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Optimization of Cultivation Conditions to Enhance the Expression of Recombinant VDAC2 from *Gallus gallus*

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VDAC2 protein identified to be over expressed in over-stunned chicken is a potential biomarker in differentiating stunned chicken and the voltage applied during the process. The VDAC2 gene was amplified by proofread DNA polymerase, then purified and inserted into pENTR-TEV-D-TOPO entry vector and transformed into One Shot TOP 10 Chemically Competent *E. coli*. The entry clone then was sub cloned into pDEST17 expression vector using LR recombinase in *E. coli* DH5α as the host. The vector construct was transformed into *E. coli* BL21-AI for protein expression. Recombinant VDAC2 protein was purified by affinity chromatography using Ni-NTA spin-column. Western blot using polyclonal anti-N terminal human VDAC2 and anti-His tag, followed by secondary antibody AP goat anti-rabbit revealed a 34 kDa protein, confirming the expression of recombinant VDAC2. Post-induction temperature, concentration of L-arabinose and post-induction time were selected for optimization studies under Response Surface Methodology (RSM). The optimum predicted cultivation conditions for the maximum expression of recombinant VDAC2 was found to be at 29°C post-induction temperature, 6.8 hour post-induction time and 0.5% (w/v) L-arabinose with a predicted expression of recombinant VDAC2 intensity of 0.496. The experimental expression of recombinant VDAC2 intensity obtained was 0.496, which was very close to the predicted value. The expression of recombinant VDAC2 improved by almost 6-fold after the optimization process. Therefore, RSM is a suitable method for optimizing recombinant VDAC2 expression in *E. coli* to provide a better understanding of its characteristics and propertiesto become abiomarker in detecting over-stunned chicken.

Key words: Chicken, electrical stimulation, *E. coli*, recombinase reaction, optimization.

Voltage-dependent anion channel (VDAC) is a protein of the porin family[1], which is the most abundant protein found in the outer mitochondrial membrane (OMM) and is responsible in the transportation of metabolites between cell organelle and the cytosol[2,3,4]. Structurally, VDAC is a porin-type α-barrel diffusion pore formed by one polypeptide with a molecular weight of about 30 kDa[5]. There are three isoforms of VDAC which are VDAC1, VDAC2 and VDAC3, each of a molecular mass of 30-35 kDa, 30-32 kDa and 31 kDa, respectively[6,7,8].

VDAC has a small amount of protein mass yet displays complex behavior, packed with many functions. Although the voltage-gating feature is highly conserved among different organisms and VDAC isoforms, it has not yet been unambiguously correlated by a biological function. Some of the functions include the regulation of cellular energy production by controlling metabolite flux across the mitochondrial outer membrane and the involvement in apoptosis[9,10] and a modulator for anti-cancer therapy[11].

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During high-level expression of recombinant protein, macromolecular crowding of proteins in the cytoplasm of *E. coli*, suggest a highly unfavorable protein-folding environment, resulting in protein aggregation[12]. In recombinant bacteria, protein aggregation is an ordinary consequence of thermal stress[13]. Therefore, by simply lowering the culture temperature during and after induction, production of soluble recombinant protein in bacteria will be enhanced[14,15]. However, low temperature will influence the growth of bacteria, resulting in reduction of expression level. Therefore, it is important to find the optimum post-induction temperature that will balance the production of the recombinant protein with the growth of the cultivation cultures. Besides reducing the temperature, recombinant genes can be placed under a variety of promoters. The *E. coli* strain BL21-AI contains a chromosomal insertion of the gene encoding T7 RNA polymerase (T7 RNAP) into the *ara*B locus of the *ara BAD* operon, placing regulation of the T7 RNAP gene under the control of the *araBAD* (or P_{BAD})promoter. So, to induce recombinant protein expression from the *araBAD* promoter, L-arabinose was used[16,17]. Consequently, the optimum L-arabinose concentration can overcome low yield of recombinant protein thus lowering the cost for expression process.

Recent study showed that VDAC2 level is elevated in the samples of muscles of chickens stunned at 0.75 Ampere, 70 Volt from 2-D proteomic profiles[18]. It was confirmed at the transcriptional level where the gene coding for VDAC2 exhibits up-regulation up to 59.87 fold. Thus, VDAC2 is a potential biomarker to detect whether the chicken has been electrically over stunned. Recombinant protein obtained from this study can be used for antibody production and further facilitates the research and development of a suitable detection kit. Research on chicken's VDAC protein is still lacking compared to other organisms and this study provides better insight of this protein.

MATERIALS AND METHODS

Female broiler chickens (*Gallus gallus*) aged 35 days and weighing approximately 1.5 kg were collected from a farm in Kuang, Selangor, Malaysia. Polyclonal anti-N-terminal human VDAC2 purchased from Aviva (USA) and anti-His tag was purchased from Millipore (USA). Alkaline phosphatase-conjugated goat anti-rabbit IgGwas purchased fromBiorad (USA).

Plasmid and expression system

E. coli BL21-AI (Invitrogen, USA) was used as the expression host. The VDAC2 gene was isolated from electrically stimulated chicken (0.75 Ampere, 70 Volt, 5 seconds) and cloned into the expression vector pDEST17 (Invitrogen, USA). This construct was named as *pDEST17-VDAC2*.

Fermentation and over-expression

Three or four transformants that grew on LB plates were selected and cultured in 5 mL LB medium containing 100 µg/mL ampicilin. The culture was grown at 37°C, 250 rpm until the OD_{600} reached 0.3. This initial culture was used to inoculate 95 mL of fresh LB supplemented with 100 ìg/mL ampicilin. The culture was grown until it reached mid-log phase (OD_{600}~0.4). Then, L-arabinose (0.2% w/v) was introduced into the culture. It was incubated further for six hours, and then cells were harvested by centrifugation at 5,000x *g*, 4°C for 15 minutes.

Visualization and intensity measurement of the VDAC2 expression

The sample from each run was purified and loaded onto the SDS gel. The gel was silver stained and scanned using LabScan 6.0 software (GE, Germany) and the intensity of the band was determined from the spots identified by the ImageMaster software (GE, Germany). This method was used to measure the expression of VDAC2 since VDAC2 is not an enzyme and its activity cannot be measured by enzyme assay method.

Validation of VDAC2 expression by Western Blot

Protein bands on the SDS-PAGE were transferred onto nitrocellulose membranes using Mini Trans-Blot® Electrophoretic Transfer Cell (Biorad, USA) at 4°C for 1 hour in a transfer buffer (0.025 M Tris, 0.192 M glycine, 20% methanol, pH 8.3-8.5). Membranes were blocked with 5% non-fat powder milk in TBST (50 mMTris–HCl, 150 mMNaCl, 0.1% Tween 20, pH 7.5) for 2 hours at 4°C. Next, the membrane was probed with 1:1000 dilutions of primary antibody, polyclonal anti-N terminal human VDAC2 (Aviva Systems Biology, USA) on the rocker. Then the membrane was washed extensively with 20 mL TBST for 5 minutes and repeated three times before incubated with

1:10,000–15,000 dilutions of alkaline phosphatase-conjugated mouse anti-rabbit IgG. Both primary and secondary antibodies were diluted in TBST. To remove the detergent, the membrane was incubated with TBS (20 mMTris-HCI, 140 mMNaCl, pH 7.5) for 5 minutes (Tris-Buffered Saline) and washed three times for 5 minutes. Finally, the color was developed using Sigma 5 tablet (Sigma Aldrich, USA) which was prepared by solubilizing each tablet in 10 mL distilled water.

Experimental design and cultivation condition optimization

Response Surface Methodology[19] was applied to design the experimental runs to optimize the cultivation conditions for the over-expression of recombinant VDAC2 in *E. coli* via Design Expert v 8.0.7.1 software (Stat-ease, USA). The optimization experiment was designed based on a rotatable central composite design (CCD) with a total of 15 experimental runs involving 4 star points and 1 replicate at the central points which is shown in Table 1. Three factors chosen for optimization study were post-induction temperature (X_1), post-induction time (X_2) and amount of inducer (X_3). The expression of recombinant VDAC2 was measured by the intensity of the band from each run in the SDS polyacrylamide gel using LabScan 6.0 and ImageMaster 2D Platinum 6.0 (GE, Germany).

For statistical calculations, the relation between the coded values and real values were as described in the following equation:

$$X_i = U_i - U_o \quad \Delta U \quad ...(1)$$

where X_i is the independent variable coded value, U_i the real value of the independent variable, U_o the real value of the independent variable on the center point, ΔU the step change and the central point was set with α of 1.68.

The significance of each coefficient whether the model is linear or quadratic was analyzed using the ANOVA test and *P*-value (probability >*F*) less than 0.05 indicated that the model terms are significant. Adequacy of the model developed was further analyzed. The optimal values were obtained by solving the regression equation and analyzing the 3D response surface plot.

RESULTS AND DISCUSSION

Validation of VDAC2 expression

The expression of recombinant VDAC2 was confirmed by Western blot (Fig. 1). Two bands with a size of ~35 kDa and ~50 kDa were observed. The 35 kDa band appears to be in agreement with the predicted size of VDAC2. However, the identity of the 50 kDa band is still elusive.

The binding affinity of most antibodies is influenced by conformational determinants, and antibodies may not bind the same protein in a denatured state[20]. Since polyclonal antibody generally is less specific compared to monoclonal antibody, therefore, it is not a surprise to observe multiple bands in Western blot analysis. VDAC2 is an isoform of VDACs and monoclonal antibody would be more likely to identify single member of protein families.

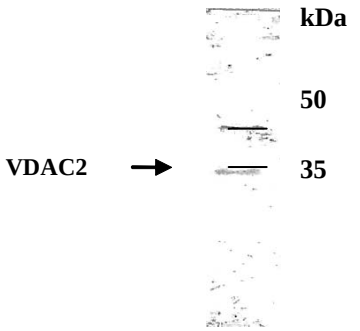

Fig. 1. The Western blotting result showed the His-tagged VDAC2 band at 35 kDa

Effects of the cultivation conditions on the expression level of recombinant VDAC2

The optimization study was to investigate three physical factors with five levels of variance which are post-induction temperature (X_1), post-induction time (X_2) and amount of L-arabinose (X_3) using Response Surface Methodology (RSM) design which is able to predict the optimum cultivation condition for the expression of recombinant VDAC2. The response is measured by the intensity (Y) of the spot detected from the band stained using silver nitrate on the 1-D polyacrylamide gel. The results obtained were analyzed using Design Expert v 8.0.7.1 software (Stat-ease, USA). The results are shown in Table 1.

From Table 1, the highest expression of VDAC2 is 0.663 intensity (Run 6), suggesting the highest expression will be obtained when post-induction temperature is 30°C, harvest 6.5 hour after induction and induce with 0.5% (w/v) L-arabinose. The actual value (Y) is the experimental value transformed according to the model requirement and compared to the predicted value generated by the model. The residual (Y-Ý) showed the difference between the actual and predicted value. The responses were analyzed by using the analysis of variance (ANOVA) and the details are shown in Table 2. The *P*-value for the model source, each model terms and interactions were detailed in the ANOVA (Table 2). The analysis of variance coupled with high value of R^2 which is 0.933 showed that the quadratic model used for the prediction was significant ($p < 0.05$). Nevertheless, this model was transformed into an inverse square root model since the ratio of maximum response (0.6625) to minimum response (0.045) is greater than 10. An actual model as shown in Equation 2 was developed to predict the optimum cultivation conditions that are to maximize the expression of recombinant VDAC2.

$$1/\mathrm{Sqrt}(Y) = 20.61281 - 1.10776\,X_1 - 0.19223X_2 - 10.73884\,X_3 - 0.032011X_1X_2 - 0.055973X_1X_3 + 4.57754E\text{-}003X_2X_3 + 0.023612X_1^2 + 0.081635X_2^2 + 12.35689X_3^2 \quad ...(2)$$

Although the R^2 is high (93.3%), nevertheless the predicted R^2 of 0.3497 is not as close to the adjusted R^2 which is 0.8173. The coefficient of determination (adjusted R^2) was calculated to be 0.8173, indicating that only 19% of the total variation were not included in the model. This value indicates a good agreement between the observed and the predicted values of recombinant VDAC2 production. Therefore, the regression model was applied to calculate the predicted values and the usefulness of the model where the predicted values are closely matched with the experimental values after the transformation. In other words, the model obtained is applicable to predict the optimum cultivation conditions that will maximize the expression of recombinant VDAC2.

Table 1. Central Composite Design (CCD) matrix, the predicted and actual values obtained from the expression of VDAC2 in *E. coli*

Run	Actual Values			Intensity			
	X_1 (°C)	X_2 (h)	X_3 (%w/v)	Actual value[a]	Predicted value (Ý)	Actual value[b] (Y)	Residual (Y-Ý)
1	30	12.5	0.5	0.073 ± 0.014142	3.88	3.70	-0.18
2	30	7.5	0	0.045 ± 0.000000	4.58	4.72	0.14
3	40	7.5	0.5	0.056 ± 0.002616	4.24	4.24	-4.667E-003
4	35	5	0.25	0.073 ± 0.004172	3.70	3.87	-0.17
5	25	5	0.75	0.123 ±0.000636	2.61	2.85	0.24
6	30	7.5	0.5	0.663 ±0.002121	1.46	1.23	-0.24
7	30	2.5	0.5	0.105 ±0.010112	3.14	3.09	-0.085
8	25	10	0.75	0.057 ±0.003889	3.79	4.19	0.40
9	30	7.5	1	0.058 ±0.004455	4.57	4.16	-0.41
10	35	10	0.75	0.084 ± 0.000354	3.25	3.44	0.19
11	25	10	0.5	0.073 ±0.000919	3.68	3.69	6.979E-003
12	20	7.5	0.25	0.098 ±0.00700	3.42	3.19	-0.23
13	35	10	0.25	0.085 ±0.010112	3.43	3.43	9.392E-005
14	25	5	0.25	0.152 ± 0.000000	2.52	2.57	0.051
15	35	5	0.75	0.065 ±0.005798	3.68	3.91	0.23

a Experimental value

b Intensity value that have been transformed (1/sqrt Actual value[a]) according to the requirement of the statistical analysis.

Post-induction temperature, post-induction time and amount of inducer are factors that are highly crucial in the expression of recombinant protein. Most of the optimization

study on the culture conditions will include these three factors. The use of contour plot is important in illustrating and interpreting the response surface method. They are simple two-dimensional and wider view of three-dimensional graphs that show interaction between two design variables, while the other variable remains constant. The two dimensional and three dimensional response surface and their corresponding contour plots for interactions between post-induction temperature and post-induction time (X_1X_2), post-induction temperature and amount of inducer (X_1X_3), post-induction time and amount of inducer (X_2X_3) are presented in Fig. 2a, 2b and 2c, respectively. Although only X_1X_2 interactionis significant, the insignificant interaction effects simplify the scale-up process for the enzyme production and are

Table 2. ANOVA for response surface quadratic model for the expression of recombinant VDAC2 from *E. coli*

Source	Sum of Squares	Degree of Freedom (DF)	Mean Square	F - Value	Prob. > F	Remarks
Model	9.31	9	1.03	7.96	0.0171	Significant
X_1(Post-induction temperature)	0.67	1	0.67	5.15	0.0725	*
X_2 (Post-induction time)	0.55	1	0.55	4.24	0.0945	*
X_3 (Amount of inducer)	7.193E-004	1	7.193E-004	5.537E-003	0.9436	*
X_1X_2	1.28	1	1.28	9.86	0.0257	**
X_1X_3	0.039	1	0.039	0.30	0.6066	*
X_2X_3	6.548E-005	1	6.55E-005	5.040E-004	0.9830	*
X_1^2	3.86	1	3.86	29.71	0.0028	**
X_2^2	2.88	1	2.88	22.20	0.0053	**
X_3^2	6.61	1	6.61	50.85	0.0008	**
Residual	0.65	5	0.13	-	-	-
Correlation total	9.96	14	-	-	-	-

desirable for most of the large-scale production[24].

For the interaction of post-induction temperature and post-induction time, the amount of inducer remained constant at 0.45% (w/v). In this study, the post-induction temperature does increase the expression of VDAC2 with post-induction time but at the some point which is at 28.6°C and after 6.7 hour the expression decreased (Fig. 2a).

Meanwhile, if the post-induction time was set constant at 6 hours, the highest post-induction temperature for maximum expression is at 28.3°C with amount of inducer of 0.5% w/v until the decrease in expression of VDAC2 after that point (Fig. 2b). Lastly, at a constant post-induction temperature of 29°C, the expression of protein will increase with the increase of post-induction time and amount of inducer until 6.3 hour and 0.5% w/v L-arabinose, where the expression start to decline (Fig. 2c).

A routine temperature in expression condition usually is constant at 37°C. However, by lowering the temperature after induction can enhance the expression of VDAC2, and the higher temperature reduces the desire expression of the protein. There are many documented studies in which many soluble proteins were expressed better in lower temperature. It is believed that a lower temperature can lift off the stress bared by the bacteria in producing the recombinant protein[15]. Moreover, in a lower temperature, the newly transcribed recombinant protein tends to fold properly due to slower rates of protein production. In T7 expression system, a large number of recombinant proteins often precipitate when expressed at 37°C, but tend to be soluble when induction temperature is lowered to 15-25°C[25]. Since VDAC2 is insoluble, decreasing the post-induction temperature has been shown to significantly reduce in protein aggregation.

In addition, the stability of plasmid in recombinant *E. coli* cultures can be affected by temperature[26,27]and thus affects production of soluble proteins[28,29]. Lower temperature coupled

with lower cell growth rate usually favor higher production of soluble protein because the cells did not pressured to compete for nutrients as the cell density is lower.

In this study, it was discovered that 0.5% w/v concentration of L-arabinose used under the optimized conditions, has greatly contributed towards attaining higher VDAC2 production. This indicated that too much of L-arabinose can decrease recombinant protein expression. However, it all depends on the type of protein expressed since different protein expression required different cultivation conditions and in this case, it is a bit higher than a normal amount which is 0.2% w/v L-arabinose[30]. Nevertheless, it does reach its maximum need of L-arabinose and decline following that. In fact it has been reported that higher concentrations of L-arabinose cause over production of recombinant protein that leads to ribosomal destruction, production of heat shock proteins and eventually cell death[31].

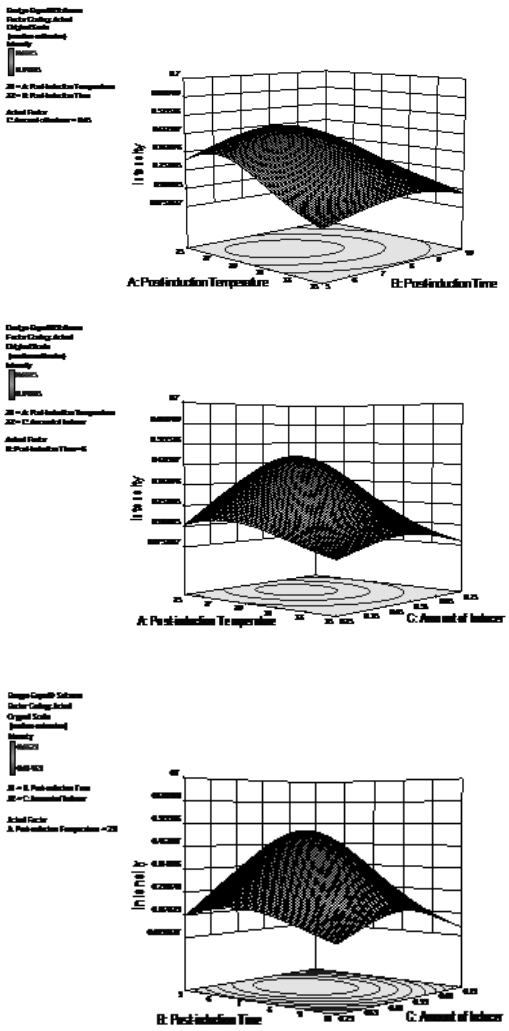

Fig. 2. The 3D response surface curves of the combined effects of post-induction temperature, inducer concentration and post induction time on VDAC2 expression. (a) Post-induction temperature and time at a fixed level of amount of inducer. (b) Post-induction temperature and inducer at fixed level of post-induction time. (c) Inducer concentration and post-induction time at fixed level of post-induction temperature

The intensity of the protein identified from SDS-PAGE analysis was chosen as an indicator of protein expression using proteomic software, ImageMaster 2D Platinum 6.0 (GE, Germany). Each run of the experiment provides different intensity which was quantified as the expression level of recombinant VDAC2 (Fig. 3). This powerful software manages multiple image analyses and offers the possibility to automate detection and matching steps. However, some disadvantages are the quality and reproducibility of the gels is sometimes mediocre. Poor resolution, high noise levels and large distortions in the protein patterns often hamper the analysis process. The reasons for this are unskilled sample preparation leads to unintended biological variability, poor sample solubilization produces insufficient resolution or dust particles or droplets lead to many spot artifacts.

With this software, the intensity is based on the highest calibrated pixel intensities in the spot from which the background has been withdrawn. The background is defined as the minimum pixel value in the spot neighborhood. This alternative technique was adopted since the VDAC2 is not an enzyme and thus its activity cannot be measured by enzyme assay. On the other hand, immunodetection is also not possible hence there is neither available ELISA kit nor antibody specific to chicken VDAC2 in the market. The antibody used in the Western blotting is not suitable to quantify recombinant VDAC2 since it is not highly specific (Fig. 1) because it is designed for human polyclonal VDAC2, and not specific for chicken VDAC2. There are several studies conducted that quantify protein by studying its intensity[32,33].

Table 3. Summary of the optimized cultivation conditions for the production ofrecombinant VDAC2

Cultivation conditions	Optimum value	Routine value
Post-induction temperature	30°C	37°C
Post-induction time	7.5h	6h
Amount of inducer	0.5% (w/v)	0.2% (w/v)

Optimization of the cultivation conditions for the expression of recombinant VDAC

The response surface plots have shown that the optimum predicted cultivation conditions for the maximum production of recombinant VDAC2 were found to be comprised of a post-induction temperature of 30°C, after 7.5 hours of induction, induced with 0.5% (w/v) L-arabinose. The maximum intensity value of expression calculated from the model according to the

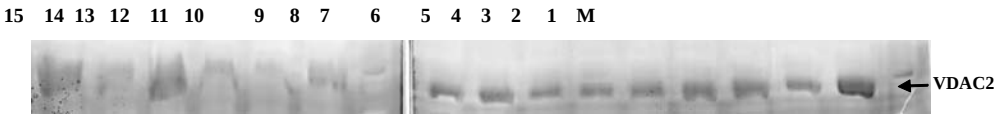

Fig. 3. The silver stained SDS-PAGE gel. The intensity of the bands is varied with each run under different culture conditions. M represents the marker and the number each represents the experiment's run

predicted optimum cultivation condition was 0.459 (OD). In order to confirm the predicted optimized cultivation condition, a validation experiment with the cultivation conditions proposed by the model had been carried out. The recombinant VDAC2 expression obtained by its intensity was 0.496 (OD), which was very close to the predicted value. The expression of recombinant VDAC2 was successfully optimized and improved about 6- fold after the optimization was carried out (Table 3). The routine was carried out under 37°C for 6 hour post induced with 0.2 % (w/v)L-arabinose.

CONCLUSION

Cultivation condition for VDAC2 expression in *E. coli* has been optimized and suggesting that the optimum conditions for the maximum expression of recombinant VDAC2 was at 29°C post-induction temperature, 6.8 hour post-induction time and 0.5% (w/v) L-arabinose with a predicted expression of recombinant VDAC2 intensity of 0.459 (OD). The experimental expression of recombinant VDAC2 intensity obtained was 0.496 (OD), which was very close to the predicted value. The expression of recombinant VDAC2 improved by almost 6-fold after the optimization process. Since VDAC2 in chicken has not been studied extensively compared to other organism, chicken VDAC2 studies provide greater challenge in understanding its characteristics and properties. It is hoped that findings of the present study can stimulate more studies to provide further insight of this interesting protein. One of the future applications is that VDAC2 can be a reliable biomarker in detecting over-stunned chicken in the market.

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