eukaryotic posttranslational modifications and the commercial availability of this expression system.

Currently, the hepatitis B virus vaccine is produced as a recombinant protein using *Pichia* expression system (Hardy *et al.*, 2000). Moreover, an Indian company has released recombinant insulin made in *Pichia* and this product has been registered in

40 countries (Chandra, 2008). In this view and in line with the development of a more efficient diagnostic tool for the detection of NDV in infected birds, the present study was initiated to examine the production of the nucleocapsid protein of NDV in *P. pastoris* expression system.

The main objectives of this study are:

i. To express the recombinant nucleocapsid protein of Newcastle disease virus strain AF2240 in *P. pastoris* expression system.

ii. To purify the recombinant nucleocapsid protein using sucrose gradient.

iii. To determine the yield of its production in this methylotrophic yeast system.