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# Obtaining of "S region" of HBV surface antigen in Pichia pastoris GS115

**S.A. Sasmakov*[1], O.N. Ashirov, J.M. Abdurakhmanov[2], Sh. Sh. Khasanov[3], F.B. Eshboev[4], A.A. Makhnev[5], Sh.S. Azimova[6]**

[1,2,3,4,5,6]Acad. S.Yu. Yunusov Institute of the Chemistry of Plant Substances, Academy of Sciences of the Republic of Uzbekistan, M. Ulugbek st. 77, 100170, Tashkent, Uzbekistan;

e-mail: [1*]sasmakov@web.de

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**ABSTRACT:** New plasmids pPIC3.5-S (8426 bp) and pPIC9-S (8680 bp) producing the "S region" of hepatitis B virus surface antigen (HBV) were cloned and the recombinant strains of Pichia pastoris GS115 were obtained on their basis.

**KEYWORDS:** Pichia pastoris, recombinant plasmids pPIC3.5-S and pPIC9-S, recombinant S protein, HBV.

## I. INTRODUCTION

The "S region" of surface protein of the hepatitis B virus (HBV) (226 amino acids, 24-26 kDa) is considered that the basis of commercial vaccines and diagnostic kits, as it contains a common determinant "A" for all genotypes of HBV [1,2]. Nowadays, various expression systems are used for obtaining this protein, including the yeast expression system [3,5]. Methylotrophic yeast Pichia pastoris is considered one of the widely used microorganisms for the production of heterologous proteins. The advantages of the Pichia pastoris expression system are considered that the accumulation of significant biomass when cultured in low-cost nutrient media, the absence of endotoxins and pyrogens, a higher level of synthesis of recombinant proteins and the ability to synthesize of recombinant proteins in the nutrient medium [6-13]. The yield of the resulting recombinant proteins is more dependent on the genetic constructs used for their expression - vectors, as well as expression regulators - promoters. Currently, various vectors are available for use in this expression system. In this study, the transfer vectors pPIC3.5 (7751 bp) and pPIC9 (8023 bp) containing the nucleotide sequences of the Pichia pastoris genome, including the AOX1 promoter and HIS4 were used in order to obtain of new recombinant plasmids encoding the S region [14]. The success of P. pastoris as a platform for the production of heterologous proteins was due to the use of the highly regulated pAOX1 promoter of the AOX1 gene. In P. pastoris, the AOX1 and AOX2 genes encode alcohol oxidases responsible for the utilization of methanol, used as the main carbon source. Moreover, 90% of the total activity of alcohol oxidases in the cell belongs to AOX1 [14]. Under appropriate cultivation conditions, the level of synthesized AOX1 may represent more than 30% of the total amount of soluble proteins [6]. Therefore, pAOX1 is used to manage and control the synthesis of heterologous proteins. In addition, the strain of Pichia pastoris GS115 (HIS4) containing about 5,313 protein-encoding genes has a mutation in the histidinol dehydrogenase gene, which allows selection of recombinant strains in a histidine-free medium.

## II. EXPERIMENTAL, MATERIALS AND METHODS

In the experiments, the enzymes, and reagents from Thermo Fisher Scientific (USA), New England Biolabs (USA), Sigma-Aldrich (Merck, Germany), SBS Genetech (China), Panreac (Germany), Biosset (Russia), Himedia (India) were used.

**Preparation of vectors and insertion (gene) for ligation.**

A bacterial strain of E. coli Neb-5α containing the transfer vectors pPIC3.5 and pPIC9 were cultured in LB medium with the presence of ampicillin (30 µg / ml) at a temperature of 37 ° C [15].

According to [14,15], 1 µg of the vector plasmids pPIC3.5 and pPIC9 were hydrolyzed sequentially with restriction enzymes of BamHI, XhoI, and EcoRI [15].

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Linear DNA with a length of 7745 bp and 7999 bp isolated after electrophoresis in a 0.7% agarose gel by electro elution [15].

**Ligation and transformation of the obtained plasmids to electrocompetent cells of E.coli Neb-5α. Identification of the obtained clones.**

Ligation was performed using a T4 phage DNA-ligase with a prepared vector into a molar ratio of 1:10 (vector: insert) according to the standard method. Then, the ligase mixture was transformed into competent cells of the Escherichia coli NEB-5. The size of plasmid DNA isolated from clones that obtained after transformation with a ligase mixture was determined by the method of agarose gel electrophoresis. Clones containing DNA with a molecular weight of 8.426 and 8.680 kb were investigated by PCR analysis using specific primers for the S region of hepatitis B virus. After that, the products of amplification were analyzed by electrophoresis in a 1% agarose gel. DNA of the recombinant plasmids that identified by PCR was also confirmed with restriction analysis. For this, a comparative analysis of the recombinant clones and the initial vector plasmids pPIC3,5 and pPIC9 was performed by the EcoRI site. The molecular size of the obtained fragments was determined by electrophoresis in a 0.7% agarose gel. 1 kb Plus DNA Ladder from Invitrogen was used as a molecular weight marker.

**Transformation of the obtained recombinant plasmids to the yeast Pichia pastoris strain GS 115 and analysis of the obtained clones.**

The transformation of the obtained recombinant plasmids containing the target genes to the Pichia pastoris GS 115 was carried out by the method of electroporation [14,15]. Integration of the target gene into the genome of Pichia pastoris was detected by PCR using the following primers:

5’ AOX1 – 5’-GACTGGTTCCAATTGACAAGC-3’,

3’ AOX1 – 5’-GCAAATGGCATTCTGACATCC -3’,

α-factor – 5’-TACTATTGCCAGCATTGCTGC -3’.

## III. RESULTS AND DISCUSSION

According to the physical map (Fig. 1), the plasmids pPIC3.5 (**I**) and pPIC9 (**II**) were processed sequentially with restriction enzymes BamHI / EcoRI and XhoI / EcoRI in order to cloning of new plasmids. The implementation of foreign DNA that encoding the amino acid sequence of the S protein in the above sites, allows to insertion it under the control of the promoter of the AOX1 gene.

BamHI: 5´-G↓GATCC-3´ (pPIC3.5)

3´-CCTAG↓G-5´

XhoI: 5’-C↓TCGAG (pPIC9)

3´-GAGCT↓C-5´

EcoRI: 5´-G↓AATTC-3´ (pPIC3.5 и pPIC9)

3´-CTTAA↓G-5´

As a result, fragments of the vectors pPIC3.5 and pPIC9 containing “sticky” ends that allowing for “directed” ligation were obtained. Linear DNA plasmids with a length of 7745 bp and 7999 bp isolated after agarose gel electrophoresis. They were processed with alkaline phosphatase for avoid the "closure" of the vectors [15].

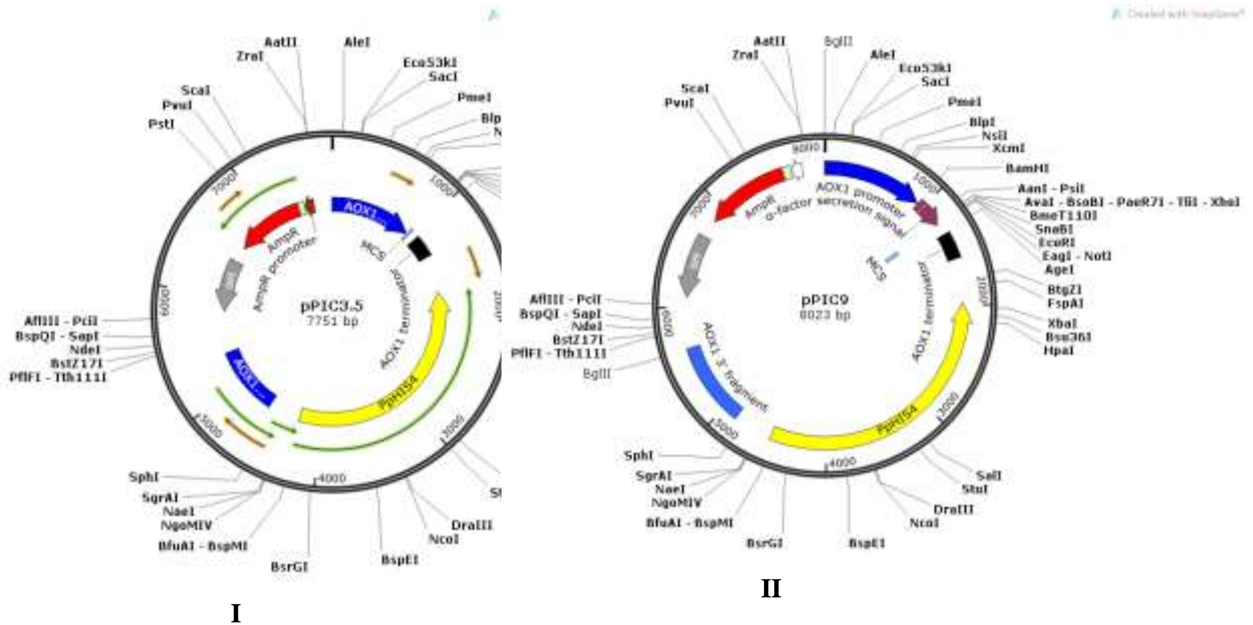



**Fig. 1. Physical maps of transfer vectors pPIC3.5 (I) and pPIC9 (II)**

cDNA of the S gene with the length of 681 bp amplified by PCR via the following primers using DNA of HBV as a matrix that isolated from human blood plasma.

1) Forward-5'-ACTGAGGATCCTGCACCGAACATG-3' (pPIC3.5);

2) Forward-5'-CTGCACTCGAGATGGAGAACATCACATCAGGAT-3' (pPIC9)

3) Reverse-5'-GGTTTATTAGAATTCAAATGTATACCCAAAGACA-3'.

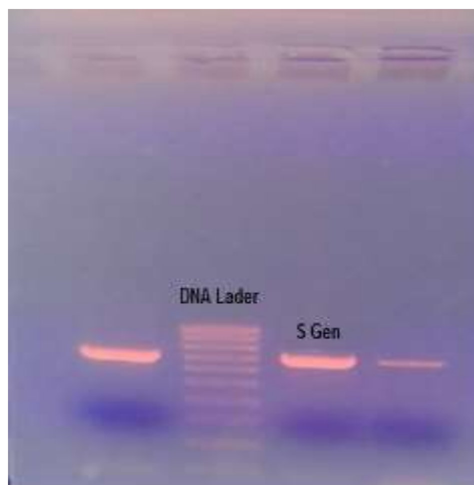



**Fig. 2. Gel electrophoresis of products of PCR.**

According to Figure **2**, fragments for DNA with a size of 681 kb were obtained, which corresponds to the calculated data as a results of amplification. Then, after elution from the agarose gel, the cDNA was processed sequentially with restriction enzymes BamHI, XhoI and EcoRI in order to the creation of specific sites for cloning into transfer vectors.

Ligation of transfer vectors with the fragment of cDNA of S region of a hepatitis B virus was carried out using T4 phage DNA ligase in a molar ratio of 1:10, respectively. After that, the obtained ligase mixture was transformed into the competent cells of Escherichia coli NEB-5α strain. The identification of the formed clones was conducted by the PCR using the specific primers for the HBV S region. PCR products were analyzed by electrophoresis in 1% agarose gel.

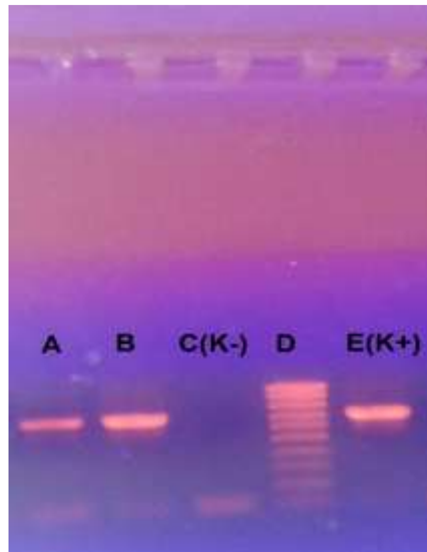
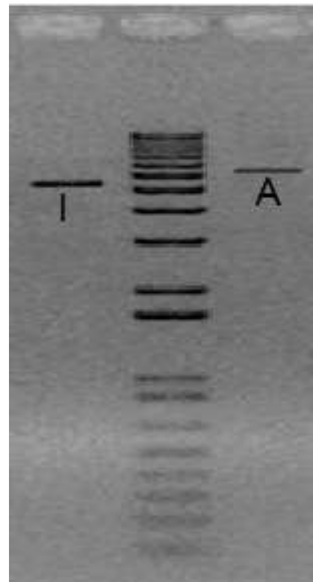



**Fig. 3.** Gel electrophoresis of transformants containing the target gene.

**A –** pPIC3.5-S; **B –** pPIC9-S; **K-**DNA that isolated from the blood of HBV negative people; **D –** marker DNA; **E** - (**K** +)-DNA that isolated from the blood of HBV "positive" patients.




**I**-pPIC3.5/EcoR I (7751 bp), **A**- pPIC3.5-S/EcoR I (8426 bp)

Fig.4. Restriction analysis of PIC3.5-S (A) (EcoR I/8426 bp)

The presence of a DNA fragment with a molecular weight of 681 kb corresponding to the mass of the DNA fragment amplified from the HBV positive sample indicated the presence of an insert (cDNA of S region of HBV) in the studied clones. Thus, clones containing the S region of DNA hepatitis B virus were detected by PCR.

The size of recombinant plasmids pPIC3.5-S and pPIC9-S were revealed by the method of electrophoresis after restriction of the EcoRI site **(Fig. 4).** It was found that the molecular weight of recombinant plasmids corresponds to theoretical calculations on physical maps of these plasmids and make up 8426 bp and 8680 bp accordingly.

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After that, the "orientation" of the inserted fragments of the S region of DNA of the hepatitis B virus was studied. For this purpose, the linearized plasmids were digested with BamHI restriction enzyme in the case of pPIC3.5-S and XhoI restriction enzyme in the case of pPIC9-S. Moreover, the fragments with a length of 7745 (pPIC3.5-S), 7999 (pPIC9-S), and 681 bp were identified by electrophoresis, which are indicated the correct orientation of the S region of cDNA the hepatitis B virus.

Then, the transformation of recombinant plasmids pPIC3.5-S and pPIC9-S to the strain of yeast P. Pastoris GS115 was carried out. For this, recombinant plasmids were linearized in the HIS4 region by SalI nuclease [14] and inserted into P. Pastoris cells via electroporation [15]. Transformants ($His^+$) were selected in histidine deficient media. The obtained recombinant clones were screened by PCR using both the above specific primers for the S region of hepatitis B virus and primers 5 'AOX1, 3' AOX1, α-factor (see Material and methods) to identify the integration and orientation of the target gene in Pichia pastoris genome.

Selected transformant colonies were cultured until the stage of the exponential phase (OD = 2 at 600 nm) in a medium containing glycerin [14]. The expression of the S gene inserted under the AOX1 promoter was induced by the addition of methanol until a final concentration of 0.5%, on a cultivation time of 120 h. The level of synthesis of recombinant protein was determined by ELISA. The “Hepatitis-B Vaccine (rDNA) 20 µg / ml, Serum Institute of India” and “Euvax B 20 µg / ml, Sanofi Pasteur Korea Ltd.” vaccines containing the protein encoded by the S region of hepatitis B virus were used as the calibration standards (Table 1).

**Tab. 1.** The results of ELISA

| The cultivation time after induction with methanol, hours | Result of ELISA (OD value at 450 nm) | The amount of S protein in the test sample |
|---|---|---|
| 24 | 0.520 | ≈ 5 ng / ml |
| 48 | 1.062 | ≈ 10 ng / ml |
| 72 | 1.992 | ≈ 20 ng / ml |
| 96 | >3.000 | ≥ 26.5 ng / ml |
| 120 | 2.800 | ≈ 25 ng / ml |
| Hepatitis-B Vaccine 20 µg/ml (rDNA), diluted (1:4000; 1:2000; 1:1000; 1:750 | 0.502<br>0.959<br>2.176<br>>3.000 | ≈ 5 ng / ml<br>≈ 10 ng / ml<br>≈ 20 ng / ml<br>≥ 26.5 ng / ml |

The results of ELISA show that the optimal cultivation time is 96 hours after induction with methanol. According to the results of electrophoresis in PAGE and immunoblotting, obtained recombinant protein has corresponded to the expected molecular weight (about 26 kDa) and it was highly specific for HBV antibodies (Fig. 5).

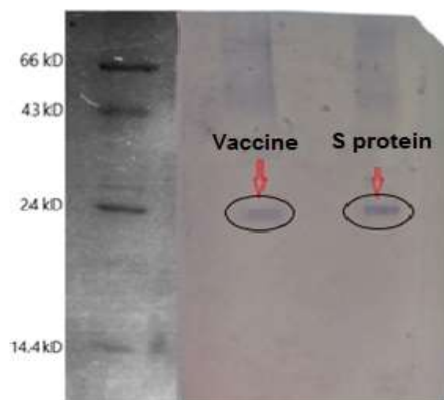


**Fig. 5. The result of the immunoblotting of recombinant S region of HBV. Vaccine -** Hepatitis-B Vaccine 20 µg/ml.

## IV. CONCLUSION

Thus, new recombinant plasmids pPIC3.5-S and pPIC9-S containing the cDNA “S region” of the HBV surface antigen were cloned and recombinant strains of Pichia pastoris GS115 producing the target protein were obtained on their basis.

## V. REFERENCES

[1] Patent. US5670630 A. Gregory P. Thill, San Diego, Calif. Expression of Hepatitis B S and Pres2 Proteins in Methylotrophic yeasts, 23.09.1997.

[2] Mohamed S Elghanam, Ahmed S Attia, Hussein A Shoeb and Abd Elgawad M Hashem. Expression and purification of hepatitis B surface antigen S from Escherichia coli; a new simple method. BMC Research Notes 2012, 5, 125.

[3] Yew Joon Tam, Nazariah Allaudin Zeenathul, Akhavan Rezaei Morvarid, Mohd Lila Mohd Azmi, Abdul Rani Bahaman, Sewn Cen Lo, Joo Shun Tan. Two-phase fed-batch modification for 48 hour peak expression of hepatitis B surface antigen in Pichia pastoris shake flask system. Central European Journal of Biology, 2014, Vol. 9(8), pp. 749-760.

[4] Deng Ning, Xiang Junjian, Zhang Qing, Xiong Sheng, Chen Wenyin, Rao Guirong and Wang Xunzhang, Production of Recombinant Humanized Anti-HBsAg Fab Fragment from Pichia pastoris by Fermentation. Journal of Biochemistry and Molecular Biology, 2005, Vol. 38 (3), pp. 294-299.

[5] Hu Bo, Liang Minjian, Hong Guoqiang, Li Zhaoxia, Zhu Zhenyu, Li Lin, Expression of Hepatitis B Virus S Gene in Pichia pastoris and application of the product for detection of Anti-HBs antibody. Journal of Biochemistry and Molecular Biology, 2005, Vol. 38(6), pp. 683-689.

[6] J.M. Cregg, J.L. Cereghino, J. Shi, D.R. Higgins. Recombinant protein expression in Pichia pastoris. Mol Biotechnol. 16 (1), 23 (2000).

[7] M. Ahmad, M. Hirz, H. Pichler, H. Schwab. Protein expression in Pichia pastoris: recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol. 98, 5301 (2014).

[8] T. Vogl, F.S. Hartner, A. Glieder. New opportunities by synthetic biology for biopharmaceutical production in Pichia pastoris. Current Opinion in Biotechnology. 24, 1094 (2013).

[9] M. Bollok, D. Resina, F. Valero, P. Ferrer. Recent patents on the Pichia pastoris expression system: expanding the toolbox for recombinant protein production. Recent Pat Biotechnol. 3, 192 (2009).

[10] B. Gasser, R. Prielhofer, H. Marx, M. Maurer, J. Nocon, M. Steiger, V. Puxbaum, M. Sauer, D. Mattanovich. Pichia pastoris: protein production host and model organism for biomedical research. Future Microbiol. 8, 191 (2013).

[11] S.C. Spohner, H. Quitmann, P. Czermak. Expression of enzymes for the usage in food and feed industry with Pichia pastoris. Journal of Biotechnology 202, 118 (2015).

[12] L.M. Damasceno, C-J. Huang, C.A. Batt. Protein secretion in Pichia pastoris and advances in protein production. Appl Microbiol Biotechnol. 93, 31 (2012).

[13] S. Macauley-Patrick, M.L. Fazenda, B. McNeil, L.M. Harvey. Heterologous protein production using the Pichia pastoris expression system. Yeast 22, 249 (2005).

[14] Pichia Expression Kit for expression of recombinant proteins in Pichia pastoris. User Guide. 2014, Publication Number MAN0000012. http://tools.thermofisher.com/content/sfs/manuals/pich_man.pdf.

[15] M.R. Green, J. Sambrook. Molecular cloning. A laboratory manual. Fourth edition. New York: Cold Spring Harbor Laboratory Press, 2012.