Kor. J. Appl. Microbiol. Bioeng.
Vol. 14, No. 6, 455 – 459 (1986)

The Production of HBsAg in the Recombinant Yeast Cells

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(Received September 3, 1986)

재조합 효모 세포내에서의 간염백신 생산

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서울공대 공업화학과

(1986년 9월 3일 수리)

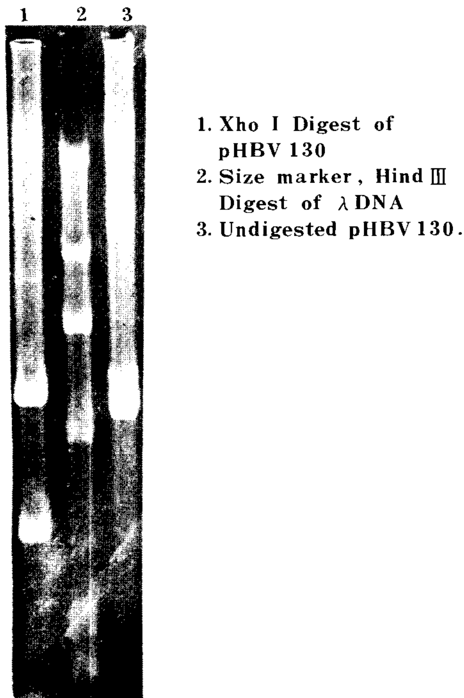
Dane particle was prepared from the plasma of chronic HBsAg carrier with high levels of HBsAg activity. DNA extracted from Dane particle core, after a DNA polymerase reaction with α-(^{32}p) dNTP, was identified as HBV DNA by liquid scintillation counter and agarose gel electrophoresis-G.M. counting. To produce Hepatitis B surface antigen for use as a vaccine against Hepatitis B virus infection, yeast strains harboring recombinant plasmid with Apase promoter was used. Recombinant plasmid was construced from pHBV 130 and pAM 82, transformed into *E. coli*, and then transferred into yeast strains. HBsAg was produced by derepression in Burkholder minimal medium with controlled inorganic phosphate concentration. The kinetics of HBsAg production was also investigated. Total HBsAg activity increased rapidly between 3 and 6 hours after transfer to phosphate-free medium and reached a maximum at around 9th hour. The transfer into phosphate-free medium after 6 hours in high phosphate cell growth medium gave maximum activity.

The advent of recombinant DNA technology made possible the cloning and expression of foreign genes in heterologous hosts to be carried out as a common laboratory practice. The techniques of gene manipulation became so well known that it has been quite a while since they ceased to be the target of well-thought scientific research and now the major attention of biotechnology is again directed toward the large-scale production using the recombinant cells. Speaking of the large-scale production, one may mean the cell growth and product formation in bioreactors, the product purification, and/or their scale-up. One of the factors that determine the mode of bioreactor design and operation is the kind of host recombinant cells being dealt with.

Recombinant host cells of microbial, plant, and animal origin have different requirements for successful bioreactor design and operation. Yeast is one of the cell species recommended as host for the gene carried on a recombinant plasmid partly because it has been around human being for such a long time, has a desirable fluid mechanical property, and has eukaryotic potential while still maintaining the simple-celled morphology.

In order to carry out a meaningful bioreactor operation for a practical gene product the HBsAg gene was cloned into an efficient yeast plasmid vector inducible with low phosphate concentration. The resulting recombinant plasmid was transformed into various yeast strains and the pattern of the gene product formation was studied in a bioreactor being run under different operating schedules.

Key words: HBsAg, Apase promoter, recombinant plasmid, bioreactor operation, yeast



1. Xho I Digest of pHBV 130
2. Size marker, Hind Ⅲ Digest of λ DNA
3. Undigested pHBV 130.

Fig. 1. XhoI digest of pHBV 130.

Materials and Methods

These are described in detail in the thesis by one of the authors[1] and in another paper[2].

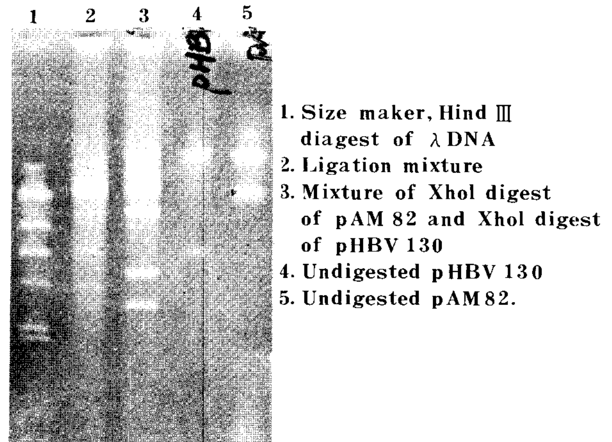
Results and Discussion

Construction of Expression Plasmid

Noting that the whole HBV DNA has a unique *Xho* I site in front of the translation start codon for the HBsAg the yeast expression vector, pAM 82, was created such that there exists a Xho I site behind the transcription start and ribosome binding sites in the regulatory segment of pho 5.

The recombinant plasmid, pHBV 130[3], carrying the Hepatitis B virus DNA of subtype adyw, was isolated from *E. coli*, digested with restriction endonuclease *Xho* I, and electrophoresed on 1% agarose gel (Fig. 1) to confirm complete digestion. *Xho* I digest of the yeast expression vector, pAM 82[4], was mixed with *Xho* I digest of pHBV 130 five times excess and ligated with T4 DNA ligase.

Electrophoresis shows the smearing band of the ligation mixture on agarose gel with higher density at large size position as compared with the bands of the digestion mixture at the corresponding position (Fig. 2).



1. Size maker, Hind Ⅲ diagest of λ DNA
2. Ligation mixture
3. Mixture of XhoI digest of pAM 82 and XhoI digest of pHBV 130
4. Undigested pHBV 130
5. Undigested pAM82.

Fig. 2. Electrophoresis of ligation mixture

Analysis of the Recombinant Plasmid

Ligation mixture was used to transform *E. coli*. Transformants were selected on LB agar plate containing 50 μg ampicillin per ml of medium, and 1,000 colonies were transferred separately to ampicillin-LB plate to conserve for the ensuing analysis. About 60 colonies per day were analyzed to select the right recombinant plasmid. Plasmids of larger size than pAM 82 were collected. Figure 3 shows the collected plasmids. To check the orientation of hepatitis B virus DNA insert, recombinant plasmids were digested with two restriction endonucleases, *Xho* I and *Pst* I. Restriction map of recombinant plasmids with HBV DNA insert in the right and

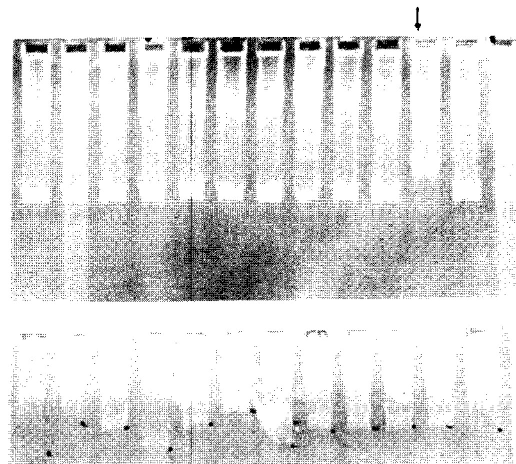


Fig. 3. Selection of recombinant from *E. coli* transformants.

(A) Screening of recombinants by rapid plasmid DNA isolation procedure
(B) Collection of selected plasmid.
Band 4: Size marker (pAM82)

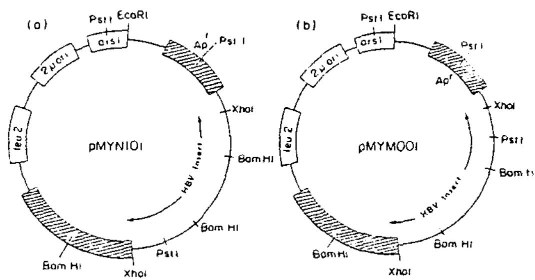


Fig. 4. Gene map of pAM 82 with insert
(a) proper orientatin (b) opposite orientation.

opposite orientations is depicted in Figure 4. When digested with restriction endonuclease *Pst* I, the recombinant plasmid with the insert in the correct orientation should have two large fragments and one small fragment. Figure 5 shows *Xho* I digest and *Pst* I digest of recombinant plasmids. In the lanes 3,4,5, and 8 of the gel run with *Pst* I digest two large fragments and one small fragment are located. These recombinant plasmids with the correctly oriented insert was named pMYN 101. Colonies numbered 3,4,5, and 8 were selected for use in the yeast transformation procedure. Yeast transformants were selected on leucine-minus syntheitic plate. Plasmid isolation from yeast transformants was performed for confirmation (Figure 6).

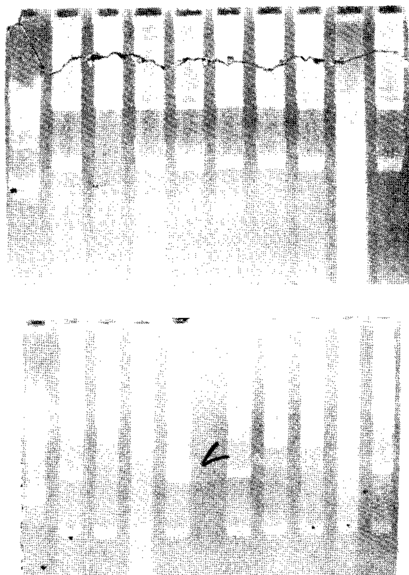


Fig. 5. Check on the orientation of the selected plasmids.
(A) Digestion with Xho I
(B) Digestion with Pst I
band II ; Size marker (Hind III digest of λDNA)

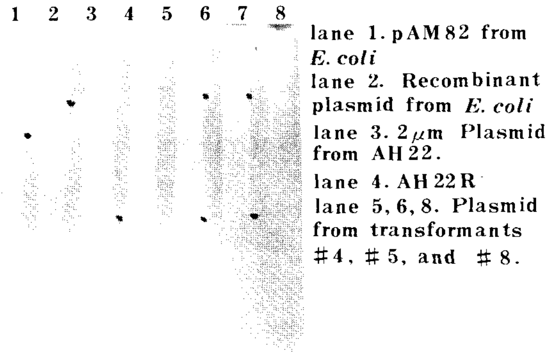


Fig. 6. Plasmid isolation from yeast transformants to confirm transformation.

Induction of Hepatitis B Virus Surface Antigen

The yeast acid phosphatases are classified into two categories, the constitutive ones (c-acp) and the repressible ones (r-acp), according to whether they are repressed by the inorganic phosphate or not. The major portions of c-acp and r-acp are encoded by pho3 gene and pho5 gene respectively and these loci are clustered together on chromosome II of yeast (Figure 7)[7]. The vector used in the current study incor-

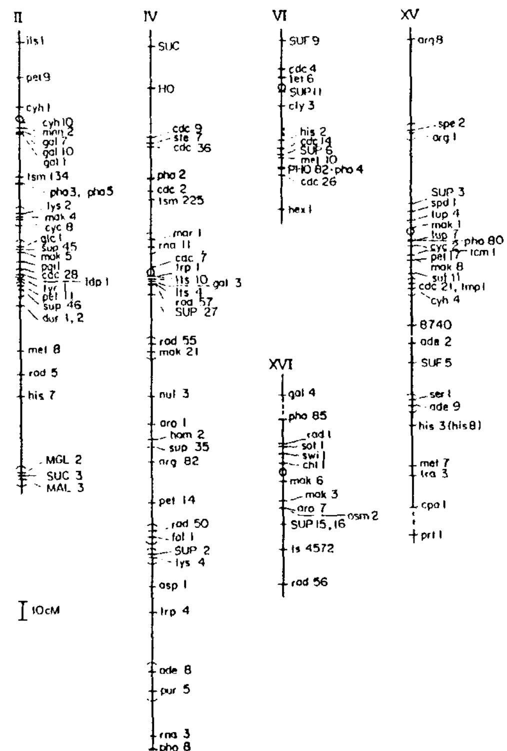


Fig. 7. Map of yeast chromosomes aecomodating yeast. Genes. cM, centi morgans

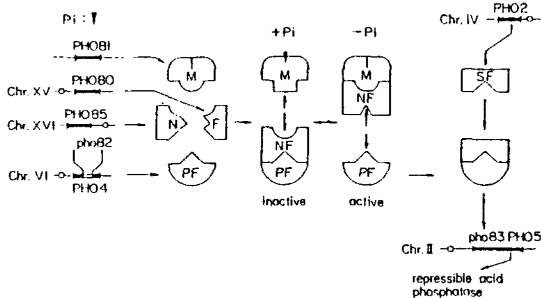


Fig. 8. A genetic model for the regulation of the expression of the pho 5 gene wich encodes the repressible acid phosphatase

porates the regulatory region of the pho5, the nucleotide sequence of which is also already determined[4]. The regulatory mechanism for the expression of pho5 involves several intermediate steps (Figure 8).[7,8]. The complex made of PHO2 and PHO4 interacts with the pho83 segment of the regulatory region for pho5. The complex NF made of PHO80 and PHO85 captures PHO4 making it unavailable for complexation with PHO2. The PHO81(M) in the absence of inorganic phosphate takes a conformation which can neutralize the NF complex thus releasing the PF factor (PHO4) to interact with the SF factor (PHO2). The overall regulatory mechanism can be made insensitive to inorganic phosphate by one or more of the mutations in pho81, pho80, pho85, and pho82 segment of pho4. The mutation in pho83 renders the pho5 expression free of all the regulatory steps.

Several media were tried to induce Hepatitis B virus surface antigen under the control of acid phosphatase promoter by varying the concentration of inorganic phosphate. Gerhard Schmidit's medium[5] and Rubin's method[6] were not appropriate. Only Burkholder minimal medium could induce Hepatitis B virus surface antigen from the recombinant

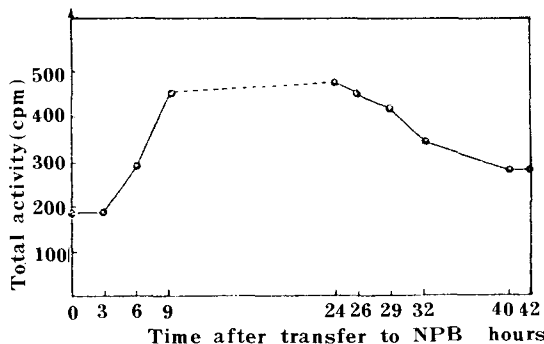


Fig. 11. Kinetics of HBsAg production: time course behaviour of total activity.

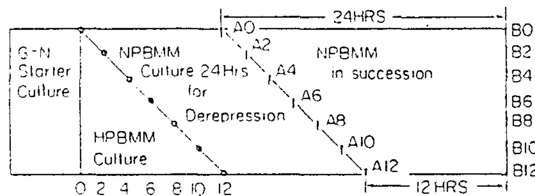


Fig. 10. Sampling schedule.

- ● medium change
  sampling
- ★ HP BMM: High phosphate burkholder min. medium
- ★ NP BMM: Phosphate free burkholder min. medium
- ★ G-N: Glucose neopeptone medium

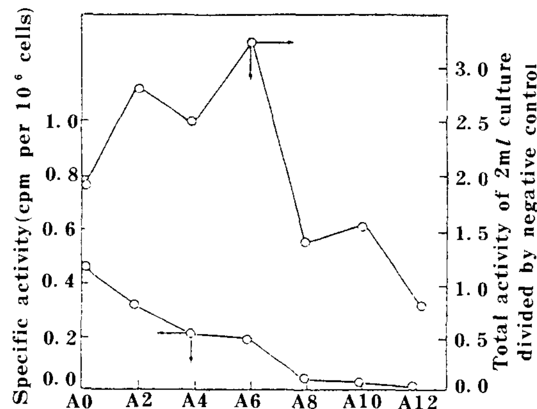


Fig. 11-a: Kinetics of HBsAg production in AH22 using constant derepression period: total activity and specific activity.

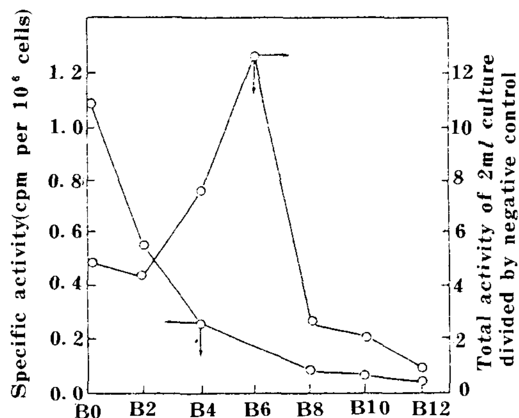


Fig. 11-b. Kinetics of HBsAg production in AH22 with extended derepression: total activity and specific activity. Samples in the order of increasing switching time and decreasing derepression Period.

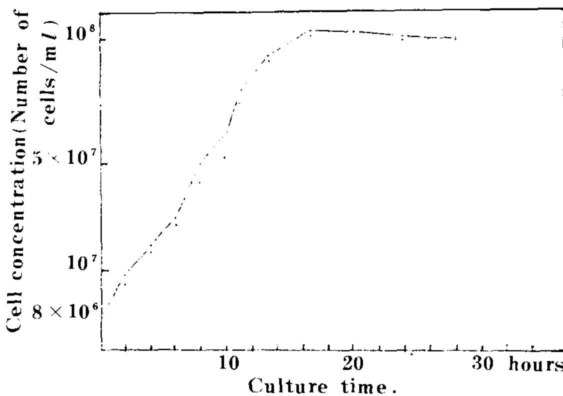


Fig. 12. Growth curve of AH22 harboring pMYN 101.

yeast cell with pMYN 101. The phosphate concentration in the low phosphate Burkholder minimal medium (20 mg phosphate per liter of medium) was not low enough for successful induction. Only phosphate-free medium gave sufficient derepression of acid phosphatase promoter.

Kinetics of HBsAg Production

10ml samples were taken from each culture flask every 3 hours. Figure 9 shows that the total activity of HB_sAg produced increases rapidly between 3 and 6 hours after transfer to the phosphate-free medium, reaches a maximum at around 9th hours, and remains at this level for up to 26 hours. To speculate on the optimal condition for the production, adaptation time in high-phosphate Burkholder minimal medium and derepression time in phosphate-free Burkholder minimal medium and derepression time in phosphate-free Burkholder minimal medium were adjusted according to the schedule represented in Figure 10. Sample numbers are designated in the drawing as A0 to A12 and B0 to B12. Figure 11 shows the gradual decrease of the specific activity (the quantity of HBsAg produced per cell) with the time of switching from HP to LP, while the maximum total activity was observed with the sample A6. Specific activity was calculated based on the growth curve shown in Figure 12. From these results one can conclude that optimal switching time does exist. Culturing for 6 hours in high phosphate Burkholder minimal medium (sample number A6) and 24 hour culture in phosphate free medium gave the maximum total activity and this trend was persistent in the extended culture[1]. The data in Table 1 indicate high expression by the repressor minus host. A suggestion that the coexistence of the 2 micron plasmid seems to hamper the expression level comes from the data in Table 2. LL20 cir 0 strain gave the best result among the host cells tested.

Table 1. Expression of HBsAg in AH22R⁻

Sample No.	Culture Condition	Counts (cpm)	
AH22R⁻	Ind	1539	
pMYN101	Non-ind.	541	Negative: 121
AH22R⁻	Non-ind.	197	Positive:4780
No plasmid			
AH22 pMYN 101		2207	
AH22	Ind	201	
AH22R pMYN101		6069	
AH22R⁻		165	Negative:135
AH22 pMYN101		393	Positive:7786
AH22	Ind.	315	
AH22R⁻ pMYN 101		1483	
AH22R		227	

Table 2. Effect of the presence of 2 μm plasmid on the expression of HBsAg in *S. cerevisiae*

Strain	Count (cpm)	Control
LL20 cir +	1004	
LL20 cir 0	1960	Negative:116
AH22 cir +	1058	Positive:4878
AH22 cir 0	1316	

Acknowledgement

This research was made possible by the generous grants from the Ministry of Education, the Ministry of Science and Technology, and the Korean Science and Engineering Foundation, for which the authors are deeply grateful.

요 약

간염 보균자의 혈액으로 부터 Dane 입자를 분리하였다. Dane 입자의 핵으로 부터 분리해낸 DNA는 α-(^{32}p) dNTP 존재하의 DNA 폴리머레이즈 반응후 액체 씬틸레이션 카운터와 한천 전기영동 및 가이거 뮐러 카운터에 의하여 간염의 DNA임이 확인되었다. 간염 바이러스에 의한 감염을 막기 위한 백신으로서의 B형 간염 바이러스 표면항원을 생산하기 위하여 산성포스파테이즈 프로모터를 갖는 새조합 프라스미드를 함유하는 효모균주를 사용하였다. 새조합 프라스미드는 pHBV 130 및 pAM 82로부터 제작되었으며 대장균에 변화되어진 후 효모균

주에 전달되었다. 간염 표면항원은 조절된 무기 인산 농도하에서 버크홀더 최소배지에서의 저해 해제로 생산되었다. 간염 표면항원의 생산 속도도 조사하였다. 전체 간염 표면항원 활성은 인산이 없는 배지에 옮겨진 뒤 3시간 내지 6시간에서 급격히 증가하였으며 9시간째에 최대에 도달하였다. 인산이 없는 배지에 옮기는 것은 고농도 인산 배지에서의 세포 배양을 6시간동안 수행한 뒤에 하는 것이 최적의 결과를 나타내었다.

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