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Short Communication

Optimized expression of capsid protein from red-grouper necrosis virus displayed on *Pichia pastoris* in shake flasks

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Abstract

Importance of the work: High antigen levels on the yeast cell surface are critical in designing oral vaccines against viruses.

Objectives: To investigate the environmental factors affecting the production of RNA2/AGα-1, a displayed fusion protein.

Materials & Methods: The recombinant *Pichia pastoris* was cultivated in shake-flask culture under varying conditions and induced with methanol for 168 hr. Total cell proteins were used as a measure of cell population growth. The expression of the RNA2 fusion protein was also characterized by western blot, immunofluorescence and enzyme-linked immunosorbent assay (ELISA).

Results: The recombinant *P. pastoris* (Mut^s) strain produced optimal growth and product yields using 72 hr post-induction and 0.5% methanol. Prior to induction, the yeast relied on glycerol as the sole carbon source to increase biomass under favorable conditions: 23 °C in an Erlenmeyer flask (5 L) containing 1 L buffered minimal glycerol complex medium (pH 6.0) with vigorous shaking (300 revolutions per minute). Using these optimal conditions, western blot analysis revealed that the RNA2 fusion protein had a molecular weight of approximately 73 kDa. These proteins were localized on the yeast cell surface, as proven based on immunofluorescence labeling at a minimum of 1,000 ng and measured using ELISA.

Main finding: The method developed successfully increased the expression of capsid proteins from the virus on the yeast cell surface in a shake flask culture. Large-scale production is required before investigating the potential use of the fusion protein as an oral vaccine in fish.

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Introduction

Nervous necrosis virus (NNV) or *Betanodavirus* belongs to the family *Nodaviridae*. The NNV virion is a non-enveloped icosahedral comprising two single-stranded RNA gene segments of positive polarity. *RNA1* and *RNA2* encode for a protein with a replicase activity and a coat protein of 42 kDa, respectively. NNV causes viral nervous necrosis (VNN) in many fish species. Affected fish show clinical signs of reduced appetite, a dark body, an abnormal swimming pattern and death mainly at the larval and juvenile stages. Disease outbreaks have the potential to seriously impact the aquaculture industry, causing significant food insecurity (Fukuda et al., 1996). Currently, there are no effective treatments available.

In the yeast surface display (YSD) system, heterologous proteins are fused to an anchor protein confined to particular domains displayed on the yeast cell surface. YSD technology has proven to be promising as an oral vaccine for the immunization of animals. This vaccine can be used for oral immunization in fish food for large-scale vaccination, especially in juvenile fish where an injection is unavailable. Oral administration of such vaccines has substantial economic value, including reducing both labor costs and vaccination pressure. Upon administration as an oral vaccine, the yeast cell wall is not digested in the stomach. Therefore, displayed antigens can efficiently reach the intestine where they induce mucosal and systemic immunity. It has been suggested that YSD-based vaccines induce immune responses more efficiently than yeast vaccine carriers expressed in the cytoplasm, even at a lower expression level (Howland and Wittrup, 2008).

Saccharomyces cerevisiae has been used as a production host in many display systems. However, *S. cerevisiae* produces high mannosylation of recombinant proteins, possibly leading to superantigen production (Yuan et al., 2021). In contrast, *Pichia pastoris* (syn. *Komagataella pastoris*) has a lower degree of mannosylation than *S. cerevisiae.* Being a methylotrophic yeast, *P. pastoris* can utilize methanol as a sole carbon and energy source. Production of recombinant proteins in *P. pastoris* is usually controlled with a strong AOX1 promoter, which is induced by adding methanol. Using an inducible promoter offers advantages superior to a constitutive promoter as the gene expression only starts under induction conditions. Therefore, the production of recombinant proteins does not interfere with normal host cell growth. *P. pastoris* has been used for antigen display and vaccine development because it offers numerous advantages, including its generally recognized safety, easy culture, cheap production and adjuvant functions. *P. pastoris* displaying antigenic proteins has been used in oral vaccines for highly pathogenic avian influenza virus hemagglutinin and *Vibrio harveyi* (Tamaru et al., 2006; Wasilenko et al., 2010; Li et al., 2015). In addition, other oral vaccines have been proposed (Schreuder et al., 1996; Shibasaki et al., 2013). Most of these experiments have produced specific, yet weak antibody responses. Although the antigens were readily detected, their concentrations may not have been sufficient to induce a robust immune response (Schreuder et al., 1996). The ability to expand more antigens targeted to APC is of great importance to the research of vaccine carriers.

The current study aimed to express the displayed antigen for oral vaccination against the red-grouper necrosis virus (RG-NNV), causing viral nervous necrosis. However, in another study, the number of antigens detected was relatively low and likely inadequate to boost any immune response when applied in fish. Thus the current work determined the environmental factors affecting the production of RNA2/AGα-1 fusion proteins displayed on the *P. pastoris* cell surface (Intamaso et al., 2018). The approach obtained under shake-flask culture should facilitate further optimization of large-scale production of the oral vaccine against RG-NNV in fish.

Materials and Methods

Strains, growth medium and plasmid

The RNA2 gene was cloned from RG-NNV and used to modify the *P. pastoris* strain GS115 (Mut^+His^-) genetically (Intamaso et al., 2018). The expression plasmid using heterologous protein expression was pPIC9K purchased from Invitrogen (Carlsbad, CA, USA). The attenable stable clones were propagated on a yeast extract peptone dextrose (YEPD) medium plate (Invitrogen; Carlsbad, CA, USA) and cultured in buffered minimal glycerol complex medium (BMGY) broth. Buffered methanol complex medium (BMMY) broth was used to induce the expression of RNA2/AGα-1 fusion protein.

Optimization of cell growth and RNA2 in a shake flask

The recombinant *P. pastoris* GS115 (Mut^s His^+) was generated by fusing the RG-NNV RNA2 gene to *S. cerevisiae* AGα1 gene coding for α-agglutinin (Intamaso et al., 2018). The recombinant and a negative control yeast containing only the AGα-1 gene were cultured on YEPD agar and incubated at 30 °C

for 72 hr. Then 1–2 colonies were picked and inoculated in a 1 L Erlenmeyer flask containing 100 mL of BMGY medium (1% yeast extract, 2% bacto-peptone, 100 mM KH_2PO_4 pH 6.0, 1.34% yeast nitrogen base (BD Diagnostics; Franklin Lakes, NJ, USA), 0.4 mg/mL biotin, 1% glycerol) overnight until reaching and optical density at 600 nm (OD600) of 2–6. The cultures were centrifuged at 3,000 revolutions per minute (rpm) for 5 min; then, the cell pellet was resuspended in 1/10 (10 mL) BMMY medium (1% yeast extract, 2% bacto-peptone, 100 mM KH_2PO_4 pH 6.0, 1.34% yeast nitrogen base, 0.4 mg/mL biotin, 40 mg/mL L-histidine (Sigma Aldrich Chemical; St. Louis, MO, USA), 0.5% methanol) to an OD600 of 1. The cultures were incubated at 30 °C with constant shaking. Absolute methanol was added to a final concentration of 0.5% daily. To determine factors impacting the efficient production of the RNA2, yeast cultivations were primarily conducted under the following conditions: temperature (23 °C, 25 °C and 28 °C), shaking speed (250 rpm and 300 rpm), and the flask size (100 mL, 250 mL and 500 mL).

The above experiments determined that the optimal conditions were 23 °C with a 500 mL flask volume and a shaking speed of 300 rpm. Samples (each 1 mL) were taken from the shake flasks every 24 hr for 168 hr to check the product yield under the optimal conditions. An equal volume of BMMY broth was added to maintain a constant volume. To scale up the culture, yeast colonies were cultured in 1 L of BMGY media in a 5 L Erlenmeyer flask adjusted to its optimal conditions for 72 hr. The protein induction was performed in 100 mL of BMMY. The samples were centrifuged at 10,000 rpm for 5 min to collect the pellets and stored at -20 °C for further study.

Protein extraction

The pellets were resuspended in 250 µL of lysis buffer pH 7.2 (Tris 10 mM, NaCl 150 mM, Triton-X 0.5% (v/v), ethylene-diamine-tetraacetic acid 1 mM, pH 7.2*)*, vortexed to homogenize the mixture and then incubated on ice for 30 min. The mixtures were sonicated (Sonicator Probe: UP50H; Hielscher; Teltow, Germany) on ice at a frequency of 20 kHz (amplitude 100%, pulse 15 s/10 s, 5 min) to obtain the cell lysate. Cell debris was discarded using centrifugation at 13,000×*g* for 10 min at 4 °C. Crude proteins (25 µL) were assessed for protein concentrations using a Pierce BCA protein assay kit (Thermo Scientific; Waltham, MA, USA) and compared with a bovine serum albumin standard curve. The proteins were stored at -80°C until used in subsequent experimentation.

Western blot and dot blot analysis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was used to estimate the molecular weight of the RNA2 fusion protein with 12% separating gel according to Laemmli (1970) by comparing with the Precision Plus Protein Dual Color Standard protein marker (Bio-Rad; Hercules, CA, USA). Twenty-five micrograms of total proteins in each lane were separated via electrophoresis using 1x running buffer (250 mM Tris, 1.92 M glycine, 1% SDS (weight per volume), pH 8.3) at 25 mA and 60 V for 35 min and 55 mA and 120 V for 1 hr 20 min. The protein bands on gel were transferred onto a nitrocellulose membrane in a transfer buffer (192 mM glycine, 25 mM Tris 10% (v/v) methanol) using 100 mA and 30 V overnight at 4 °C. The membrane was blocked for 1 hr in blocking buffer (20 mM Tris-HCl, 0.15 M NaCl pH 7.5, 3% skimmed milk powder). The rabbit anti-NNV primary antibody (ab26812; 1:5000) diluted in Fast Western Antibody Diluent (Thermo Scientific; Waltham, MA, USA) was added to bind the target protein overnight at 4 °C. After rinsing with 1x Fast Western wash buffer (Thermo Scientific; Waltham, MA, USA), optimized horseradish peroxidase reagent working solution diluted (1:5000) in Fast Western Antibody Diluent (Thermo Scientific; Waltham, MA, USA) was added and incubated at room temperature with gentle shaking for 30 min. The expected protein targets (73 kDa) were visualized using KPL 1-Component TMB Membrane Peroxidase Substrate (Seracare; Milford, MA, USA). For the dot blot analysis, 1 µL of the crude proteins was dropped on the membrane and baked overnight at 80 °C, before further processing, as described above.

Immunofluorescence

Yeast cells were washed twice in sterile distilled water using centrifugation at 6,000 rpm for 5 min. Cells were dispersed into a single-cell suspension and spread on a coverslip at a density of 3×10^8 cells. After air drying at room temperature, monolayer cells were fixed with acetone for 3 min and washed with 1× phosphate buffer saline (PBS). Finally, cells were stained with rabbit anti-NNV antibody (ab26812; 1:5000) diluted in 1× PBS at 4 °C, followed by Anti-Rb IgG (H+L), F(ab')2 Fragment (AlexaFluor® 488 Conjugate; 1:3,500) diluted in 1× PBS. Fluorescence-positive cells were observed under a fluorescence microscope (D-LEDI; Nikon Corporation; Minato, TYO, Japan).

Enzyme-linked immunosorbent assay

The rabbit anti-NNV antibody (ab26812) was serially prepared at various concentrations (1,000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.90 or 1.95 ng/mL) in 1× PBS containing 1% Bovine serum albumin. The solutions (each 100 µL) of serially diluted antibody were incubated with yeast cells at a density of 1 × 10^8 cells per well, with gentle shaking at room temperature for 1 hr. Following washing with 1× PBS, the goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Invitrogen; Carlsbad, CA, USA) at 1:5000 was added and incubated in the dark at room temperature for 1 hr. Finally, 40 µL TMB Substrate Solution (Thermo Scientific; Waltham, MA, USA) was added and incubated in the dark for 20 min. The reactions were stopped with 40 µL Stop Solution (Thermo Scientific; Waltham, MA, USA). After centrifugation, the amount of RNA2 fusion proteins displayed on the yeast surface was quantified by measuring the absorbance at 450 nm.

Statistical analysis

A paired Student's t-test was used to evaluate the different protein expression levels between *P. pastoris* displaying the RNA2/AGα-1 fusion protein strain and the control under the same condition. The total protein production of the recombinant strain in different conditions were analyzed using analysis of variance following by mean comparison using t test. The statistical significance was set at $p < 0.05$.

Ethics statement

This study was approved by the Ethics Committee of BUU-IBC Burapha University (Approval no.26/2558).

Results and Discussion

Intamaso et al. (2018) reported that RNA2 capsid proteins of RG-NNV linked to AGα-1 of *S. cerevisiae* were displayed at low levels on the *P. pastoris* cell surface. However, the current study demonstrated that growing yeast under optimized conditions successfully increased the levels of fusion proteins displayed on the yeast cell surface.

Analysis of shake flask cultivations of P. pastoris

In bioprocessing, various parameters may affect the yield and quality of each recombinant protein product. Experiments were performed under various conditions to determine the optimal growth conditions for the cultivation of *P. pastoris* displaying RNA2 fusion proteins. Temperature has been reported as one of the critical environmental factors (Laurent et al., 2000; Hong et al., 2002; Woo et al., 2004). *P. pastoris* generally grows at temperatures in the range 20–30 °C. Thus, in the current study, temperature was initially screened for its effect on the cell growth of *P. pastoris* by measuring cell population growth. The total proteins harvested from cells cultured at the studied temperatures of 23 °C, 25 °C and 28 °C were 57.7 mg/mL, 55.65 mg/mL and 54.44 mg/mL, respectively (Fig. 1A). The results indicated that *P. pastoris* grown at 23 °C in the respective media, had better cell growth than at the other temperatures (25 °C and 28 °C).

At a low induction temperature, the solubility of oxygen gas increases to oxidize methanol, thereby increasing recombinant protein expression. Ensuring a low temperature reduced the chances of cell death and degradation of recombinant proteins (Li et al., 2001; Jahic et al., 2003; Sinha et al., 2005). In another study, at a lower cultivation temperature, *P. pastoris* produced a three-fold increase in recombinant protein production (Dragosits et al., 2009). On the other hand, elevated temperature extends the accumulation process of the nascent proteins in the endoplasmic reticulum (ER), leading to overload of the ER and ultimately cell death (Zhong et al., 2014). This result was in accordance with other studies (Li et al., 2001; Joseph et al., 2022), in which the cell density decreased at the end of fermentation when grown at 30 °C compared to 23 °C.

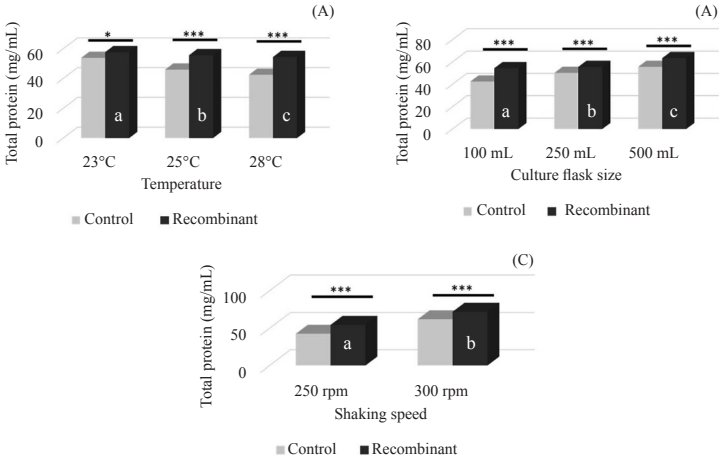

Fig. 1 Comparison of total proteins harvested from *Pichia pastoris* displaying RNA2/AGα-1 fusion proteins when grown under different conditions of: (A) temperature; (B) culture flask size; (C) shaking speed, where yeast strain containing only AGα-1 gene was used as a negative control; *, *** denote significant ($p < 0.05$) and highly significant ($p < 0.001$) different between control and treatment; different lowercase letters at each bar denote significant ($p < 0.05$) different among treatments

In addition, the temperature can significantly influence protein folding (Mattanovich et al., 2004) and enhance the proteolysis of recombinant protein (Li et al., 2001; Masuda et al., 2004; EFSA Panel on Food Additives and Flavourings et al., 2021).

With *P. pastoris* systems, it has been shown that an increase in expression strongly depends on the extent of aeration (Villatte et al., 2001; Fredericks et al., 2010). The current study investigated the effect of aeration conditions on cell growth. Culture flasks (100 mL, 250 mL and 500 mL) each with the same amount of BMGY medium (10 mL) were inoculated with the yeast strains. As seen, the total proteins extracted from harvested cells increased with the increasing volume of the flasks (protein concentrations of 54.44 mg/mL, 55.58 mg/mL and 63.48 mg/mL, respectively), as shown in Fig. 1B, illustrating that increasing the aeration by increasing the vial size with the same medium volume enhanced cell growth. Based on the fact that methanol metabolism utilizes oxygen as a carbon and energy source, the amount of oxygen in the medium is related to the space in the culture flask available for air circulation or for the oxygen dissolved in the liquid. Generally, *P. pastoris* produces a high protein expression level in vials containing medium filling up no more than 10–30% of the vial; thus, the aeration ratio does not exceed 3:10 (Invitrogen™ Life Technologies Corporation, 2020). Finally, the agitation speed was varied from 250 rpm to 300 rpm to monitor the effect of shaking speed on cell growth. The result illustrated that the total protein content from cell cultivation at 300 rpm was greater than that at 250 rpm (71.99 mg/mL and 54.44 mg/mL, respectively), as shown in Fig. 1C. In all experiments, the protein content levels of *P. pastoris* displaying RNA2 fusion proteins were significantly greater than the control recombinant strain containing only AGα-1 grown under the same conditions. The current results indicated that increasing the amount of oxygen from the increased flask size and the shaking speed increased the cellular respiration capacity of yeast, especially in the methanol pathway. Besides being used for respiration, molecular oxygen is also utilized for the initial oxidation of methanol by the AOX enzyme to produce formaldehyde and hydrogen peroxide (Higgins and Cregg, 1998; Cereghino and Cregg, 2000). When cells are exposed to oxygen limitation, formaldehyde accumulates within the cell, triggering cell death. The proteases are then released, causing surrounding protein degradation.

Time course analysis of yeast cell growth and NNV RNA2 fusion protein expression under optimized induction conditions

Due to the disruption of the AOX1 gene, the Mut^s (methanol utilization slow) strain used in the current study must depend on the weaker AOX2 promoter for methanol metabolism; consequently, it grows slowly on a carbon source (Cregg et al., 1987). One potential advantage of the Mut^s phenotype strains is that they utilize less methanol and sometimes express greater levels of recombinant protein than wild-type (Mut$^+$) strains, especially in shake-flask cultures (Cregg and Madden, 1987). The current study investigated the time course of yeast cell growth under the optimized conditions. In a 500 mL Erlenmeyer flask, 10 mL of yeast cell suspension in BMMY broth at pH 6.0 were incubated in an incubator shaker (300 rpm) at 23 °C under methanol induction for 168 hr. The results showed that the total protein from the yeast displaying fusion proteins, in addition to the control strain, reached the highest concentrations at 72 hr post-induction of 107.22 mg/mL and 54.84 mg/mL, respectively (Fig. 2A). At the same time, the maximum level of NNVRNA2 expression was measured using dot blot analysis (Fig. 2B). The level of these proteins was correlated to the growth of *Pichia pastoris* based on measuring the total protein concentration.

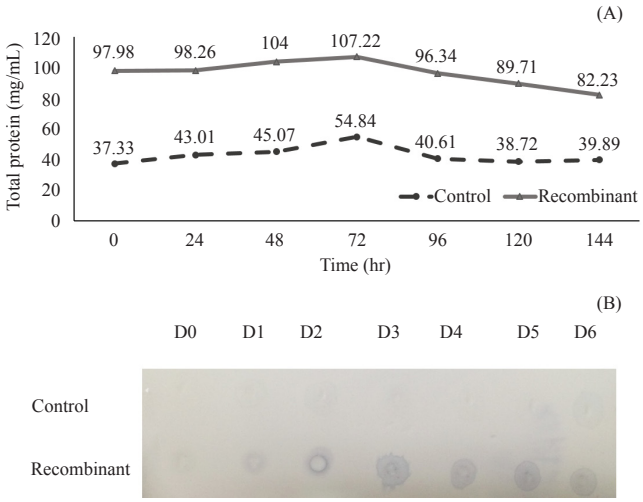

Fig. 2 Time course analysis of yeast cell growth under optimized conditions: (A) total proteins harvested from *P. pastoris* displaying RNA2/AGα-1 fusion proteins at indicated times; (B) dot blot analysis of cell extracts using anti-RG-NNV antibody, where D0–D6 = number of days

Scaling nervous necrosis virus capsid protein displayed on P. pastoris

The initial cell concentration at the commencement of induction provides the most effective method to increase heterologous protein expression levels (Fredericks et al., 2010). In general, strains are initially grown in a BMGY medium containing glycerol as its carbon source. The increasing volume of the BMGY medium enhances the biomass density after reaching the stationary phase. Thus, the recombinant *P. pastoris* strains were cultured in 1 L of BMGY in a 5 L Erlenmeyer flask and adjusted to the optimal conditions. During this period, biomass accumulated, but heterologous

gene expression was completely repressed. Finally, methanol in 100 mL of the BMMY medium was fed to the culture to induce high transcription levels for 168 hr. The highest expression level was observed at 72 hr post-induction. The total protein extracted from the surface-displayed strain was markedly higher in concentration (119.34 mg/mL) than that of the control strain (72.45 mg/mL), as shown in Fig. 3A. The western blot result confirmed the expression of the RNA2/AGα-1 fusion proteins with the expected molecular weight of approximately 73 kDa (Fig. 3B). On the other hand, these proteins could not be detected in the control strain expressing only AGα-1 proteins. Notably, no traces of degradation products (56 kDa) were observed on the western blots on cultured yeast at a lower temperature (23 °C) than at 28 °C in another study (Intamaso et al., 2018).

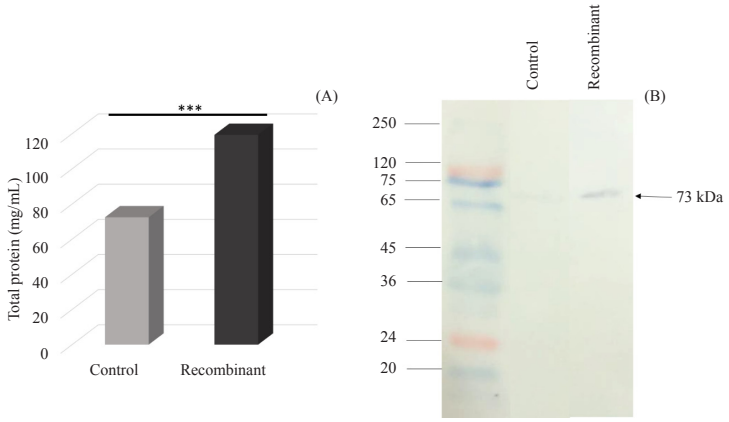

Fig. 3 Scaling nervous necrosis virus (NNV) capsid protein displayed on *P. pastoris* under optimized conditions: (A) total protein harvested from *P. pastoris* displaying RNA2/AGα-1 fusion proteins at 72 hr post-induction; (B) western blot analysis of cell extracts using an anti- red-grouper necrosis virus (RG-NNV) antibody, where lane 1 = *P. pastoris* control strain, Lane2 = *P. pastoris* displaying RNA2 fusion proteins, compared to a protein marker (Bio-Rad, Hercules, CA, USA); *** denote highly significant ($p < 0.001$) different between control and recombinant

ELISA was used to quantify the amount of RG-NNV RNA2 fusion proteins displayed on the yeast surface. The results demonstrated that the yeast strain displayed a total yield of the fusion protein of at least 1,000 ng on its surface (Fig. 4). Using flow cytometry, the exact amount of the RNA2 fusion protein on the yeast cell surface could be quantified (Ananphongmanee et al., 2015). Utilizing a yeast surface display system in vaccine applications, correct protein folding and surface localization are also critical characteristics of an effective vaccine that ensures interaction with its antibody. Immunofluorescent labeling of cells with anti-NNV RNA2 IgG revealed that cells expressing the RNA2/α-agglutinin fusion gene had a high fluorescence intensity, whereas the control cells did not (Fig. 5). Nonetheless, the intensity differed from cell to cell, probably due to differences in the expression levels among the individual cells. These results confirmed that the fusion proteins had correctly folded on the yeast cell walls. In a yeast cell surface display system, a target protein attached to a cell-membrane-bound glycosylphosphatidylinositol (GPI)-anchor protein was successfully displayed on the yeast cell surface, with sizes in the range 93–136 kDa (Kondo and Ueda, 2004). In the current study, the 73 kDa RG-NNV RNA2/AGα-1 fusion protein had a lower molecular weight. Nevertheless, based on the tertiary structure prediction from the I-TASSER server, the RNA2 fusion proteins folded into correct epitopes that could be recognized by the immune system (Intamaso et al., 2018).

The current study successfully increased the expression of the RG-NNV RNA2/AGα-1 fusion protein on the *P. pastoris*

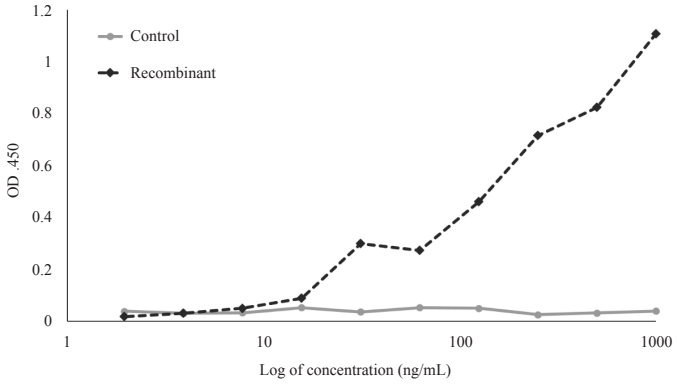

Fig. 4 Quantitation of NNVRNA2/AGα-1fusion proteins displayed on yeast surface based on enzyme-linked immunosorbent assay using an anti-red-grouper necrosis virus antibody, where OD.450 = optical density at 450 nm

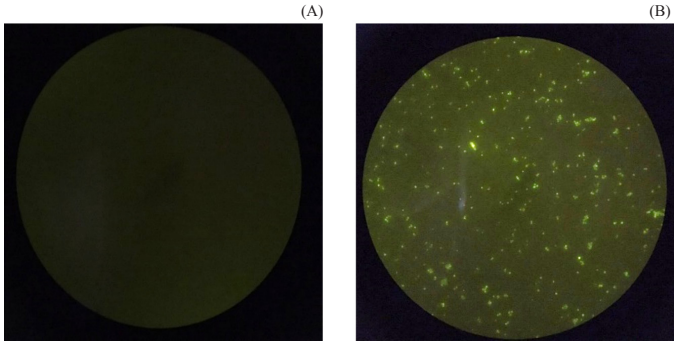

Fig. 5 Immunofluorescence staining using an anti-RG-NNV antibody for: (A) control yeast strain; and (B) *P. pastoris* displaying RNA2 fusion proteins

cell surface. In a shake-flask culture, the *P. pastoris* (Mut^S) strain grew well at 23°C in an Erlenmeyer flask (5 L) containing 1 L BMGY media at pH 6.0, with vigorous shaking (300 rpm). The product yields on the surface protein also maximally increased when induced with the methanol concentration at 0.5% (v/v) for 72 hr. The findings in the current study obtained from shake-flask culture should facilitate further optimization of the large-scale production of the fusion proteins. Antisera binding to expressed gene products is necessary prior to further studies for their efficacy in protecting fish against RG-NNV. If yeast displaying RNA2 fusion proteins proves an effective vaccine candidate, it may be taken orally to prevent fish from being infected with RG-NNV.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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