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Evaluation of Phycocyanin Promoter Function in Bacteria by Investigating the Expression of HBsAg

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MANY items affect the yields of every recombinant protein production. Promoters are one of the key regulatory elements which control the level of recombinant protein expression in the host. Although in some studies (Hepatitis B Surface Antigen) HBsAg was cloned in *E. coli*, in many of them common promoters were used. In this study two co-vectors (P^{HK} and P^{HGK}) were designed and used. Both of them were shuttle types based on phycocyanin-specific promoter and each was a combination of two types of vectors: a plasmid and a transposon. ELISA results, in line with Western blot results, showed the ability of phycocyanin promoter as well as revealed the expression level of HBsAg in transgenes from the Top10 P^{HK} vector is higher than those of Top10 P^{HGK}. The expression of HBsAg under the phycocyanin promoter in the present study is 7.5 µg/lit. In the current study as a pilot step, our attitude and objective of bacteria expression are to evaluate the function of the phycocyanin promoter based on the HBsAg gene. Due to the nature of the shuttle vector, in continuation of the present study to reduce the non-responsive populations, high antigen expression, meet the need for renewal and, other benefits, expression of HBsAg in other than bacterial host is under investigation.

Keywords: Bacteria, *Escherichia coli*, Expression, Phycocyanin, Promoter Function.

Introduction

Hepatitis B is a potentially fatal liver inflammation and an important health problem worldwide caused by the hepatitis B virus (HBV) [1]. Hepatitis B results in chronic inflammatory changes and high mortality risk due to cirrhosis and liver cancer. The WHO statistics reported 257 million cases of chronic hepatitis B infection (defined as Hepatitis B surface antigen (HBsAg) positive) in 2015 and estimated that 887 000 deaths occurred in this year. Most of these deaths are caused by cirrhosis and hepatoma that is the primary liver malignancy [2]. So the goal is the removal of viral hepatitis as an important threat to public health by 2030; A 90% reduction in the prevalence of HBV compared with the 2015 baseline would be equivalent to 0.1% incidence of infection in children 5 years old in 2030 [3].

The first HBsAg-based hepatitis B vaccine (HepB) was on the market in 1982. HepB vaccine composed of HBsAg derived from the HBV carriers' plasma [4] has been applied for almost

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two decades as an effective vaccine. Although plasma-derived vaccines are effective and safe, they have several disadvantages: (i) Possibility of transmitting other diseases such as AIDS; (ii) High production costs (a major barrier to public access); (iiis) Limitation of human plasma supply; and (iv) Requires a 6-month trial period on chimpanzees. Advancing in biotechnology and genetic engineering allowed the first subunit hepatitis B vaccine produced in recombinant yeast *Saccharomyces cerevisiae* [5]. For more than 20 years, the HBsAg expression has been reported in multiple species of yeast (*Saccharomyces cerevisiae*, *Pichia pastoris,* and *Hansenula polymorpha*), as well as Chinese hamster ovary (CHO) cells [4].

Many items affect the costs and yields of every recombinant protein production. To achieve an optimal production system, some issues should be considered: (i) host organism (ii) expression vector (iii) characteristics of regulatory elements, such as promoters or terminators (iv) appropriate condition like growth and production medium for the host cell (v) compatibility of foreign protein (vi) purification strategy. As clear, promoters are one of the key regulatory elements which control the level of recombinant protein expression in the host [5, 6]. Although in some studies HBsAg was cloned in E.coli, many of these common promoters, such as *lac* were used [7]. Expression and production of an antigen against a new bacterial strain would take less than seven days. Moreover, increasing batch size of production in bioreactors would establish the generation of a large number of doses within a period of less than 24 hours i.e., five hundred million doses of vaccine per month [8].

The phycocyanin-specific promoter sequence, which is the strongest promoter in *Spirulina* (*Arthrospira platensis*) and 20-50% of the *spirulina* proteins weight, is expressed by this promoter [9-12].

Phycobiliproteins (PBPs) include allophycocyanin (AP) and phycocyanin (PC) [9]. PBPs related to certain linker polypeptides form the light-harvesting structure, which is called phycobilisomes (PBSs) [13]. Pilot & Fox reported the C-phycocyanin gene sequence for the first time in 1984.

A problem with the Hepatitis B vaccine is that, even after 3 doses, up to 15% of persons don't react sufficiently [14, 15]. So the need to consider a new host and the possibility of reducing the unresponsive population is noticeable.

Unquestionably, our attitude and objective of bacteria expression as an intermediary host are to evaluate the function of the phycocyanin promoter. Due to the nature of the shuttle vector, in continuation of the present study, to reduce the non-responsive populations, high antigen expression, meet the need for renewal (booster dose), and other benefits, expression in another host is under investigation by our team.

Materials and Methods

Selecting vectors and sequences

In this study, two co vectors were designed and used. Both of which were of the shuttle type and each was a combination of two types of vectors: a plasmid and a transposon. As can be seen in Fig. 1 and 2, the design of both composite vectors includes the primary vector of pBluescript IISK (+) plasmid as the backbone and the secondary vector of a Tn5 transposon. The secondary vector components, from 5 'to 3', are *EcoR*I restriction site, ME left sequence, phycocyanin operon, the targeted genes, ME right sequence, and *Hind* III restriction site, respectively

In the first vector (P^{HGK}), the transposon contains a gene structure of 2539 bp including HBsAg (NCBI Gene Bank KR028431.1), Green Fluorescent Protein, and Kanamycin resistance genes. In the second vector (P^{HK}), the transposon consists of a gene structure of 1843 bp including 6xHis-tagged HBsAg, Kanamycin resistance genes.

Optimized for your reference

Codon Optimization was done using our non-free software with the parameters as follows: codon efficiency cut off was chosen to be 15%: codons less than 15% were deleted except for sites with robust secondary structures (to mitigate the problem, less frequent codons were employed). A built-in M-fold module was used to check the secondary structure. Internal ribosome binding sites were deleted. Optimization parameters optimize various critical parameters to the gene expression efficiency, which include (but not restricted to): bias of codon use, guanine-cytosine content, the content of CpG dinucleotides, secondary structure of mRNA, sites of cryptic splicing, premature polyA sites, chi sequence sites, ribosome binding sites, negative CpG

islands, RNA instability motif (ARE), repetitive sequences (direct repetition, reverted repetition, dyad repetition), and restriction sites interfering with creation of clones.

Bacterial Strain Preparation

Two bacterial strains were prepared for transformation: *E. coli* Top10 strain and genotype of BL21 (PLYsS) strain *E.Coli* BL21, which was prepared from the bacterial collection of Pasteur Institute of Iran.

Competent cell preparation and transformations

Competent cell Preparation using $CaCl_2$ method: the bacterium was cultured in LB broth medium and cooled on ice for 10 minutes as it reached OD600 = 0.6-0.8. After centrifugation and removal of the supernatant, 10 ml of cold $CaCl_2$ was added to the bacterial sediment (0.1-0.05 M). After a 20-minute break in the cold and centrifugation, the plate was dissolved in 5 ml of cold 0.1 M CaCl2 solution and 20% glycerol. Competent cells were stored in the freezer until use [16, 17].

Transformation: 5µl of plasmid was added to the melting component. Then, it was run on a continuous heat program including 30 minutes in cold, 2 min in Bain Marie at 42°C and 5 minutes in cold. After Broth LB (600µl) addition, the sample was incubated at 37 °C/150 r/1h. The solution containing the transgenes was transferred to LB agar Amp + in dilutions of complete, 1/10, 1/100, 1/1000 and 1/10000, and the plates were incubated at 37 °C/18-24h [16, 17].

Quantitation of total protein and purified HBsAg

Quantitation of total protein was measured using the Bradford assay [18]. The expression of protein was confirmed initially on the SDS-PAGE and finally by western blot.

Sample preparation for ELISA

The bacterial culture was centrifuged for 15 minutes at 4000 rpm to spin down the cells. The plate was diluted with Tris HCl-buffer (pH 7.0). Add to 1 volume cells, 4 volumes buffer. The solution was sonicated 60 times 30 seconds at 20% power while keeping the sample in ice water to prevent heating. The protein suspension was centrifuged for 15 minutes at 4700xg at 4^0C. The upper layer was further used as the protein extract [16, 17].

Western blot analysis

Bacteria were lysed using a lysis Buffer solution (1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM Ethylene glycol tetraacetic acid, and 1 mM NaF) containing 1 mM Na3VO4, 1 mM phenylmethanesulfonyl fluoride or phenylmethylsulfonyl fluoride and protease inhibitor pellet [16, 17, 19].

To western blotting, the lysates were mixed with the same volume of 5X Laemmli sample buffer. Bradford assay was used to measure protein quantitation. After a 5 min boiling, 15 µg lysates were subjected to SDS-PAGE. They were then transferred to a 0.2 µm immune-Blot™ polyvinylidene difluoride (PVDF) membrane (Cat No: 162-017777; Bio-Rad Laboratories, CA, USA). The 5% BSA (Cat No: A-7888; Sigma Aldrich, MO, USA) was used to block the membranes for 1 h (in 0.1% Tween 20). The membranes were then incubated together with anti-Hepatitis B virus X antigen-antibody (Cat. No: ab39716-Abcam antibody) for 1 h at room temperature. The membranes were then rinsed with TBST, and incubated with goat anti-rabbit IgG H&L (HRP) (Cat No: ab205718-Abcam antibody) as a secondary antibody. Finally, the membranes were placed in an incubator with enhanced chemiluminescence (ECL) for 1–2 minutes [16-20].

Affinity Chromatography

The recovery and purification of the recombinant protein was carried out using affinity chromatography through its histidine tag. The protocols were optimized, based on the QIAexpressionist ™ and Katalani et al. [21-23].

Results

1-3 Vectors and gene construct design and synthesis Synthesis of structures and confirmatory sequencing were performed by Biomatik Canada.

To reconfirm the synthesized structures, enzymatic digestion based on two EcoRI and HindIII sites was performed (Fig. 3 and 4).

Transformation Results

Growth in a selective medium

After transformation, the culturing was performed in Amp $^+$& Kan $^+$ selective medium. Despite the replication of the process between *E. coli* Top10 and *E. coli* BL21 strains, Top10 strain always showed a better response to the transformation process (Fig. 5 and 6).

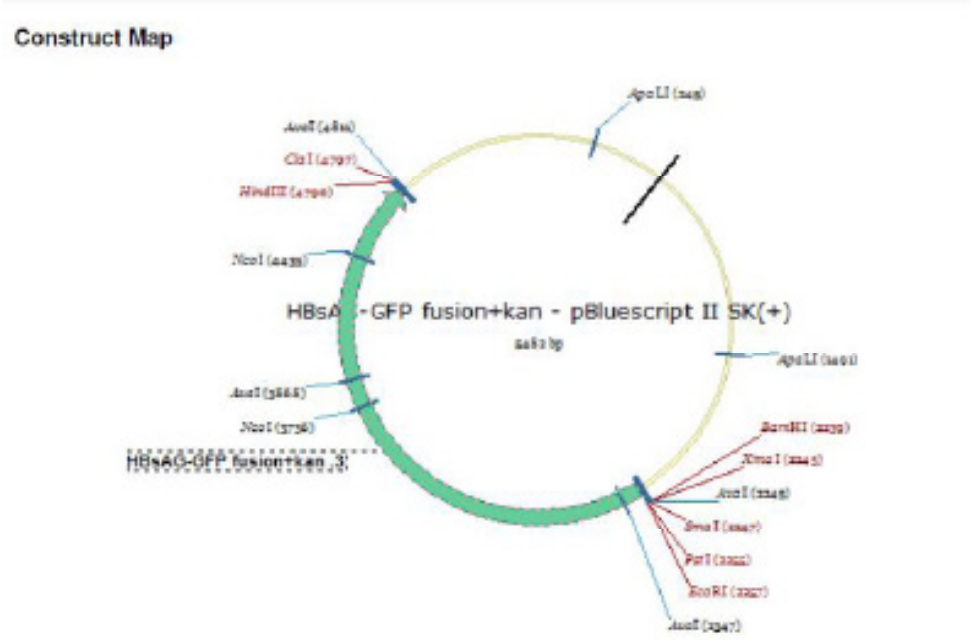

Fig.1. is the first shuttle composite vector (P^{HGK}) containing a specific sequence of the target gene construct. In the designed gene construct, an operon, based on the phycocyanin promoter gene family structure and RBS, is placed in the Tn5 transposable sequence known as the secondary vector which was also embedded into a pBluescript II SK (+) plasmid as the primary vector.

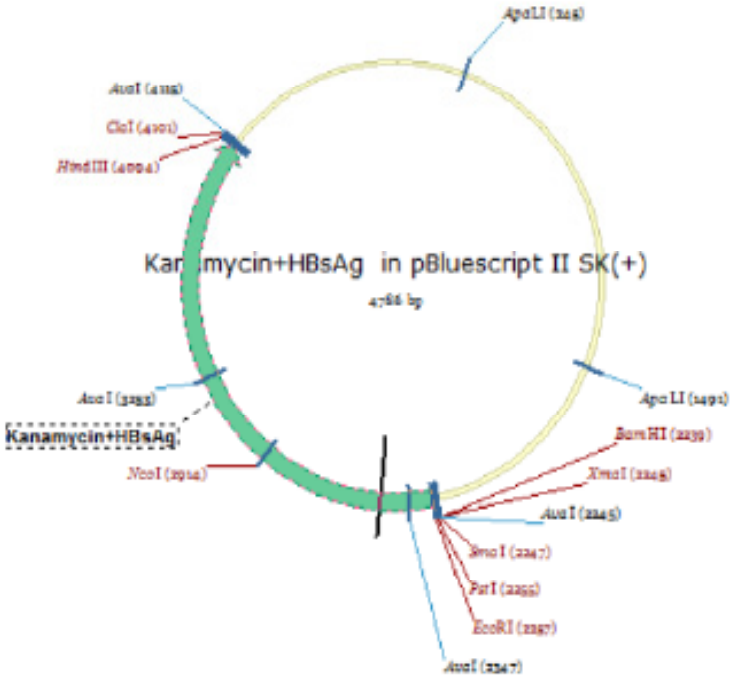

Fig.2. Is the second shuttle composite vector (P^{HK}) containing a specific sequence of the target gene construct. The designed gene construct was embedded into the Tn5 transposable sequence known as the secondary vector. This secondary vector was also embedded into a pBluescript II SK (+) plasmid as the primary vector.

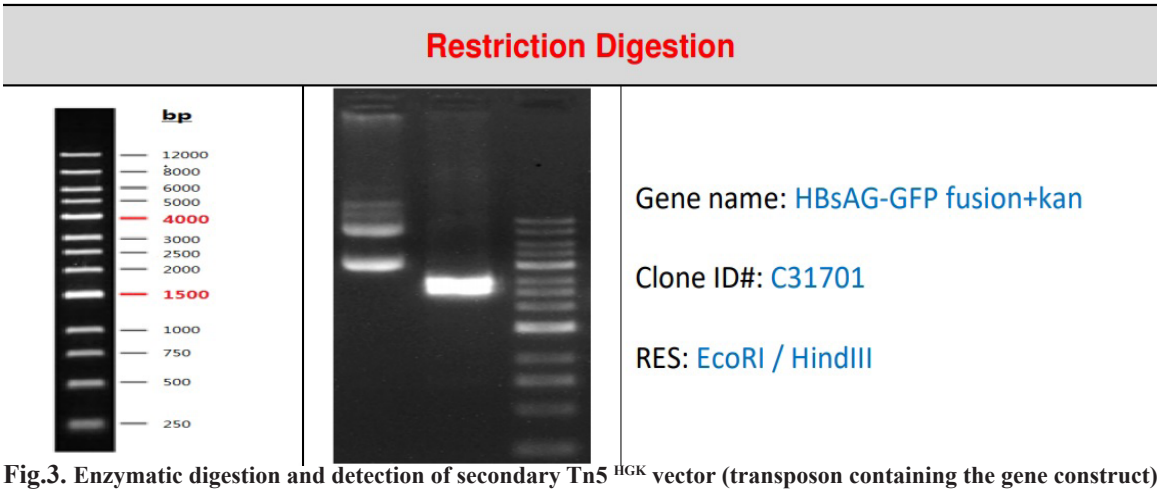

Fig.3. Enzymatic digestion and detection of secondary Tn5 ^{HGK} vector (transposon containing the gene construct) on gel.

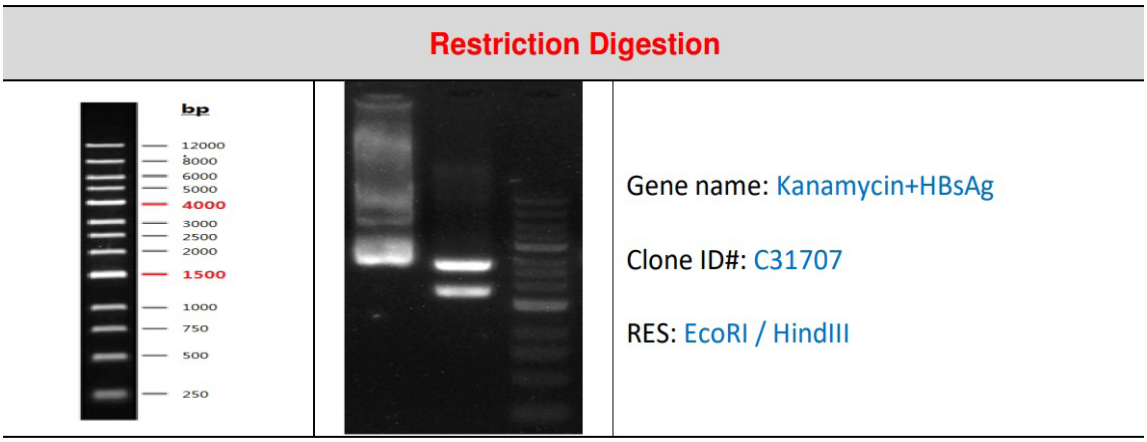

Fig.4. Enzymatic digestion and detection of secondary Tn5^{HK} vector (transposon containing the gene construct) on gel.

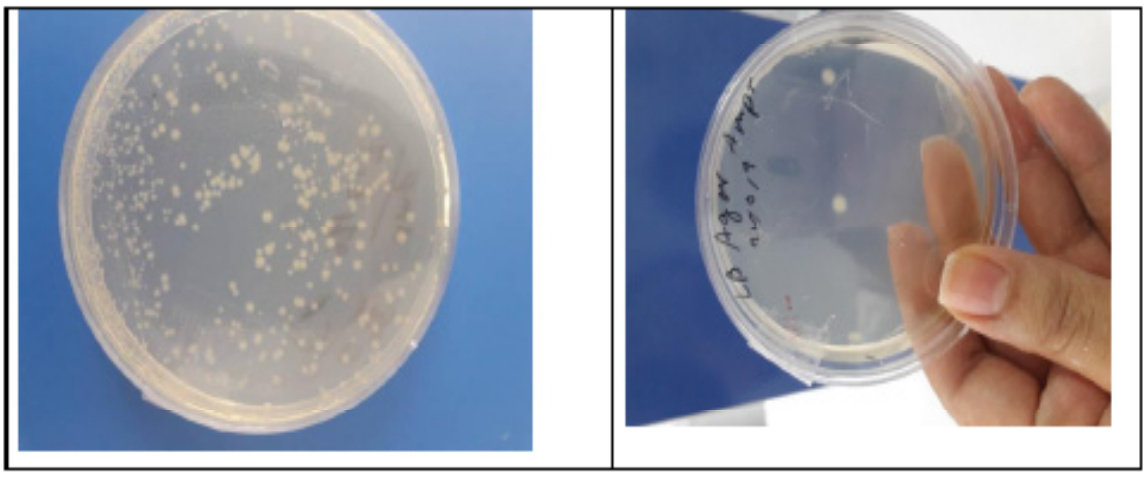

Fig.5. Cloned vector expression image in the host in the selected culture medium.

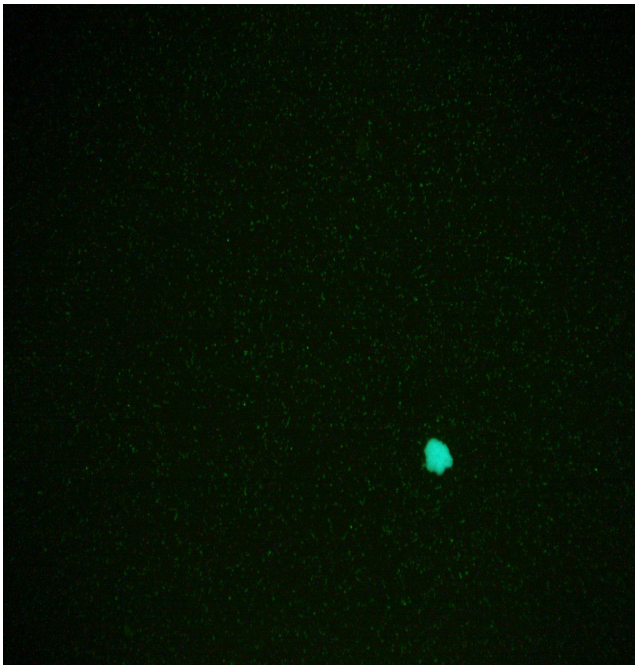

Fig.6. GFP expression Image in the P^{HGK} vector cloned in the host.

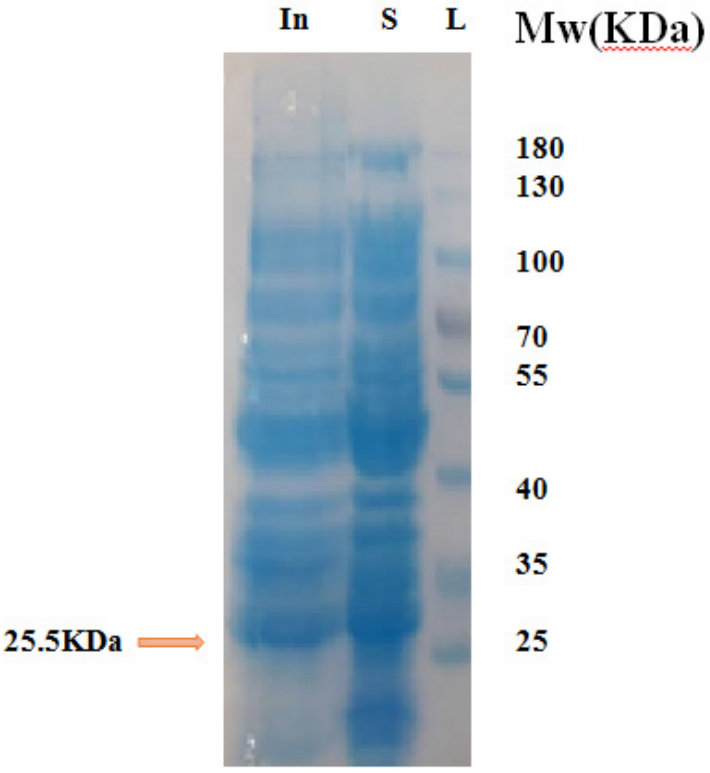

Fig.7. Investigation of the presence of protein in the soluble phase and Inclusion bodies by SDS-PAGE.
In: Inclusion bodies; S Soluble Protein; L: Ladder.

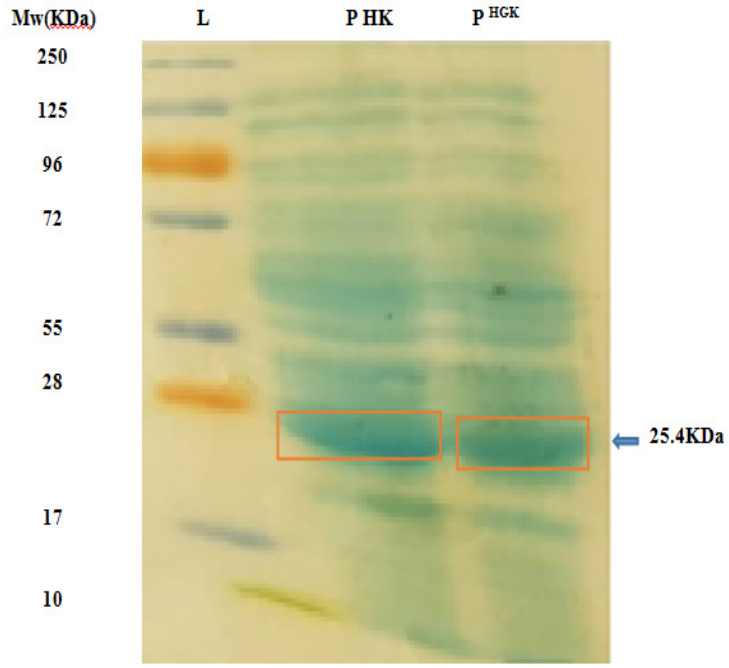

Fig.8. The HBsAg illustration with 25.4 KDa size.

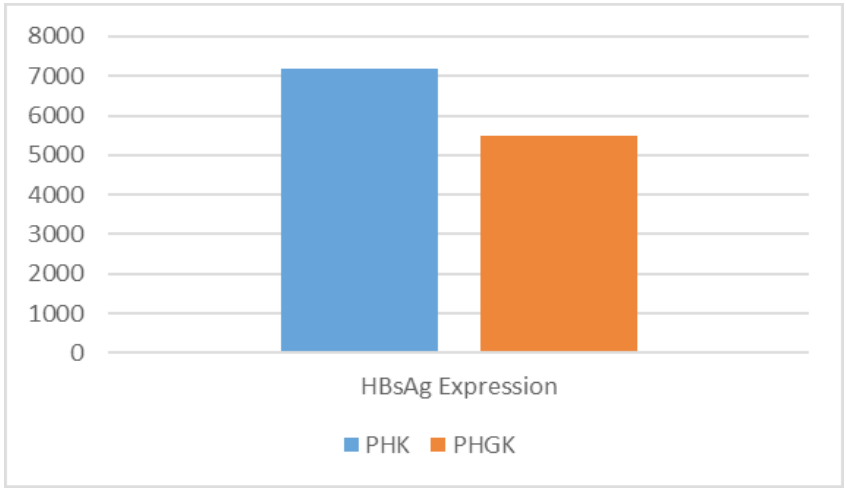

Fig.9. The diagram of band area and intensity differences in Western blot.

TABLE 1. The area and intensity of bands obtained from Western blot.

Samples	Area and intensity of bands
Top10 P ^{HK}	7187
Top10 P ^{HGK}	5495

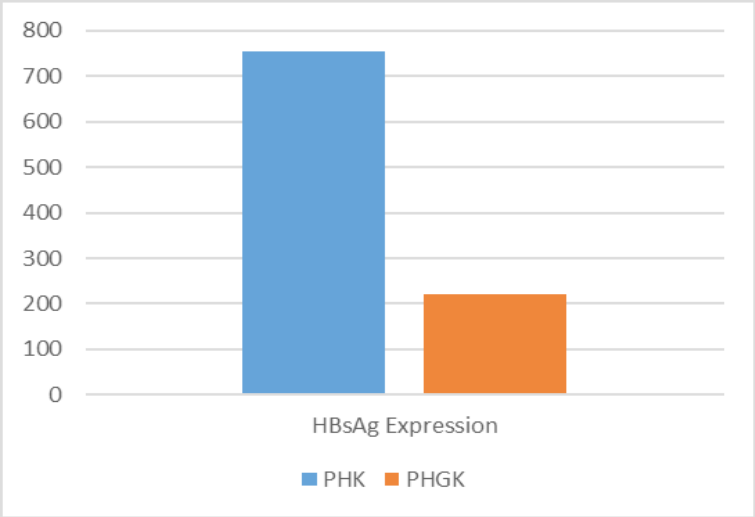

Fig.10. Diagram of band intensity differences in ELISA.

TABLE 2. Results of HBsAg expression analysis of ELISA.

Samples	HBsAg (*p*g/ml)	HBsAg (µg/lit)
Top10 P^{HK}	7548	7.5
Top10 P^{HGK}	2217	2.2

2-2-3 SDS-PAGE and Western blot results

To confirm the HBsAg expression in both transgenic bacteria with P^{HGK} and P^{HK}, SDS-PAGE and Western blot tests were conducted. The findings are presented in Fig. 7, 8, 9 and Table 1.

ELISA

ELISA was performed to confirm the expression of HBsAg in both transgenic bacteria with P^{HGK} and P^{HK}. The results are presented in Table 2 and Fig.10.

Discussion

The hepatitis B virus, as one of the contagious viruses, can spread rapidly across the world's population, so early diagnosis and prevention of Hepatitis B disease are one of the top priorities to prevent the spread of this disease [24]. The Hepatitis B vaccine has shown to have some shortcomings in inducing adequate immune response; furthermore, following observations of the difficulty in obtaining this antigen from serum, research into the cloning and expression of this protein in various hosts began.

The HBsAg protein expression in *Pichia pastoris* is more similar to that of other eukaryotes, such as Saccharomyces cerevisiae, in terms of glycoprotein structure [25]. Among the major advantages of prokaryotic host cells for HBsAg gene expression is their ability to regulate protein expression using molecular engineering techniques such as gene promoter adjustments. *Escherichia coli* has been used for HBsAg gene expression due to its ability to produce a high amount of this antigen in a short time with low costs [26]. In the present research, we further studied the possible potential of *Escherichia coli* as a host. The phycocyanin promoter has previously shown the ability to substantially enhance product yields [27].

According to Jeamton et al. 2017, our results revealed the functionality of this chimeric operon. Moreover, the obtained results showed that the phycocyanin promoter of *Arthrospira platensis* was capable of expressing both HBsAg and GFP genes in *E. coli* [28]. As it can be seen (table 1 and

Fig.9), the intensity and area of the band in the transgene obtained from the Top10 PHK vector is higher than that of Top10 PHGK.ELISA results (table 2 and figure10), like Western blot results, show that the expression level of HBsAg in transgenes from the Top10 PHK vector is higher than those of Top10 PHGK. This difference is far greater than the results of Western blotting. In a 2012 study, Elghanam and his colleagues cloned and expressed the GST tagged-HBsAg(S) protein into *Escherichia coli*. They used vector PRC/CMV-HBs(S) with CMV promoter as the S gene source encoding the HBsAg(S). The HBsAg gene expression in *E. coli* under the common Lac promoter showed to be 140 (µg/lit) [26]. While the expression of HBsAg under the phycocyanin promoter in the present study is 7.5 µg/lit. According to the results in the present study, the phycocyanin promoter can express HBsAg in *E.coli* bacteria. But quantitatively, if the goal is to improve this promoter's performance, the amount of expression needs to be further optimized and studied. Another study published in 2019 investigated the HBsAg gene expression in *E. coli*. The expression vector used was pET-28a with T7 promoter. *E. coli* cells were transformed with the confirmed recombinant pET28a+HBsAg clone. Afterward, protein expression and purification were carried out. The existence of a 27 kDa protein band in *E. coli* indicated the HBsAg protein expression, which was further confirmed by western blot. In *E. coli*, our findings revealed a 25.4 KDa protein, which was also validated by western blot [29].

In a 2016 study, Mina Mirian and her colleagues employed pcDNA to assess the expression of HBsAg. The pcDNA/HBsAg vector was transformed in *E. coli* TOP10F, and digestion of the plasmid with BglII was carried out. The digested plasmid was transfected to the HEK293T cell line and was treated with hygromycin B. The RT-PCR demonstrated an expression of 2275 copies of mRNA in each cell. Although this study in contrast with our study the was expressed in the membrane of HEK293T cell, the RT-PCR and flow cytometry demonstrated a significant expression of HBsAg, which could show the potential of using cell line hosts to elevate the expression of HBsAg by phycocyanin promoter in our research [30].

To the best knowledge of the authors of this article, this is the first research on the phycocyanin promoter effect on the expression of HBsAg in bacteria. We recognize that our study has certain limitations, such as low levels of HBsAg expression; however, this study provided a detailed examination of *E. coli* as a host and the phycocyanin promoter's role in HBsAg expression.

Conclusion

Since the present study aimed to investigate the expression in *E.coli* only as an intermediate host, the promotion of expression in the final host in future studies is considered the primary goal.

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Conflict of Interest

None

Funding statement

Nil

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