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Continuous Secretion of Human Epidermal Growth Factor Based on *Escherichia coli* Biofilm

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Abstract: Human epidermal growth factor (hEGF) holds significant importance in the fields of medicine and cosmetics. Therefore, it becomes imperative to develop a highly efficient fermentation system for hEGF production. In this study, a stable hEGF-secreting expression strain was created by integrating the hEGF gene into the genome of *Escherichia coli* (*E. coli*) BL21, and an immobilized fermentation system was developed based on biofilm to facilitate continuous hEGF production. After optimization of fermentation conditions and gene dosage, the production of hEGF was increased from 13.9 mg/L to 52.4 mg/L in free-cell fermentation. Moreover, genetic modifications targeting dgcC, csgD, bcsA, and bcsB proved to enhance biofilm formation. When the *bcsB* was overexpressed in BL21-hEGF-C5, the biofilm-forming ability was enhanced by 91.1% and the production of hEGF was increased by 28% in biofilm-immobilized continuous fermentation. In conclusion, this study successfully confirms the feasibility of continuous hEGF production through the biofilm system of *E. coli*, providing valuable insights for the development of other proteins in the field of continuous biomanufacturing.

Keywords: human epidermal growth factor; *Escherichia coli*; protein secretion; biofilm-immobilized continuous fermentation

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1. Introduction

hEGF exhibits potent abilities to stimulate epithelial cell proliferation, differentiation, and tissue repair [1]. These remarkable functions have rendered it invaluable for therapeutic interventions in many diseases and for the formulation of cosmetics. Genetic engineering has been employed to achieve large-scale production of hEGF. By harnessing the power of genetic manipulation techniques, researchers can engineer host organisms to express hEGF in substantial quantities [2–4].

E. coli has become the favored host expression system for recombinant protein production owing to its advantageous features, such as efficient gene manipulation and ease of culture [5]. However, the intracellular environment of *E. coli* often leads to the production of insoluble aggregates such as inclusion bodies, and achieving extracellular production of recombinant proteins could overcome this obstacle [6]. Signal peptides play an important role in protein secretion, and previous investigations have demonstrated that the secretion efficiency and production of hEGF were enhanced by employing diverse signaling peptides in *E. coli* [7,8]. Another critical factor that demands attention is gene dosage, which has been observed to positively correlate with increased protein production in a specific range [9].

Conventional free-cell fermentation is widely used for hEGF production in *E. coli*, which has inherent limitations such as shear and other unfavorable conditions affecting cell

viability, the inability of the cells to be repeatedly utilized, high production costs, and a long fermentation period. However, the implementation of an immobilized fermentation system based on biofilm offers a promising solution to overcome these challenges and enhance hEGF production. Biofilm refers to an organized and structured community of cells that proliferate and persist as cohesive units during their growth and development [10]. The extracellular polymeric substances (EPS) produced by microorganisms play a crucial role in the formation of biofilms, rendering them cohesive and viscoelastic [11]. Compared with free-cell fermentation, biofilm-immobilized fermentation offers advantages such as high cell density, a short fermentation period, high metabolic activity, recycled cells, and greater tolerance to environmental factors such as heavy metals, pH fluctuations, and temperature changes [12,13]. Biofilm-immobilized fermentation has found successful applications in the production of small-molecule chemicals such as ethanol and butanol [14], but its application in the field of macromolecular protein synthesis needs to be further studied and developed. Previously, we used plasmid expression of *hEGF* and established a continuous fermentation process for hEGF production based on biofilms. However, the production of hEGF was affected during long-term fermentation because of plasmid loss.

In this study, we engineered a stable expression system for hEGF production by integrating the hEGF gene into the genome of *E. coli* BL21. Building upon this foundation, we employed genetic manipulation techniques to express key genes involved in biofilm formation, ultimately yielding highly efficient strains capable of biofilm formation. Leveraging the advantages offered by biofilms, we successfully achieved continuous and sustained secretion of hEGF (Figure 1). By integrating biofilm formation mechanisms and harnessing the intrinsic capabilities of microbial communities, we have established a novel platform for the production of hEGF. This approach offers improved stability, enhanced production rates, and the potential for continuous secretion. Our findings contribute to the advancement of biofilm-based fermentation systems and highlight their potential for the production of complex biomolecules.

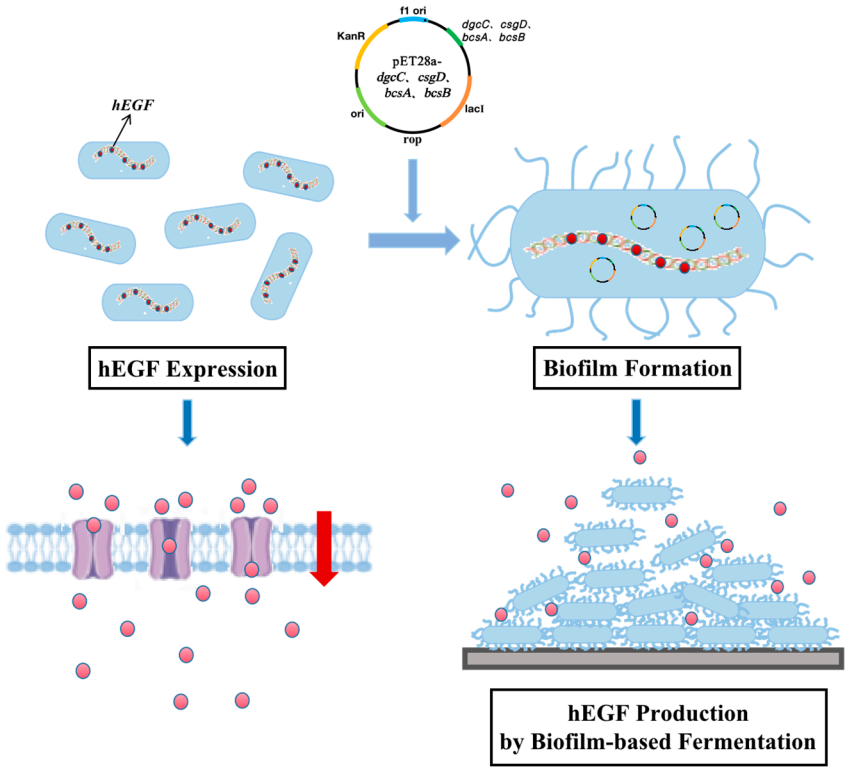

Figure 1. Graphical illustration of the continuous secretion system of hEGF in this study.

2. Materials and Methods

2.1. Strains and Plasmids

E. coli BL21(DE3) was used in this study. All strains and plasmids used in this work are listed in Table 1. Primers used in this study are listed in Table S1. To achieve efficient expression of *hEGF* in *E. coli* BL21(DE3), the CRISPR technology was employed. Each *hEGF* expression cassette was integrated at a specific locus of the *E. coli* BL21(DE3) genome.

Table 1. Strains and plasmids used in this study.

Strains or Plasmids		Relevant Characteristics	Sources
Strains	*E. coli* BL21(DE3)	Host strain	Invitrogen
	BL21-*hEGF*-C1	Genome-integrated expression of *hEGF* in one locus	This study
	BL21-*hEGF*-C2	Genome-integrated expression of *hEGF* in two loci	This study
	BL21-*hEGF*-C3	Genome-integrated expression of *hEGF* in three loci	This study
	BL21-*hEGF*-C4	Genome-integrated expression of *hEGF* in four loci	This study
	BL21-*hEGF*-C5	Genome-integrated expression of *hEGF* in five loci	This study
	BL21-*hEGF*-*dgcC*	Plasmid-based expression of *dgcC*	This study
	BL21-*hEGF*-*csgD*	Plasmid-based expression of *csgD*	This study
	BL21-*hEGF*-*bcsA*	Plasmid-based expression of *bcsA*	This study
	BL21-*hEGF*-*bcsB*	Plasmid-based expression of *bcsB*	This study
	BL21-*hEGF*-pBb-*bcsB* *	Plasmid-based expression of *bcsB*	This study
Plasmids	pET30a-PelB-*hEGF*	*hEGF* expression	[15]
	pCas	CRISPR editing	[16]
	pTarget	CRISPR editing	[16]
	pET28a	Expression vector	Invitrogen
	pBbE1a	Expression vector	[15]
	pET28a-*dgcC*	Plasmid-based expression of *dgcC*	This study
	pET28a-*csgD*	Plasmid-based expression of *csgD*	This study
	pET28a-*bcsA*	Plasmid-based expression of *bcsA*	This study
	pET28a-*bcsB*	Plasmid-based expression of *bcsB*	This study
	pBbE1a-*bcsB*	Plasmid-based expression of *bcsB*	[15]

* Compared with other modified strains overexpressing biofilm-related genes in this study, the strain used plasmid pBbE1a to express *bcsB*.

Locus of *hEGF* gene integration were *yjjM*, *yddE*, *yfbL*, *arpA*, and *yjcF*. Each integration event generated a distinct strain capable of *hEGF* expression, denoted as BL21-*hEGF*-C1, BL21-*hEGF*-C2, BL21-*hEGF*-C3, BL21-*hEGF*-C4, and BL21-*hEGF*-C5, indicating the presence of 1 to 5 copies of the *hEGF* expression cassette. The *hEGF* expression cassette with T7 promoter and T7 terminator, derived from the *hEGF* expression plasmid pET30a-*hEGF* [15], was amplified and characterized. To generate the donor DNA for CRISPR-mediated integration, two homologous arms, each approximately 500 base pairs in length, were selected upstream and downstream of the targeted insertion site. The *hEGF* expression cassette was ligated with the upstream and downstream homologous arms using overlap PCR, yielding the donor DNA required for the integration process.

To facilitate the formation of biofilms, the genes responsible for biofilm development, namely, *dgcC*, *csgD*, *bcsA*, and *bcsB*, were amplified from the genomic DNA of *E. coli* MG1655. Each of the biofilm genes was cloned into either the pET28a or pBbE1a expression plasmid using the ClonExpress II One Step Cloning Kit C112 (Vazyme, Nanjing, China), and they were then introduced into BL21-*hEGF*-C5. By incorporating each of the biofilm genes into strain BL21-*hEGF*-C5, strains BL21-*hEGF*-*dgcC*, BL21-*hEGF*-*csgD*, BL21-*hEGF*-*bcsA*, BL21-*hEGF*-*bcsB*, and BL21-*hEGF*-pBb-*bcsB* were generated.

2.2. Media and Growth Conditions

Before the fermentation conditions were optimized, strains were inoculated into 5 mL Luria-Bertani (LB) liquid medium and cultured at 37 °C and 200 rpm for 12 h to obtain seed culture. Following this, the 2 mL seed culture was transferred to 200 mL Terrific Broth

(TB) liquid medium in 500 mL conical flasks and grown at 37 °C to OD_{600} of 0.6–0.8. The cells were then induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (S11086, Yuanye Bio-Technology, Shanghai, China) at 25 °C and 200 rpm.

The LB medium comprised 5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl, and the TB medium contains 11.8 g/L tryptone, 23.6 g/L yeast extract, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, and 4 mL/L glycerol. Solid media were prepared by adding 1.5% (*w*/*v*) agar.

For strains expressing the biofilm gene using the pET28a plasmid, cultivation was performed with 50 μg/mL of kanamycin (P659139, Aladdin, West Palm Beach, FL, USA). For strains expressing the biofilm gene using the pBbE1a plasmid, cultivation was performed with 100 μg/mL of ampicillin.

2.3. Fermentation and Analytical Methods

In free-cell fermentation, a single colony was chosen and transferred to 5 mL LB liquid medium at 37 °C and 200 rpm for 12 h to obtain seed culture. Subsequently, the seed culture was transferred to TB medium (the 50 mg/mL kanamycin for strains contained biofilm gene expressing plasmid pET28a, and the 100 mg/mL ampicillin for strains contained biofilm gene expressing plasmid pBbE1a) at 1% (*v*/*v*) inoculum. This culture was further incubated at 37 °C and 200 rpm. Once the optical density (OD_{600}) reached a range of 0.6 to 0.8, the inducer IPTG was added to the culture. The fermentation process was then continued in a shaker at 25 °C. For biofilm-based immobilized fermentation, 40 g/L of cotton fibers were placed within a conical flask and sterilized at 115 °C for 20 min with the fermentation medium. At the end of each batch, the fermentation broth was replaced by a fresh fermentation medium and other procedural steps remained consistent with free-cell fermentation.

In this study, the secretion of hEGF was the focus of analysis. To determine the presence and quantity of hEGF in the culture medium, the supernatant obtained after centrifugation was subjected to two analytical techniques: polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC).

For qualitative assessment of hEGF in the supernatant, SDS-PAGE was employed. The supernatant was mixed with a 4× SDS loading buffer, and the resulting mixture was subjected to a heat treatment at 95 °C for 5 min. The prepared samples were subsequently loaded onto a protein gel, which was prepared according to the instructions provided by the Tricine-SDS-PAGE Gel Preparation Kit (C641100, Sangon Biotech, Shanghai, China). Gel electrophoresis was performed according to the instructions of the Tricine-SDS-PAGE Gel Preparation Kit, and the standard of hEGF (105-04B, Prime Gene, Shanghai, China) was loaded into the seventh lane with a volume of 10 μL of 30 mg/L. The gel was then visualized using a gel imaging system to identify the presence of the target band corresponding to hEGF, and the results were recorded for further analysis.

To determine the expression level of hEGF more quantitatively, HPLC was employed. The analysis utilized an Agilent 300SB-C18 (USA) analytical column with dimensions of 4.6 × 250 mm. The mobile phases employed were mobile phase A, consisting of 0.1% trifluoroacetic acid (TFA) in water, and mobile phase B, consisting of 0.1% TFA in acetonitrile (CAN). A gradient elution method was employed, commencing with a mixture of 90% A and 10% B, gradually transitioning to 100% B over a period of 38 min. A volume of 50 μL of fermentation broth supernatant was injected into the HPLC system for detection of hEGF production. The analysis was conducted at an operating temperature of 25 °C, with a detection wavelength set at 280 nm. The flow rate of the mobile phase was maintained at 0.8 mL/min. This HPLC method allowed for the determination of hEGF expression levels and provided quantifiable data for further evaluation and comparison.

2.4. Characterization of Biofilm Formation

In the experimental procedure, a 96-well plate was utilized to culture the strains in LB medium for 48 h, facilitating the formation of biofilms on the bottom surface of each

well. Following this, the cells were fixed using a 4% paraformaldehyde solution to preserve the integrity of the biofilm structure. To visualize and quantify the biofilms, the fixed cells were subjected to staining with a crystal violet solution (A100528, Sangon Biotech). Subsequently, the excess staining solution was carefully removed by gently washing the wells with phosphate-buffered saline (PBS). The amount of 150 μL of 33% glacial acetic acid solution was added to each well in order to dissolve the crystal violet bound by the biofilm cells. To obtain quantitative data, the absorbance of each well was measured using a microplate reader, specifically at a wavelength of 570 nm (A570). This measurement reflected the amount of crystal violet dye retained by the biofilm cells and provided a means of assessing the biofilm formation and growth.

2.5. Congo Red Assay

The bacterial strain was cultured in an LB medium, and the cells present in the fermentation broth were collected through centrifugation. Subsequently, the cells were washed twice with PBS buffer to remove contaminants, followed by adding 1 mL of PBS buffer to the cells, and they were thoroughly mixed. Then, 10 μL of a Congo Red solution (A600324, Sangon Biotech), with a concentration of 10 mg/mL, was introduced to the cell suspension. The resulting mixture was incubated in a shaker at 150 rpm and 25 °C for 10 min. The purpose of this incubation was to allow for the interaction between the Congo Red dye and the bacteria. To separate the cells from the supernatant, the mixture was subjected to centrifugation. The resulting supernatant was analyzed by scanning at full wavelength, capturing the spectral profile. Specifically, the residual amount of Congo Red in the supernatant was determined by measuring the absorbance at a wavelength of 485 nm (OD_{485}).

Furthermore, the Congo Red binding ratio, denoted as CR%, was calculated using the following equation:

$$CR\% = 1 - \frac{OD_{485}}{OD_{485(PBS+CR)}} \quad (1)$$

In this equation, OD_{485} represents the absorbance of the sample at 485 nm, while OD_{485}(PBS+CR) represents the absorbance of a control solution consisting of PBS and Congo Red without any microbial cells at 485 nm.

By observing the combining effect of Congo Red and the bacteria, it becomes possible to characterize whether the overexpression of specific genes can enhance the biofilm formation ability of the strains by increasing the amount of cellulose. This assessment helps to elucidate the impact of gene expression on the biofilm-forming capacity of the strains under investigation.

2.6. Confocal Laser Scanning Microscope

The biofilm formation on the carrier was visualized through a confocal laser scanning microscope, a powerful imaging technique. The strains were cultured in an LB medium, and the optical density of the bacterial cell suspensions was determined. To ensure standardized conditions, the suspensions were adjusted to a final OD_{600} value of 1.0 by adding sterilized water. For the experimental setup, the laser confocal petri dish (J04121, JingAn Biological, Shanghai, China) was utilized. The amount of 4 mL of LB liquid medium and 400 μL of the appropriately diluted bacterial solution were added to the petri dish. The petri dish was then incubated at a temperature of 28 °C for 48 h, allowing sufficient time for biofilm development to occur. After the designated incubation period, the culture medium was carefully drained, and the cells were fixed using a 4% paraformaldehyde solution to preserve their morphology and structure. To facilitate staining and visualization of the biofilm, a solution of DAPI dye (C1006, Beyotime, Shanghai, China), which specifically binds to DNA, was added to the fixed cells. After staining, the samples underwent an additional wash step with PBS buffer to remove excess dye. Subsequently, the prepared samples were subjected to analysis using a laser confocal microscope.

3. Results

3.1. Screening Results of High Production Strain

In our previous work, *hEGF* was expressed and secreted from *E. coli* BL21(DE3) with the pET30a plasmid and *pelB* signal peptide; the production of hEGF reached 24 mg/L in a shake flask [15]. To avoid the problem of plasmid loss during the long-term continuous fermentation, the hEGF gene was integrated into the genome of *E. coli* BL21(DE3) through CRISPR gene editing technology. Five specific sites within the BL21(DE3) genome, namely, *yjjM*, *yddE*, *yfbL*, *arpA*, and *yjcF*, were selected for iterative integration of multiple-copy *hEGF* genes. Genome-integrated expression strains were generated, which harbored 1–5 copies of the hEGF gene and were named BL21-*hEGF*-C1, BL21-*hEGF*-C2, BL21-*hEGF*-C3, BL21-*hEGF*-C4, and BL21-*hEGF*-C5, respectively.

Gene dosage on hEGF production was evaluated. Figure 2a illustrates the results obtained through SDS-PAGE analysis, which revealed the presence of protein bands in the five strains that corresponded in size to the expected hEGF standard size of 6.2 kilodaltons, suggesting that the five strains were successful in secreting hEGF. The gray value of the target protein bands increased as the copy number increased, indicating that the production of hEGF increased with the copy number. The quantification of hEGF secretion levels in the recombinant strains was accomplished using HPLC. The results showed that the recombinant strain BL21-*hEGF*-C5 had the highest hEGF production, reaching 29.6 mg/L at 96 h, which was 2.13 times higher than that of BL21-*hEGF*-C1 (13.9 mg/L) (Figure 2b).

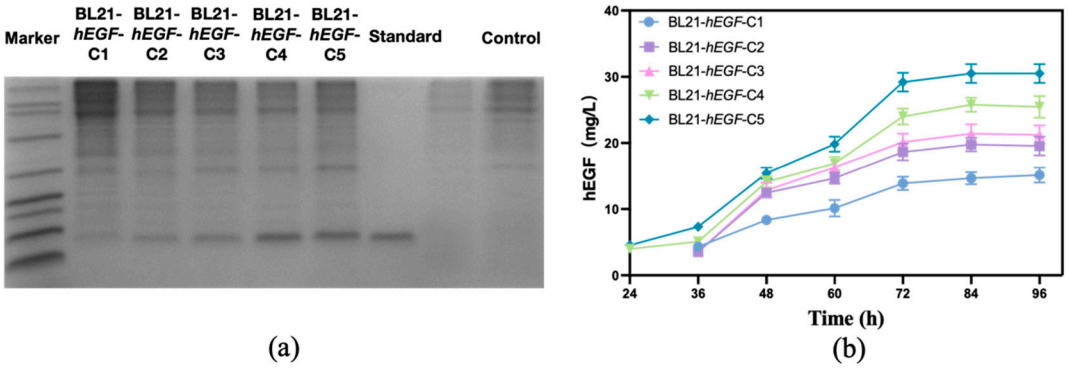

Figure 2. Effect of gene dosage on hEGF secretion. (**a**) SDS-PAGE results of recombinant strains. Lane 1 was Marker, with a sample volume of 5 μL; lanes 2–6 were modified strains, with a sample volume of 20 μL; lane 7 was the standard of hEGF with a sample volume of 10 μL of 30 mg/L; lane 9 was the control strain BL21(DE3), with a sample volume of 20 μL. (**b**) Production of hEGF was secreted from a recombinant strain.

To further increase the secretory expression of hEGF, a single-factor optimization experiment was conducted to obtain the conditions favorable for the recombinant strain BL21-*hEGF*-C5. It has been observed that an increase in temperature during induction adversely affects protein folding and that low-temperature induction attenuates bacterial metabolic activity, thereby reducing the likelihood of protein misfolding [17]. The induction temperatures of 20 °C, 25 °C, 30 °C, and 35 °C were compared in this study. The result showed that the production of hEGF was significantly higher at 20 °C. Furthermore, high concentrations of IPTG have been found to have toxic effects on bacteria [18]. Therefore, the concentration of IPTG was compared at 0.1, 0.5, 1.0, 1.5, and 2.0 mM. The result showed that the production of hEGF was significantly higher at 0.5 mM IPTG. Furthermore, the specification of shake flasks and the volume of medium were investigated for hEGF production. These results showed that a 500 mL flask with a baffle, containing 100 mL of TB medium was the best condition for hEGF production (Figure 3c,d). The use of flasks with baffles and appropriate reduction of medium loading increased the amount of dissolved oxygen. Increased dissolved oxygen favors cell growth and enhances protein expression, ultimately leading to increased hEGF production. Through careful consideration and

optimization of these variables, the production of hEGF experienced an increase of 77.0%, elevating the yield from 29.6 mg/L to 52.4 mg/L (Figure 3e).

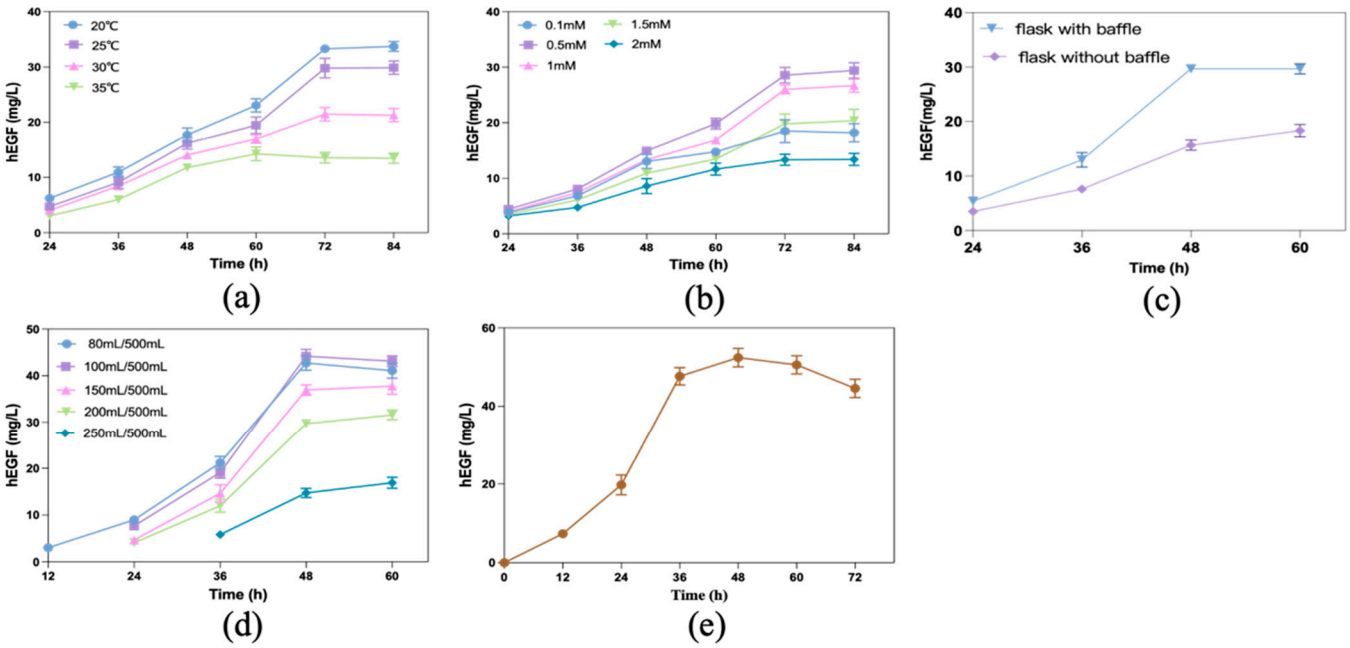

Figure 3. Effect of fermentation conditions on hEGF secretion. (**a**) Effect of induction temperatures on hEGF secretion; (**b**) effect of IPTG concentration on hEGF secretion; (**c**) specification effect of shake flasks on hEGF secretion; (**d**) effect of medium volume on hEGF secretion; (**e**) hEGF secretion after the optimization of fermentation conditions.

3.2. Effect of Gene Modification on Biofilm Formation

In order to improve the biofilm-forming ability and construct a continuous fermentation process based on biofilms, biofilm-related genes of *dgcC*, *csgD*, *bcsA*, and *bcsB* were overexpressed in BL21-*hEGF*-C5. The biofilm-forming ability was measured by crystal violet staining in 96 microtiter plates. The experimental results showed that the overexpression of these genes enhanced the formation of biofilm by actively promoting cellular aggregation and adhesion. Specifically, the overexpression of *bcsA* and *bcsB* genes demonstrated a pronounced influence on biofilm formation, leading to more substantial enhancements compared to the other genes tested. Compared to the strain BL21-hEGF-C5, recombinant strains BL21-*hEGF-bcsB* and BL21-*hEGF*-pBb-*bcsB* exhibited significantly increased biofilm formation by 91.1% and 80.2%, respectively (Figure 4). Recombinant strains BL21-*hEGF-dgcC*, BL21-*hEGF-csgD*, and BL21-*hEGF-bcsA* demonstrated enhanced biofilm formation by 63.9%, 68.2%, and 74.6%, respectively.

Congo red is a widespread amyloid-binding dye that binds to curli as well as cellulose [19]. Higher amounts of curli and cellulose mean that the strain was more able to form biofilm. In this study, recombinant strains were stained by Congo Red dye, which observed that the color of the bacterial cells is redder and the supernatant is clearer compared with the strain BL21-*hEGF-C5* (Figure 5). These results highlight that the overexpression of *dgcC*, *csgD*, *bcsA*, and *bcsB* genes enhanced cellulose synthesis, leading to increased binding ratio of the modified strains to Congo Red compared to the control strain, Congo Red binding of the modified strains was 1.27 to 1.95 times higher than that of the control strain BL21-*hEGF*-C5 (Table 2). Overexpression of *dgcC*, *csgD*, *bcsA*, and *bcsB* genes enhanced biofilm formation by promoting the synthesis of cellulose.

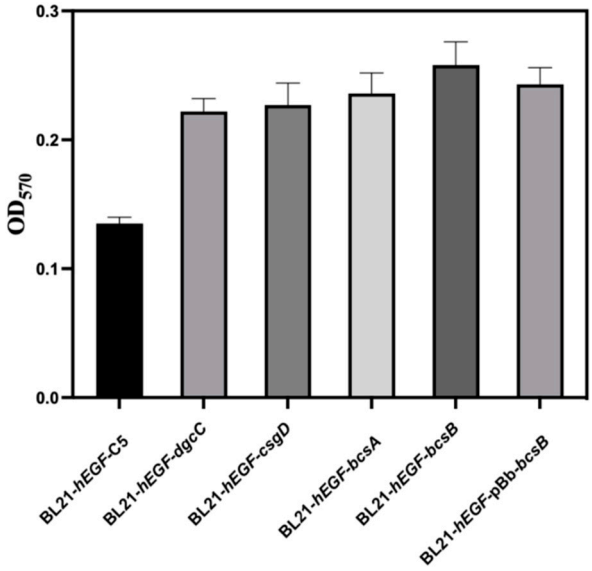

Figure 4. Crystal violet staining-based quantification of biofilm biomass. Strains were inoculated into a 96-well plate and incubated at 28 °C for 48 h; the cells were stained with CV, and the absorbance was determined using a microplate reader at A 570. These values represent the biofilm formation of the strains in the well plates, with higher values indicating more biofilm biomass.

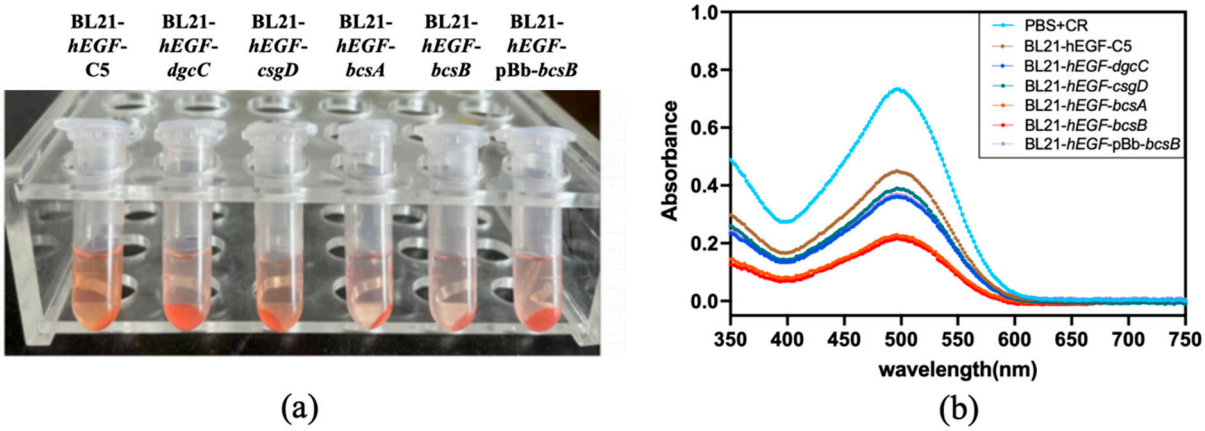

Figure 5. Binding assay of Congo Red with biofilm matrix. (**a**) Congo Red adsorption with different strains overexpressing biofilm-related genes; (**b**) residual amount of Congo Red in the supernatant, measured as absorbance at 485 nm.

Table 2. Congo Red binding ratio of strains overexpressing biofilm-related genes.

Strains	Congo Red Binding Ratio
BL21-*hEGF*-C5	0.37
BL21-*hEGF-dgcC*	0.51
BL21-*hEGF-csgD*	0.47
BL21-*hEGF-bcsA*	0.69
BL21-*hEGF-bcsB*	0.72
BL21-*hEGF*-pBb-*bcsB*	0.51

Further analysis using a confocal laser scanning microscope demonstrated that the modified strains had more biofilms in 96-well plates than the control strain, indicating increased biofilm formation and significant cellular aggregation. In contrast, the control strain displayed a lower adhesion amount and a more dispersed state within the 12-well plates (Figure 6). These findings support the notion that the modified strains possessed

stronger adhesion capabilities, with the overexpression of *bcsA* and *bcsB* genes particularly contributing to a significantly enhanced biofilm formation ability.

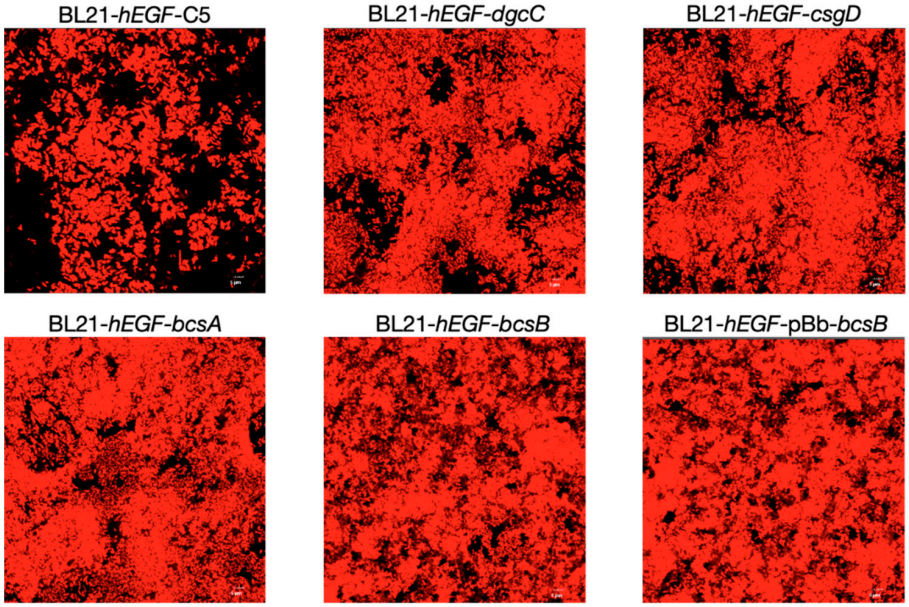

Figure 6. The results of confocal laser scanning microscope. Strains were inoculated into the laser confocal petri dish, incubated at 28 °C for 48 h, and then photographed after DAPI staining.

3.3. Effects of Overexpression of Biofilm-Related Genes on hEGF Secretion

Biofilm formation represents a multifaceted process with the potential to impact various aspects of cellular physiology and metabolism [20]. Therefore, the influence of overexpressing biofilm-related genes on hEGF production and strain growth were evaluated first through conventional free-cell fermentation. The engineered strains successfully secreted hEGF, and the production of hEGF reached the highest level at 48 h. Overall, overexpression of biofilm-related genes did not significantly reduce hEGF production, and in particular, overexpression of the *csgD* and *bcsB* genes increased hEGF secretion to some extent (Figure 7a). Specifically, the strains BL21-*hEGF-csgD* and BL21-*hEGF-bcsB* exhibited hEGF production levels of 60.4 mg/L and 62.6 mg/L, respectively. These values represented an increase of 15.3% and 19.5% compared to the control strain BL21-*hEGF*-C5, which produced hEGF at a level of 52.4 mg/L. These findings highlight the potential of manipulating biofilm-related genes to modulate hEGF production in the context of free-cell fermentation, but the mechanisms of regulation need to be further explored.

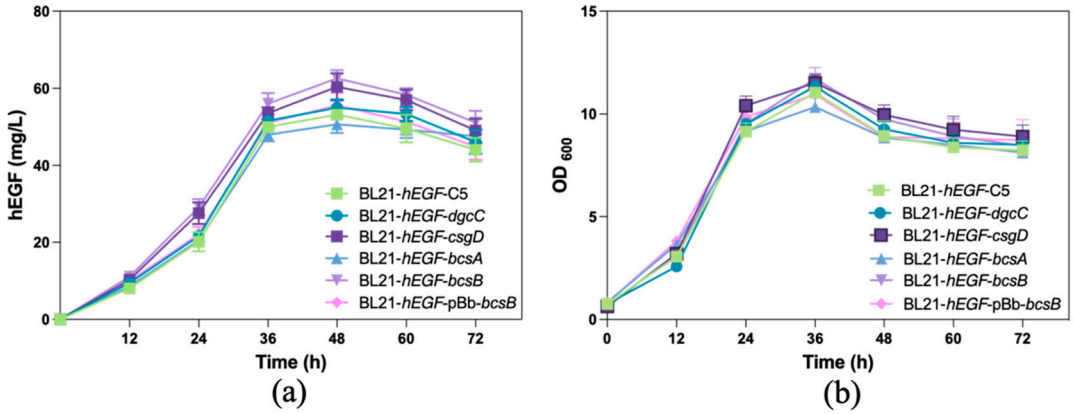

Figure 7. Effect of overexpression of biofilm-related genes on the strains. (**a**) Effect on hEGF secretion; (**b**) effect on strain growth.

3.4. Production of hEGF by Biofilm-Immobilized Continuous Fermentation

Biofilm-immobilized continuous fermentation was conducted to assess the capability of recombinant strains for continuous hEGF production. The evaluation of bacterial adsorption onto the surface of the immobilized carrier serves as an indicator of the efficiency of bacterial cell immobilization. Compared with the conventional free-cell fermentation, the OD_{600} values of the fermentation broth were substantially reduced during the biofilm-immobilized continuous fermentation, this means all strains were successfully adsorbed on the carrier with a good immobilization effect (Figure 8a). Compared with the strain BL21-*hEGF*-C5, biofilm-forming strains showed significantly lower OD_{600} values of fermentation broth during biofilm-immobilized continuous fermentation, indicating a higher degree of modified strain absorption onto the carrier surface, suggesting the effectiveness of the biofilm-related gene modification. The turbidity of the fermentation broth gradually increased in the first few batches and then stabilized. In the initial batch of immobilized fermentation, all strains exhibited low cell density in the fermentation broth, primarily due to the adsorption state of cells. In the second batch, the biofilm developed to a relatively mature stage, leading to a significant increase in cell density within the fermentation broth. In subsequent batches, the trend of cell density changes remained relatively consistent, indicating the establishment of a consistent and stable biofilm structure. During each batch of immobilized fermentation, the density of bacteria in the fermentation broth initially increased, followed by a noticeable decline due to continuous cell adsorption onto the carrier surface. The best immobilized of all the strains were BL21-*hEGF-bcsA* and BL21-*hEGF-bcsB*. In the fifth, sixth, and seventh batches of immobilized fermentation, the number of bacteria in the fermentation broth of BL21-*hEGF-bcsA* and BL21-*hEGF-bcsB* strains exhibited a reduction compared to previous batches. This reduction in free cells implies that the overexpression strains carrying the *bcsA* and *bcsB* genes demonstrated increased stability in immobilization, becoming less susceptible to desorption as the batches progressed. These findings highlight the successful immobilization and sustained stability of the modified strains, specifically those overexpressing the *bcsA* and *bcsB* genes, in the immobilized fermentation system.

The results depicted in Figure 8b exhibit a decreasing trend in hEGF production initially; followed by a stabilization phase as the fermentation batch number increased. This trend provides compelling evidence of the feasibility of employing the modified strains for immobilized continuous production; emphasizing the potential of this approach. Biofilm formation is considered to be a protected growth pattern for microorganisms to adapt to harsh environments [21]. Compared to the control strain; the modified strains were more capable of hEGF production. Further details can be found in Table 3; which presents a comprehensive overview of hEGF secretion by the modified strains relative to the control strain. Across seven batches of immobilized continuous fermentation; the average hEGF production levels of strains BL21-*hEGF-dgcC*; BL21-*hEGF-csgD*; BL21-*hEGF-bcsA*; BL21-*hEGF-bcsB*; and BL21-*hEGF*-pBb-*bcsB* were 41.2 mg/L; 47.5 mg/L; 44.2 mg/L; 49.2 mg/L; and 46.4 mg/L; respectively. These values increased by 7.2%, 23.8%, 14.5%, 28.0%, and 20.6%, respectively; compared with the control strain BL21-*hEGF*-C5 (38.6 mg/L). These findings highlight the superior performance of the modified strains overexpressing *dgcC*; *csgD*; *bcsA*; and *bcsB* genes in continuous hEGF production compared to the control strain. BL21-*hEGF-bcsB* had the highest yield of hEGF; and the potential it showed in immobilized continuous production was due to the formation of more biofilms.

Moreover, the results obtained from scanning electron microscopy (SEM), as depicted in Figure 9, reveal that the modified strains formed more extensive biofilms on the cotton fiber carrier. This observation indicates that an increased biofilm presence facilitated continuous hEGF production.

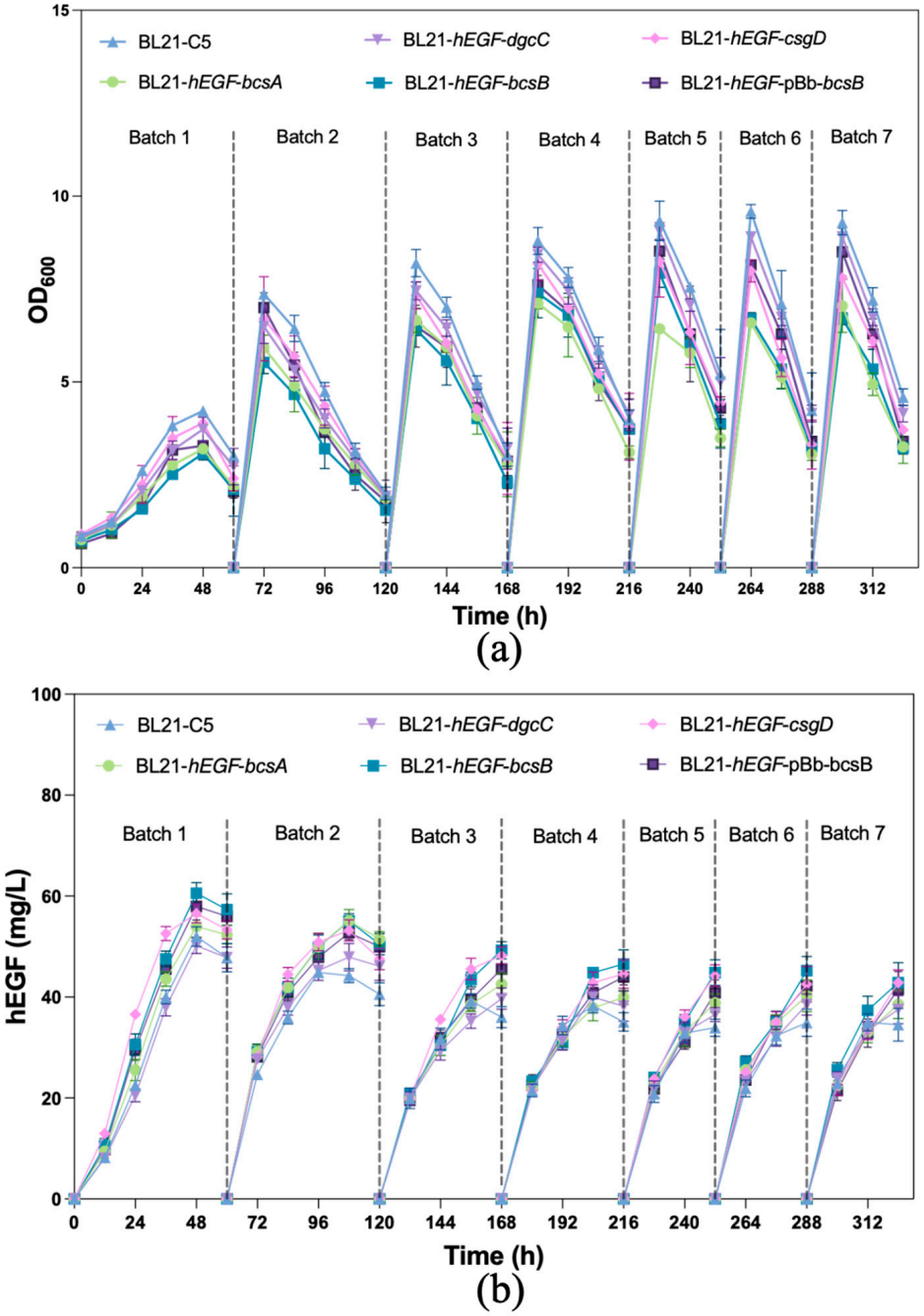

Figure 8. Biofilm-immobilized continuous fermentation. (**a**) Comparison of adsorption of the strains in immobilized continuous fermentation; (**b**) comparison of hEGF secretion of the strains in immobilized continuous fermentation.

Table 3. hEGF secretion of the modified strains compared to the control strain.

Strains	hEGF Secretion of the Modified Strains Compared to the Control Strain (%)							
	1st	2nd	3rd	4th	5th	6th	7th	Average
BL21-*hEGF-dgcC*	−3.5	8.4	10.2	8.9	7.7	10.3	8.7	7.2
BL21-*hEGF-csgD*	8.8	20.3	33.9	27.0	30.4	21.8	24.1	23.8
BL21-*hEGF-bcsA*	3.8	24.5	18.1	13.9	14.5	15.3	11.7	14.5
BL21-*hEGF-bcsB*	16.5	24.5	36.7	32.7	32.2	29.6	24.1	28.0
BL21-*hEGF*-pBb-*bcsB*	11.5	19.2	26.4	25.3	20.4	21.0	20.4	20.6

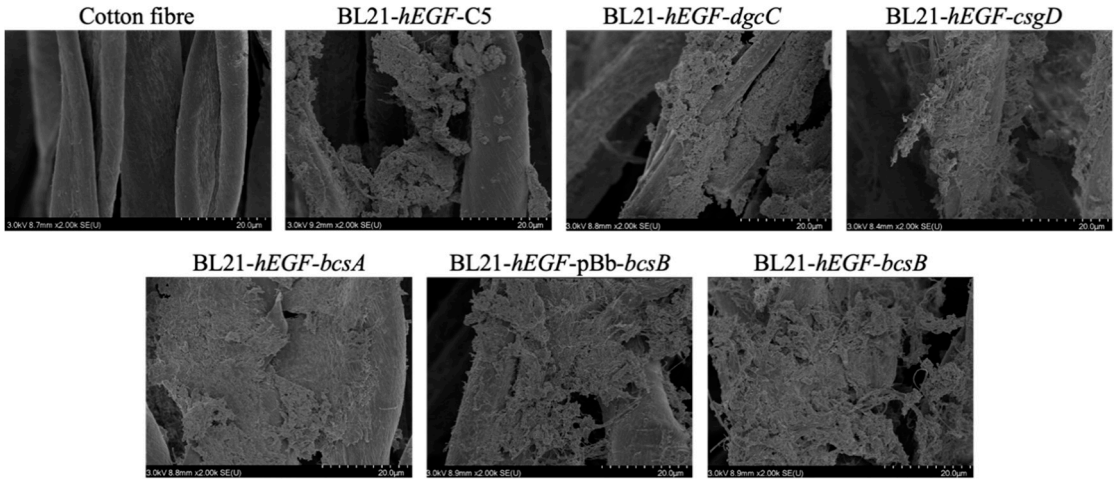

Figure 9. SEM image of the carrier in immobilized continuous fermentation. Biofilm on the carrier after immobilized continuous fermentation; image taken by SEM. Scale bar, 20 μm.

4. Discussion

Secretory production of hEGF in *E. coli* has been developed using traditional batch fermentation. Here, to further improve the production efficiency, the present study aims to develop a continuous hEGF secretion system based on biofilm formation. In our previous study, plasmid-expressing strains for hEGF production were constructed. However, one of the limitations of plasmid-based expression systems was that the plasmids were prone to loss over time, rendering them unsuitable for sustained, long-term production. To overcome this challenge, the present study took a different approach by expressing *hEGF* genes through multiple genomic integrations to obtain long-term gene stability and sustainability of hEGF production. A recombinant strain expressing five-copy genome-integrated *hEGF* genes was constructed, and it gave the best hEGF production. After fermentation optimization, it produced 52.4 mg/L of hEGF, much higher than the previous plasmid-based production of 24 mg/L [15]. Cellulose is one of the major constituents of the biofilm matrix. To enhance biofilm formation in *E. coli*, the potential cellulose-forming genes *bcsA*, *bcsB*, *dgcC*, and *csgD* were incorporated into the BL21-*hEGF*-C5 strain. The catalytic subunit of cellulose synthetase, encoded by *bcsA*, forms a catalytically active complex along with periplasmic proteins anchored to the cell membranes, encoded by *bcsB*. This complex is responsible for the synthesis and transport of cellulose [22]. Moreover, the *dgcC* gene, encoding the diguanylate cyclase, serves as a facilitator for promoting cellulose synthesis. Increased c-di-GMP levels lead to increased cellulose synthesis and biofilm formation, and *csgD* is an important component of the c-di-GMP signaling network and a master regulator of biofilms [23]. Crystal violet staining, Congo Red assay, and confocal laser scanning microscopy all indicated that cellulose production was increased and biofilm formation was enhanced after overexpression of these genes. Finally, by employing the immobilized continuous fermentation technique, we were able to examine the productivity and stability of continuous hEGF secretion by the modified strains. The results showed that the modified strains successfully achieved continuous production of hEGF. In immobilized continuous fermentation, the modified strains produced more hEGF than the control strain.

While the highest reported production of hEGF by *E. coli* has reached 506 mg/L in a 50 L high-density fermenter, here an average production of 49.2 mg/L of hEGF was obtained by immobilized continuous fermentation in flasks. Considering the existing gaps between the achieved hEGF production level and the reported levels, subsequent scale-up fermentation using large fermenters will be carried out. Large fermenters offer improved control over fermentation conditions such as pH and dissolved oxygen, providing more favorable fermentation processes. With these improved controls, it is anticipated that hEGF production can be significantly increased in the future.

5. Conclusions

In this study, a stable hEGF secretion system was constructed using *E. coli* as the host. Building upon this achievement, the study further developed highly efficient *E. coli* strains capable of robust biofilm formation, enabling the realization of immobilized continuous fermentation for hEGF production. This innovative approach harnessed the advantages offered by biofilms, ultimately leading to the establishment of an immobilized continuous fermentation system based on biofilm.

By integrating the concepts of stable *hEGF* expression, efficient biofilm formation, and immobilized continuous fermentation, this study introduces a novel framework for the production of hEGF and offers insights into the advancement of continuous biomanufacturing technologies for diverse proteins.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation10040202/s1 Table S1. Primers used in this study. Figure S1. Elution profile. (a) hEGF standard (25 mg/L); (b) BL21-hEGF-C5 (37 mg/L); (c) BL21 (DE3).

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