The Myanmar Health Sciences Research Journal, Vol. 19, No. 1, 2007

Establishment of a simple procedure to detect the yield of recombinant HBsAg protein expressed by transformed *Hansenula polymorpha* yeast cells (MCB)

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For large scale production of recombinant Hepatitis B vaccine, recombinant *Hansenula polymorpha* yeast cells containing the HBsAg -expressed gene (Master Cell Bank) was processed under cultivation in seed and main fermentors ,followed by cell disruption and purification by using concentrator, homogenizer, ultracentrifuge, column chromatography , and sterilization filtration to have a purified HBsAg bulk. The whole process takes about 2 months. In our study, a single colony of MCB strain was cultured in the simple culture tube with methanol feeding followed by glass bead disruption of recombinant yeast cells. The released HBsAg protein was determined by using the AUSZYME test kit. The identity and purity of recombinant HBsAg protein was confirmed by SDS PAGE and Western blot hybridization. The whole procedure took about one week. It also indicates the viability of recombinant *Hansenula polymorpha* yeast cells and stability of HBsAg - expressed gene in the MCB. This is a simple and rapid procedure to predict the yield of HBsAg protein expressed by recombinant *Hansenula polymorpha* yeast cells prior to the actual production process of recombinant HB vaccine in the Plant.

INTRODUCTION

In Myanmar, hepatitis B (HB) viral infection is one of the important national health problems with 10.36 % of HBsAg carrier rate [1] and 35-60 % of infection rate [2]. As there is no cure for chronic HB carriers, prevention is extremely important. The HB vaccine is the best protection against HB viral infection [3]. Safe and effective plasma-derived HB vaccine was successfully developed in Department of Medical Research (Lower Myanmar) in collaboration with WHO/ UNDP and locally distributed in 1997. In 2004, yeast-derived recombinant HB vaccine was successfully developed in the WHO GMP standard Plant, DMR (LM) with a capacity to produce 5 million doses annually under the EDCF Loan provided by the Republic of Korea. In production process of the recombinant HB vaccine, Master Cell Bank (i.e., recombinant HBsAg-expressed transformed *Hansenula polymorpha* yeast cells) was under cultivation in seed and main Fermentors, followed by cell harvesting, diafiltration, concentration, homogenization, pH precipitation, ultracentrifugation, gel chromatography and sterile filtration to have a final purified HBsAg bulk. This whole process takes about 8 weeks to identify the HBsAg protein in the product. Before starting the actual production process, it is necessary to know the productivity of HBsAg proteins expressed by MCB in time. In this study, MCB was cultured in our laboratory and HBsAg yield was detected by appropriate test methods. This study was

conducted with an aim to establish the test procedure to predict the HBsAg protein productivity expressed by MCB prior to the actual production process of recombinant HB vaccine.

MATERIALS AND METHODS

MCB strain (i.e lyophilized HBsAg-expressed recombinant *Hansenula polymerpha* yeast cells) was cultured in an appropriate media with addition of methanol to express HBsAg protein intracellularly. These cells were lysed by using physico-chemical procedures. The expressed HBsAg protein in media was determined by AUZYME ELISA test kit and confirmed by SDS PAGE and Western blot by hybrid-dization. The whole procedure was as follows:

Cell culturing and sampling

First, MCB was reconstituted with 1ml of autoclaved distilled water and 100 ul was cultured on 0.7% Yeast Nitrogen Base (YNB) with 2% glucose media culture plate at 30˚C for 48 hours. On the 3rd day, a single colony was inoculated into 10ml of Yeast extract Peptone (YP) broth media with 1% glucose in 50ml culture tube and incubated at 30˚C for 24 hours by using a shaking incubator with 250 rpm. On the next day, culture media broth was centrifuged at 3000 rpm for 15 minutes at room tempera-ture(RT) followed by discarding supernatant and collecting pellets. These cells were resuspended in 20ml of YP media without glucose. After taking a sample volume of 1ml, culture tube containing these cells were incubated at 30˚C in shaking incubator with 250 rpm for 72 hours. During the incubation period, sampling of 1ml immediately followed by addition of calculated volume of methanol to make final concentration of 0.6% v/v at every 12 hours. (i.e. 0, 12, 24, 36, 48, 60 and 72 hours). Each sample was centrifuged 12000 rpm at RT for 2 minutes followed by discarding supernatant and collecting pellets.

Cell washing

Each sample was resuspended with same volume of autoclaved DW water followed by centrifugation at 4000 rpm for 10 minute at RT. Supernatant was discarded and pellet was collected, followed by resuspended with same volume of autoclaved distilled water. The above washing cycle was repeated 5 times for each sample to have complete cell washing.

Cell lysis

Each test tube containing pellets were weighed into 100 ug of cell mass (pellet), 50 ug of glass bead and 400 ul of buffer D solution (i.e. ratio of 2:1:8) were added followed by vortexing for 30 sec and keeping stand in ice berg for 30 sec. The above cell disruption cycle was repeated 10 times for each sample. Finally, all samples were centrifuged 12000 rpm for 15 min at RT, followed by discarding pellets and collecting supernatants which were kept at -20˚C till analyzed.

Determination of total protein and HBsAg contents

Each cultured sample was determined for total protein content and HBsAg content by using the Bradford Assay and the AUZYME test kit Monoclonal EIA (ABBOTT Lab) procedure respectively [4,5].

Identification of HBsAg protein by SDS-PAGE

Some cultured samples were subjected under SDS-PAGE by using SeeBlue Plus 2 protein standards and Silver X press staining Kit to detect the presence of HBsAg protein at the desired level of kDa.

Confirmation of HBsAg protein Southern blot hybridization

Finally, HBsAg protein containing SDS PAGE Gel was transferred onto the nylon membrane and subjected under Western blot hybridization procedure using specific antibodies to confirm the presence of HBsAg protein in the culture samples.

RESULTS

Total proteins and total HBsAg contents expressed by recombinant *Hansenula polymorpha* yeast cells at different times on cultivation with methanol feeding are shown in Table 1. Considerable amounts of total proteins and HBsAg were only expressed at 36 hours of cultivation, followed by increasing trend up to the 60 hours of cultivation. After that, the yields of total proteins and HBsAg became declined. The pattern of the HBsAg protein expression is clearly illustrated in Fig.1. It shows the productive capability of recombinant *Hansenula polymorpha* yeast cells in MCB to express HBsAg protein .

Table 1. Determination of total proteins and HBsAg contents in different times of cultivation with methanol feeding

Sample No.	Time of cultivation	Total volume(ul)	Total proteins(ng)	Total HBsAg (ng)
1	0 hr	40	44	2.60
2	12 hr	32	36	2.24
3	24 hr	24	15	2.58
4	36 hr	78	34	13.2
5	48 hr	80	87	79.8
6	62 hr	120	108	82.8
7	72 hr	188	39	12.2

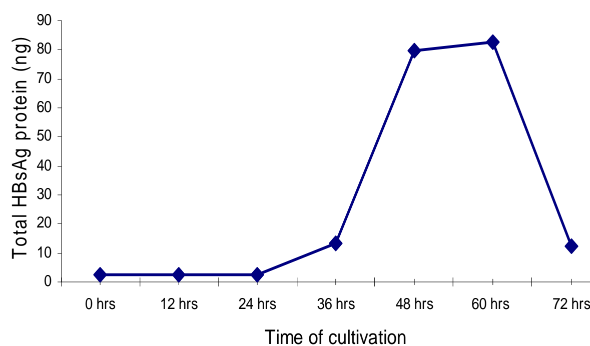


Fig.1. Pattern of the HBsAg protein productivity expressed by *Hansenula polymorpha* yeast cells in cultivation with methanol feeding

SDS-PAGE determination of the HBsAg proteins samples at various time intervals of cultivation was illustrated in Fig. 2. Since the volumes and amounts of total protein and HBsAg expressed at 0, 12, and 24 hours of cultivation were too low, these samples were insufficient to apply in SDS-PAGE determination. However, distinct bands were identified in lane 2, 3, 4 and 5 at the level of 24 kDa which is the theoretical molecular weight consistent with that of HBsAg protein, thus representing the HBsAg proteins of 36, 48, 60 and 72 hours of cultivation respectively. It confirms the presence of the HBsAg protein in these samples expressed by MCB.

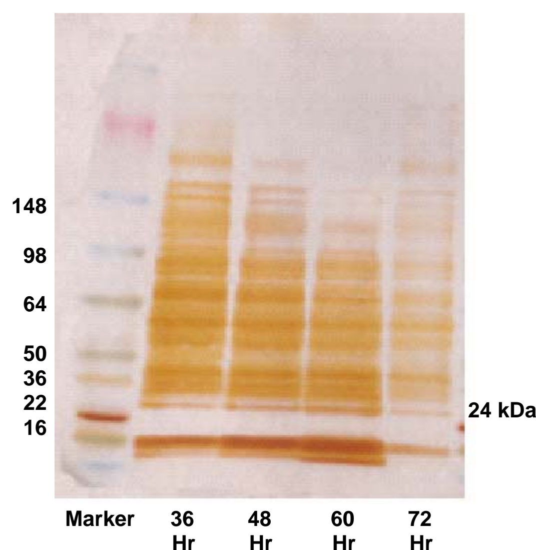


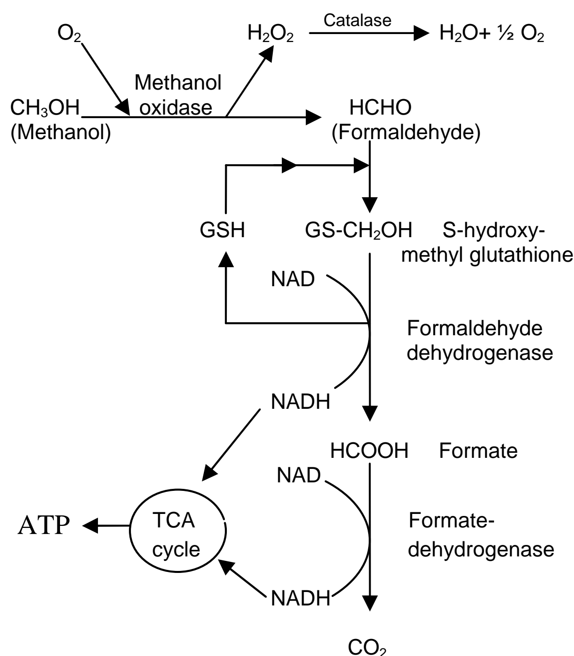
Fig. 2. SDS-PAGE determination of HBsAg protein in various cultured samples at different time intervals.

After transfer of HBsAg protein bands containing SDS-PAGE gel onto the nylon membrane, these bands were identified by using specific probes for HBsAg protein in Western blot hybridization technique. It was found that clear and distinct HBsAg bands were observed in samples of cultivation for different time intervals thus directly confirming the HBsAg productivity of recombinant *Hansenula polymorpha* yeast cells (Figure not showing).

DISCUSSION

For production of recombinant HB vaccine in the Hepatitis B Vaccine Plant, MCB containing HBsAg expressed-*Hansenula polymorpha* yeast cells (MCB) provided by

CJ Corporation, Republic of Korea has been used as a starting material, followed by fermentation and purification processes by using sophisticated machines and complicated procedures to obtain a purified HBsAg bulk. It is well documented that *Hansenula polymorpha* is a methylotrophic yeast and is a very useful system for manufacturing recombinant proteins [6, 7]. Because of the presence of Methanol oxidase (MOX) promoter gene in chromosomal DNA of transformed *Hansenula polymorpha* yeast cell containing the HBsAg structural gene (MCB), it is capable of metabolizing methanol as its sole carbon and energy source.



A schematic presentation of methanol metabolism in transformed *Hansenula polymorpha* yeast cell

The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme methanol oxidase (MOX), expressed by the MOX promoter gene present in the HBsAg expression vector incorporated in the chromosomal DNA of *Hansenula polymorpha* yeast cells. This reaction also generates hydrogen peroxide which is very toxic to the host cell and is degraded by catalase. Formaldehyde first condenses non-enzymatically with reduced glutathione (GSH) to form 5-hydroxy methyl glutathione ($GS\text{-}CH_2OH$). This product is hydrolyzed to formate and oxidized to carbondioxide by formaldehyde dehydrogenase and formate dehydrogenase respectively. This process generates NADH molecules which are required for formation of high energy rich compound, Adenosin Triphosphate (ATP) through the Tricarboxylic acid cycle used for biosynthetic purpose.

Therefore, in this study methanol was added as an inducer for MOX promoter gene incorporated in the chromosal DNA of these cells to express the desired protein during cultivation period. Since the expression is intracellular, lysis of these cells was required. For this purpose, vortexing with glass beads was performed for rupturing of cell wall in our study.

On studying the productive capability (yield) of HBsAg protein expressed by MCB in our laboratory, it was found that the MCB expressed the detectable level of HBsAg protein from the time of beginning, throughout the cultivation period, and up to 72 hours, obtaining the maximum expression at 60 hours of cultivation from which HBsAg expression quantity became declined. The expressed HBsAg proteins were identified in SDS-PAGE and confirmed by Western blot analysis.

The procedures of cell cultivation, cell disruption and HBsAg protein identification in this study were very simple, and only routinely used laboratory instruments and equipment were required to obtain the desired and expected protein. In addition, the whole procedure took about one week whereas the actual production process lasts about two months to identify the HBsAg protein expressed by the starting MCB in the intermediate product.

Recently, viability of the HBsAg protein-expressed recombinant *Hansenula polymorpha* yeast cells and stability of the

recombinant HBsAg expression vector in genomic DNA of *Hansenula polymorpha* yeast cells in MCB were already studied and reported [8, 9]. The results of our findings directly confirmed that the yield expressed by MCB was found to be the expected HBsAg protein. It also indirectly confirmed that lyophilized recombinant *Hansenula polymorpha* yeast cells in MCB stored for a certain period were found to be viable and the HBsAg protein expressed gene incorporated in chromosomal DNA was also tested to be stable in MCB. Therefore, this simple and rapid procedure could be used to predict the yield of HBsAg protein expressed by recombinant *Hansenula polymorpha* yeast cells of MCB prior to the actual production process of recombinant HB vaccine in the Plant.

ACKNOWLEDGEMENTS

This research work was performed at the Research and Development Centre of Pharmaceuticals, Institute of Science and Technology, CJ Corporation, Ichon, Republic of Korea during my training program for basic molecular biology and recombinant DNA technology for production of recombinant hepatitis B vaccine, funded by EDCF loan agreement between governments of the Union of Myanmar and the Republic of Korea.

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