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Amplification of Bone Morphogenetic Protein 2 (BMP2) in Avian Genome and Cloning it into Pichia Pink α-HC and Express it in *Pichia pastoris*

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Key words: Bone Morphogenetic Protein 2, BMP2, Demineralized Bone Matrix, DBM, *Pichia pastoris*, Protein expression and PCR

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Abstract: This study was conducted to detect the presence of Bone Morphogenetic Protein 2 (BMP2) gene within the avian genome and cloned it into *Pichia pastoris* for future usage. DNA was extracted from feathers and preceded to BMP2 gene detection via Polymerase Chain Reaction (PCR). Agarose gel presented positive result for BMP2 gene amplification. Then BMP2 gene was then cloned into pCR2.1-TOPO plasmid and sent for sequencing to further confirm the presence of BMP2 gene. Followed by, inserted the BMP2 gene into *Pichia* Pink α-HC then allowed the BMP2 gene to express the BMP2 protein with *Pichia pastoris* expression System. SDS-PAGE results shown presence of the BMP2 protein and it was further confirm using Chromogenic Development method.

INTRODUCTION

Bone Morphogenetic Proteins (BMPs) are multifunctional growth factor that belong to Transforming Growth Factor beta (TGFβ) family according to Chen *et al*.[1] and they has many roles in the body, however, their activities are regulated at different molecular levels. Whereby, BMPs will act together with different types of chemical signaling within the body to express different effects at the respective parts of body from time to time. The chemical signaling effects include development of both central and peripheral nervous system in vertebrates according to Liu and Niswander[2], cardiac cells differentiation[3], bones and cartilage formation in fracture wound[4].

Demineralized Bone Matrix (DBM) was first discovered by Senn and it was further confirm by Urist[5] that it possess the capability to induce bone formation due to presence of Bone Morphogentic Protein (BMP) within the DBM. An *et al*.[6] also confirmed that DBM graft able to lead to hard callus formation in bone fractures. With that DBM is used for augmentation of bony defects in clinical cases. Conversely, Jalila[7] was the first to prove that Avian DBM is as effective as mammalian DBM.

Whereby, new bone callus formation was observed on birds graft using chicken and turkey bone via radiologic examination. By Jalila[7] also confirmed that Avian DBM is suitable for bone graft substitutes for birds especially in avian fracture treatment as the DBM possess the osteoinductive and osteoconductive properties. However, Boden *et al*.[8] and Ulmanen *et al*.[9], managed to proven that BMP2 alone also has the ability to induce bone formation in mammals.

Chen *et al*.[1] also reported that both preclinical and clinical studies have shown that BMP-2 can be utilized for various therapeutic intervention such as bone defects, non-union fractures, spinal fusion, osteoporosis and root canal surgery. Harvinder also has shown successful results of using BMP2 to treat long bones fractures and spinal fusion.

At the same time, Nakagawa*et al*.[10] also managed to shown osteocyte-like cells and osteoblast-like cells after 14 days of implantation of 5 μg mL^{-1} of Recombinant Human BMP2 (rhBMP2) into the rats calf muscle. According to Yoshida *et al*.[11], BMP2 has shown significant effects in inducing new bone formation especially in fracture gap studies. Therefore, the aim of this study was to amplify the BMP2 gene in avian genome and cloned it into Pichia Pink α-HC for BMP2 protein production in *Pichia pastoris*.

MATERIALS AND METHODS

Amplification of BMP2 gene within avian genome: In order to amplify the BMP2 gene from the avian genome, a set of primer (BMP2F and BMP2R) was designed based on published sequence of Gallus gallus BMP2 (Accession: NM_204358.1). Then, DNA was extracted from broiler chicken's feathers, proceed to PCR using both BMP2F (5'-ATGGTTGCCGCCACCCGCTC-3') and BMP2R (5'-TCAGCGGCACCCGCAGCCCT -3') primers. The PCR final reaction mixture of 25 μL was prepared as follows: 1×PCR buffer; 0.25 mM dNTP; 1 U/25 μLTaq DNA polymerase; 1 pmol μL^{-1} of primer; 0.2 μg/25 μL DNA template and topped up with ddH_20.

The PCR was performed in a MyCycler (Bio-Rad, USA) throughout the experiment. The PCR mixture were then subjected to the following temperature profile: Initial denaturation at 95°C for 2 min, followed by 35 cycles of amplification of denatureation at 95°C for 30 sec, optimized annealing temperature at 71.6°C for 30 sec and extension at 72°C for 2 min. The reaction was then concluded with a final extension at 72°C for 10 min and the reaction was hold at 10°C.

The PCR products were then separated by agarose gel electrophoresis and analysed by 1% (w/v) agarose gel in 1×TAE buffer. The 1% agarose gel was pre-stained with 0.530 μL mL^{-1} of midori green (Nippon Genetic, Japan) before the electrophoresis process. Then 5 μL of 1 kbp DNA marker (Vivantis, USA) were loaded into the agarose gel and 5 μL of PCR products were mixed with 2 μL of 6x loading dye (Fermentas, USA) and then loaded into the respective well on agarose gel. This was followed by subjected to electrophoresis at 80 V (Power Supply EPS 600, Pharmacia, Biotech) for an hour with 1×TAE buffer (40 mM Tris-base, 20 mM Acetic Acid, 1.8 mM EDTA at pH 7.6). The agarose gel was then visualized under Gel Doc System (Bio-Rad, USA). The expected PCR products sizewas 1179 bps.

Cloning of BMP2 gene into pCR2.1-TOPO plasmid: The PCR products were then purified following the protocol provided by PCR clean-up Gel extraction kit (Macherry-Nagel, Germany). Then it was inserted into pCR2.1-TOPO plasmid following the protocol from the pCR2.1-TOPO plasmid kit (Invitrogen, USA). The plasmid was then sent for sequencing, and the sequencing results were blast against the National Center for Biotechnology Information (NCBI) data bank. The BMP2 gene sequence were then translated into amino acid sequence and sent to GeneArt for codon optimization due to the original nucleotide sequence consists of high GC content and also to ensure it is optimized for protein secretion during the expression stage. The new synthesize BMP2 gene that corresponding to the BMP2 protein expression was then labeled as BMP2v2 and was cloned into Pichia Pink α-HC plasmid with Restriction Enzyme Kpn1 (Fermentas, USA) and EcoR1 (Fermentas, USA) followed the protocol from Pichia Pink Expression System (Invitrogen, USA). Subsequently, another set of primer was designed to confirm the presence of BMP2 gene in the Pichia Pink α-HC plasmid. Which were GNF (5'-GAAGAAGGGGTATCTCTCGAGAAA-3')and GNR(5'-AATTACATGATATCGACAAAGGAA-3') with the same PCR profile but the annealing temperature was set at 63°C.

Transformation of Pichia Pink α-HC into *Pichia pastoris* for protein expression: Then, the Pichia Pink α-HC with the BMP2v2 gene insert was then linearizedagain using restriction enzyme Kpn1 again and subjected to heat shock transformation into chemically competent *Pichia pastoris*. Then it was spread on the PAD agar for colonies selection, only white colony was selected for the protein expression. The selected white colony was then cultured in BMMY culture media at 28°C and agitation rate of 250 revolution per minute (rpm) for 24 h and then followed by centrifuged it at 3927 rpm for 5 min to pallet it and followed by transferred the pallet into in BMMG culture media at 28°C and agitation rate of 250 revolution per minute (rpm) for another 24 h.

SDS PAGE Gel Eltectrophoresis: The cultured protein was then analyzed using SDS PAGE gel electrophoresis using Bolt® Mini Gel Tank (Invitrogen, USA) and using commercially available protein ladder SeeBlue® Plus2 Pre-Stained Standard (Invitrogen, USA) as protein marker. A total of 26 μL of protein sample were mix with 10 μL of Bolt® LDS sample Buffer and 4 μL of Bolt® Sample Reducing Agent. Then the protein mixtures were heated up to 70°C for 10 min. Both protein samples and protein markers were then loaded into pre-cast gel Bolt® 4-12% Bis-Tris Plus (Invitrogen, USA) that consists of 10 wells and immersed in MES SDS Running Buffer (Invitrogen, USA) and run at 165 Volt for 30 min in Bolt® Mini Gel Tank.

Transfer protein samples onto nitrocellulose membrane: SDS-Page gel results were then transferred onto nitrocellulose membrane using iBlot Dry Blotting System (Invitrogen, USA). Whereby, nitrocellulose membrane was pre-soaked in deionized water and followed by placed on top of the SDS-Page gel then the protein was transferred onto the nitrocellulose membrane via. iBlot at 20 Volt for 7 min then it was subjected to staining and destaining of the nitrocellulose membrane.

Western Blotting and Immunodetection BMP2 protein: The nitrocellulose membrane was then treated with 5 stages of immune detection following the protocol from iBlot Western Detection Kit (Invitrogen, USA). Which were blocking the protein on the nitrocellulose membrane, binding the primary antibody, binding the secondary antibody, washing of the nitrocellulose membrane and finally chromogenic detection of protein on the nitrocellulose membrane. The BMP2 primary antibody BMP-2(N14) (Santa Cruz Biotechnology, USA) used was an affinity purified polyclonal antibody derived from goat, it was diluted into working concentration of 1:100 ratio. The secondary antibody used was donkey polyclonal secondary antibody to goat IgG-H&L (AP), (Abcam, UK) which was diluted into 1:5000 ratio. Then the nitrocellulose membrane was incubated in Novex AP Chromogenic Subtrate (BCIP/NBT), (Invitrogen, USA) for 30 min on a rotary shaker to allow the development of purple bands of BMP2 proteins on the nitrocellulose membrane.

RESULTS AND DISCUSSION

As the PCR results shown in Fig. 1, the primer BMP2F and BMP2R was able to amplify the BMP2 gene within the avian genome and the products also shown the expected size of ~1179 bps in 1% agarose gel. DNA marker used for Fig. 1, M1 was 1 kbps DNA ladder (Vivantis, USA) and the other DNA ladder marker in M2 was 100 bps DNA ladder from (Vivantis, USA). The annealing tempfor this PCR was 71.6°C.

As presented in Fig. 2, the alignment results of both amino acid sequences were congruent to each other however the BMP2v2 amino acid sequences is slightly longer as compared to the original sequence. That also explains that the expected size of GNF and GNR in Fig. 3 as ~1193 bps instead of 1179 bps in Fig. 1.

Codon optimization was considered as many studies had shown that it help to improve the quality and quantity of the protein being expressed. Yavada and Ockenhouse[12] managed to prove that codon optimization has superior yield of protein being express as compared to the original codon sequence. According to Young and Alper[13], codon optimization is the common approach especially dealing with synthetic biology and metabolic

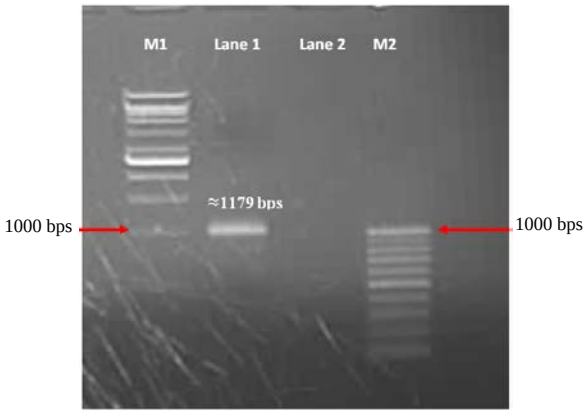

Fig. 1: Diagram shown the PCR result of BMP2F and BMP2R which was used to detect the presence of BMP2 gene in avian genome. M1: 1 kbps DNA ladder from Vivantis; Lane1: BMP2 gene (≈1179 bps); Lane 2: negative control; M2: 100 bps DNA ladder marker from Vivantis

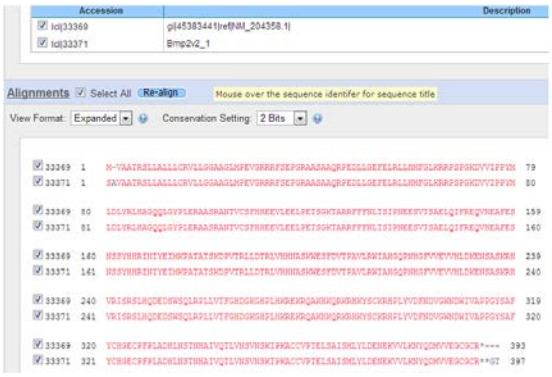

Fig. 2: Translation of BMP2 gene sequence into amino acid sequence. Subject 33369 was the original sequence from NCBI and subject 33371 was the BMPv2 gene synthesize by GeneArt

cellular engineering context. Lanza *et al*.[14] also claimed that codon optimization is a standard tool for protein expression framework.

This result had confirmed that both GNF and GNR primers were able to amplify the BMP2v2 gene within the selected white colonies *Pichia pastoris* on the PAD agar plate and the positive PCR results also indicated that successful transformation of BMPv2 in Pichia Pink α-HC into *Pichia pastoris* and then the selected white colony was then transferred into BMGY culture media. According to Cereghino *et al*.[15], the glycerol within the BMGY acts as the carbon source for Pichia cells and it was incubated in incubator shaker at 28°C in aerobic condition which is the suitable condition for the Pichia

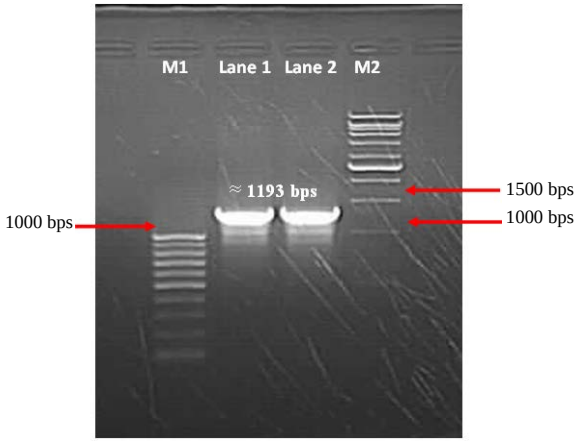

Fig. 3: PCR results of GNF and GNR primers, which showed the amplification of BMP2v2 gene that has been successful transformed into *Pichia pastoris*. M1: 100 bps DNA ladder from Vivantis; Lane1: BMP2v2 plasmid; Lane 2: BMP2v2 plasmid extract from Pichia pink yeast strain 1 at day 4 after the transformation; M2: 1 Kbps DNA ladder from Vivantis

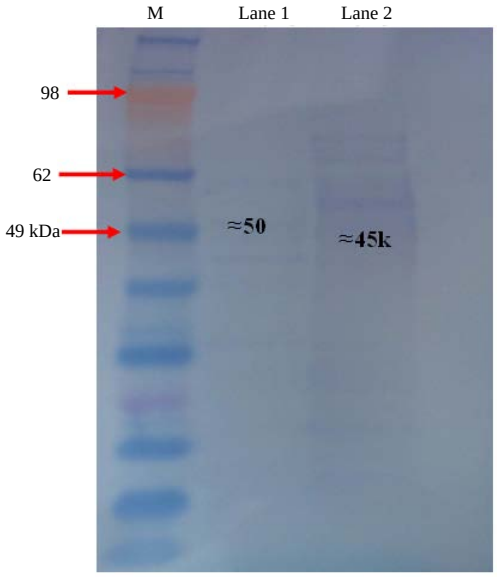

Fig. 4: SDS-PAGE results shown presence of protein within the BMMY cultured media. M: Molecular weights of SeeBlue Plus2 Pre-Stained Standard; Lane 1: Protein mixtures of negative control (*Pichia pastoris* that carries Pichia pink α-HC plasmid); Lane 2: Protein mixtures of BMP2v2

cells to grow until all glycerol were used up which it normally about 24 h. Then the Pichia cells was then

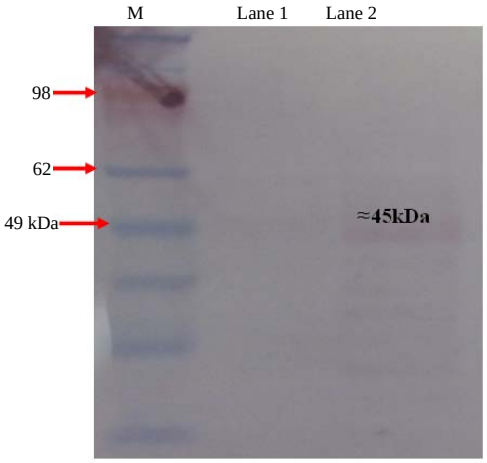

Fig. 5: The chromatography result show expected BMP2 protein size was ~45 kDa. M: Molecular weights of SeeBlue Plus2 Pre-Stained Standard; Lane 1: Protein mixtures of negative control (*Pichia pastoris* that carries Pichia pink α-HC plasmid); Lane 2: Protein mixtures of BMP2v2

palleted by centrifugation at 3927 rpm for 10 min to remove the death cells and the rupture cells by discard the BMGY slowly follow by adding BMMY to induce the Pichia cells to express the BMP2 protein under the same condition. According to Li *et al*.[16], the methanol induction and the level dissolved oxygen % within the culture medium are closely related in the expression of protein.

Upon transferred SDS-PAGE results onto nitrocellulose membrane, it was subjected to staining process to detect presence of protein samples on the nitrocellulose membrane.

As shown in Fig. 4, both Lane 1 and 2 also had shown some faint bands of proteins. It was then subjected to destaining before proceed with 5 stages of immune detection. As presented in Fig. 5, no visible bands were detected in Lane 1 as it was the negative control. Howeverin Lane 2, there were presence of multiple faint bands but the more distinctive band will be the expected products size band which was the ~45 kDa band. According to Alejandra *et al*.[17] multiple bands might not be due to lack of specificity.

However, it may represent same target antigen with post-translational modification, breakdown product or splice variants into small fragments due to proteolytic activities. Li *et al*.[16] also confirmed that the protein production rate slowing down over time has the direct relation to the accumulation of protease and also increased of proteolytic activity within the culture media.

According to Zhang, protein stability could be improved by setting the temperature within the range between 25-27°C. Jahic[18] also commented that decrease temperature shown significant rate of reduction in proteolytic activity.

CONCLUSION

In summary, BMP2 gene was successfully amplified from the avian genome using BMP2F and BMP2R primers. The BMP2 gene was then sent for codon optimization to ensure better yield of BMP2 protein production in expression stage. The newly synthesize BMP2v2 carry lesser GC content as compared to the original BMP2 gene sequence. Which explained the differences between the annealing temperature of BMP2F and BMP2R at 71.6°C while GNF and GNR annealing temperature at 63°C. Both SDS-PAGE and western blot results also had shown the presence of the BMP2 protein but relatively faint. The BMP2 protein then can be produced at any point of time using *Pichia pastoris* and the application of the BMP2 protein also has great potential usage in avian fracture treatment therapy. Thus, the future studies of this will be reduce the incubation temperature instead of 28°C and adding protease inhibitor into the culture could be an option to reduce the proteolytic activity. Produce a series of BMP2 concentration and to determine the optimum BMP2 dosage to for clinical application.

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