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Integration Stability of *sHBsAg*-Multi Expression Cassettes in *Pichia pastoris* GS115 during Methanol Induction

Patricia Gita Naully[1, 7], Neni Nurainy[2], Elvi Restiawaty[3, 6], Dessy Natalia[4, 6], Debbie Soefie Retnoningrum[5, 6], Wardono Niloperbowo[1, 6], Ernawati Arifin Giri-Rachman[1, 6*]

[1]*School of Life Science and Technology, Institut Teknologi Bandung, Bandung, Indonesia*
[2]*PT Biofarma (Persero), Bandung, Indonesia*
[3]*Faculty of Industrial Technology, Institut Teknologi Bandung, Bandung, Indonesia*
[4]*Department of Biochemistry, Faculty of Mathematics and Natural Science, Institut Teknologi Bandung, Bandung, Indonesia*
[5]*School of Pharmacy, Institut Teknologi Bandung, Bandung, Indonesia*
[6]*Bioscience and Biotechnology Research Centre, Institut Teknologi Bandung, Bandung, Indonesia*
[7]*School of Health Sciences, Jenderal Achmad Yani, Cimahi, Indonesia*

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ABSTRACT

Hepatitis B is the major health problem worldwide including in Indonesia. Vaccination is the best prevention strategy for the disease. For the purpose of vaccine development and to decrease drug import, production of Hepatitis B Virus (HBV) small surface antigen (sHBsAg) from Indonesian HBV subtype is needed. The recombinant protein production can be conducted by integrating multi expression cassettes of *sHBsAg* gene in *Pichia pastoris* chromosome using gene replacement method. Such integration method turns out to allow loss of foreign gene from chromosome by excisional recombination-mediated looping out. This research was aimed to determine integration stability of four copies of *sHBsAg* expression cassette in *P. pastoris* GS115 chromosome inducted with 2% methanol in FM22 medium. The methanol induction was conducted twice at 63-h and 75-h. Integration stability determination was conducted qualitatively using PCR and quantitatively using qPCR absolute quantification. A band of 208 bp with similar intensity was observed after amplification of genomic DNA. All samples generated the same Ct value of around 22 with four copies of *sHBsAg* gene per genome. The result from this experiment shows that integration of four copies of sHBsAg expression cassette in *P. pastoris* GS115 chromosome is stable during methanol induction.

1. Introduction

Hepatitis B is an infectious disease affecting hepatocytes and is caused by Hepatitis B Virus (HBV). Indonesia has high prevalence of Hepatitis B, the second highest among South East Asian Region (SEAR) countries after Myanmar. In a study and blood donor screening of Indonesian Red Cross, it was estimated that 18 million Indonesian people have been infected by Hepatitis B (Ministry of Health of Indonesia 2014). Hepatitis B can be prevented by vaccination which contains HBV's small surface antigen (sHBsAg). Presence of sHBsAg in the form of Virus-Like Particle (VLP) in blood can induce antibody to eliminate HBV (Lunsdorf *et al.* 2011). Hepatitis B vaccine has been available in Indonesia since 1997, but up until now Indonesia still imports sHBsAg protein to fulfil its need. Therefore, production of sHBsAg protein that matches Indonesia's HBV genotype is much needed. A study from Giri-Rachman *et al.* 2015 showed that predominant HBV genotype in Indonesia is B3.

Production of sHBsAg recombinant protein from Indonesian isolate can be conducted by expressing sHBsAg gene in certain organism. Organism chosen as expression platform must be able to produce protein with correct structure and folding (Bo *et al.* 2005). The prerequisite cannot be fulfilled by *Escherichia coli* and *Saccharomyces cerevisiae* (Daly and Hearn 2005; Balamurugan *et al.* 2007). Other microorganism than can be utilised as expression platform is *P. pastoris* (Bo *et al.* 2005). Expression of *sHBsAg* gene in *P. pastoris* can be increased in several ways such as using strong and regulated promoter (AOX promoter), inducing promoter with optimum methanol concentration, and increasing number of integrated expression cassette in chromosome.

* Corresponding Author
E-mail Address: erna@sith.itb.ac.id

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In previous study, four copies of *sHBsAg* expression cassette from HBV genotype B3 has been constructed using pAO815 expression vector (Giri-Rachman *et al.* 2015). Expression cassettes were integrated to *P. pastoris* GS115 using gene replacement method. The method allows multiple copies of the gene integrated in the same locus so that the chromosomal repetition of genes occurs sequentially (Balamurugan *et al.* 2007). However, through his research, Schwarzhans *et al.* (2016) proves that integration with such methods can occur in some loci so that there are integrants that contain higher number of gene copies and some are lower than they should be. In addition, according to Wang *et al.* (1996), the gene replacement integration method can lead to excisional recombination so that foreign genes can be detached from the chromosome (looped out). Instability of gene integration in *P. pastoris* chromosome due to excisional recombination can be affected by methanol presence in the medium (Curvers *et al.* 2001). The stability of the integration of gene should be of concern because a recombinant microorganism can be used in vaccine production if it has a stable genetic condition (WHO 2013). Therefore, this research aimed to determine integration stability of four copies of *sHBsAg* expression cassette in *P. pastoris* GS115 during cultivation and 2% methanol induction in FM22 medium qualitatively and quantitatively using qPCR absolute quantification.

2. Materials and Methods

2.1. Strains, Plasmids, and Reagents

P. pastoris GS115 transformant culture, pAO815 plasmid harbouring single copy and four copies of *sHBsAg* expression cassette (pAO-HBs1 and pAO-HBs4) was obtained from previous study (Giri-Rachman *et al.* 2015). Yeast DNA purification kit were purchased from Epicentre. All primers and restriction enzyme used in this study were purchased from Invitrogen. qPCR supermix were purchased from Bio-Rad. All other reagents used were of the purest grade commercially available.

2.2. Analysis of *P. pastoris* GS115 Integrant by Polymerase Chain Reaction (PCR)

Genomic DNA from *P. pastoris* whose chromosome has been integrated with *sHBsAg* gene (*P. pastoris* integrant) was isolated using MasterPure™ Yeast DNA Purification kit. Purified DNA concentration was measured by fluorometer (Invitrogen). PCR was performed using two sets of primer (Table 1). PCR mastermix composition for each reaction of 25 µl was as follow: 18.5 µl deionised water; 2.5 µl 10X reaction buffer; 0.5 µl 10 mm dNTPs; 0.5 µl Taq DNA Polymerase 5U/µl; 0.5 µl 10 mm *sHBsAg1* primer or AOX1 primer; 0.5 µl 10 mm *sHBsAg2* primer or AOX2 primer; and 2 µl genomic DNA. Amplification of *sHBsAg* gene was performed under condition of initiation (94°C, 5 minutes), denaturation (94°C, 30 seconds), annealing (55°C, 1 minute), elongation (72°C, 2 minutes), and final elongation (72°C, 5 minutes).

2.3. Cultivation of *P. pastoris* GS115 Integrant and Methanol Induction in FM22 Medium

Single colony of *P. pastoris* whose chromosome has been integrated with *sHBsAg* gene (*P. pastoris* integrant) was inoculated into 100 ml of Minimal Glycerol (MGY) medium. MGY medium contains 13.4 g/l YNB, 10 g/l glycerol, and 0.4 mg/l biotin. The culture was incubated in shaker incubator (Innova® 42 New BrunSwick Scientific) in 30°C with 250 rpm agitation for 48 hours. 10 ml of 48 hours culture was inoculated into 100 ml of MGY medium and was incubated for 24 hours. The 24 hours culture was aliquoted into 200 ml of FM22 medium until OD_{600}=1. FM22 medium contains 42.9 g/l KH_2PO_4, 5 g/l $(NH_4)_2SO_4$, 1 g/l $CaSO_4{\cdot}2H_2O$, 14.3 g/l K_2SO_4, 11.7 g/l $MgSO_4{\cdot}7H_2O$, 40 g/l glycerol, 1 ml Pichia Trace Metals 4 (PTM4) (Higgins and Cregg 1998), and adjusted into pH 4.5 using KOH. Culture in FM22 medium was incubated for 87 hours. Induction with 2% methanol was conducted in 63-h and 75-h.

Table 1. Sequences of primers used in this study

Primer	Utility	DNA Sequence 5'-3'	Amplicon size (bp)
sHBsAg1 sHBsAg2	Integrant analysis (PCR)	5'-GGAGAACATCGCATCAGGAC-3' 5'-CCTCGAGTCAAATGTATACCC-3'	700
AOX1 AOX2	Integrant analysis (PCR)	5'-GACTGGTTCCAATTGACAAGC-3' 5'-GCAAATGGCATTCTGACATCC-3'	1,000
qsHBsAg1 qsHBsAg2	Integration Stability Test (PCR and qPCR)	5'-CTTCTAGGACCACTGCTTGTGT-3' 5'-GACACATCCAACGATAACCAGGAC-3'	208

2.4. Qualitative Test for Integration Stability by PCR

Genomic DNA was aliquoted into PCR reaction mix of 25 µl total volume consisting 19.9 µl deionised water; 2.5 µl 10X reaction buffer; 0.5 µl 10mm dNTPs; 0.5 µl Taq DNA Polymerase 5U/µl; 0.3 µl 10 mm *qsHBsAg1* primer (Table 1); 0.3 µl 10 mm *qsHBsAg2* primer; and 1 µl genomic DNA with final concentration of 50 ng/µl. Deionzed water and genomic DNA from *P. pastoris* whose chromosome has not been integrated with SHBsAg gene (non-integrant *P. pastoris*) were used as negative controls. Amplification of *sHBsAg* gene was performed in 30 cycles of initiation (94°C, 5 minutes), denaturation (94°C, 30 seconds), annealing (63°C, 30 seconds), elongation (72°C, 15 seconds) and final elongation (72°C, 1 minute).

2.5. Quantitative Test for Integration Stability by qPCR

Genomic DNA was aliquoted into qPCR reaction mix of 10 µl total volume consisting 3 µl deionised water; 5 µl 2X SsoFastTM EvaGreen® Supermix; 0.5 µl 10 mm *qsHBsAg1* primer; 0.5 µl 10 mm *qsHBsAg2* primer (Table 1); and 1 µl genomic DNA with final concentration of 1.8 x 10^{-9} g/ml. Negative controls used were no-template control (NTC), control without DNA Polymerase, and genomic DNA of *P. pastoris* non-integrant. Gene amplication was performed in 40 cycles of initiation (95°C, 30 seconds), denaturation (95°C, 5 seconds), annealing and elongation (63°C, 15 seconds). Melting curve was analysed with temperature gradient of 60–95°C for 5 seconds with temperature increment of 0.5°C. Actual copy number of sHBsAg gene in genome can be calculated in two steps as follow:

1. Calculation of sHBsAg gene copy number per ml Sample's Ct value from qPCR reaction is calculated using equation from standard curve.
2. Calculation of P. pastoris GS115 genomic DNA per ml Genomic DNA copy number is calculated with equation (Whelan *et al.* 2003): Genomic DNA copy = $6.02x10^{23}$ (copy/mole) x DNA mass (g) / DNA size (bp) x 660 (gr/mole/bp).

3. Results

3.1. Analysis of *P. pastoris* GS115 Integrant by PCR

Electropherogram (Figure 1) shows that *sHBsAg* gene in *P. pastoris* chromosome has been amplified using sHBsAg primer and AOX primer. In PCR using sHBsAg primer, 700 bp DNA band was amplified from *P. pastoris* genome (Lane 1) and positive control (Lane 3). Positive control used was pAO-HBs4 plasmid. Similar result was obtained from PCR using AOX primer. Fragment was obtained around 1,000 bp from *P. pastoris* genome (Lane 7) and positive control (Lane 5). The result showed that AOX promoter, *sHBsAg* gene, and terminator originated from pAO815 plasmid were present in *P. pastoris* chromosome.

3.2. Determination of *P. pastoris* GS115 Integrant Growth Curve

Growth curve of *P. pastoris* in FM22 medium (Figure 2) showed a gradual increase of biomasss during 63 hours of growth. Dry weight of *P. pastoris* increased from 0.13 g/l into 4.58 g/l with growth rate of 0.17 g/l/hour. After first methanol induction at hour-63, relatively high biomass increase was observed. *P. pastoris* dry weight increased from 4.58 g/l into 6.67 g/l with growth rate of 0.34 g/l/hour. Different result was observed after the second methanol induction at hour-75. *P. pastoris* biomass increase was almost unobservable until 87 hours.

3.3. Qualitative Determination for Integration Stability of *sHBsAg* Gene in *P. pastoris* GS115 Chromosome during Cultivation and Methanol Induction

Electropherogram (Figure 3) showed a DNA band of 208 bp amplified from *P. pastoris* chromosome (Lanes 5-8). All bands had similar size and intensity with positive control (Lane 2). The result showed that *P. pastoris* chromosome was still integrated with *sHBsAg* gene until second methanol induction.

3.4. Quantitative Determination for Integration Stability of *sHBsAg* Gene in *P. pastoris* GS115 Chromosome

To obtain more accurate result, we used quantitative measurement of integration stability using qPCR absolute quantification. The method's accuracy was determined by standard curve. The obtained qPCR standard curve was equation y= -3.2063x + 41.083 with R^2 of 0.9906 and E of 105% (Figure 4). Standard curve is used to further ensure presence of 4 copies of *sHBsAg* gene in genomic DNA of *P. pastoris* integrant.

In *sHBsAg* gene amplification curve (Figure 5a), all samples of *P. pastoris* genomic DNA yielded similar C_t value of around 22. Similar C_t values

indicates same copies of *sHBsAg* gene in genomic DNA samples. The qPCR process was performed well without contamination or sample missdilution. This was showed from no fragment amplified in three negative controls used. Furthermore, quality of amplification product was analysed using melting curve. In melting curve graph (Figure 5b), only a single PCR product was obtained. Result showed that all amplification products can completely denature at same temperature, 80°C.

The graph of *sHBsAg* gene copy number (Figure 6) showed that there were four copies gene integrated in *P. pastoris* chromosome. The copy number did not change during activation in MGY medium, cultivation in FM22, and methanol induction.

4. Discussion

In this research, cultivation was divided into three stages. First stage was activation or pre-starter aiming to activate genes involved in cell division in order to accelerate cell number increase in FM22 medium. MGY medium was used as activating medium due to

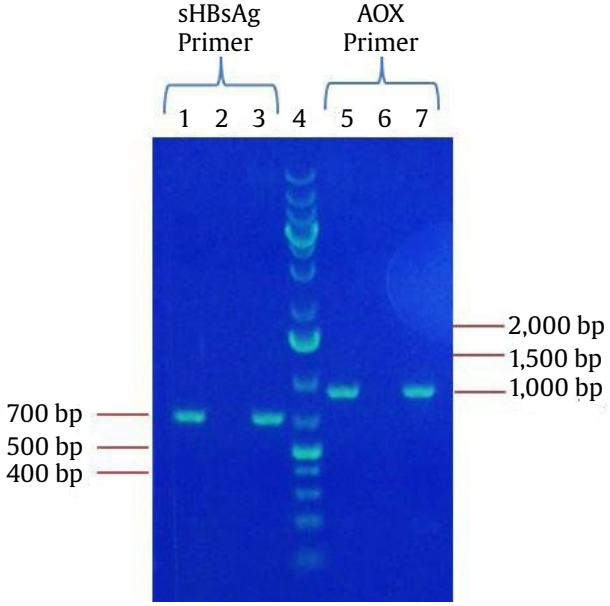

Figure 1. PCR analysis of *P. pastoris* GS115 transformant. Lane 1: Genomic DNA from *P. pastoris* GS115 transformant, lane 2: deionised water (negative control), lane 3: pAO-HBs4 plasmid (positive control), lane 4: thermoscientific 1 kb plus ladder, lane 5: pAO-HBs4 plasmid (positive control), lane 6: deionised water (negative control), lane 7: genomic DNA from *P. pastoris* GS115 transformant

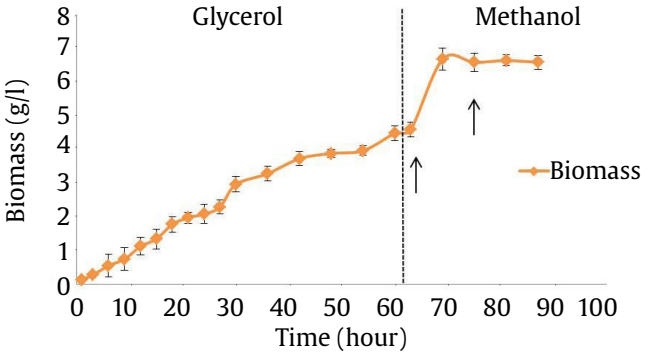

Figure 2. Growth curve of *P. pastoris* GS115 integrant in FM22 medium. First arrow indicates first methanol induction at hour-63. Second arrow indicates second methanol induction at hour-75. Data was presented as mean ± SD, n = 3

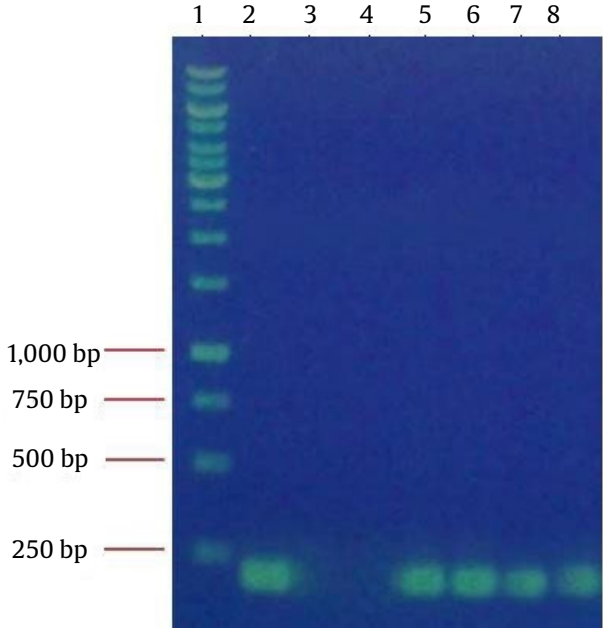

Figure 3. PCR analysis of the stability of integrated *P. pastoris* GS115 with four copies of *sHBsAg* expression cassette. Lane 1: thermoscientific 1 kb Ladder, lane 2: pAO-HBs4 plasmid (positive control), lane 3: genomic DNA from non integrant *P. pastoris* GS115, lane 4: deion water (negative control), lane 5: genomic DNA from *P. pastoris* GS115 integrant during activation in MGY medium, lane 6: genomic DNA from *P. pastoris* GS115 integrant during cultivation in FM22 medium, lane 7: genomic DNA from *P. pastoris* GS115 integrant during first methanol induction, lane 8: genomic DNA from *P. pastoris* GS115 integrant during second methanol induction

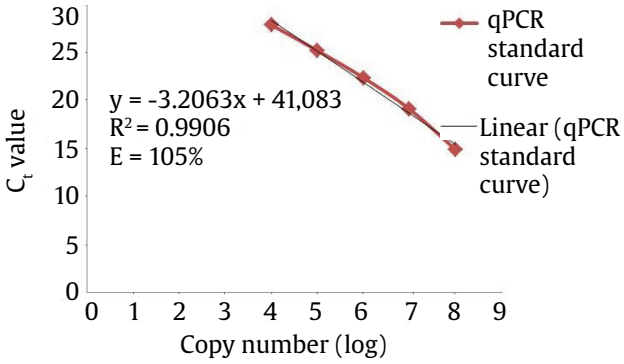

Figure 4. Standard curve for *sHBsAg* DNA concentrations. The standard curve were established with 10-fold serial dilutions of pAO-HBs1. Copy concentrations of 104–108 copies/µl were arbitrarily assigned to 10-14 to 10-10 dilution of initial pAO-HBs1. Each standard dilution was amplified by realtime qPCR using q*sHBsAg* primer pairs in triplicate. For each gene, determined C_t values were plotted against the logarithm of their initial copy numbers (n = 3). A standard curve was then generated by linear regression through these points

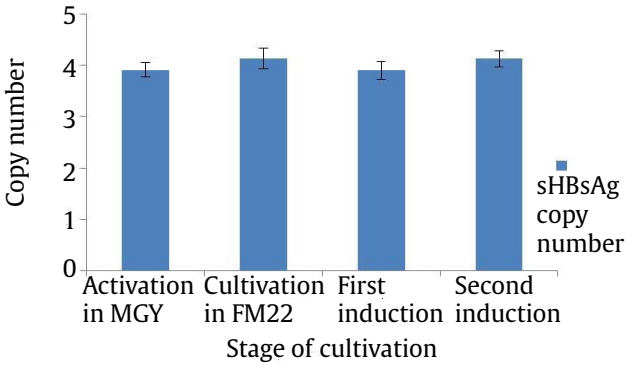

Figure 6. Copy number of the *P. pastoris* GS115 integrant during activation in MGY medium, cultivation in FM22 medium, and methanol induction. This graph shows that integration of four copies of *sHBsAg* expression cassette is stable during cultivation and 2% methanol induction in FM22 medium. Data was presented as mean ± SD, n = 3

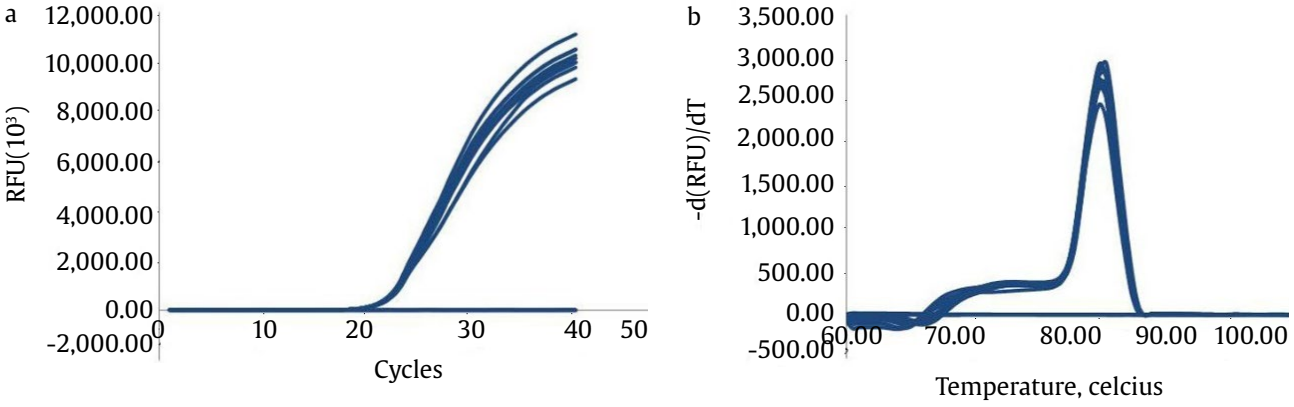

Figure 5. (a) qPCR analysis of the stability of integrated *P. pastoris* GS115 with four copies of *sHBsAg* expression cassettes. Genomic DNA was prepared from the *P. pastoris* GS115 integrant during activation in MGY medium, cultivation in FM22 medium, and methanol induction. All of genomic DNA has the similar C_t value, (b) qPCR melting curve analysis. This curve shows that only a single peak of 80°C was obtained

its property as enriched medium. MGY has additional nutrients such as YNB therefore is able to increase *P. pastoris* growth rate (Higgins and Cregg 1998). Second stage was starter aiming to grow *P. pastoris* cells to reach sufficient biomass before being induced by methanol. FM22 medium was chosen because its formulation enables *P. pastoris* to reach high cell density in fed-batch culture (Higgins and Cregg 1998). Component of FM22 is similar to Basic Salt Medium (BSM), but FM22 contains higher potassium than BSM. Both FM 22 and BSM are generally used for high cell density fermentation of *P. pastoris* (Cos *et al.* 2006). FM22 also contains 40 g/l of glycerol (Higgins and Cregg 1998). The glycerol concentration was chosen because higher than 40 g/ concentration can inhibit *P. pastoris* growth. Chiruvolu *et al.* (1998) successfully detected 0.5-2.4% of ethanol when using glycerol concentration higher than 70 g/l. Ethanol presence can repress enzymes involved in methanol metabolism, thus hindering the enzyme involved in methanol metabolism and affects recombinant protein expression negatively (Zhang *et al.* 2000). Last stage was methanol induction. This stage aims to induce *P. pastoris* to produce sHBsAg protein using 2% methanol. The concentration has been showed by Giri-Rachman *et al.* (2015) to have positive effect on

growth and sHBsAg protein expression level. Besides, Santoso *et al.* (2012) showed that 2.5% methanol can increase human erythropoietin gene expression in *P. pastoris*.

Growth curve of *P. pastoris* in FM22 medium showed than *P. pastoris* GS115 integrant had no adaptation phase because of activation in MGY medium. *P. pastoris* directly entered exponential phase in 60 hours of growth. It is important to note that from hour-63 to hour-75, growth rate was higher than previous stage. It is especially interesting because in no other researches have shown growth rate increase during methanol induction (Pais *et al.* 2003; Ren *et al.* 2003; Jungo *et al.* 2006). Generally, growth rate during methanol induction is lower than during glycerol cultivation (Cos *et al.* 2006). The event can be influenced by two factors, the methanol concentration and *P. pastoris* strain used. When glycerol in FM22 medium started to deplete, *P. pastoris* growth rate started to decrease. However, during first induction (hour-63), *P. pastoris* started to utilize methanol added as sole carbon source. Relatively higher methanol used (20 g/l) allows *P. pastoris* to use methanol to increase the growth rate and express sHBsAg protein. Such *P. pastoris* can use methanol for growth due to its Muts phenotype. The *P. pastoris* has undergone mutation in its AOX1 gene (Balamurugan *et al.* 2007). In this research, the AOX1 gene has been replaced by *sHBsAg* gene. However, *P. pastoris* still possess AOX2 gene encoding alcohol oxidase enzyme responsible in methanol metabolism (Balamurugan *et al.* 2007). During second methanol induction (hour-75 to hour-87), biomass tended to neither increase nor decrease. This might due to methanol accumulation in the medium that inhibits *P. pastoris* growth. In *P. pastoris* peroxisome, methanol is metabolised into formaldehyde and hydrogen peroxide that are toxic (Yurimoto *et al.* 2011).

Gene integration stability in different cultivation condition was determined qualitatively using PCR. Qualitative determination was based solely on the size and intensity of DNA band produced. However, actual copy number of *sHBsAg* gene cannot be determined by PCR. Therefore, this research used quantitative measurement using qPCR absolute quantification. The method is better than Southern Blot because its analysis time is relatively short and it does not need genomic DNA in high quantity (Abad *et al.* 2010). qPCR absolute quantification is based on standard curve calculated from standard with known concentration to determine actual copy number of target gene. Accuracy of qPCR standard curve is an important aspect and can affect analysis result. Dhanasekaran *et al.* (2010) showed that linear plasmid is the best standard due to its stability in serial dilution and ability to be used in long-term study. Therefore this study utilised linearised pAO-HBs1 plasmid as standard.

This study showed that consecutive repetition of heterologous gene in chromosome and methanol presence in medium do not always caused heterologous gene excision from chromosome. This is also demonstrated by Ohi *et al.* (1998). In his research, only 0.01% *P. pastoris* integrated with two copies of the Human Serum Albumin (HSA) genes that lost the gene after 2% of methanol induced on the Yeast Pepton Methanol (YPM) medium for 72 hours. Zhu *et al.* (2009) also proved that the integrated *P. pastoris* 6 copies of the Porcine Insulin Precursor (PIP) gene did not experience a reduction in the number of gene copies after induced 0.8% methanol in medium Basic Salt Medium (BSM) medium for 96 hours. However, *P. pastoris* that integrates 29 copies of PIP genes had different outcome. The integrants contain only 15 copies of the PIP gene at the end of the induction phase. The results showed that the higher the number of copies of foreign genes integrated in the *P. pastoris* chromosome, the higher the likelihood of loss of foreign genes due to excisional recombination. On the other hand, the study of Marx *et al.* (2009) proves that the integration of 11 copies of the human gene Cu/Zn superoxide dismutase on the *P. pastoris* chromosome can be stable for up to 28 generations if no methanol induction on the BSM medium is employed. In addition, there are studies that prove the low number of copies of foreign genes does not guarantee that integration will always be stable. Continuous cultivation of integrated *P. pastoris* integrated with one copy of human chymotrypsinogen B lost the gene faster when cultivated on bioreactor containing 7 L shake flask growth medium (SF) with methanol concentration of 4 g/l (Curvers *et al.* 2001). Stability of integration of foreign genes on *P. pastoris* chromosome not only influenced by the method of integration and the presence of methanol in the medium, but also influenced by the number of copies of foreign genes, methanol concentrations, and culture volumes used. High gene copy number should be balanced with low methanol concentration and culture volume. In this research, *P. pastoris* was induced with high methanol concentration but gene

copy number integrated in chromosome is relatively low and culture medium volume used was only 200 ml. This condition does not disturb integration stability of four copies of *sHBsAg* gene in *P. pastoris* chromosome. Metabolic burden in used *P. pastoris* was not too high, therefore still allows growth as well as maintenance of foreign gene in chromosome.

In conclusion, this study shows that integration of four copies of *sHBsAg* expression cassette in *P. pastoris* GS115 chromosome is stable during 63 hours of cultivation and 15 hours of 2% methanol induction in FM22 medium.

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Conflict of Interest

None.

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