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Stability of the recombinant hepatitis B surface antigen expression vector in genomic DNA of *Hansenula polymorpha* yeast cells (Master Cell Bank)

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For production of recombinant hepatitis B (HB) vaccine, fermentation of the transformed *Hansenula polymorpha* yeast cells containing hepatitis B surface antigen (HBs Ag) expression vector stored as lyophilized form (Master Cell Bank) at 4ºC followed by several purification steps has to be performed. In our study, the existence of the structural HBsAg gene and URA 3 selective marker gene in the HBsAg expression vector, integrated in chromosomal DNA of recombinant *Hansenula polymorpha* yeast cell was determined. First, genomic DNA of *Hansenula polymorpha* yeast cell was extracted from MCB and purified by using QIAGEN genomic DNA extracting kit, followed by restriction enzyme digestion, PCR amplification, agarose gel electrophoresis and Southern blot hybridization. The nucleotide sequence of HBsAg DNA segment was also confirmed by DNA Genetic Analyser. In addition, the presence of the URA3 marker gene in the HBsAg expression vector was also identified by culturing of transformed *Hansenula polymorpha* colonies on the FOA containing culture plate for their survival. The results indicated that both structural HBsAg expressed gene and URA 3 marker gene were found to be stable in chromosomal DNA of *Hansenula polymorpha* yeast cells at 4ºC on long-term storage.

INTRODUCTION

After transfer of technology from the Institute of Science and Technology, CJ Corporation, Republic of Korea, recombinant hepatitis B vaccine was successfully developed by the Hepatitis B Vaccines Plant, DMR (LM) in 2004 by using *Hansenula polymorpha* yeast cells as an effective host system. In this recombinant DNA technology, antigen expression vector (pH-HBs) was prepared by step-wise construction consisting of hepatitis B surface antigen (HBsAg) structural gene (678 base pairs), methanol oxidase (MOX) promoter gene (961 bp), URA 3 marker gene (451 bp), Hansenula Autonomously Replicating Sequence (HARS) functional gene (440 bp) and Transcription terminator gene (320 bp). This vector was then transformed and incorporated into the chromosomal DNA of *Hansenula polymorpha* yeast cells. After cultivation of these cells in selective media, they were freezed-dried followed by aseptic sealing in glass vials which were kept at 4ºC for long-term storage as Master Cell Bank (MCB) before used for the production process [1].

In our study, the existence the HBsAg expression vector, integrated in the genomic DNA of *Hansenula polymorpha* yeast cells (MCB) stored at 4ºC was determined with an aim to confirm its stability for using as a starting material in process of recombinant HB vaccine production.

MATERIALS AND METHODS

The lyophilized form of MCB containing the recombinant HBsAg expressed *Hanse-*

nula polymorpha yeast cells, stored at 4ºC for about 4 years, a product of CJ Corporation, Republic of Korea was first reconstituted with 1 ml of autoclaved distilled water. The suspension was smeared on 0.7 % Yeast Nitrogen Base (YNB) culture plate which was kept at 30ºC incubator for 48 hours. On day 3, a single colony from the above culture plate was inoculated into 5 ml of 0.7 % YNB broth, with 2 % glucose and it was then incubated at 30ºC with 250 rpm for 24 hours.

1. Identification of HBsAg expressed gene

From the above cultured broth containing transformed *Hansenula polymorpha* yeast cells, a genomic DNA was extracted and purified by using the QIAGEN Genomic DNA Extraction kit [2].

1.1. Southern blot identification

The extracted whole chromosomal DNA segment was digested by Restriction Enzyme Dra I. Then, the cut DNA fragments were identified by using agarose gel electrophoresis. Finally, this HBsAg expressed gene was confirmed by Southern Hybridization by using a specific probe prepared with specific primers; AY 21 F (5'- ATTGAGAACATC ACATCAGGA-3') and AY 21 R (5'-TTGG TAACAGCGG TAAAAAGGGAC.3') in PCR, binding with sequence of recombinant HBsAg gene labeled with digoxiginin-dUTP.

1.2. DNA sequencing analysis

The extracted genomic DNA containing the gene of interest was also amplified by thermocycler using specific primers: AY 21 F (5'- ATGGAGAACATCACATCAGGA-3') and CTR (5'-CTCTTTGTTTTGTTAGGGT -3'). Then, DNA segments were purified by using PCR purification QIAGEN test kit [3]. The purified HBsAg gene was confirmed by agarose gel electrophoresis, and nucleotide sequencing analysis was performed by using the ABI Prism 3100 Genetic Analyzer [4].

2. Determination of URA 3 marker gene

Serial dilutions (i.e. 10^{-2}, 10^{-4}, 10^{-6} & 10^{-8}) of the above culture in 0.7% YNB broth were done with distilled water and smeared on Yeast Peptone Dextrose (YPD) culture plates. After 48 hours incubation at 30ºC, 100 colonies were picked up from these plates and transferred on to the YPD plates containing 5–Fluoroorotic acid (5–FOA) and also on to the YPD plate without 5-FOA. After cultivation of these plate at 30ºC for 48 hours, number of viable colonies in each culture plate media was counted.

RESULTS

Figure 1 shows the agarose gel electrophoretic pattern of chromosomal DNA segments extracted from MCB, cut by Restriction Enzyme Dra I. Many partially cut DNA segments were observed indistinctly along the lane of digested test sample whereas uncut (i.e. undigested control) whole DNA segment was detected at the level of 12 Kb pairs. Southern hybridization of these DNA segments by using specific probe revealed that distinct DNA band of HBsAg expression vector containing the HBsAg structural gene (~700 bp) and part of MOX promoter gene (~400 bp) were observed at the level of 1.1 Kbp(cut) and 12 Kbp (uncut) indicating the existence of the HBsAg expression vector in genomic DNA of *Hansenula polymorpha* yeast cell (Fig. 2).

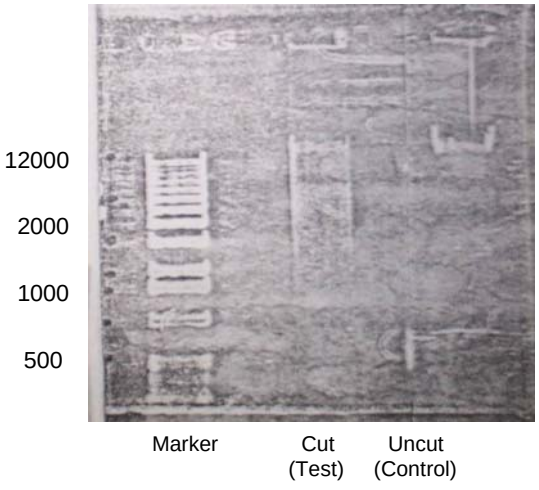

Fig .1. Agarose gel electrophoretic pattern of chromosomal DNA segment of H*ansenula polymorpha* yeast cell, digested by Restriction Enzyme Dra I

Agarose gel electrophoretic identification of the HBsAg gene extracted from the chromosomal DNA of *Hansenula polymorpha* yeast cells amplified by specific primers followed by PCR purification is illustrated in Fig 3. The distinct DNA band was observed at about 700 base pair level totally identical to that of the theoretical HBsAg gene, confirming the identity between number of base pair of HBsAg gene in expression vector and that of the gene of interest.

Figure 4 represents the nucleotide sequence of the HBsAg DNA segment, extracted from the chromosomal DNA of *Hansenula polymorpha* yeast cells analysed by using the genetic analyzer. The nucleotide sequences obtained were found to be totally identical to that of the HBsAg gene.

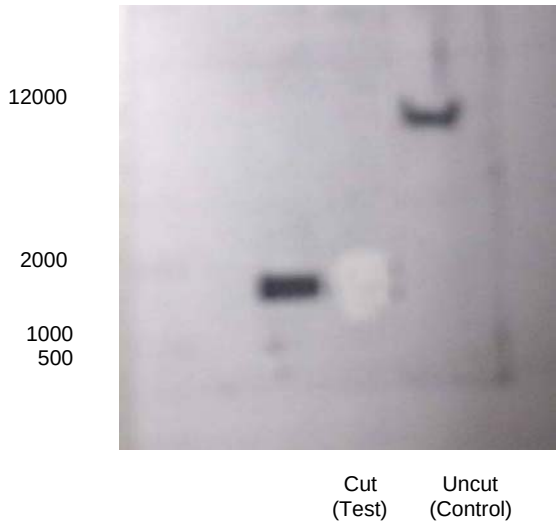

Fig .2. Southern hybridization of chromosomal DNA segments after digestion with RE Dra I by using a specific probe

Figure 5 depicts the growth of transformed *Hansenula polymorpha* colonies cultured on YPD plate with 5-FOA and plate without 5-FOA. All colonies cultured on YPD without FOA plate showed 100 % survival where as those cultured on YPD plate containing 5-FOA showed no survival. It indicated the coexistence of URA 3 functional gene with the HBsAg structural gene in the plasmid DNA segment integrated in genomic DNA of *Hansenula polymorpha* yeast cell.

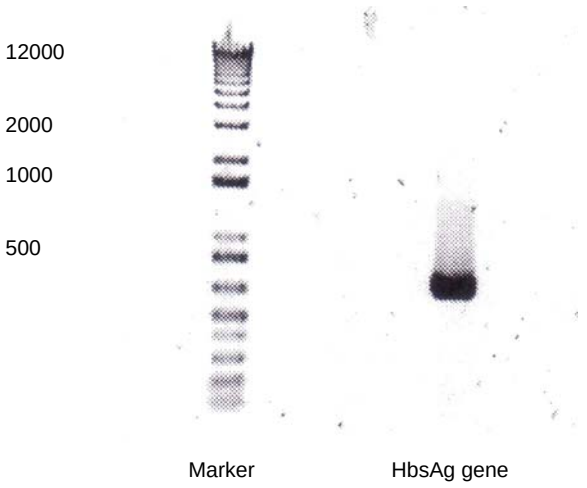

Fig. 3. Agarose gel electrophoretic identification of the HBsAg gene extracted from the chromosomal DNA of *Hansenula polymorpha* yeast cells

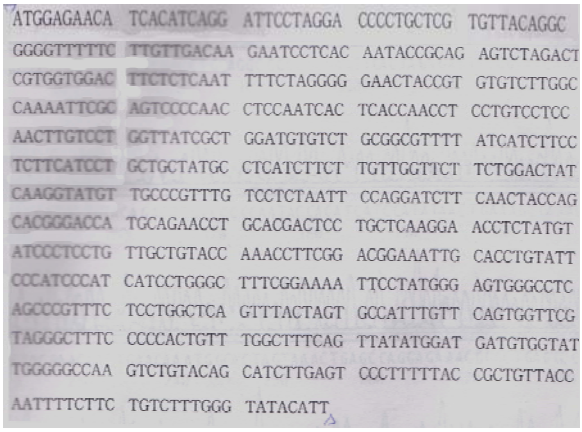

Fig. 4. Nucleotide sequence of the HBsAg DNA segment, extracted from the chromosomal DNA of *H. polymorpha* yeast cells

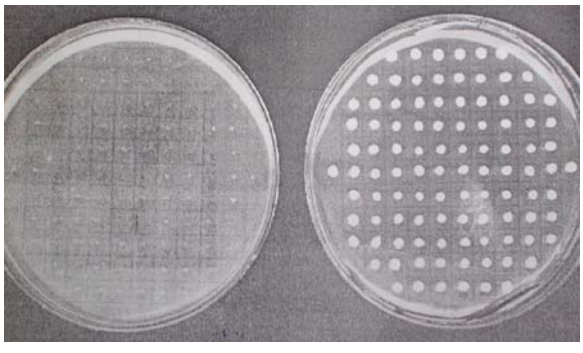

Fig. 5. Growths of transformed *Hansenula polymorpha* colonies in FOA containing media and FOA free media

DISCUSSION

It is well documented that *Hansenula polymorpha* yeast cell is the latest and efficient expression system for manufacturing recombinant HB vaccine. It is highly acceptable for mass production because of its preference to massive cultivation and control of protein expression from the recombinant *Hansenula polymorpha* yeast cell under control of MOX promoter gene. In addition, since the expression vector is incorporated in the chromosomal DNA, it is more stable than that of other vectors which are extra-chromosomal after transformation [5].

Because of the lack of orotidine 5-phosphate decarboxylase (OPD) which controls the uracil synthesis in *Hansenula polymorpha* yeasts, wild type (i.e untransformed) cells can not survive in uracil free- media. The plasmid of the transformed these yeast cell contains URA 3 gene which controls uracil synthesis in cells by expression of OPD. It transforms 5-fluro orotic acid (5-FOA) into cytotoxic 5 fluro uracil (5 FU). Therefore, transformed *Hansenula polymorpha* yeast cell containing HBsAg expression vector can not survive on media containing 5-FOA [1]. Based on these characteristics, stability of URA 3 gene was evaluated in our study by culturing of transformed colonies on FOA containing media and FOA free media. Our finding showed that all transformed cells could not survive on media containing FOA, thus indicating the existence of URA 3 maker gene in the HBs Ag expression vector of *Hansenula polymorpha* yeast cell.

This study also showed that, both HBsAg structural gene and MOX maker gene were identified in the expression vector integrated in genomic DNA of *Hansenula polymorpha* yeast cell. In addition, studying the stability of HBs Ag gene in *Hansenula polymorpha* yeast cells, the existence of the gene of interest was indicated by agarose gel electrophoresis followed by Southern hybridization as well as by nucleotide sequencing analysis.

In Myanmar, the HB Vaccine Plant, DMR (LM), Ministry of Health has been producing recombinant HB vaccine after transfer of technology from CJ Corporation, Republic of Korea since 2004. The lyophilized form of MCBs (i.e. transformed *Hansenula polymorpha* yeast cell) containing HBsAg expression vector provided by CJ corporation was used as a starting material for the fermentation and purification processes of HB vaccine production. It is necessary to determine the existence and stability of HBsAg expression vector in MCB periodically (i.e. once a year or prior to each and every batch of production process) [6].

The results of our study showed that the structural HBs Ag gene, MOX promoter gene and URA3 marker gene were identified in MCB containing transformed *Hansenula polymorpha* yeast cells. Therefore it could be concluded that the recombinant HBsAg expression vector was totally integrated and stable in genomic DNA of *Hansenula polymorpha* yeast cell (lyophilized form) kept at 4ºC for long-term storage, indicating that MCBs could be used effectively and safely as a starting raw material for production of recombinant HB vaccine in the HB Vaccine Plant, Myanmar.

REFERENCES

1. CJ Pharmaceutical Company (2000). Safety and Effectiveness of CJC 50100, hepatitis B vaccine (Recombinant), *Product Manual,* Republic of Korea. 2000; 1- 55.
2. *Handbook* of *the QIAGEN Genomic DNA* 1995; 1-50.
3. *User's guide of the Bioneer AccuPrep Plasmid Extraction* Kit 1999: 1-13.
4. *Sequencing Chemistry Guide for ABI Prism 3100 Genetic Analyzer*. HITACHI. 1-21.
5. Barnett JA, Payne RW & Yarrow D. In: *Yeasts*: *characteristics and identification*, published by the Press Syndicate of the University of Cambridge USA 1983; 7-17.
6. Requirements for hepatitis B vaccines made by recombinant DNA technology. *WHO Technical Report Series* 1989; no. 786:38-60.