ORIGINAL ARTICLE

Cloning and Expression of Recombinant Human Growth Hormone in *E. coli*: High-Yield Production and Biological Activity Assessment

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ABSTRACT

Background: *The Human Growth Hormone (HGH) is a pituitary gland secretion hormone that stimulates cell division, growth, and repair in humans and animals. It Fis a protein with a molecular weight (MW) equal to 22 kDa.* ***Objectives:*** *Production of HGH in prokaryotic expression system via synthetic gene coding design for HGH.* ***Methodology:*** *Cloning the HGH-designed coding sequence in a pET-3a expression vector for protein synthesis in BL21 DE3 E. coli strain. The expressed recombinant HGH (rHGH) at the batch fermentation level was purified according to HGH MW and its biological activity was assayed.* ***Results:*** *24 h batch fermentation showed a bacterial growth at OD_{595} equal to 1.6 ± 0.023 and the wet cell weight (WCW) was 16.4 ± 0.32 gm/L. Immunodetection confirmation of rHGH using Western blot showed a 22 kDa band at the expected MW. Purification of HGH using anion exchange chromatography revealed a concentration of purified rHGH of about 480.22 µg/ml. Using normal Vero cells, the activity of purified rHGH was 0.228 IU/mg.* ***Conclusion:*** *Native rHGH protein was produced at a large scale with potential biological activity compared to standard HGH; somatotropin, using modern technologies in recombinant DNA.*

INTRODUCTION

Human Growth Hormone (HGH) is a protein with four helical configurations, 191 amino acids, and a molecular weight of 22 KDa [1]. It influences several facets of human metabolism, including lipolysis, the stimulation of protein synthesis, and the suppression of glucose metabolism [2]. It is necessary for proper human growth and development [3]. Enhancing eventual adult height is the main goal of GH therapy in children with GH deficit because of its anabolic agent function [4]. The only growth hormone available for treating GH deficit worldwide is recombinant Human Growth Hormone (rHGH) [5].

For medicinal purposes, pituitary-derived Human Growth Hormone from cadavers was superseded by biosynthetic Human Growth Hormone in 1985 [6]. In 2005, various recombinant growth hormones were approved by the FDA including Omnitrope and Nutropin Depot[7], but their use was suspended until resumption in 2005 [8].

By applying innovative biotechnology processes, Egyptian pharmaceutical companies can use these technologies to produce critically important therapeutics[9]. The acquisition of crucial drugs, leveraging Egyptian expertise and lab-scale production, can generate substantial commercial income, save millions of dollars, and ensure the availability of essential therapeutics [10].

The work aims to clone and express the gene coding for Human Growth Hormone. Cloning the HGH gene from well identified clone and transform this gene into expression vector for protein synthesis, using the prokaryotic system of *E. coli*. The expressed rHGH, will be purified and its biological activity will be assayed at lab batch fermentation level.

METHODOLOGY

HGH gene design

Gene retrieval from database

The NCBI database provides the rHGH full sequence protein with amino acids sequence obtained from the translation of gene coding sequences.

Bioinformatical gene optimization.

The Expasy online tool was employed to study the open reading frame (ORF) of HGH [11], The gene was developed to produce HGH full CDs into the pET-3a

expression vector, containing a stop codon (TAG) at the 3′ end and an additional initiator methionine codon (ATG) at the 5′ end. [12]. Furthermore, the chosen gene was supplemented with distinct restriction endonuclease sequences at the 5′ and 3′ ends, *Nde*I and *BamH*I, respectively to subclone it into the pET-3a vector [13].

Synthetic fragments and primers set designing

Proper primer integrity is essential for determining the length, melting temperature, and hairpin length of an effective amplification [14]. The designed primers were performed using SnapGene® v2.1 software, the HGH gene (~573 bp) was amplified by creating appropriate primers. To conclude, Primer-BLAST tool, was used to assess primer validation and determine the specificity of the primers. Practically, to produce a distinct single band, several annealing temperatures (between 55 and 60°C) were explored in the PCR process using 2x PCR master mix (Thermo Fisher, USA Cat. No. K0171) and the designed primers were purchased from Thermo Fisher [15].

Molecular cloning of the designed HGH gene

Synthetic construct restriction digestion

To prepare the pET-3a expression vector (Biomatik,Cat. No. 69418) for ligation with the digested HGH synthetic construct, it was digested using *Nde*I and *BamH*I restriction enzymes (Thermo Fisher Scientific,Cat. No. ER0581 and ER0051). In a 37 ºC water bath, the digestive processes were incubated for 2 h. Agarose gel electrophoresis was used to examine the digested DNA of both samples and GeneJET Gel Extraction Kit (Thermo Fisher Scientific,Cat. No. K0691) was used to purify the HGH gene and digested pET 3a vector [16].

Synthetic HGH gene insertion into pET-3a

The mass between vector and insert was calculated using an online tool (www.nebiocalculator.neb.com/#!/ligation) to determine the exact concentration of insert needed for ligation (vector to insert, 1:3, 1:6, and 1:7), in the presence of T4 DNA ligase (Thermo Fisher Scientific, Cat. No. EL0011). The reactions were then incubated for 2 h at 16 °C [17].

Ligation product transformation into E. coli competent cells

The synthetic HGH DNA was maintained by transforming recombinant pET-3a vector into TOP10 chemically competent cells (Thermo Fisher Scientific, Cat. No. C404010). Expressing the gene in *E. coli* cells using BL21 DE3 competent strains, (Thermo Fisher, Cat. No. EC0114). The chosen colonies were confirmed using conventional PCR with specific primers for HGH, and the positive clones were stored as glycerol stocks at -80 ºC [18].

rHGH protein expression in prokaryotic cells

Using suitable LB media containing antibiotics that are specific to pET-3a (ampicillin 50 µg/ml and chloramphenicol 25 µg/ml, Sigma Aldrich, Cat. No. A5354 and 56-75-7), induction was carried out [19]. The Cultivated medium was incubated in an environmental shaker at 37 °C and 250 rpm of shaking. Each culture's bacterial growth was tracked using turbidity measurement until it reached an OD_{600} of roughly 0.6 [20]. Induction was then carried out by adding β-D-1-thiogalactopyranoside; IPTG (Sigma Aldrich, Cat. No. 67-93-1) to a final concentration of 2 mM and letting the cultures incubate for at least 3 h while shaking them again at 250 rpm in an environmental shaker [21]. The cultures were then centrifuged for 10 minutes at 7000g and 4ºC. Using 12% SDS-PAGE, the recombinant protein expression was determined, and the gel was stained using the Commassie staining method to detect the protein bands [22].

Identification of the expressed rHGH by WB

The 12% SDS-PAGE was transferred to an immobilized nitrocellulose membrane (Sigma Aldrich, Cat. No. HATF07850) using 1× transfer buffer (25 mM Tris-HCL, 192 mM glycine, and 20% (V/V) methanol) at 80 V for 2 h. After that, the membrane was blocked for two hours with 5% bovine serum albumin; BSA (Sigma Aldrich, Cat. No. 9048-46-8) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05 % Tween 20). Next, the primary antibody (rabbit poly anti-HGH, Abcam, Cat. No. ab126882) was incubated for 1 h at room temperature with gentle stirring [23]. After three TBST washes, the blot was incubated for 1 h at room temperature with a secondary antibody (antirabbit IgG alkaline phosphatase conjugate, Abcam, Cat. No. ab6722) with dilution of 1:15000. After washing with TBST three times, the membrane was submerged in an NBT/BCIP-ready solution (Sigma Aldrich, Cat. No. 72091) for color development [24].

Optimization of batch fermentation for rHGH production

Using benchtop Bioflo110 bioreactor 7.5 L, batch fermentation was carried out. The agitation speed, temperature electrode, pH electrode, and dissolved oxygen (DO_2) of the bioreactor were optimized for large-scale rHGH production [25]. The inoculum of BL21 DE3 cells harboring pET-3a/HGH was prepared (500 ml) for 1:10 dilution in LB medium containing suitable antibiotics (5 L). To adjust pH throughout batch fermentation, two buffers, 25% H3PO4 (acid) and 30% NH4OH (base), were connected to the bioreactor. All environmental parameters were adjusted to the required set points; standard settings were 37 °C, pH 7.0, 500 rpm of agitation, and 100% air saturation with DO_2 [26]. Samples obtained at 3 h intervals were analyzed for OD_{600} to track growth, and polyacrylamide gel analysis was used to find the expression of rHGH. The following formula was used to determine the growth rate (µ) of the batch fermentation process: $\mu = (\ln OD_2 - \ln OD_1)/T_1\text{-}T_2$ [27].

The induction of rHGH expression was performed using 2 mM IPTG. Cells harvest was performed through centrifugation for 15 minutes at 4 °C and 10,000 g.

FPLC chromatography of rHGH.

Solubilization, and refolding of rHGH inclusion bodies (IBs)

Following induction, the Palmer and Wingfield approach was followed to disrupt the cells containing rHGH aggregates to purify IBs using a strong chaotropic agent [28]; GdmCl, and then in vitro refolding was done using rapid dilution method in a particular reaction solution including L-arginine, which stabilizes proteins against aggregation, and a redox buffer (3 mM reduced glutathione and 1 mM oxidized glutathione) for the creation of disulfide bonds [29]. The Bradford assay was utilized to determine the total protein content in the solubilized IBs and refolded protein using the BSA standard curve [30].

Purification rHGH using ion exchange chromatography

Firstly, centrifugation was carried out at 20.000g, 4 ◦C, and 30 min to remove unfolded proteins to purify refolded rHGH. According to pI of HGH, three columns were examined to detect the best purifying method; HiTrap-DEAE, Hitrap QFF and Hitrap QxL (Cytiva, USA). The buffers utilized were **A:** 20 mM tris base with suitable pH and **B:** 20 mM tris base with 1 M NaCl at a flow rate of 1 ml/min for 20 column volumes, the sample volume injected was 500 ul of rHGH [31].

Bioassay for rHGH protein employing in vitro cell proliferation.

A modified methodology was followed to examine the cell proliferation induction of rHGH on a normal mammalian cell line; Vero cells (Vacsera, ATCC: CCL-81) [32]. Cells were cultured as adhering cells in a tissue culture flask that contained 30 ml of liquid growth medium of Dulbecco's modified Eagle medium (DMEM) with L-glutamine and Earle's salt, 10% foetal bovine serum (FBS), 1% antibiotic (streptomycin/penicillin) fungizone solution, and 1% HEPES buffer (Lonza – Bioscience). In a humidified environment, the cells were cultured at 37 °C in a 5% CO_2 incubator. At the semi-confluent stage, Vero cells were subcultured in 2% DMED (including 2% serum, 1% antibiotic, and 1% HEPES), in 96-well tissue culture plate in volume of 100 µl containing 7000 cells/well and 5000 cells/well. The cells were treated with 50 µl of the tested sample and standard [33]. Two-fold serial dilutions of standard protein; Somatropin (SEDICO), and the produced rHGH were made, ranging from 150 µg/ml to 1.17 µg/ml. were then incubated (5% CO2). After the incubation at 37 **º**C for 24 h in a 5% CO_2 incubator, crystal violet (5 mg/ml) was used to stain the cells. The optical density measurements were measured at 490 nm when the plate was placed in the BioTek EL808 Microplate ELISA Reader. Every sample was examined three times [34].

Statistical analysis

The study used GraphPad Prism 8 for data analysis, tracking fermentation processes using multiple regression analysis, and paired t-test for significance, determining a p-value of less than 0.05 [35].

RESULTS

HGH gene design

Gene retrieval from database

The pharmaceutical rHGH full sequence 191 amino acids retrieved from NCBI (Synthetic construct growth hormone gene, complete coding sequence (CDs) from GenBank: ID: MF093689.1, and the coding gene sequence (573 b.p).

Bioinformatical gene optimization.

The open reading frame (ORF) is fully optimized without interruption and chimeric contaminations to the Standard Prokaryotic (Bacterial) Expression system. The designed gene was optimized using a pairwise sequence to match with triplet codon and amino acids for *E. coli* expression (Codon Usage) as follows:

The optimized HGH Nucleotide's sequence

ATG TTT CCC ACA ATC CCA TTA TCC CGC CTT TTT GAC AAC GCT ATG
CTT CGT GCT CAT CGC CTT CAC CAA CTG GCG TTC GAC ACG TAC CAG
GAG TTC GAA GAG GCG TAC ATT CCC AAA GAG CAA AAA TAC AGT TTC
TTA CAA AAT CCT CAG ACG TCC CTG TGC TTC AGC GAG TCC ATC CCC
ACC CCC TCA AAC CGC GAA GAG ACT CAA CAA AAG TCC AAT CTG GAG
TTG TTG CGT ATT TCT CTT TTG CTG ATT CAA TCT TGG CTG GAA CCC
GTG CAG TTC CTG CGT TCT GTA TTC GCA AAC TCC CTG GTG TAC GGT
GCA AGC GAC AGT AAC GTC TAC GAT CTG CTT AAG GAC CTG GAG GAA
GGG ATT CAG ACA CTT ATG GGG CGT TTG GAG GAC GGG TCA CCT CGT
ACA GGA CAA ATT TTT AAG CAG ACG TAC TCG AAA TTT GAC ACC AAC
TCT CAC AAC GAT GAT GCC TTG CTG AAG AAC TAC GGT TTA TTG TAC
TGT TTT CGC AAA GAT ATG GAT AAG GTT GAG ACG TTC TTG CGT ATT
GTG CAG TGC CGC AGC GTA GAG GGC AGC TGC GGC TTT **TGA**

The optimized HGH protein's sequence

MFPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPTPSNREETQQKSNLELLRISLLLIQ
SWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYC
FRKDMDKVETFLRIVQCRSVEGSCGF

The Results showed that 116 codons were changed to match *E coli* bacterial expression system, so that it can be translated more accurately and efficiently.

Synthetic fragments and primers set designing

The "SnapGene" software was used for fragmentation and primer design, dividing the DNA into five fragments with varying sizes. The first fragment was located from base 1 to base 138, the second from base 139 to base 253, the third from base 254 to base 355, and the fifth from base 469 to base 585.

The designed primers were used to synthesize HGH gene of interest. **Fig 1.** Results obtained from SnapGene Software showed that 18 primer pairs with melting temperatures ranging from 54 ºC to 58 ºC, as shown in **Table 1**.

The optimized annealing temperature for the chosen primers was determined to be 58 ºC, where a sharp band of 0.585 kb was identified corresponding to HGH coding gene sequence MW based on the temperature grade. **Fig 2(a)**

Molecular cloning of the designed HGH gene

Synthetic construct restriction digestion

NdeI and *BamHI* restriction enzymes were used to digest *pET-3a* into a linear form for ligation with digested HGH-PCR, and the HGH gene sequence was purified for further ligation. **Fig 2(b,c)**

The concentration of cleaned digested HGH DNA was 74.7 µg/µl and the concentration of digested pET-3a was 72.5 µg/µl. The ideal molar ratio for gene: vector to be ligated was 1:3, respectively. However, the ratio 1:6 gave a sufficient number of transformed colonies after ligation as the given equation formula: 0.016 x 3 x 6 = 0.288/0.19. The final ratio was 1.5 µl PCR: 3 µl plasmid.

rHGH protein expression in prokaryotic cells

After transformation in TOP10 cells, the plasmid was isolated and purified for characterization. Two clones were analyzed and revealed a band at the expected MW ~ 5183 bp as shown in **Fig 2(d)**. PCR using specific HGH primers on positive clones showed a distinct band observed with MW of 588 bp **Fig 2(e)**. The digested recombinant pET3-a with both *Nde*I and *BamH*I was compared with the undigested pET-3a and checked by running on 1% agarose gel electrophoresis to show the complete digestion confirmed by detection of HGH band at the expected MW. **Fig 2(f)**

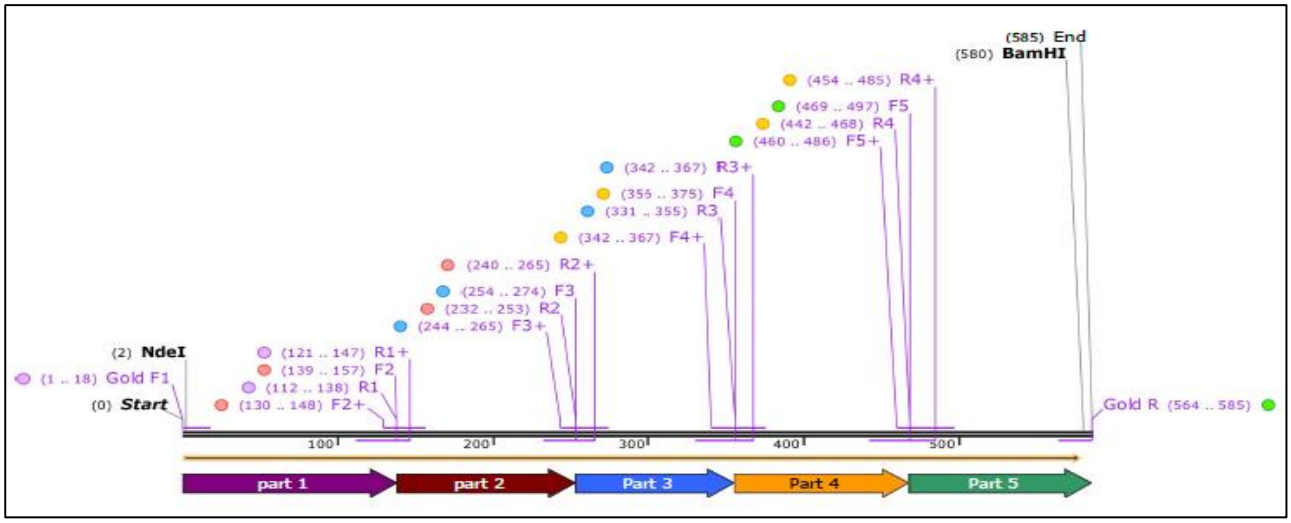

Fig 1: The HGH gene was split into 5 fragments, each covered by overlapping primers to assess gene coverage and locate restriction enzymes NdeI and BamHI. The primers included the product length (579 bp), stop codons and restriction enzyme cutting loci.

Table 1: Primers designed for synthetic optimized HGH coding gene sequence

#	Primer	Length	Sequence	Binding Sites	Tm
1	Gold F1	18-mer	CATATGCCGACCATTCCG 56% GC / 5419.6 Da	1 .. 18	56°C
2	R1	27-mer	CAAAAAGCTATATTTCTGTTCTTTCGG 33% GC8239.5 Da	112 .. 138	55°C
3	R1+	27-mer	CGGATTCTGCAAAAAGCTATATTTCTG 37% GC / 8273.5 Da	121 .. 147	57°C
4	F2+	19-mer	AGCTTTTTGCAGAATCCGC 47% GC / 5778.8 Da	130 .. 148	57°C
5	F2	19-mer	CAGAATCCGCAGACCTCAC 58% GC / 5726.8 Da	139 .. 157	57°C
6	R2	22-mer	GAATCAGCAGTAAGCTAATGCG 45% GC/6792.5 Da	232 .. 253	57°C
7	R2+	26-mer	CTAACCAACTCTGAATCAGCAGTAAG 42% GC / 7932.2 Da	240 .. 265	58°C
8	F3+	22-mer	CTGCTGATTCAGAGTTGGTTAG 45% GC 6796.5 Da	244 .. 265	55°C
9	F3	21-mer	AGAGTTGGTTAGAACCGGTTC 48% GC / 6501.3 Da	254 .. 274	56°C
10	R3	25-mer	CCAGATCCTTTAACAGATCATACAC 40% GC / 7554.0 Da	331 .. 355	56°C
11	F4+	26-mer	GTTAAAGGATCTGGAAGAAGGCATTC 42% GC / 8083.4 Da	342 .. 367	58°C
12	R3+	26-mer	GAATGCCTTCTTCCAGATCCTTTAAC 42% GC7856.2 Da	342 .. 367	58°C
13	F4	20-mer	AAGAAGGCATTCAGACCCTG 50% GC 6135.1 Da	356 .. 375	57°C
14	R4	27-mer	TGCATCATCATTATGACTATTGGTATC 33% GC / 8239.5 Da	442 .. 468	54°C
15	R4+	32-mer	CCATAATTTTTCAGTAATGCATCATCATTATG 28% GC / 9747.5 Da	454 .. 485	56°C
16	F5+	27-mer	GATGATGCATTACTGAAAAATTATGGC 33% GC8346.5 Da	460 .. 486	56°C
17	F5	29-mer	TTACTGAAAAATTATGGCTTACTGTATTG 28% GC 8920.9 Da	469 .. 497	54°C
18	Gold R	22-mer	GGATCCTTAAAAGCCACAAGAG 45% GC 6761.5 Da	564 .. 585	55°C

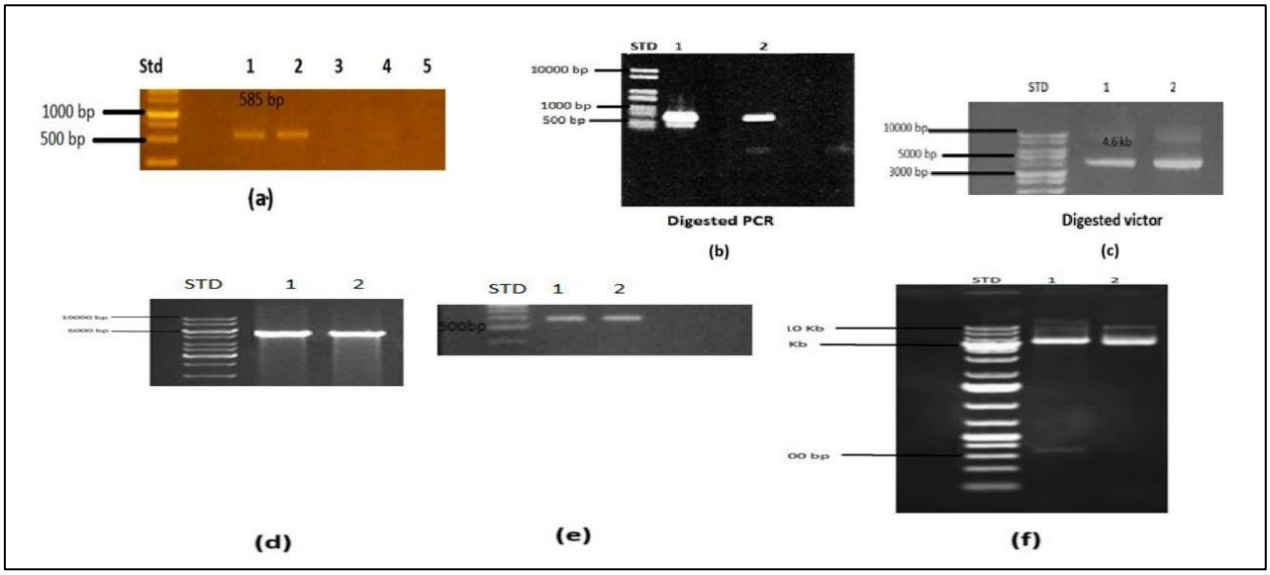

Fig 2(a): 1.5% Agarose gel electrophoresis of HGH gene obtained by gradient PCR appeared at molecular size 585bp. STD: 10 kb DNA Ladder. Lanes 1-5: PCR amplified products using different annealing temperatures (lane 1: 56 ºC, lane 2: 58ºC, lane 3: 50 ºC, lane 4: 55ºC, lane 5: 60 ºC)**. (b,c):** 1% agarose gel electrophoresis of digested HGH and pET-3a for further ligation. **(b)** Digested HGH (PCR): Lane STD: DNA ladder of 1 kb. Lane 2: Cleaned HGH gene with the MW of 579 bp. **(c)** Digested pET-3a vector: Lane STD: DNA ladder of 1 kb. Lane 1 & Lane 2: Cleaned pET-3a vector with a 4.6 kb molecular size. **(d):** 1% Agarose gel electrophoresis of plasmid miniprep purification: STD: 1Kb DNA ladder, Lane 1 and 2: pET-3a vector containing HGH gene at MW 5183 bp. **(e)**: Agarose gel electrophoresis (2%) of the HGH gene isolated by PCR appeared at molecular size 588 bp. STD: 1 kb DNA Ladder, Lanes 1&2: PCR amplified product using annealing temperature 58 ºC with a molecular size of 588 bp, lane 3: negative control.**(f):** Electrophoresis on 1% agarose gel showed digested cloning vector with NdeI and BamHI restriction enzymes. STD: 1 Kb DNA ladder. Lane 1: Digested recombinant plasmid of MW size 4640 bp and a band of HGH at 580 bp. Lan 2: undigested products show a plasmid of 5228 bp.

After induction using 2 mM IPTG, a sharp protein band of 22 KDa was achieved. The protein was identified in the pellet lysate of bacterial-induced clones, but no protein band was discovered in the non-induced bacteria. **Fig 3(a)**

Identification of the expressed rHGH by WB

Confirmation of successful recombinant HGH expression using a specific antibody showed that a single band with a MW of 22 kDa was identified on the membrane. These findings show that recombinant rHGH was produced in the lysate of bacterial cells carrying the recombinant plasmid pET-3a/HGH. Thus, this data verified the HGH gene's cloning, and expression in the prokaryotic *E. coli* system.

Optimization of batch fermentation for rHGH production

Culture samples from the bioreactor showed a decrease in DO2 value from 0h to 24h, **Table 2**, indicating nutrient medium consumption by cells. WCW increased directly with time, **Fig 4(a)**, and statistical analysis showed high significance between DO2, OD600, and WCW parameters. rHGH expression was evaluated using 12% SDS-PAGE, **Fig 3(c)**.

Table 2: The batch fermentation parameters of pET 3-a/ rHGH

Parameter	0h	3h	6h	9h	12h	24h
DO_2	98	62.6	61.8	42.4	52.5	100
OD_{600}	0.048	0.41	1.3	1.6	1.99	1.6
(WCWg/L)	2.1	6.3	8.31	10	14.8	16.4

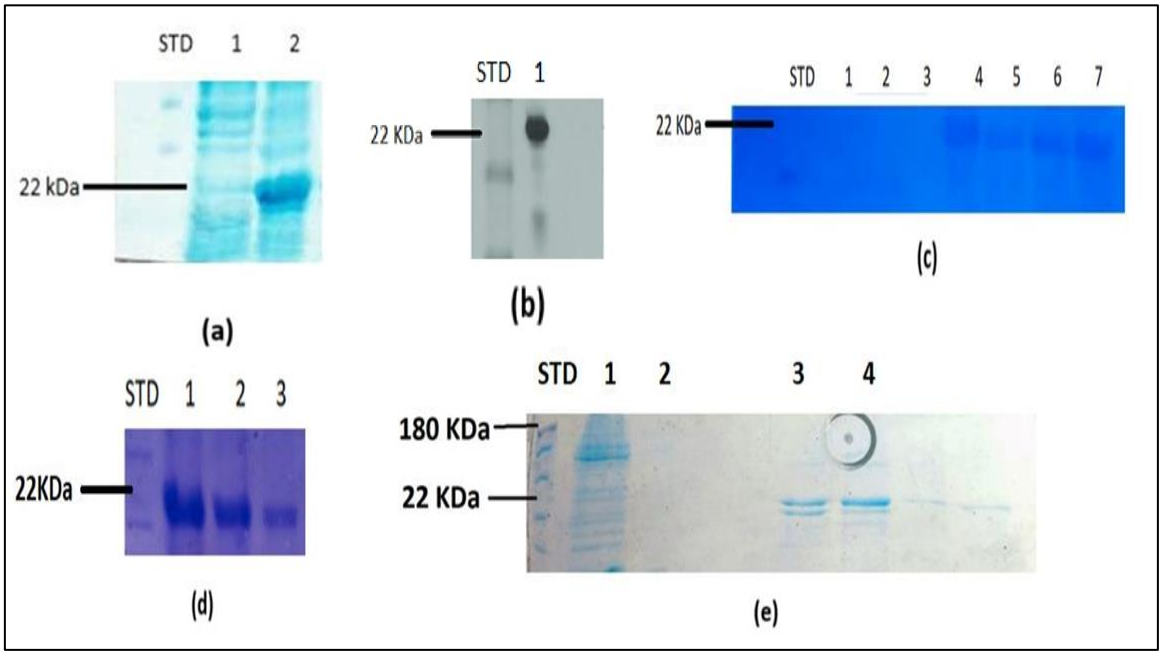

Fig 3: Shows the Results of SDS-PAGE of the expression of rHGH,**(a)** after Induction by IPTG in a bacterial cell lysate **(c)** Fermentation Samples in 3h Intervals**, (d)** Solubilization of IB in 8M GdmCl and, **(e)** purification on a HiTrap-column. **(b)** Shows the Western blot analysis in Bl21 DE3 expression system. they all Show The expected Band at 22 kDa.

Gel-filtration chromatography of rHGH.

Solubilization, and refolding of rHGH inclusion bodies (IBs)

5 gm of WCW of induced bacterial pellet obtained from batch fermentation yielded about 1.5 gm of IBs which comprise 30% of total weight. Three concentrations of GdmCl were tested for IBs solubilization; 6, 7, and 8 M GdmCl. The refined IBs containing rHGH were entirely dissolved at 8 M GdmCl as shown in **Fig 3(d)**. After centrifugation, the dissolved IBs was measured to be 480.22 mg/ml.

Purification rHGH using ion exchange chromatography

According to the isoelectric point (pI) of HGH which is 5.12, an anion exchange chromatographic column was employed under several evaluated experimental circumstances to purify the rHGH.The sample had a protein content of 480.22 mg/ml in buffer with pH 8. Either HiTrap-DEAE column were used for the anion exchange chromatography procedure. **Fig 4(b)** revealed that three peaks were separated: two of the peaks represented all proteins, and one of the peaks was a good peak that represented the pure rHGH protein. Following the washing process, these fractions were removed from the column as indicated by the chromatogram in **Fig 4(b)**.

Fig 3(e) Shows the three peaks obtained from Akta were separated on 15% SDS-PAGE. Using a Nanodrop 2000C spectrophotometer, the concentration of isolated rHGH protein from the HiTrap - DEAE column was determined. The ultimate concentration of concentrated and purified rHGH protein was 480.22 µg/ml, according to the results.

Bioassay for rHGH protein employing in vitro cell proliferation.

The rHGH bioassay demonstrated the safety and promotion of Vero cell proliferation with dosages ranging from 1.17 µg/ml to 150 µg/ml, using only 150 and 75 µg/ml concentrations. (**Table 3**). The study found that purified rHGH, with a higher activity than somatropin at all tested doses, exhibited better proliferation activity and was more active than ordinary rHGH, with a concentration of 18.75 µg/ml.

Table 4 demonstrate a clear dose-dependent decline in the biological activity of purified rHGH with decreasing concentration. Furthermore, rHGH exhibits higher specific activity at a seeding density of 5000 cells compared to 7000 cells, indicating enhanced responsiveness at lower cell density.

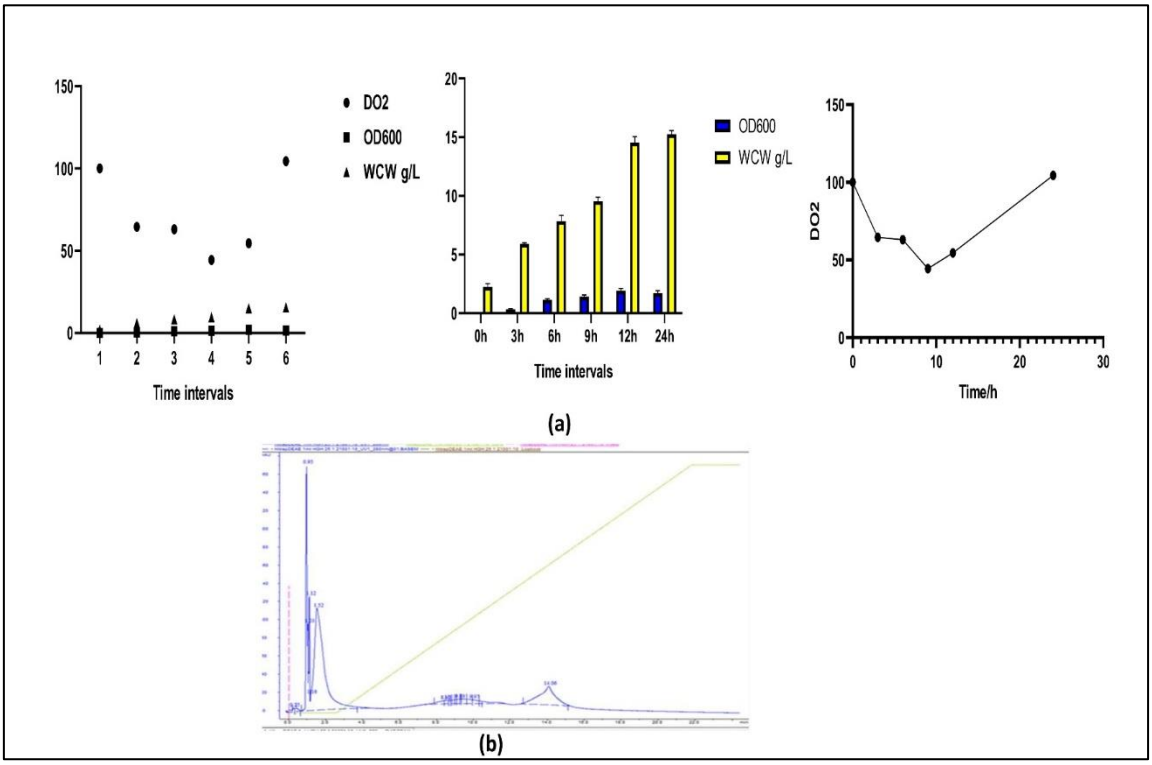

Fig 4(a): Correlation between physical and fermentation parameters in accordance with time. **(b):** Chromatogram for rHGH protein purification using HiTrap-DEAE column, Chromatographic purification of the refolded rHGH using column (1ml). The chromatographic chart measured at 280 nm. The chromatogram showed that the target peak harboring the rHGH was shown at RT 14.05 min. The Y-axis represents OD 280 and X-axis represents time in min; green line represents the gradient illusion buffer.

Table 3: Comparison between the Effect of Standard and purified rHGH on cell proliferation at 2 cell counts

Concentration µg/ml	OD492 (7000 cells/well) Standard rHGH	Mean of the cell proliferation ratio Standard rHGH	OD492 (7000 cells/well) Purified rHGH	Mean of the cell proliferation ratio Purified rHGH	OD492 (5000 cells/well) Standard rHGH	Mean of the cell proliferation ratio Standard rHGH	OD492 (5000 cells/well) Purified rHGH	Mean of the cell proliferation ratio Purified rHGH
100	0.162	2.13	0.23	4.02	0.101	1.88	0.16	3.03
50	0.141	2.31	0.21	3.66	0.078	1.73	0.17	3.01
25	0.113	1.77	0.14	3.18	0.099	1.62	0.14	2.80
12.5	0.084	1.42	0.122	2.15	0.071	1.33	0.07	1.77
Cell control	0.047	-	0.05	-	0.060	-	0.060	-

Table 4: Activity of purified rHGH in IU at two cell counts 7000 and 5000 cells generated from the equations obtained from the standard curve of standard rHGH readings.

Concentration µg/ml	Activity of standard in IU	Activity of purified rHGH in IU at count 7000cells	Activity of purified rHGH in IU at count 5000cells
100	1000	2604.17	4694.934
50	500	2256.94	4419.604
25	250	1736.11	1941.63
12.5	125	763.89	289.65

DISCUSSION

Therapeutic protein medications are crucial for patients seeking innovative treatments. Recombinant protein therapies have been authorized for several clinical indications, including cancer, autoimmunity/inflammation, infectious agents, and genetic diseases [36].

Recombinant HGH is currently produced through microbial expression systems, predominantly *Escherichia coli*, due to its rapid growth rate, well-characterized genetics, and ability to express heterologous proteins at high yields. A commonly reported production yield is 1.6 g/L of rHGH using an *E. coli* system with a biomass of 25 g dry cell weight per liter [37].

In the present study, codon optimization of the HGH gene was carried out to enhance expression efficiency in *E. coli*. Rare codons were systematically mutated to match the host's codon usage bias, resulting in a gene sequence with 79% identity to the wild-type HGH gene. The expressed rHGH protein was successfully identified via Western blot analysis, and batch fermentation achieved a cellular growth rate of 42.4% under optimized conditions [38].

Our workflow began with the retrieval of the human growth hormone (HGH) gene sequence from the NCBI database, followed by bioinformatic optimization to adapt the coding sequence for expression in Escherichia coli. Subsequently, we designed a primer set to enable the synthesis of the optimized HGH coding region. This strategy is comparable to that employed by Dolgova *et al*. (2017) for the synthesis of the Fel d 4 gene, which, at 581 base pairs, is similar in size to our target sequence. In their study, the sequence of Fel d 4 was retrieved from GenBank and codon-optimized for E. coli expression using the DNAWorks software. Oligonucleotides were then designed and manually refined using the OligoAnalyzer tool [39]. In contrast, we utilized SnapGene software for both optimization and primer design. While both approaches share the same conceptual pipeline—comprising sequence retrieval, codon optimization, and oligonucleotide design—the variation in software tools may influence design efficiency, primer characteristics, and downstream synthesis fidelity.

Hao Song et al. achieved high-purity expression of recombinant human growth hormone (rHGH) in *Escherichia coli* using the phoA promoter. This promoter is repressed in the presence of excess phosphate and is activated under phosphate-limited conditions. In addition to regulating gene expression, phosphate also enhances glucose assimilation and stimulates microbial growth. Unlike the current study, which utilized a different *E. coli* strain, Song et al. employed the *E. coli* W3110 strain. Notably, they omitted the inclusion body solubilization step, as the heterologous protein was secreted in a soluble form. Their reported final yield was 1.4 mg/L.[40]

A. Doozandeh et al. employed a similar workflow for the expression of recombinant human growth hormone (rHGH); however, several methodological differences were noted. For gene optimization, they utilized the GeneScript online tool. Additionally, they selected different restriction enzymes—*XhoI* and *NcoI* for cloning. The expression vector used in their study was pET22b. During the purification process, they employed separate dialysis bags, a Poly-Prep chromatography column, and a Ni–NTA agarose matrix. To evaluate the bioactivity of the purified rHGH, they applied the classical sandwich ELISA method. Their reported final yield was approximately 1 g/L [41].

Shaowang Hu et al used HepG2/IGF-1 cell to measure the Biological Activity of PEGylated Recombinant Human Growth Hormone[42], while Wenrong Yao et al used human embryonic kidney 293 (HEK293) cell line to measure the Bioactivity Determination of Long-Acting Fc-Fusion Recombinant Human Growth Hormone [43]

CONCLUSIONS

This study successfully produced physiologically active rHGH using *E. coli* bacteria, which can be used for therapeutic purposes and scientific research due to its high expression, purity, and cell proliferation activity.

Declarations:
Consent for publication: Not applicable
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