Production of excreted human epidermal growth factor (hEGF) by an efficient recombinant *Escherichia coli* system


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Running Title: Excretory hEGF production by *E. coli* K-12

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Abstract

Recombinant *Escherichia coli* JM101 strains harboring plasmids pWKW2 or lacUV5par8EGF, both encoding human epidermal growth factor (hEGF), were used in fermentations to optimize levels of excreted hEGF. Medium composition, inducer level, growth stage at induction, and culture conditions, were optimized with respect to volumetric production of the recombinant protein. MMBL medium, with glucose at 5g/l, and tryptone as nitrogen source, was chosen. Isopropyl-β-D-thiogalactopyranoside (IPTG) concentrations of 0.1mM for *E. coli* JM101[pWKW2] and 0.2mM for *E. coli* K-12 JM101[lacUV5par8EGF], were found to give the best hEGF production levels. The volumetric yields of hEGF were maximal when the cultures were induced in the mid-logarithmic phase. Growth temperature had a significant effect on hEGF yield. A simple continuous fed-batch process for cultivation of *E. coli* JM101[pWKW2] was developed. The maximum attained concentration of excreted hEGF in continuous fed-batch cultivation was 325mg/l, as compared to 175mg/l in batch cultivation. The hEGF produced from the continuous fed-batch cultivation was substantiated by SDS-PAGE and immunoblotting.

Introduction

Human epidermal growth factor (hEGF), which is considered to be identical with human urogastrone [1], is a small polypeptide of mol wt 6201, with 53 amino acid residues. It is a potent inhibitor of gastric acid secretion [2] and promotes epithelial cell proliferation both in vitro and in vivo [3]. Many previous studies [4-8] have attempted to address the technical challenge of achieving high volumetric hEGF productivity. It is desirable that recombinant product be secreted into the cell growth
medium [9-15]. Purification would then be simpler than for an intracellular protein as the product would not be contaminated with cytoplasmic components. In addition, the formation of inclusion bodies would be avoided and possible toxic effects of the hEGF polypeptide product on the host cell would be reduced. An ampicillin (Amp)-resistant E. coli strain JM101[pETacEGF] was used to produce secreted hEGF [4,11]. A synthetic gene for hEGF was fused with the signal peptide of E. coli alkaline phosphatase to direct mature hEGF to the E. coli periplasm [5]. Excreted proteins are usually stable and not significantly degraded, because of low levels of extracellular protease activity [9]. In 1998, the cost of commercially available hEGF ranges from US $210 to US$1020 per milligram. Growing evidence that hEGF may be used to accelerate the healing of epithelial tissues, and the potential of hEGF for cosmetic applications [16, 17] has created considerable excitement in this field. The expense of commercially available hEGF has led to the search for a new economical source.

A DNA vector, suitable for use in E. coli, and enabling heterologous proteins to be excreted to the culture medium, has been described by us previously[12, 15]. With this construct, the excretion of heterologous proteins from E. coli can be achieved consistently and for a wide variety of proteins, without assistance from carrier proteins or membrane-lytic agents, and by using "healthy" hosts rather than mutant strains having defective outer membranes. Here, this system was used to achieve hEGF excretion from the E. coli JM101 strain. Culture and induction conditions were investigated and optimized with respect to volumetric production of the protein. As an approach to developing a better system for large-scale production, we focussed on batch and fed-batch cultivations using glucose as carbon source. Fed-batch fermentations have been favoured for production of heterologous proteins by recombinant microorganisms. Such fermentations afford tight control over
environmental parameters, and hence improve overall product yield compared with simple batch cultures [18-21]. In this work a simple feeding strategy for fed-batch recombinant cell fermentation was developed to maintain high excretory production of hEGF.

Materials and Methods

**Strains and plasmids**

*E. coli* strain JM101 [22] was the chosen host. Two plasmids encoding hEGF were used in this work. Plasmid lacUV5par8EGF [Huang et al. manuscript in preparation] contains the hEGF gene (preceded by the signal sequence of the *E. coli* ompA gene) under the control of the lacUV5 promoter, on a plasmid which also encodes the lac repressor (thus: the plasmid contains the lacI' gene). Plasmid pWKW2 lacks the plasmid-borne lac repressor, but contains a plasmid stabilization system (ytl2-incR) encoded in a large plasmid, pSLT, normally resident in *Salmonella typhimurium* [15].

**Culture media**

2xYT medium contained 16g/l Difco tryptone, 10g/l Difco yeast extract, and 10g/l NaCl, and (sometimes supplemented with 70µg/ml Amp), was used for fermentor inoculum preparation. For solid medium, Difco Bacto-agar was added to the medium to a final concentration of 1.5% (w/v).

A modified version of the MBL medium [4] abbreviated as MMBL was used in both shake flask and fermentor experiments. The medium composition was: glucose,
(variable); Difco tryptone, 20g/l; Difco yeast extract, 10g/l; KH₂PO₄, 3.5g/l; K₂HPO₄, 5g/l; (NH₄)₂HPO₄, 3.5g/l; NaCl, 5g/l; MgSO₄·7H₂O 1g/l; ampicillin (when used) 70µg/ml; trace metal solution, 3ml/l. The trace metal solution was prepared as follows: FeCl₂·6H₂O, 0.162g/l; ZnCl₂·4H₂O, 0.0144g/l; CoCl₂·6H₂O, 0.12g/l; Na₂MoO₄·2H₂O, 0.012g/l; CaCl₂·2H₂O, 0.006g/l; CuSO₄·5H₂O, 1.9g/l; H₃BO₃, 0.5g/l and HCl (37%, w/v), 37ml/l.

_Shake flask experiments_

Seed cultures were prepared by inoculating single colonies from a freshly spread plate into 30ml of 2xYT liquid medium with 70µg/ml Amp. The inocula were grown at 30°C for about 8h on a rotary shaker at 200rpm. The final A₅₆₀ was typically about 1-2. Flask cultures of 30ml were inoculated (10%(v/v)) (in 250ml flasks) and shaken at 30°C at 200rpm in an orbital shaker. Induction with IPTG, at various concentrations, was effected at different cell densities.

_Inoculum preparation for fermentor experiments_

Inocula for fermentors were obtained by two-stage cultivation. In the first stage, a 250ml flask containing 30ml of 2xYT medium was inoculated with a single colony from a freshly-spread plate, and cultured for 8h at 30°C at 200rpm. In the second stage, a 1l flask containing 300ml of 2xYT medium was inoculated with the culture from the first stage and incubated for 3h at 30°C at 200rpm.
Fermentation studies

All fermentations were carried out in 2l bench top fermentors (B Braun BIOSTAT B). Cultures were grown in the MMBL medium. Antifoam A (Sigma) was used. Each fermentor initially contained 900ml medium and was inoculated with 100ml of culture (freshly prepared as described above). The dissolved oxygen level was determined with a steam-sterilizable polarographic electrode, and the culture pH measured with a steam-sterilizable pH probe (both electrodes: Ingold Electrodes, Inc.). The pH of the medium was controlled at pH 6.8 until induction by the automatic addition of H₂SO₄ and NaOH. Cultures were induced with IPTG at various concentrations when biomass attained a level corresponding to A₅₀₀ value of 8.0. During all tests, the agitation speed and air flow rate were adjusted to keep the dissolved oxygen level above 5% relative to saturation. Samples were taken at various intervals to measure cell concentration, hEGF level, reducing sugar level and plasmid stability. The samples were centrifuged at 12000g for 5min to prepare cell supernatants.

In intermittent fed-batch tests, batch cultivation was continued until the consumption of reducing sugars in the fermentation medium was complete, after which 3g of glucose and 0.6g of MgSO₄·7H₂O were added. These additions were repeated when the residual sugar concentration in the medium was again exhausted.

In constant fed-batch cultures, the growth medium initially contained 2g/l glucose. Fed-batch fermentation was carried out by adding concentrated medium to the fermentor, when the glucose in the initial batch had been completely consumed. The feeding solution consisted of 200g/l glucose and 20g/l MgSO₄·7H₂O. The flow rate of
the feeding solution was adjusted so that the pH was maintained at about 6.8, based on the observation that the pH begins to rise when the principal carbon substrate is depleted [23, 24]. To compensate for possible pumping errors, on-the-spot on-line calibration was performed at the beginning of the feeding phase.

Analytical procedures

Cell density ($A_{600}$) was measured spectrophotometrically. Glucose concentration (g/l) in the culture medium was determined using a clinical glucose analyzer. (YSI 2300 STAT PLUS; YSI Instruments Inc., OH.)

Plasmid stability was assessed by spreading diluted culture samples (in duplicate) on selective (70μg/ml Amp) and nonselective 2xYT agar plates. The agar plates were incubated at 30°C for 20h. After incubation, colonies on each plate type were counted (using a suitable dilution of the original culture). The plasmid stability was calculated by taking the ratio of the average colony counts on the 2xYT medium agar plate with Amp and the 2xYT medium agar plate without Amp.

Assay of hEGF

The hEGF secreted by bacterial cultures was assayed directly from culture supernatants after removal of bacterial cells by centrifugation. The ELISA assay was performed according to a standard procedure [25] using rabbit anti-hEGF antibody from CalBiochem (Cambridge, MA), and goat anti-rabbit IgG conjugated with alkaline phosphatase (Pierce, Rockford, IL).
Western blot analysis

The protein samples were first resolved on a 15% tricine-SDS-polyacrylamide gel [26], then transferred on to a polyvinylidene difluoride membrane (No. 162-0184, Bio-Rad) and subsequently reacted with a mouse anti-hEGF serum (Berkeley Antibody Co.) according to a protocol described previously[25].

Results and Discussion

Expression of hEGF by the different plasmid constructs

Two plasmid constructs were studied to optimize expression of hEGF by E. coli JM101 in shake flasks. Initially, 2xYT medium with Amp was used. The inoculated medium was incubated at 30°C and shaken to an A_{590} value of 3.0. The culture was split into two portions. One portion (induced culture) received IPTG to a final concentration of 0.1mM whereas the other portion (non-induced culture) did not. Both cultures were then incubated as before and samples withdrawn at suitable intervals. Significant hEGF activity levels (Fig. 1A) were detected in supernatant samples from induced cultures, thereby demonstrating the ability of the OmpA signal peptide to direct hEGF to the periplasm, from where the protein was excreted to the supernatant. The hEGF excreted by an induced culture of E. coli JM101[pWKW2] was ca. 10-fold greater than the amount excreted from a non-induced culture; with E. coli JM101[lacUV5par8EGF] the hEGF ratio (induced/non-induced) was ca. 6. E. coli JM101[pWKW2] expressed 31.5mg/l hEGF at 20h post-induction (Fig. 1A), while E. coli JM101[lacUV5par8EGF] expressed only 8.5 mg/l hEGF at this time.
The plasmid stabilities (Fig. 1B) in, and cell viabilities (Fig. 1C) of, induced cultures, are also shown. Very high levels of cell viability and plasmid stability (ca. 80%) were maintained in cultures of \textit{E. coli} JM101[lacUV5par8EGF], to 20h post-induction. While \textit{E. coli} JM101[pWKW2] gave better levels of hEGF, only ca. 60% of \textit{E. coli} JM101[pWKW2] cells maintained the plasmid 20h post-induction, and cell viability dropped to ca. 5% at this time (Fig.1B and 1C). Even though Amp was present in the growth media, the antibiotic would be gradually destroyed by \(\beta\)-lactamase, allowing eventual elimination of plasmid from host cells [12,15]. The reduction in proportion of plasmid-containing cells is part-explained by the fact that such cells have a growth rate lower than that of cells without plasmid and thus, plasmid-free cells become enriched on prolonged culture [27,28]. The low viability of induced \textit{E. coli} JM101[pWKW2] cells 20h post-induction is similar to an effect noted previously, where a deleterious effect of induction on cell viability was shown when the synthesis of a secretable foreign protein (a \textit{Cellulomonas fimi} exoglucanase) was induced in \textit{E. coli} JM101 [9]; this may be attributable to alterations in the cytoplasmic membrane of the induced cell.

As both \textit{E. coli} JM101[pWKW2] and \textit{E. coli} JM101[lacUV5par8EGF] showed useful properties post-induction (high hEGF production in the case of \textit{E. coli} JM101[pWKW2]; high plasmid stability and cell viability with \textit{E. coli} JM101[lacUV5par8EGF]), both strains were investigated further.

\textit{Effect of medium composition on growth properties and hEGF production}

Production of hEGF by \textit{E. coli} JM101[lacUV5par8EGF] and \textit{E. coli} JM101[pWKW2] was compared, in shake flasks at 30°C, using the MMBL medium (see Materials and
Methods) containing variable amounts of glucose. Cultures were induced with 0.1 mM IPTG in mid-logarithmic phase (A\textsubscript{600} = 8.0). With glucose at 5g/l, expression of hEGF was greater than that achieved in the 2xYT medium (Fig. 2). Thus, E. coli JM101[lacUV5par8EGF] produced ca. 15mg/l hEGF, while the figure for E. coli JM101[pWKW2] was ca. 120mg/l. Various nitrogen sources (casein acid hydrolysate, casein enzymatic hydrolysate, casamino acids and tryptone) in the MMBL medium were tested for effects on hEGF expression; no significant differences either in production of hEGF, cell viability post-induction, or plasmid stabilities, were found (data not shown). Tryptone was selected as the preferred nitrogen source for further work, as cultures with tryptone foamed less than did cultures with other nitrogen sources, and foaming was of major consideration in the planned fermentor experiments. Different glucose concentrations, in MMBL medium, were next tested for optimization of hEGF. Maximum hEGF expression, from both constructs, was observed when the initial glucose concentration was 5g/l (Fig.2), while plasmid stabilities were also at-or-near optimal at this glucose level. Glucose concentrations higher than 5g/l severely inhibited cell growth (data not shown) and hEGF production. Thus, MMBL medium with 5g/l glucose was used in further experiments.

Effect of inducer concentration on hEGF expression

The effect of the concentration of IPTG (levels of 0, 0.05, 0.1, 0.2, 0.3, and 0.4 mM were tested) on the expression of hEGF in both constructs, grown in shake flasks, was determined in an optimal medium (MMBL with 5g/l glucose) as described above. The hEGF production was maximal when the inducer concentration was 0.1mM for E. coli JM101[pWKW2] and 0.2mM for E. coli JM101[lacUV5par8EGF]. Higher concentrations of inducer did not significantly improve the secreted hEGF levels (data
not shown). In other work [29,30], the expression levels of cloned proteins varied markedly with IPTG concentrations, showing that the effect of IPTG concentration on recombinant protein production is dependent on the specific characteristics of the recombinant protein, the host cell, and the culture conditions. The effect of induction start time on hEGF production levels was also examined for both constructs. IPTG was added at biomass levels corresponding to $A_{590}$ values of 4.0, 8.0 and 12.0. Maximal hEGF production was observed when the inductions were performed at $A_{590} = 8.0$, at which stage the cells were in approximately mid-logarithmic phase (data not shown).

**Fermentor experiments-batch culture**

To test the optimized conditions (MMBL medium, tryptone as nitrogen source, glucose at 5g/l, IPTG induction at 0.1mM or 0.2mM (depending on the strain), and induction at $A_{590} = 8.0$) that were developed in shake flask cultures, laboratory scale fermentations were next performed. The effect of different temperatures (28-36°C) on hEGF expression in MMBL medium was assessed (Table 1). Although the optimal temperature for the growth of recombinant *E. coli* was 36°C (at which temperature the highest specific growth rates were seen for both strains), the optimal temperatures for the maximal production of hEGF were 32°C for strain *E. coli* JM101[lacUV5par8EGF], when hEGF productivity was 2.77mg/l/h, and 34°C for *E. coli* JM101[pWKW2] (hEGF productivity 29.2mg/l/h). With both strains, plasmid stabilities decreased with growth temperature.

Results from batch fermentations of *E. coli* JM101[pWKW2] (grown at 34°C) and *E. coli* JM101[lacUV5par8EGF] (grown at 32°C) are shown (Fig. 3). There is a rapid
loss of plasmid following induction (most acute with *E. coli* JM101[pWKW2], where only *ca.* 20% of cells retained the plasmid 10h post-induction). Also, loss of cell viability of *E. coli* JM101[pWKW2] was very marked post-induction, with only-*ca.* 0.5% residual viability 6h post-induction. Despite these shortcomings, hEGF yield from the *E. coli* JM101[pWKW2] fermentation was much higher, and faster, than that seen using *E. coli* JM101[lacUV5par8EGF] (175mg/l in 6h with *E. coli* JM101[pWKW2], compared to 28mg/l in 10h using *E. coli* JM101[lacUV5par8EGF] (Fig. 3).

*Fed-batch culture*

As hEGF production was consistently better from *E. coli* JM101[pWKW2], compared to *E. coli* JM101[lacUV5par8EGF], fed-batch fermentation of the former strain was explored, using, first, intermittent addition of glucose and MgSO₄·7H₂O (see Materials and Methods). All the fed-batch experiments were conducted at 32°C since the cell viability, product concentration, and hEGF stability, were lower at 34°C using fed-batch protocols (data not shown). The magnesium was included in the feed to minimize precipitation of salts [30]. The depletion of glucose in the medium increased medium pH, which was used as an index to determine the timing of glucose addition (this index was validated by direct measurement of medium glucose). There was no significant increase in production of hEGF (compared to levels obtained in batch cultures with MMBL medium, as described above) and the plasmid was not stable (data not shown). Glucose was not consumed after the first 3 feeds, which might reflect (a) a reduction in viable cell number, and/or (b) production of by-products (organic acids) inhibiting protein production.
In the next experiment, glucose and MgSO$_4$$
\cdot$7H$_2$O were fed constantly, commencing
after the glucose present in the original medium was consumed. The feeding medium
flow rate was adjusted so that the pH was maintained at ca. 6.8, which balanced the
consumption rates of the carbon and nitrogen sources. An unbalanced consumption
rate may cause the accumulation of an undesirable substance (such as ammonium ion)
in the medium, with a consequent reduction in product yield [31,32]. There was a
notable increase in hEGF production and productivity (Fig. 4). A maximum hEGF
production of 325mg/l was now achieved 10h post-induction, which corresponds to a
productivity of 32.5mg/l/h. Unlike the case with the batch culture fermentations (see
above), plasmid stability was maintained at ca. 50% to 10h post-induction. Cell
viability decreased to ca. 5% at this time, but the decrease was more gradual than that
noted in batch fermentations. Samples of supernatants from a continuous fed-batch
culture collected at different time points were analysed by SDS-PAGE and Western
blotting (Fig. 5). Comparison of relative staining intensities indicated a yield of about
300 mg/l was produced by an induced E. coli JM101[pWKW2] culture 10h post-
induction (Fig. 5). The results are in line with the ELISA data (Fig. 4 and Fig. 5). An
induction for 10h was found to be optimal under the specified conditions. A longer
duration resulted in higher levels of contaminating proteins in the supernatant, and in
lower yields of biologically active hEGF (data not shown).

*Performance of the E. coli JM101[pWKW2] clone in producing hEGF*

The time-course study of a fed-batch culture of an induced *E. coli* JM101[pWKW2]
strain, growing in MMBl medium, revealed a gradual increase in production of hEGF in
the first 10h post-induction, to give a yield of 325 mg/l (Fig. 4). Based on previous
estimations [11], the 325 mg/l of extracellular hEGF could represent over 90% of the
hEGF produced by the cells. From 4h post-induction, expression and excretion of hEGF was detectable by SDS-PAGE, and soon the product became the major protein species in the culture supernatant (Fig. 5A), despite the presence of some intracellular proteins resulting from the lysis of cells.

This high rate of production of hEGF by the *E. coli* JM101[pWKW2] clone up to the 10h post-induction time point (32.5 mg/l/h), has rendered this clone a better choice for use over other systems [4,7], which have also been reported to express hEGF in the mg/l scale during the productive phase. The levels of hEGF produced by an engineered *Bacillus brevis* strain [7] and by the *E. coli* JM101[pETacEGF] clone [11], grown under optimized conditions [4], were 1.1 g/l/6 days and 250 mg/l/32 h, respectively. These long growth times required for production of hEGF may be expected to adversely affect the activity of the product, and would also elevate the production cost and prolong the occupancy of the fermentation equipment. All these deficiencies may become even more difficult to tackle on large-scale production.

The high stability (15) and productivity, of the pWKW2 construct would be expected to facilitate the scale-up production of hEGF. Despite the co-existence of quite a high background of contaminating proteins in the supernatant of an induced JM101[pWKW2] culture, the hEGF may be readily purified by conventional chromatographic methods (Huang *et al.*, manuscript in preparation) and likely by immunoabsorption (Fig. 5B). Additional points supporting the application of the *E. coli* JM101[pWKW2] clone are that the recombinant hEGF has been confirmed to possess the expected N-terminus and has been shown to share a similar bioactivity level to that of a commercial hEGF sample (Huang *et al.*, manuscript in preparation).
Acknowledgements

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References


Figure Legends

Figure 1. Expression of hEGF by, plasmid stability in, and cell viability of *E. coli* JM101[pWKW2] and *E. coli* JM101[lacUV5par8EGF]. The 2 strains were grown at 30°C in ampicillin-supplemented 2xYT medium to an A₅₅₀ value of 3.0, and then induced with IPTG (0.1 mM final concentration). A: hEGF expression; B: plasmid stability; C: cell viability (100% viability corresponded to 3x10⁶ cells/ml). The tests were performed 3 times, and standard error bars are shown. Solid lines: data from JM101[pWKW2]; dashed lines: data from JM101[lacUV5par8EGF].

Figure 2. Expression of hEGF by, and plasmid stability of, *E. coli* JM101[pWKW2] and *E. coli* JM101[lacUV5par8EGF], upon growth in MMBL medium with varying glucose concentrations. The cultures were grown at 30°C in ampicillin-supplemented MBL medium to A₅₅₀ values of 8.0 and then induced with IPTG to a final concentration of 0.1 mM. The hEGF values and plasmid stabilities were measured 20h post-induction. A: hEGF expression; B: plasmid stability. The tests were performed 3 times, and standard error bars are shown. Solid lines: data from JM101[pWKW2]; dashed lines: data from JM101[lacUV5par8EGF].

Figure 3. Batch cultivation of *E. coli* JM101[pWKW2] and *E. coli* JM101[lacUV5par8EGF] in MMBL medium. The working volume was 1 l, the inoculum was 10% (v/v), the initial glucose level was 5 g/l, the fermentation temperature was 32°C for *E. coli* JM101[lacUV5par8EGF] and 34°C for *E. coli* JM101[pWKW2], and the pH was controlled at 6.8 until induction. The cultures were grown to A₅₅₀ values of 8.0 and then induced with IPTG to a final concentration of 0.1...
mM (\textit{E. coli} JM101[pWKW2]) or 0.2mM (\textit{E. coli} JM101[lacUV5par8EGF]). A: hEGF production; B: plasmid stability; C: cell viability (100% viability corresponded to 8\times10^9 cells/ml). The tests were performed 3 times, and standard error bars are shown. Solid lines: data from JM101[pWKW2]; dashed lines: data from JM101[lacUV5par8EGF].

\textbf{Figure 4.} Fermentation of \textit{E. coli} JM101[pWKW2] with the constant feeding of medium. The working volume was 1l, the inoculum was 10\% (v/v), the initial glucose level was 2g/l, the fermentation temperature was 32°C, and the pH was controlled at 6.8 throughout fermentation. The cultures was grown to A_{590} values of 8.0 and then induced with IPTG to a final concentration of 0.1mM. The feeding medium was 200g/l glucose and 20g/l MgSO_{4-7H_2O}, and was pumped at 7.2ml/h after the initial glucose was consumed. A: hEGF production; B: plasmid stability; C: cell viability (100\% viability corresponded to 8\times10^9 cells/ml). The tests were performed 3 times, and standard error bars are shown.

\textbf{Figure 5.} Analyses of excreted hEGF produced by a continuous fed-batch culture of \textit{E. coli} JM101[pWKW2]. (A). SDS \cdot PAAGE on a 15\% gel, which was stained with Coomassie Brilliant Blue R-250. Lanes: M, molecular weight markers (Seeblue\textsuperscript{TM} pre-strained standards, Novex\textsuperscript{TM}); 1, 1\mu g of commercial hEGF (Promega); 2, sample collected just before induction; 3-7, samples collected at 2,4,6,8 and 10h post-induction. Lanes 2-7 each contains 28\mu l of culture supernatant. (B). Western blot analysis of the samples in (A); the lane order is as in (A).
Table 1. Effect of temperature on growth and hEGF production by recombinant *E. coli* JM101 strains grown in optimized MMBL medium in batch cultures. The incubation times shown were chosen to optimize hEGF production; incubation beyond these times did not yield further increases in excreted hEGF levels. The tests were performed 3 times; averages are shown. Individual values were within 10% of these means.

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th><em>E. coli</em> JM101[lacUV5par8EGF]</th>
<th><em>E. coli</em> JM101[pWKW2]</th>
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<td>30</td>
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<tr>
<td>Incubation time, h</td>
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<td>0.42</td>
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* The plasmid stability was calculated by taking the ratio of the average colony counts on the 2xYT medium agar plate with Amp and the 2xYT medium agar plate without Amp.