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A Taguchi Approach for Optimizing the Expression of Recombinant Human Growth Hormone in *Escherichia Coli*

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Abstract

Expression of recombinant human growth hormone in *E. coli* requires specific measures to be more efficient. In this study, two strains of *E. coli*, one with a genomic expression of T7 and arabinose promoters and the other with a plasmid expression (T7 promoter), were examined. In this regard, the appropriate time for sampling strains carrying the rhGH gene in genomic or plasmid form was tested. First, the best time to add the inducer to the culture medium and different concentrations of arabinose and IPTG inducers were investigated. Separate and simultaneous use of both inducers in strains with both promoters was investigated. Next, the strains were studied in five different culture media, and TB was selected as the optimal culture medium. Finally, the optimal expression of recombinant protein in TB medium was investigated using the Taguchi test for three parameters of peptone casein, yeast extract, and glucose at three different levels. Results showed the best sampling time in genomic strains was overnight; however, in plasmid strains, it was 4 hours after induction. The best time to add the inducer in genomic strains was at the beginning of the exponential phase of bacterial growth. Furthermore, lower amounts of antibiotics were associated with higher amounts of recombinant protein production in genomic strains. In the strains that had both are and T7 promoters, simultaneous induction with both inducers, i.e., arabinose and IPTG resulted in more protein expression than the single inducer.

1. Introduction

Just 50 years after Watson and Creek discovered the double-stranded helix structure of DNA, new advances in the field revolutionized the pharmaceutical industry. These new gene technology and biotechnology methods have created a wide range of new drug compounds (Hou, 2005). Recent advances in genetics, proteomics, and bioinformatics have facilitated the use of recombinant DNA technology to obtain the required proteins. After insulin, the human growth hormone is the second most biotechnological product. This product was originally developed and commercialized by

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Genetec (Ascacio-Martínez & Barrera-Saldaña, 2012; Leader et al.,.008) and is now used in the treatment of short stature due to pituitary insufficiency, chronic renal failure, Turner syndrome, adults with growth hormone deficiency or acquired immunodeficiency syndrome (HIV) (Ecamilla-Treviño et al., 2000).

This hormone is synthesized and secreted by anterior pituitary cells called somatotrophs and, as a crucial endocrine factor, is involved in postnatal growth. In general, the molecular weight of the growth hormone is 22 kDa and is made from 191 amino acids, including 4 alpha helix structures and 2 disulfide bands, and does not require post-translational changes (Ascacio-Martínez & Barrera-Saldaña, 2012; Dao et al., 2019). though the production of recombinant human growth hormone protein is possible in various bacterial, fungal, yeast, and mammalian cell hosts (Baldi et al., 2007; Doran, 2000), the bacterial culture system is more cost-efficient (Andersen & Krummen, 2002).

Easy manipulation, the ability to grow in simple environments, high volume efficiency, and fast and cheap are the advantages of choosing Escherichia coli as a host for recombinant protein production (Swarts, 2001). However, challenges such as insolubility of the product, formation of inclusion bodies, instability of mRNA, and purification are encountered when using microbial cells to express recombinant proteins. Although combining several strategies, such as the use of appropriate mutant host strains, the simultaneous expression of chaperones, and the addition of tags (Demain & Vaishnav, 2009; Kamionka, 2011) improves the expression of E-coli soluble proteins, predicting strongly soluble protein production processes remains a challenging necessity (Jana & Deb, 2005). The three characteristics that should always be considered as determining factors are the successful interaction between gene expression, protein solubility, and the purification step (Chatterjee & Esposito, 2006). In this regard, expression vector, mRNA stability, efficiency, and differences in codon usage of foreign genes play an important role in regulating efficacy (Jana & Deb, 2005; Schumann et al., 2004). In addition, the industrial production of recombinant proteins requires the optimization of various conditions, including paying attention to the amount of dissolved oxygen and the amount of aeration of the system, the composition of the culture medium (especially the amount and type of carbon source), temperature and induction conditions, the concentration and type of the inducer (Kiany et al., 2003; Patra et al., 2000; Seyfi et al., 2019). Different strains of E-coli should be selected based on the biochemical and biophysical properties of the recombinant proteins. For example, the BL21 strain, which produces less acetate at high cell densities, is a good option for producing rhGH. BL21-derived strains such as rosetta-gami (DE3) are also suitable hosts for producing recombinant proteins that have a disulfide bond.

In this research, several parameters were evaluated, including strains carrying the rhGH gene in genomic and plasmid form, effective induction time, inducer concentration, and antibiotic concentration. In addition, the TB culture medium and some of its components, such as carbon source, nitrogen source, etc., were optimized using the Taguchi algorithm.

2. Materials and methods

2.1. Chemicals

The chemicals rC6H12O6, C3H5(OH)3, K2HPO4, MgSo4.7H2o, acrylamide and

bis-acrylamide, Sodium dodecyl sulfate, deoxycholate, and L-arginine analytical grade were purchased from Merck (Germany). IPTG (isopropyl-β-D-thiogalactopyranoside) was purchased from Sigma (USA). The culture medium used in this experiment is LB, including Nutrient broth, M9a, M9b, and terrific broth. The compounds of which are described below. BL21, 3DH5α, and Rosetta-gami B (DE from Novagen) were the studied E.Coli stains. The pET28a and pKD46 vectors were developed by our laboratory.

2.2. Design and development of gene cassettes and genetic manipulation

First, the *Nco*l cleavage site and Fh8-His-tag were added to the 5' end of rhGH, respectively. The specific locus of the enterokinase enzyme is located at the beginning of the gene and the 3' end of the His tag. Next, the *Xho*I cleavage site was integrated at the 3' end of rhGH. The His tag was then embedded to facilitate purification and enterokinase to separate rhGH from the FH8 and 6xHis tags.

Afterward, to insert the rhGH gene into the host genome at the arabinose operon site, the fh8-rhgh-kan cassette was amplified by PCR from the cloned rhGH gene in the pET28 vector using two primers, each of which had 50 nucleotides of homology with the target site. Then, this cassette was transformed into the host carrying the Lambda-RED system by electroporation, i.e., the pKD46 vector, which was previously induced to facilitate recombination. In the recombinant strains, the rhGH gene was under the control of the arabinose promoter. In addition, another pair of primers was used to amplify the T7 promoter of the pET28 vector along with the rhGH gene and the kanamycin resistance gene. These primers had 50 homologous nucleotides. Other steps were performed as before to obtain recombinants where the rhGH gene is located in the genome downstream of the T7 promoter. In the latter case, the resulting strains were simultaneously inducible through arabinose and T7 promoters. In addition to the above recombinant cases, the pET28-Fh8-hgh vector was transformed into the Rosetta-gami strain to check the episomal expression.

2.3. Culture conditions, expression, and SDS page

Stocked bacteria frozen with 20-30% sterile glycerol were transferred to 5 ml fresh LB and cultured. The sample was incubated for about 14-16 h while being shaken at 180 rpm at 37° C with an appropriate antibiotic. One percent of the above culture was transferred into a 250 ml container containing 50 cc of the desired culture medium containing appropriate antibiotics. When the Optical Density (OD) of the culture medium reached 0.5-0.8 at 600 nm, a related inducer (arabinose or IPTG) was added to the culture medium at the desired concentration.

Strains 6 and 7 were induced with 20% arabinose, and strain 59 and the plasmid were induced with IPTG 0.25 mM. General culture conditions in this study were 37° C, 180-200-rpm, and a PH = 7. The antibiotic used in this study was kanamycin. The concentration used for genomic recombinant strains was 10-15 μg/ml and 60 μg/ml for plasmid recombinants.

After a specified induction time (for genome strains, 16-18 hours and plasmid strains, 4 hours), cultures were sampled, and the cell growth rate was calculated by measuring the OD of the culture medium at 600 nm with a spectrophotometer. At the

end of the incubation period, about 1 ml of each culture was poured into 2 ml microtubes, and the cells were collected by centrifugation at 5000 rpm for 5 minutes at 4 ° C. The discarded supernatant and the collected cells were mixed directly with a suitable volume of Sample Buffer diluted 2.5X and, after boiling for 10 minutes, electrophoresed in 12% denatured acrylamide gel (SDS-PAGE). Non-transformed bacteria were used as the control. As a negative control, non-transformed bacteria were cultured in the same experimental conditions at the same time as the other samples, and sampling and analysis were performed on the SDS PAGE.

2.4. Taguchi test design method

In order to optimize the TB culture medium for the expression of human growth hormone in the bacterial system, experiments were designed based on the Taguchi method. The variables affecting protein expression in the TB medium were identified, and their levels were determined before deciding on the experiment design. In this regard, the effect of three variables on protein production in the TB medium was investigated, including the amount of casein peptone, yeast extract, and glucose (each in three levels) (Table 1). It should be noted that all experiments were performed in an environment containing antibiotics with appropriate concentrations (genomic strains 10 µl/ml and plasmid strains 60 µl/ml) at 37 ° C, 180 rpm, and pH = 7. Using the Taguchi method, several factors can be evaluated simultaneously (15, 30). For example, Ghane et al. (2008) used this method to optimize the expression of interferon beta protein in the *E. coli* host.

Table 1: The variables and levels used in the Taguchi method.

	level 1	level2	level3
A: Peptone casein (%)	0.6	1.2	3.6
B: Yeast extract (%)	1.2	2.4	7.2
C: Glucose(gr)	4	8	16

Table 2: Experiments designed using the Taguchi method.

number of test	A peptone casein	B yeast extract	C Glucose
1	1	1	1
2	1	2	2
3	1	3	3
4	2	1	2
5	2	2	3
6	2	3	1
7	3	1	3
8	3	2	1
9	3	3	2

3. Results and Discussion

3.1. Optimization of rhGH production

The following explains the details of the evaluation of optimal sampling time in genomic and plasmid strains.

Genomic recombinant strains (clones 6 and 7) were cultured with Kanamycin (15 µg / ml) and induced by 20% arabinose after reaching the logarithmic phase (OD= 0.6-0.9). Recombinant plasmid strain pET28aFh8-hgh was induced by IPTG when the culture reached the logarithmic phase (OD: 0.8-1).

Sampling was performed 4, 8, 15, and 20 hours after induction. As shown in (Fig 1)',

the highest expression was detected in genomic strains at 15 and 20 hours after induction using the SDS page. The amount of protein at time T4 did not show a significant difference with T8 and T15. Likewise, in the episomal expression, the amount of protein produced at 4, 8, and 15 hours after induction showed no difference. (Fig 1).

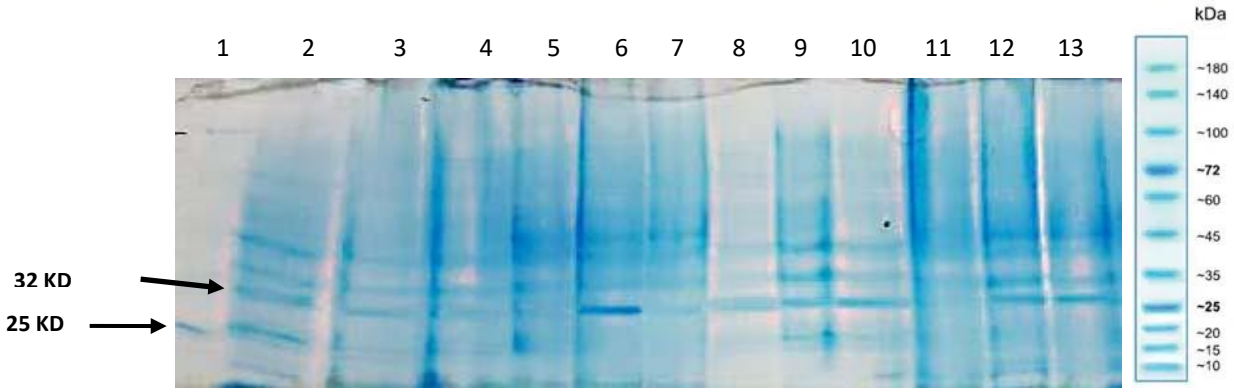


Figure 1. The expression of integrated rhGH in the E.colo chromosome under the arabinose promoter (clones 6 and 7) and episomal rhGH were used to obtain the best sampling time. 1 represents the protein ladder; 2, 3, 4, and 5 are the T20, T15, T8, and T4 of clone 6, respectively. 6 and 7 represent T20 and T8 of clone 7. 8 represents the positive control, 11 the negative control, and 9, 10, 12, and 13 stand for the T20, T15, T8, and T4 of plasmid strain pET28aFh8-hgh, respectively.

3.2. Investigating the best time to add an inductor to the culture medium

The expression of genomic clones 6 and 7 was examined under two different conditions.

a. Arabinose was added to the culture medium from the beginning and at the time of inoculating the bacteria.

b. Arabinose was added after the bacteria reached the logarithmic phase, that is, at OD=0.6-0.9.

Protein expression in the induced bacteria was evaluated 8 and 16 hours after induction. Bacterial density was measured by spectrometry at 600 nm, and expression was evaluated on SDS-PAGE.

(Fig 2) shows that the expression was higher in bacteria that were induced after entering the logarithmic phase.

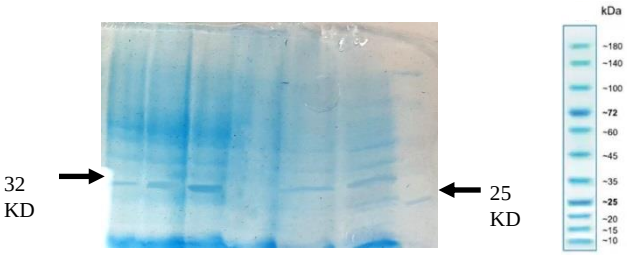


Figure 2. Expression of integrated rhGH in *E.colo* chromosome under the arabinose promoter (clones 6 and 7) was used to investigate the best time of adding an inducer to the culture medium (clones 6 and7). 1 represents 7a, 2 is 7b, 3 is the positive control, 4 is the negative control, 5 is 6a, 6 is 6b, and 7 is the protein ladder.

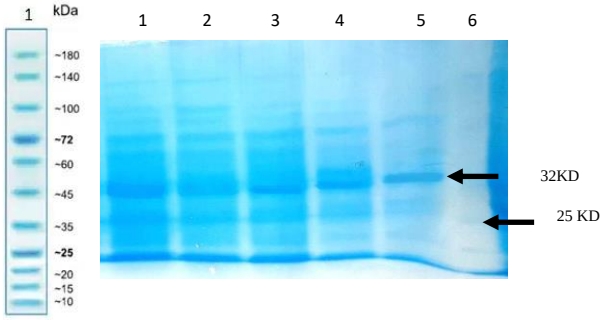
3.3. Investigation of the impact of different arabinose and antibiotics concentrations on the expression of rhGH

In order to investigate the effect of arabinose amount on human growth hormone protein expression, the rhGH expression of clone 6 with different concentrations of arabinose (0.2% and 0.4%) was studied. Also, to determine the effect of antibiotics on rhGH expression, 5 and 15

ug/ml of kanamycin were added to the rhGH in clone 6.

In both experiments, an arabinose inducer was added to the culture medium after reaching the logarithmic phase. Sampling was performed 16 hours after induction, and the result was observed on SDS-PAGE. The results showed that increasing the amount of arabinose did not affect protein expression, but reducing the use of antibiotics increased protein expression (Fig 3).

Figure 3. Expression of integrated rhGH in *E.colo* chromosome under the arabinose promoter(clone 6) used to investigate the impact of different arabinose and antibiotic concentrations. 1



represents the antibiotic 5 µg, 2 is the antibiotic 15µg, 3 is the positive control, 4 is arabinose 0.2%, 5 is arabinose 0.4%, and 6 is the protein ladder.

3.4. Investigation of the use of arabinose and IPTG inducers, individually and in concurrent in genomic 59 and plasmid strains that have both arabinose and T7 promoters

At this stage, the effect of IPTG and arabinose inducers was investigated separately and simultaneously on two strains with both arabinose and T7 promoters (strain 59 and plasmid strain). For this purpose, culture was performed from the 59 and plasmid strains, and each of the strains was induced with arabinose (0.2%) and (IPTG .250 mM) individually and concurrently. Induction was performed after reaching the logarithmic phase, and sampling was performed 4 and 18 hours after induction. Examination of the results on SDS-PAGE showed that the expression of protein when using both inducers simultaneously is higher than when one of the inducers is used alone. In other words, the simultaneous use of two inducers increases the expression (Fig 4).

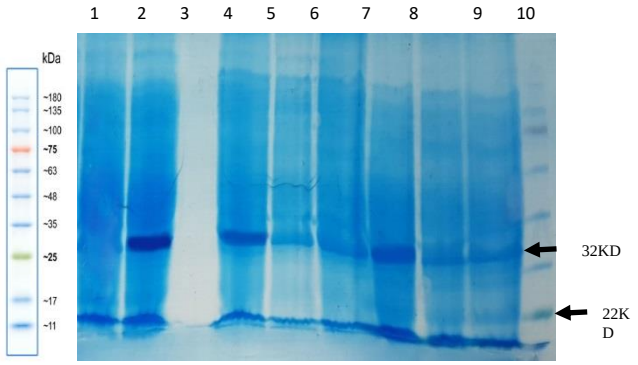


Figure. 4. Expression of integrated rhGH in chromosome under the T7 promoter(clone59) and recombinant pET28aFh8-hgh(plasmid strain) used to investigate the use of arabinose and IPTG inducers, individually and concurrently. 1 is the negative control, 2 is the positive control, 4 is the plasmid strain with IPTG and arabinose inducer, 5 is the plasmid strain with IPTG inducer, and 6 is the plasmid strain with arabinose inducer. 7 represents genomic strain 59 with IPTG and arabinose strain, 8 is genomic strain 59 with arabinose inducer, 9 is a genomic strain with an IPTG inducer, and 10 is the protein ladder

3.5. Investigation of rhGH expression in recombinant genomic strains and plasmid strains with five types of culture medium and selection of the optimal culture medium

The five culture media used in this research were LB, Nutrient broth, TB (Terrific Broth), M9a, M9b, prepared as follows.

Terrific broth (TB) consisted of Peptone and casein 1.2%, Yeast extract 2.4%, KH2Po4 17mM, K2HPo4 72mM, MgSo4 1g/lit, Glucose 8g/lit.

M9a consisted of Na2HPo4 6g, KH2Po4 3g, NaCl 0.5g, NH4Cl 10 g, Tryptone 30 g, Yeast extract 20 g, Glycerol 100% 20 ml, CaCl2 0.5M 200µl, Magnesium sulfate heptahydrate 1M 1ml.

Na2HPo4, KH2Po4, NaCl, NH4Cl, Tryptone, and Yeast extract were weighed to 978.8 ml. Then Glycerol, CaCl2, and Magnesium sulfate heptahydrate Sterile were added separately to the main culture medium.

M9b consisted of Na2HPo4 6g, KH2Po4 3g, NaCl 0.5g, NH4Cl 10 g, casein 5g, Yeast extract 5 g, Glucose 40% 25 ml, CaCl2 0.5M 200µl, Magnesium sulfate heptahydrate 1M 2ml.

Na2HPo4, KH2Po4, NaCl, NH4Cl, Casein, and Yeast extract were weighed to 972.8 ml. Then Glucose, Magnesium sulfate heptahydrate, and CaCl2 were sterilized separately and added to the original culture medium.

To determine the appropriate culture medium for protein expression, strains 59, 7, and the strain carrying the recombinant plasmid were cultured at 37°C and 180 rpm, and samples were taken 20 hours after induction. Analysis of the protein expression in these bacteria showed that the TB culture medium is more suitable than other culture mediums (sampling was performed in the plasmid strain after 4 hours).

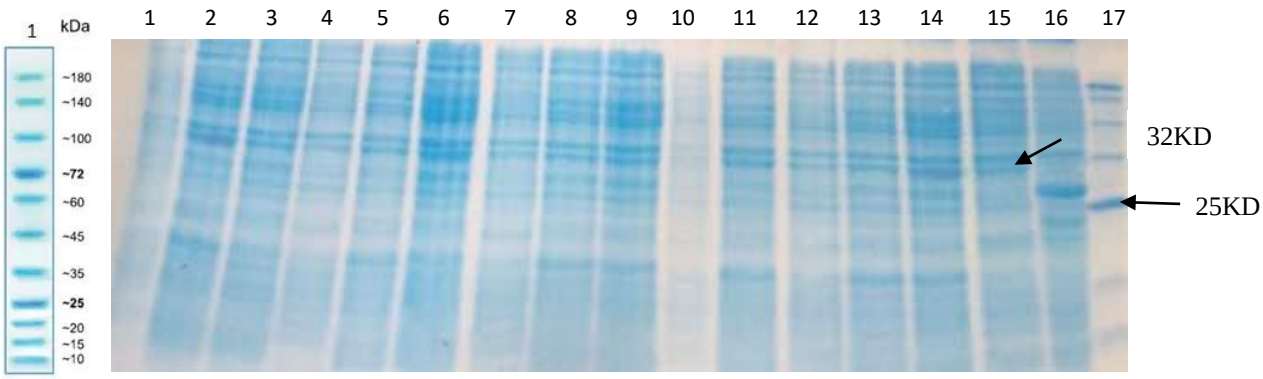


Figure. 5. Expression of integrated rhGH in E.colo chromosome under the arabinose and T7 promoter (clones 7 and 59) and recombinant pET28aFh8-hgh (plasmid strain) with five types of culture medium to select the optimal culture medium. 1 represents 7 LB, 2 is 7TB, 3 is7NB, 4 is 7M9a, 5 is 7M9b, 6 is 59TB, 7 is 59LB, 8 is 59NB, 9 is 59M9a, 10 is 59M9b, 11 is plasmid strain(P)NB, 12 is PLB, 13 is PM9a, 14 is PTB, 15 is M9b, 16 is the negative control, and 17is the protein ladder.

3.6 Qualitative study based on Taguchi's design

Examination of SDS-PAGE gels (Fig 6-8) showed that strain 6 (induced by arabinose) had the best expression in Taguchi 2, and strain 59 and the episomal strain (induced by IPTG) had the best expression in Taguchi 1.

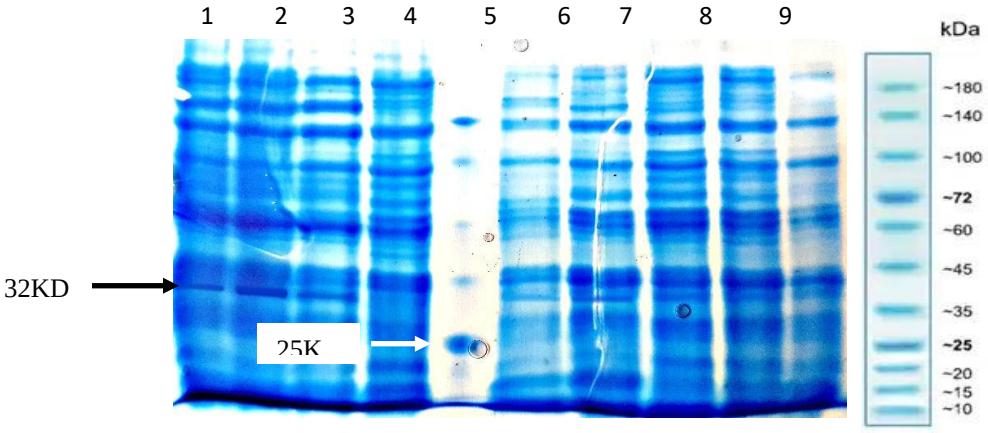


Figure. 6. Expression of human growth hormone integrated into the chromosome of Rosetta gami under the arabinose promoter (clone 6), according to the Taguchi table. 1, 2, and 3 represent Tests 1 - 3 of the table, 4 is the negative control, and 5 is the protein ladder. 6, 7, 8, 9, and 10 represent Tests 4 - 9 of the table.

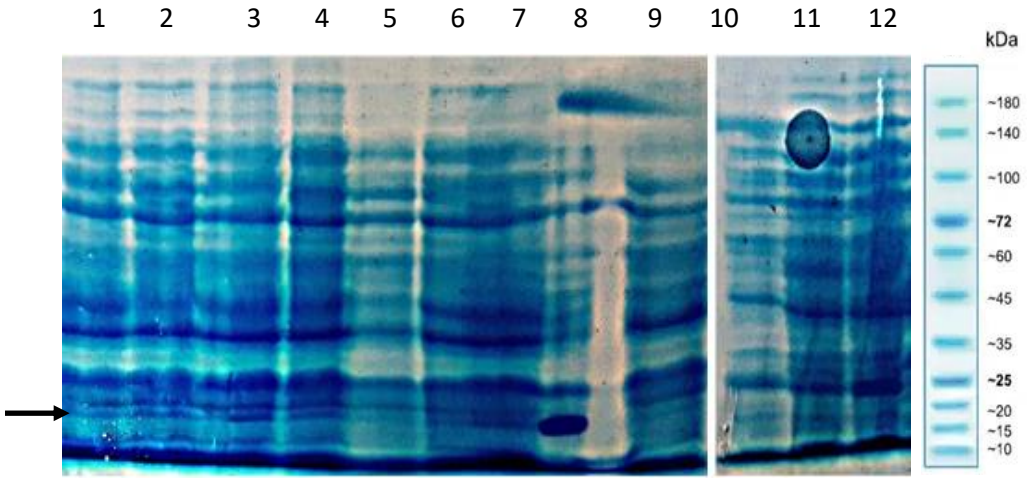


Figure.7. Expression of the human growth hormone integrated into the chromosome of BL21 under the control of the T7 promoter (clone 59), according to the Taguchi table. 12, 11, 10, and 9 show Tests 1 to 4 of the table. 8i s the protein ladder, 7 is the positive control, and 5 is the negative control. 1, 2, 3, 4, and 6 represent Tests 9 to 5 of the table.

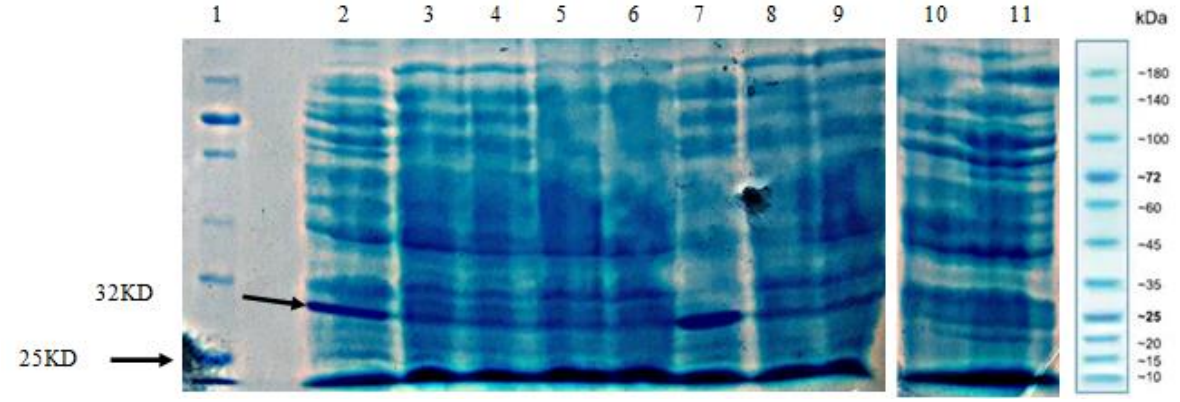
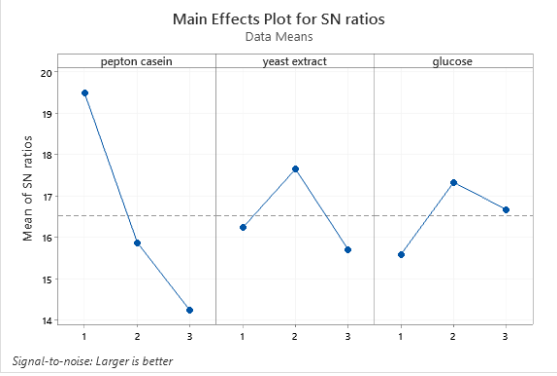


Figure. 8. Expression of human growth hormone by pET28-a Fh8-hgh plasmid in Rosetta-gami, according to the Taguchi table. 1 is the protein ladder, 2, 3, 4, 5, and 6 are Tests 1 to 5 of the table. 7 is the positive control. 8, 9, 10, and 11 are Tests 6 to 9 of the table

Below are the graphs and P-value tables resulting from Taguchi's analysis.

A.

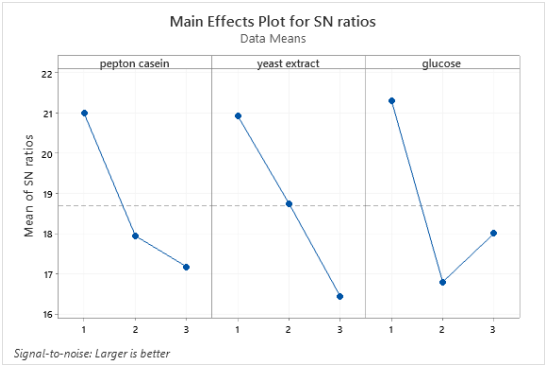


Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pepton casein	2	113.19	56.597	6.53	0.133
yeast extract	2	20.37	10.184	1.18	0.460
glucose	2	16.30	8.148	0.94	0.515
Error	2	17.33	8.664		
Total	8	167.19			

Figure. 9. Signal to Noise Ratio graphs and analysis of variance tables for the three investigated factors at the three tested levels based on Taguchi analyses. A) Genomic strain under the arabinose promoter, B) Genomic strain under the T7 promoter, and C) Plasmid strain under the T7 promoter.

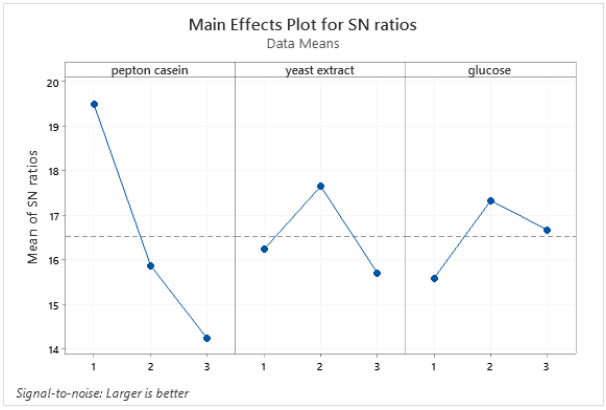
B.



Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pepton casein	2	34.280	17.140	4.43	0.184
yeast extract	2	8.123	4.062	1.05	0.488
glucose	2	5.962	2.981	0.77	0.565
Error	2	7.744	3.872		
Total	8	56.109			

C.



Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pepton casein	2	45.67	22.833	2.70	0.270
yeast extract	2	48.26	24.130	2.85	0.259
glucose	2	54.44	27.222	3.22	0.237
Error	2	16.91	8.454		
Total	8	165.28			

Figure. 9. (Continue)

Discussion

The cost-benefit and mass production of rhGH is always challenging and critical. However, there are many ways to improve the quality and quantity of recombinant protein production in the laboratory and manufacturing stages. In this study, we investigated different growth times to understand the best suitable condition for rhGH expression in bacteria and plasmids. Our finding showed that bacteria with genome-integrated rhGH and rhGH harboring plasmid under the arabinose promoter expressed the highest amount of proteins after overnight and 4 hours of growth in LB medium, respectively. Similar to our finding, Babaei et al. found four hours post-induction as the best time for overexpression in the episomal expression (Keshavarz et al., 2021).

Following that, the best inducer's induction time and concentration were evaluated by adding the inductor to the renewal and, after reaching the logarithmic phase, studying the genomic clone with an arabinose promoter (clone 6) in two simultaneous cultures. Additionally, the impact of multiple different induction times on protein expression showed that the best induction time in this recombinant strain (clone 6) is the beginning of the logarithmic phase and at OD600nm = 0.8-1. Also, the amount of inducer was found to have little effect on production efficiency, but since IPTG is a toxic substance, high concentrations can be detrimental to cells. This result was in agreement with several other studies (Aghaeepoor et al., 2017; Babaeipour et al., 2010;

Kelley et al., 2010; Malakar & Venkatesh, 2011; Muntari et al., 2012; Seyfi et al., 2019). It is necessary to point out that although an increase in the concentration of the inducer, i.e., IPTG, does not have much effect on the expression of the recombinant protein, an increase slows down the growth of the bacteria due to the toxic effect of this compound on the bacterial cell and probably leads to a decrease in the product (Lecina et al., 2013). Knowing the toxic effect of IPTG as well as the high cost of this compound, especially for large-scale production, it is recommended that the lowest optimized concentration, i.e., 0.25 mM in the present study or even less, should be added to the culture medium of recombinant bacteria

In eukaryotes (including humans), there is much evidence of the existence of multiple promoters to regulate the expression of a gene, which leads to the complex control of the expression of that gene in the cell (https://doi.org/10.1186/1471-2164-9-349). Based on our literature search, this arrangement of promoters to regulate gene expression in prokaryotes is rare, if it exists at all. To experience conditions like this in human cells with more than one promoter for one gene, two completely different promoters (namely ara and T7) would have to be placed separately and simultaneously upstream of the growth hormone gene.

Therefore, to investigate this topic and compare the role of promoter type in the expression of growth hormone in two episomal and genomic forms of this gene in *E. coli*, constructs were designed in such a way that both the T7 promoter and the ara promoter were located alone and simultaneously upstream of the rhGH gene. Examining the expression in these conditions showed that the presence of two promoters in the construct resulted in more expression compared to a single promoter, probably due to the ability to use two transcription control systems for a single gene. Moreover, the use of the lowest concentration of antibiotics in the production of recombinant proteins, especially in large-scale production, is necessary for two reasons: firstly, to reduce the cost of production and, secondly, to prevent the involuntary spread of antibiotic resistance among microorganisms. Considering these needs, different concentrations of kanamycin were investigated.

In this experiment, both genomic and episomal rhGH gene carrier strains were cultured in the presence of different concentrations of kanamycin (5,15 µg/ml). The findings, especially in the strain carrying genomic rhGH, showed that the result of protein expression is better in lower amounts of antibiotics (5 µg/ml). It seems that the presence of kanamycin in the culture medium, regardless of its role in maintaining the plasmid, slows down its growth process by imposing an additional metabolic load on the cell, which leads to a decrease in production. Therefore, from this point of view, the first priority is to use a recombinant bacterium carrying rhGH in its chromosome, which does not need kanamycin to maintain the gene.

It is clear that the contents of the culture medium can affect the production of recombinant protein due to its direct effect on bacterial growth. A rich culture medium can support the cell in easy access to its needs. Therefore, the effect of changing the type and amount of Terrific Broth culture medium compounds was compared. Results showed that LB culture media (Luria–Bertani) is one of the most common mediums for recombinant protein expression. However, this medium does not have a specific carbon source, so the bacterial cell density does not reach more than OD600nm2–4, making it an unsuitable option for recombinant protein production. On the other hand, the TB medium has characteristics that make it a good basic culture medium for the production of recombinant protein. In this regard, the recent culture medium was changed and examined to improve the production of recombinant protein. Then, the Taguchi algorithm was used to determine the optimal conditions of this cultivation environment and its changes, and the best conditions were obtained.

The results of the investigation with Taguchi's algorithm showed that the induction of the recombinant bacteria carrying rhGH under the control of the T7 promoter in the plasmid and genome with IPTG had the best expression in the culture medium with Peptone Casein (0.6%), yeast (2.4%), and Glucose (8gr) [all 3 factors at level 1]. Ot should be noted that the induction of bacteria carrying the rhGH gene in the genome with arabinose shows the best expression in the culture medium with the combination of Peptone Casein (0.6%), yeast (1.2%), Glucose (4gr) [pepton casein at level 1 and yeast extract and glucose at level 2]. The most important difference between these two culture mediums, except for the inducer, is the amount of glucose. In recent bacteria, the optimal amount of glucose in the culture medium is half its value in induction with IPTG. It seems that the cause of this significant difference is related to the regulation of the arabinose operon. In this setting, in the presence of a significant amount of glucose and as a result of the lack of cAMP formation, the regulatory protein "cAMP Actinating Protein (CAP)" does not bind to its specific sequence in the regulatory part of the arabinose operon. Therefore, it leads to less transcription, which results in less recombinant protein production.

Although the analysis of variance (P > 5%) did not show the results of these changes to be significant, this investigation on an industrial scale can be considered with the lowest amount of levels to reduce the total cost of production. This decision is consistent with the first level of the factors defined in the studies of this research, regardless of the results of the analysis of variance.

Undoubtedly, the TB culture medium is one of the most common culture mediums in the cultivation of bacteria for the production of recombinant proteins. In this culture medium, the carbon source is glycerol, which was replaced with glucose in the present study, leading to a better expression of rhGH. It seems that the quick and easy consumption of this sugar causes more bacteria growth, resulting in more expression of recombinant protein, which agrees with Kumar et al. (2008) and Malakar and Venkatesh (2011). Furthermore, the increased peptone in this culture environment, as well as the buffering conditions caused by phosphate buffer, provide the basis for better production of recombinant protein (Keshavarz et al., 2021, Kumar et al., 2008; Malakar and Venkatesh, 2011).

4. Conclusions

The results showed that the carbon source, induction time, and antibiotic concentration are important parameters for growth hormone production in *E. coli*. In addition, the simultaneous use of two promoters for the same gene, which has rarely been observed in other studies, and the induction of both together was also investigated and shown to have a tangible effect on expression compared to one promoter and one inducer. Interestingly, the decrease in antibiotic concentration in the genomic strain is associated with an increase in expression. And finally, the presence of glucose in the culture medium instead of glycerol also increases the expression.

Conflict of Interest

The authors declare that there is no conflict of interests.

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Ethical approval

This article does/does not contain any studies with human participants or animals performed by any of the authors. This article does/does not contain any studies with human participants or animals performed by any of the authors.

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