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Construction, Expression and Characterization of Multi Cassettes Encoding Indonesian Small Hepatitis B Surface Antigen (s-HBsAg) in Methylotropic Yeast *Pichia pastoris*

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A B S T R A C T

Hepatitis B is the most common liver infection worldwide. The recombinant Virus Like Particles (VLP) of small Hepatitis B surface antigen (s-HBsAg) vaccine provides excellent protection against Hepatitis B Virus (HBV). In this study, we have generated a construct of *Pichia pastoris* recombinant which comprises multi expression cassettes encoding s-HBsAg which belongs to HBV B3 genotype predominantly found in Indonesia. An expression cassette containing a synthetic codon optimized open reading frame of HBV B3 genotype/adw serotype s-HBsAg was cloned into pAO815 to generate pAOHBs1 in *Escherichia coli*. The expression cassette from pAOHBs1 was subsequently isolated and ligated with recombinant previously obtained, generated recombinant plasmid containing 2, 3 and 4 direct repeats of HBsAg expression cassette. The resulted recombinant containing 4 expression cassettes was integrated to *P. pastoris* genome. Positive integrants and stability during cultivation were identified by PCR and qPCR. The expression of s-HBsAg was induced by methanol and analyzed by SDS PAGE and western blotting. Nucleotide sequencing analysis showed that the inserted fragment encodes amino acids with 100% similarity compared to that designed for HBV B3 genotype. The PCR and qPCR analysis showed that stable *P. pastoris* integrated with 4 s-HBsAg expression cassette was successfully obtained with methanol utilizing slow phenotype. A protein band with apparent molecular weight which similar to s-HBsAg size was detected based on a SDS PAGE and western blot analysis. A stable integrant of *P. pastoris* containing four expression cassettes of HBV B3 genotype s-HBsAg capable of producing a vaccine candidate against Hepatitis B was generated.

Key words: Multi expression cassettes, HBV B3 genotype, s-HBsAg, *Pichia pastoris*

INTRODUCTION

Hepatitis B is the most common liver infection worldwide including Indonesia. Hepatitis B is caused by Hepatitis B Virus (HBV) and often leads to chronic liver disease, cirrhosis and liver cancer. It is estimated that more than one third of the world's population has been infected with HBV and approximately 70-100 millions of people death due to hepatocellular carcinoma and liver cirrhosis (Elgouhari *et al*., 2008; Khedmat and Taheri, 2009). In Indonesia, the carrier rates among apparently healthy populations have been reported to range from 4.0-20.3% (Khan *et al*., 2004; Mulyanto *et al*., 2009). The HBV has been classified into 10 genotypes (A-J) according to genome sequence divergence. The HBV genotypes have a distinct geographical distribution. As chronic HBV infection is endemic in the Asian region, genotypes B and C prevail predominantly and genotypes A and D are mainly found in the western world and Europe. In Europe and Asia, most patients with genotypes A and B have acute hepatitis B. The HBV strains were also categorized into nine subtypes based on serological analysis of antigenic determinants and sub determinants of their HBsAg. B genotype is mostly present in Indonesian ethnic populations and their distribution following the ethno linguistics structure of the population suggest that unlikely that these sub-genotypes have been introduced in recent time. In the subgenotype level, from 440 HBV isolates in Indonesia, B3 is the major sub-genotype (Juniastuti *et al*., 2011). The combination of amino acid residues in position 122 (d/y) and 160 (w/r) which flank the 'A' determinant provide four major subtypes of HBsAg: adw, adr, ayr and ayw. The nationwide study about distribution of serotype in Indonesia is indicated that serotype adw is predominant in Indonesia (Mulyanto *et al*., 1997). In addition, HBV B3 genotype is also found in some other countries such as China (Shi *et al*., 2012).

Vaccination is the most effective prevention strategy and dramatically reduced hepatitis B virus transmission. Hepatitis B vaccination has been introduced globally and become an international immunization program (Shepard *et al*., 2006). The global hepatitis B vaccine market now exceeds $1 billion dollars annually and is expected to continue to grow as more countries adopt World Health Organization recommendations for the vaccination of newborns, teenagers, healthcare workers and other 'At-risk' populations. In addition, demand of combination vaccine containing Pentavalen (DTP-HB-Hib) vaccine and hexavalent (DTP-HB-Hib-SiPV) will rise as many countries adopt WHO recommendation for Hib and sIPV vaccination (WHO Position Paper on HaemophilusInfluenzae type B (Hib) conjugate vaccines, weekly epidemiological report by WHO. (2006).

Effective immunization and protection from HBV infection have been achieved by intramuscular vaccination of yeast derived recombinant small hepatitis B surface antigen (s-HBsAg). The s-HBsAg is the surface particle from the hepatitis B virus which is composed of the S-domain consisting of 226 amino acids (Bruss, 2007; Liang, 2009). It comprises of Major Hydrophilic Region (MHR) which is also named as 'A determinant', the most important region in antigen recognition that reside in amino acid 100-160. To be immunogenic, s-HBsAg polypeptide must assemble into Virus like Particles (VLP) with 20-22 nm diameter (Cabral *et al*., 1978). The VLP of s-HBsAg does not appear to occur in *E. coli* based expression systems (Charnay *et al*., 1980; Edman *et al*., 1981; Fujisawa *et al*., 1983), yet it could appear in yeasts i.e., *Saccharomyces cerevisae, P. pastoris* and *Hansenulla polymorpha* (Vietheer *et al*., 2007).

In recent years, the methylotrophic yeast *P. pastoris* has emerged as a powerful heterologous system for the production of high levels of functionally active recombinant proteins. This system could generate highly expressed heterologous protein with a strong and tightly regulated methanol-inducible alcohol oxidase (AOX1) promoter (Cereghino and Cregg, 2000). Therefore, it would be advantageous to develop *P. pastoris* as an inexpensive expression system for the production of hepatitis B vaccine in large quantities (Bo *et al*., 2005; Vassileva *et al*., 2001).

We have previously successfully generated a *P. pastoris* strain KM71 integrated with single copy of s-HBsAg gene (Kusumawardani *et al*., 2010). However, the s-HBsAg expression level remained low, even though the amount of methanol as an inducer was optimized and the copy number of plasmid integrated in the *P. pastoris* genome was increased by elevating the level of zeocin (Maryama *et al*., 2012).

The increase in copy number of s-HBsAg expression cassette in *P. pastoris* GS115 results in a proportional elevation in the steady state levels of the s-HBsAg-specific mRNA, which in turn increase the total levels of the s-HBsAg protein (Vassileva *et al*., 2001). Based on those results, we regenerated new recombinant multi Indonesian s-HBsAg expression cassettes which was designed and synthesized based on the most HBV genotype and serotype found in Indonesian areas, B3 genotype and adw serotype (Narita *et al*., 2013). This resulted recombinant was subsequently used in *P. pastoris* transformation and expressed in order to gain higher Indonesian s-HBsAg production.

MATERIALS AND METHODS

Plasmids and bacterial strains: Plasmid pAO815 purchased from Invitrogen (San Diego, USA) is a shuttle vector in *E. coli*/*P. pastoris.* An Indonesian s-HBsAg Open Reading Frame (ORF) B3 genotype and adw serotype was previously codon optimized (Narita *et al*., 2013), subsequently synthesized and constructed in pJ204 to generate pJHBsAg by DNA 2.0 (Menlo Park, USA).

Construction of up to four expression cassettes (pAOHBs1, pAOHBs2, pAOHBs3 and pAOHBs4): The end-*Eco*RI sites of Indonesian s-HBsAg ORF was introduced to facilitate subcloning into pAO815. DNA ligation was performed as described by Sambrook *et al*. (2001). In short, target and plasmid DNA were digested with the *Eco*RI and digested plasmid was subsequently dephosphorylated by alkaline phosphatase. Ligation reaction was then performed at 4°C overnight using 1U T4 ligase buffer and variation of vector inserts. *Escherichia coli* strain TOP 10 was used as a host strain and transformation was performed by heat shock method. The cloning strategy to generate up to 4 copies of Indonesian s-HBsAg expression was following. Two kilo bases (kb) HBsAg expression cassette of pAOHBs1 which also contained of AOX promoter and transcription terminator was cleaved by *Bgl*II and *Bam*HI and reinserted into *Bam*HI site of the same plasmid to generate pAOHBs2. This method was repeated to generate pAOHBs3 and pAOHBs4. All of transformation and cloning processes were completed in *E. coli* TOP 10, either by heat shock or electroporation method.

Characterization of plasmid recombinant: Plasmid DNAs from 3 mL overnight cultures were isolated using High Speed Plasmid Mini Kit (Geneaid, Germany). Briefly, bacterial cells were lysed using alkaline lysis method and the lysate was subsequently applied to spin columns. After repeated washing, plasmid DNA was eluted using elution buffer and stored at -20°C until required. Restriction endonuclease digestion was performed to characterize resulted recombinant plasmids and digestion results were analyzed by agarose gel electrophoresis. Inserts from plasmid with expected size were subsequently sequenced (Macrogen, South Korea).

Transformation of *P. pastoris* GS115 multi copies of expression cassettes: Preparation of *P. pastoris* GS115 competent cells and transformation were performed using *Pichia* EasyComp™ kit (Invitrogen). *Pichia pastoris* GS115 was grown overnight in liquid YPD (1% (w/v) yeast extract, 2% (w/v) pepton, 2% (w/v) dextrose) and shaken at 200 rpm, 30°C. Culture was then transferred into new YPD medium until OD_{600} 0.2 and grown until OD_{600} 0.8 was reached. *P. pastoris* cells were harvested by centrifugation 500×g, 5 min at room temperature. Cells then were washed, resuspended in 1 mL solution I and aliquoted in 50 μL. Competent GS115 cells were mixed with 5 μg of *Bgl*II linearized HBsAg expression plasmid. The cells were heat shocked at 42°C and 10 min. Transformants harboring the plasmid-borne *HIS*4 marker were selected on minimal medium lacking histidine (2% dekstrosa; 1.34% YNB, dan 0.0005% biotin, 1.6% bakto agar). Subsequently, colonies were picked and resuspended with 20 μL SCED buffer (1 M sorbitol, 10 mM Natrium sitrat, 10 mM EDTA pH 7.5, 10 mM DTT) supplemented with 2 μg mL^{-1} liticase and incubated in 30°C for 1 h. Samples were then boiled using thermo mixer

at 99°C 300 rpm for 15 min, incubated on ice for 10 min and centrifuged with 12,000×g for 5 min. The 10 μL of lysates were then diluted 10× prior to PCR analysis.

Expression of HBsAg in *P. pastoris* GS 115: In order to induce the expression of HBsAg, *P. pastoris* GS115/pAOHBs1-4 were sequentially inoculated into YPD medium for 20 h, BMGY medium for 16-18 h and BMMY medium. Methanol was added to a final concentration of 0.5%, 2 and 3% methanol every 24 h for 144 h and cultured at 30°C until 100 OD_{600} were reached. Cells were centrifuged 5 min, 3000 g, washed with phosphate buffer pH 7.2 and mixed with lysis buffer (10 mM bufer posfat pH 7.2; 5 mM EDTA, 500 mM NaCl, gliserol 8%) and vortexed with 0.6 g glass bead. For SDS-PAGE analysis, 50 μL of lysate was used. For Western blotting analysis, after test samples were subjected to SDS-PAGE, protein was transferred onto blotting paper pre-wetted with blotting buffer using i-blot system (Invitrogen). Blots were then incubated with polyclonal antiserum against HBsAg (Abcam). After several washings, blots were incubated with horseradish peroxidase conjugated Rabbit antibodies directed against mouse immunoglobulins diluted 1:4000 in 3% milk powders solution for 1 h at room temperature. After washing, proteins were visualized using NBT-BCIP.

Isolation of genomic DNA for qPCR: Genomic DNA was prepared from cultures *P. pastoris* in various medium expressing HBsAg using standard protocols (Epicentre kit). Briefly, the washed cell pellet was incubated lysis solution and RNAase and subsequently added with MPC solution. The genomic DNA was then recovered by precipitation with isopropanol. Finally, genomic DNA was resuspended with 35 μL TE buffer.

Stability characterization by real time PCR: The primer design was performed using NCBI software. The primers had similar melting temperatures (Tm) (50-65°C). The amplicon size settings were defined between 200-300 bp. Real-time PCR amplification was performed using thermocycler qPCR (BioRad). For SYBR Green examinations, 200 nM of each primer were added to the 1x Power SYBR® Green Master Mix. reactions were performed in triplicate. Genomic DNA were added in a reaction volume of 10 μL comprising of 5 μL 2X SsoFastTM EvaGreen® Supermix (BIO-RAD); 0.5 μL 10 mM primer forward qsHBsAg; 0.5 μL 10 mM primer reverse qsHBsAg. The thermal profile was performed with 40 cycles of initiation 30 sec at 95°C, denaturation 5 sec at 95°C, annealing and elongation 15 sec, 63°C. Melting curve analysis was performed in temperature gradient between 60-95°C.

RESULTS

Construction, transformation and expression of multi s-HBsAg expression cassette in *P. pastoris* GS115: Indonesian s-HBsAg encoding gene was designed based on

the sequence corresponds to HBV B3 genotype and adw serotype (Narita *et al.*, 2013). The codons were optimized until the best codon adaptation indexed was achieved. The expression cassette containing s-HBsAg ORF was subsequently synthesized and constructed in pJHBsAg. Prior to further sub-cloning, the expression cassette was sequenced and shown to be identical to that designed previously.

Plasmid pJHBsAg was subsequently cleaved with *Eco*RI and the 0.7 kb fragment was gel purified and then ligated into the pAO815 expression vector cut with the same enzyme to generate pAOHBs1, as it was schemed in Fig. 1a. An s-HBsAg expression cassette was gained by inserting the HBsAg gene (0.7 kb) into the unique *Eco*RI site of pAO815 which lies in between the AOX promoter and transcription termination regulatory elements.

Prior to further construction, the insert of pAOHBs1 was sequenced and nucleotide sequencing analysis showed that the insert has one silent mutation at nucleotides 315, from adenine to guanine (Fig. 1c). However, translation analysis revealed that there is no amino acid substitution and therefore the insert encodes amino acids with 100% similarity compared to that designed previously and ligated in the correct frame. The sequencing result also revealed that the insert has correct Kozak sequence and reading frame (Fig. 1a and b). The HBV used in this research corresponds to B3 genotype and due to the presence of amino acid residues Lys, Lys and Pro at position 122, 160 and 127 (Fig. 1c), the HBV was determined to be subtype adw.

The pAOHBs1 was subsequently used for further cloning to generate pAOHBs2, pAOHBs3 and pAOHBs4. Prior to further pAOHBs2 construction, plasmid pAOHBs1 was cleaved with *Bam*HI and *Bgl*II, resulted a 2-kb *Bgl*II-*Bam*HI fragment which is included promoter and terminator for AOX. The fragment was reinserted into the *Bam*HI site of the same plasmid to create a tandem repeat of two copies.
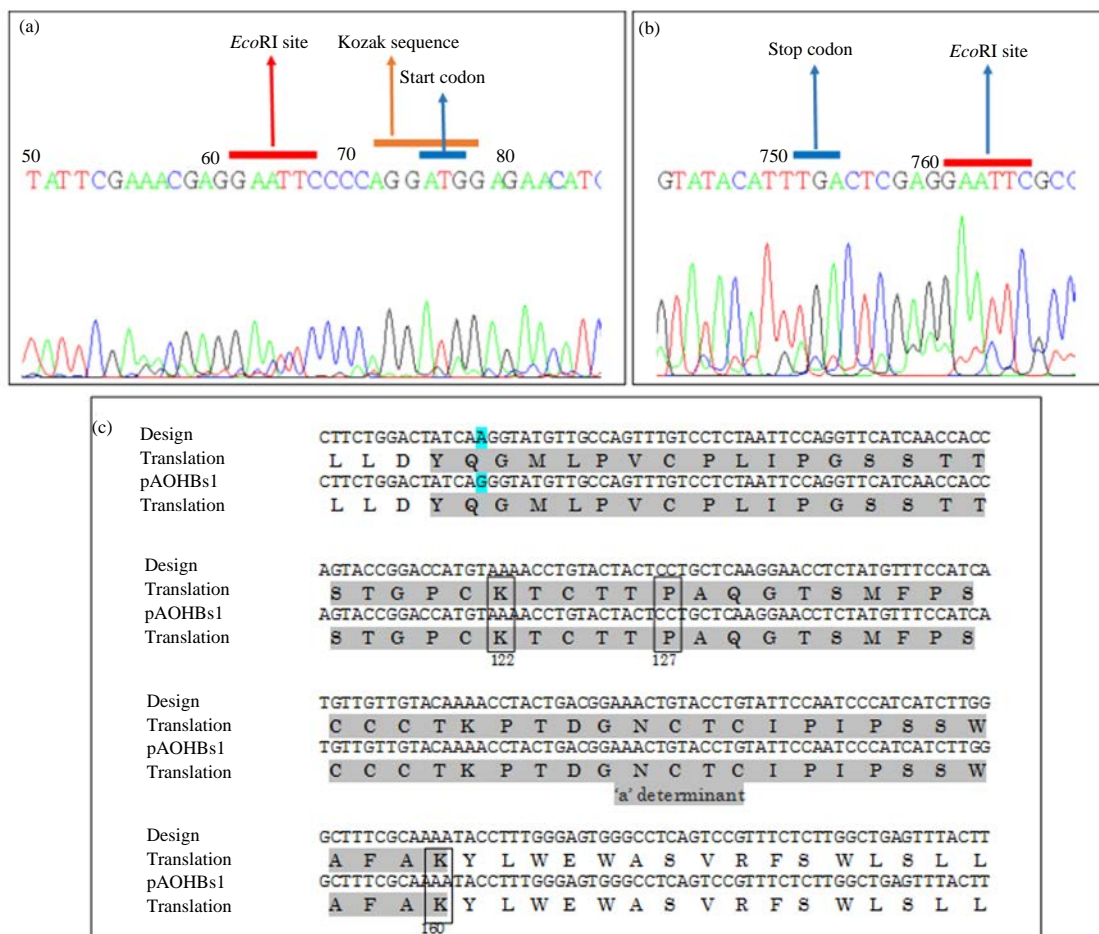

Fig. 1(a-c): Chromatogram and sequence analysis of Indonesian s-HBsAg inserted in pAOHBs1 (a, b) The sequencing result shown that the insert Indonesian s-HBsAg encoding gene has correct Kozak and correct reading frame and (c) Alignment of nucleotide sequences of encoding Indonesian 'a determinant' s-HBsAg in pAOHBs1 with previously designed that the insert has one silent mutation at nucleotides 315, from adenine to guanine (indicated in blue)
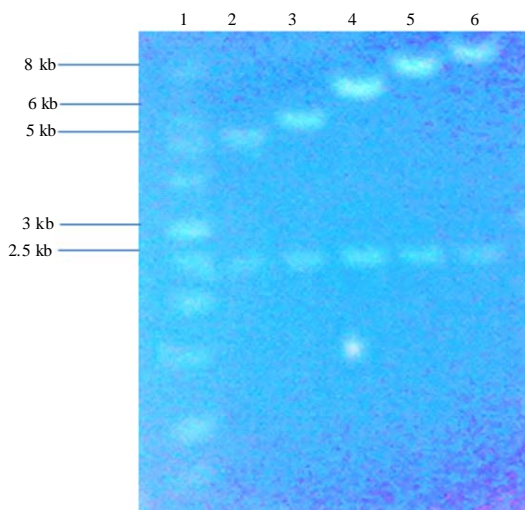
Fig. 2: Restriction analysis of pAOHBs using *Bgl*II Lane 1: Ladder 1 kb plus, lane 2: pAO815><*Bgl*II, lane 3: pAOHBs1><*Bgl*II, lane 4: pAOHBs2><*Bgl*II, lane 5: pAOHBs3><*Bgl*II, lane 6: pAOHBs4 ><*Bgl*II
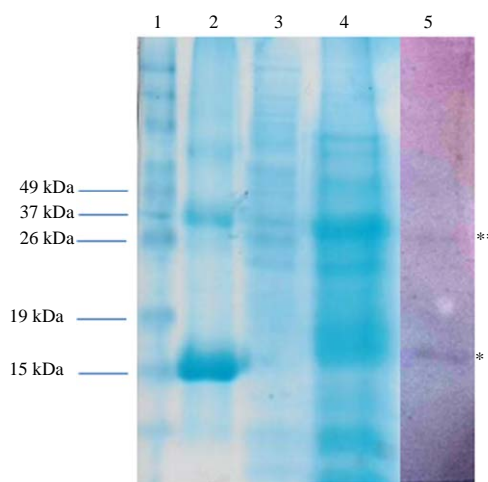

Fig. 3: Expression of sHBsAg in integrated *P. pastoris* GS115 induced with methanol. Lane 1: Molecular Weight Marker Lane 2: Control positive HBsAg, Lane 3: *P. pastoris* GS115/pAO815, Lane, 4: *P. pastoris* GS115/ pAOHBs4, Lane 5: Western Blotting of *P. pastoris* GS115/pAOHBs4

The same strategy was also applied to generate recombinant containing up to 4 copies of the HBsAg expression cassete. The restriction analysis of the resulted recombinant pAO815 without s-HBsAg ORF, pAOHBs1, pAOHBs2, pAOHBs3 and pAOHBs4 produce the bands of DNA with expected size 2.3 kb for ampicillin resistance genes and 5.3, 6, 7.2, 8.4 and 9.6 kb for remaining plasmids of empty pAO815 (Fig. 2, lane 2) and 1-4 expressions casseteS, respectively (Fig. 2, lane 3-6). This result showed that recombinant plasmids were successfully generated and could be used for subsequent transformation and s-HBsAg Virus Like Particles (VLP) expression in *P. pastoris*.

Next, recombinant pAOHBs1, pAOHBs2, pAOHBs3 and pAOHBs4 were used in *P. pastoris* strain GS115 transformation. The strain is a histidine requiring auxotroph that has an intact *AOX*1 gene and is capable of utilizing methanol efficiently (Vassileva *et al*., 2001). Single and multicopy HBsAg expression cassettes were integrated into the *P. pastoris* genome in the *AOX locus* using a gene replacement strategy. Prior to transformation, Indonesian HBsAg expression plasmids were cleaved with *Bgl*II, resulted one of the fragment contained of Indonesian HBsAg expression cassette and *His*4 selection marker which are flanked by sequences homologues to the 59 and 39 ends of the *AOX*1 locus (Vassileva *et al*., 2001). The fragments were subsequently purified and used for further transformation. Transformation resulted the acquisition of the wild-type *HIS*4 marker and transformants were selected on minimal plates lacking histidine. *Pichia pastoris* GS115 transformed with integrated sHBsAg could grow in the medium lacking histidine and subsequently identified by PCR.

In order to obtain expression of HBsAg, *P. pastoris* GS115/pAO815 and *P. pastoris* GS115/pAOHBs4 were

induced by the addition of methanol to a final concentration of 2%. Figure 3 (lane 4 and 5) shows that after induction, a protein with apparent molecular weight which similar to HBsAg as a positive control (lane 2) were achieved.

Characterization of the integrated HBsAg Expression cassettes in *P. pastoris*: For the production of recombinant proteins, it is important to ascertain if the expression cassettes in *P. pastoris* transformant would remain stably integrated. The stability were monitored at periodic intervals and examined by PCR and qPCR analysis for the continued presence of the HBsAg expression cassette. The results obtained using the integrated *P. pastoris* with 4 copy expression cassettes are shown in Fig. 4 and 5. The predicted 208 bp of partially s-HBsAg amplicon and similar Ct (22) could be identified during periodic intervals.

The copy number of HBsAg expression cassette is calculated by interpolating its Ct values to standard curve. The calculations show that there are four copies of HBsAg expression cassette is integrated in the chromosome of *P. pastoris*. Figure 6 shows that the integration of four copies of HBsAg expression cassette is stable during periodic intervals.

DISCUSSION

Indonesian s-HBsAg encoding gene was designed based on the s-HBsAg nucleotide sequence corresponds to the most HBV genotype and serotype present in Indonesian areas (Narita *et al*., 2013). Some of published data related to Indonesian genotype and subtype have been reported. Norder *et al*. (2004) was found that the subgenotypes of B and
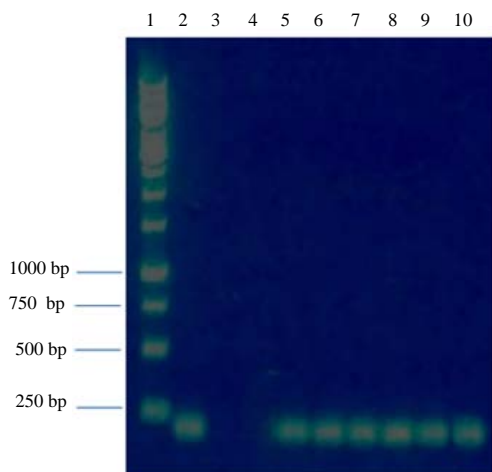
Fig. 4: PCR analysis of the stability of integrated *P. pastoris* with four copies of HBsAg expression cassettes. Genomic DNA was prepared from the *P. pastoris* transformant of 1, 21, 42, 63, 84 and 105 generation (lane 5-10). Lane : 1: 1 kb ladder, lane 2: pAO-HBs4 as positive control, lane 3 and 4: DNA Genome *P. pastoris* GS115 without Integran and reaction without template, respectively
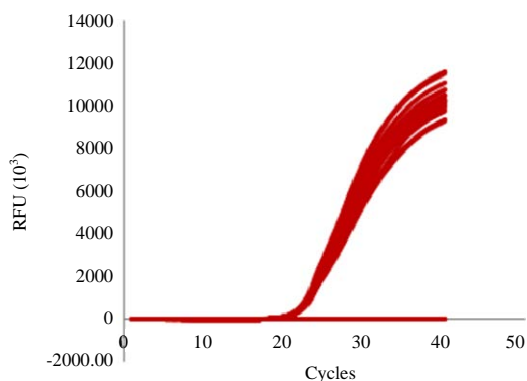

Fig. 5: qPCR analysis of the stability of integrated *P. pastoris* with four copies of HBsAg expression cassettes. Genomic DNA was prepared from the *P. pastoris* transformant of 1, 21, 42, 63, 84 and 105 generation. All of genomic DNA have the similar Ct value

C differed in their geographical distribution. Subgenotype B1 is dominating Japan, while B2 is dominating China and Vietnam and B3 is confined to Indonesia.

Another study by Mangold *et al*. (1995), revealed that all HBV isolates obtained from HBsAg-positive HBV carriers, including healthy blood donors, patients with acute hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma; and patients on hemodialysis all located in Surabaya belonged to genotype B with more than 90% of them being classified into subtype adw. Another survey in Yogyakarta was found
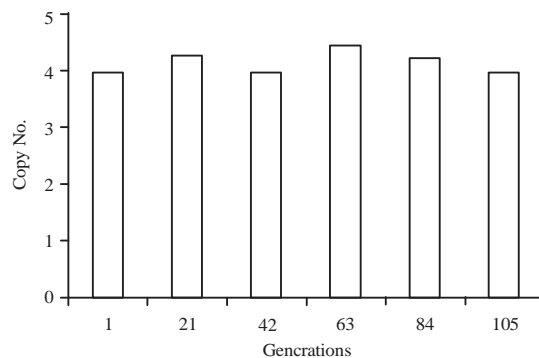

Fig. 6: Copy number of the integrant *P. pastoris* during time course of interval. This graph shows that integration of four copies of HBsAg expression cassette is stable up to 105 generations

that subtype adw was found in 34 (74%) of 46 HBV samples and adr in five (11%), compound subtypes, such as adyw and adyr were detected in the remaining seven (15%) (Juniastuti *et al*., 2011).

HBsAg is a cysteine-rich, lipid-bound protein with 226 amino acids (Zhao *et al*., 2006). Evidence supporting the mechanism that the correct disulfide bond pairing is the molecular basis for shaping up the native virion-like epitopes (Zhao *et al*., 2011). The importance of the correct intra and intermolecular disulfide bonds is very important to the immunochemical and biological function of the HBsAg subviral particles and reduction of these disulfide bonds greatly decreases or abolishes both the antigenic and immunogenic properties of this protein complex (Zhao *et al*., 2006). Cys-48, Cys-65 and Cys-69 which are located outside MHR and Cys-107, Cys-138 and both of Cys-121-Cys-124 which are located inside the region, were found to be essential for s-HBsAg VLP assembly (Mangold and Streeck, 1993; Mangold *et al*., 1995, 1997).The deduced amino acid sequence analysis confirmed all of the cystein residues were also found in inserted s-HBsAg encoding gene in pAOHBs1.Therefore, they provide a structural architecture facilitating disulfide-bonded conformational epitopes that is important for virus like particle formation and neutralizing viral infection.

T-cell epitopes at sequences 21-28, 124-147, 165-172 and 215-223 also reside in deduced amino acid sequence of s-HBsAg in pAOHBs1. One minimal essential epitope which corresponds to a peptide candidate for the binding to HLA-A2 determinant and activate T CD8+ lymphocytes also reside in deduced amino acid at sequence 172-180. In addition, 3 antigenic peptides which indicated as a dominant B cell epitopes also reside in deduced amino acid sequence of s-HBsAg in pAOHBs1 in sequence 14-32, 291-300 and 307-314 (Meng *et al*., 2013).

In the past 15 years, a wide range of HBV mutants have been found including many amino acid substitutions, insertions and deletions. One of the mutation, Gly/Arg145 substitution, alters the projecting loop (aa 139-147) of the

Major Hydrophilic Region (MHR), also namely "A" determinant, hence it was termed as vaccine-escape mutant (Coleman, 2006; Purdy, 2007). Other substitutions, Gln/Arg129 and Met/Leu 133 also in Indonesian HBV isolates, were also identified (Jinata *et al.*, 2012). If the nucleotides of HBsAg encoding gene mutated and encode different amino acid in these areas, protein expressed will not induce the proper anti-HBs which could neutralize the wild type HBV. Since the allignment of the DNA sequences of the MHR from pAOHBs1 and corresponding region in the NCBI database was shown to be identical, all of those mutants in the MHR were absent. Based on those results, this Indonesian HBsAg expression cassette is a promising candidate for generating wild type HBV vaccine.

In *P. pastoris*, expression of the native viral HBsAg gene leads to translocation of the protein into the Endoplasmic Reticulum (ER) where it assembles, at least, partly, into defined multi layered lamellar structures (Lunsdorf *et al.*, 2011). As mentioned previously, it was shown that after induction, a protein with apparent molecular weight which similar to HBsAg as a positive control were achieved. This was similar to the estimated monomer and dimer of HBsAg protein, that is possible due to the formation of lamellar structures of HBsAg prior to VLP formation during downstream processing.

Related to the stability of the *P. pastoris* transformant, result suggested that Mut^s host is capable of assembling all of the HBsAg produced by the four copy insert quite efficiently. Our studies also revealed continued integrity of the HBsAg expression cassette during the course of an extended induction phase. Four copies of HBsAg expression cassette does not lead to excess metabolic load so that the *P. pastoris* integrant is able to maintain the presence of HBsAg gene on chromosome. Low copy integrant have a higher level of gene stability.

CONCLUSION

A stable integrant of *P. pastoris* containing four expression cassettes of HBV B3 genotype s-HBsAg capable of producing a vaccine candidate against Hepatitis B has been generated.

ACKNOWLEDGMENT

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