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Production of human epidermal growth factor by an ampicillin resistant recombinant *Escherichia coli* strain

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Summary

The effect of medium composition and initial glucose concentration on production of hEGF by recombinant *E. coli* cells was investigated. Optimum hEGF production was observed in a yeast extract/acid hydrolysed casein/salts media containing an initial glucose concentration of 10 $g.l^{-1}$. A maximum hEGF titer of 250 $mg.l^{-1}$ was obtained in this medium after 32 h in laboratory fermenters with pH, temperature, agitation and aeration set at 6.8, 30°C, 500 rpm and 2 vvm, respectively.

Introduction

Mature human epidermal growth factor (hEGF) is a polypeptide consisting of 53 amino acids with three internal disulfide bonds which stimulates the growth and differentiation of various epidermal tissues *in vivo* and *in vitro*. It has the potential therapeutic properties of accelerating healing and repair of epithelial tissue following, for example, serious burns, skin grafting or corneal surgery. This potent mitogenic factor has also been shown to have specific stimulating effects on protein and DNA synthesis, glucose uptake, and ion fluxes and calcium transport (Barnes and Colowick, 1976; Carpenter and Cohen, 1979; Rozengurt & Hoppel, 1975). hEGF is produced in the human duodenum and salivary glands and is found in many body fluids including milk, colostrum, urine and seminal fluid (Carpenter, 1980, 1985; Oka and Orth, 1983).

Exploitation of recombinant DNA technology to produce EGF in microorganisms provides a possible source of supplying large quantities of hEGF. Attempts have been made at the molecular level to optimize EGF expression and product yields (Clements *et al.*, 1991; Engler *et al.*, 1988; Sumi *et al.*, 1985). Cell densities for plasmid-containing *Escherichia coli* systems expressing foreign proteins are often lower than levels observed with non-plasmid containing *E. coli* and this has led to studies on optimization of growth of recombinant *E. coli* (Kracke-Helm *et al.*, 1991; Yee and Blanch, 1992, 1993a, 1993b). Production and secretion of organic acids, especially acetic acid, have been reported to lead to growth inhibition and low product yields (Landwall and Holme, 1977; Luli and Strohl, 1990; Pan *et al.*, 1987; Yee and Blanch, 1993b). Most studies on hEGF production by *E. coli* have focused on molecular biology aspects, including gene construction and plasmid copy number. In available reports on hEGF production, yields of less than 100 $mg.l^{-1}$ have typically been reported (Engler *et al.*, 1988; Chalmers *et al.*, 1990; Morioka-Fujimoto *et al.*, 1991; Batchihoon *et*

al., 1992). In this report, culture conditions leading to efficient production of hEGF using an ampicillin-resistant recombinant *E. coli* strain are described.

Materials and Methods

Bacterial strain and culture maintenance

An ampicillin resistant *E. coli* strain JM101, transformed with plasmid pETAC (obtained from Allelix Biopharmaceuticals, Inc.), was maintained on YT medium containing agar 15 $g.l^{-1}$ agar (Table 1). Inoculated YT-agar plates, containing ampicillin, were incubated for 18-24 h at 30°C. Individual colonies (2-3) were used to loop-inoculate 1L and 2L non-baffled Erlenmeyer flasks containing a 20% volume of YT medium containing ampicillin. The culture flasks were incubated at 30°C on an orbital shaker set at 200 rpm for 12-14 h and used to prepare glycerol stock cultures for long term storage at -70°C or for fermenter inoculation.

Fermentation conditions

A 10% v/v culture inoculum, prepared as described above, was used to inoculate 7L Chemap laboratory fermenters containing 5L of medium described in Table 1. The fermentation was carried out at 30°C and agitation and aeration were set at 500 rpm and 2 vvm, respectively. pH was controlled at 6.8 until glucose was completely utilized. When glucose level dropped below 0.75 $g.l^{-1}$, the culture was induced with 1 mM isopropylthiogalactoside (IPTG). Culture samples were centrifuged at 10,000 x g for 15 min to prepare supernatants for hEGF analysis or cells for cell disruption.

Table 1. Components of *E. coli* JM101 growth media

Component per litre	YT	UC	MBL
Yeast extract (g)	10	10	10
Tryptone (g)	20	-	-
Acid hydrolysed casein (g)	-	20	20
Glucose (g)	5	5	5-35
KH_2PO_4 (g)	-	-	3.5
K_2HPO_4 (g)	-	-	5
$(NH_4)_2HPO_4$ (g)	-	-	3.5
$MgSO_4.7H_2O$ (g)	-	-	3.5
NaCl (g)	5	5	5
Trace metal solution (ml)	-	-	3
Ampicillin (mg)	0.07	0.07	0.07

Trace metal solution contains $FeCl_3.6H_2O$, 0.162 $g.l^{-1}$; $ZnCl_2.4H_2O$, 0.0144 $g.l^{-1}$; $CoCl_2.6H_2O$, 0.12 $g.l^{-1}$; $Na_2MoO_4.2H_2O$, 0.012 $g.l^{-1}$; $CaCl_2.2H_2O$, 0.006 $g.l^{-1}$; $CuSO_4.5H_2O$, 1.9 $g.l^{-1}$; H_3BO_3, 0.5 $g.l^{-1}$ and HCl, 37 $ml.l^{-1}$.

Cell disruption

Cells were resuspended in 50 mM Tris-HCl buffer (pH 7.5) and sonicated using a Braun-Sonic 2000 ultrasonicator. The sonicated suspension was centrifuged at 10,000 x g for 15 min at 4°C and the supernatant was tested for hEGF activity. The cell-free supernatant, recovered by centrifugation at 10,000 x g for 15 min, was used for hEGF analysis.

Analysis of hEGF

hEGF in fermentation broths was routinely analyzed using reverse phase HPLC, a method which was validated by use of a hEGF receptor binding assay (Yadwad *et al.*, 1993). The reverse phase HPLC method utilized a Shimadzu system (two single gradient pumps, model LC-600; SCL-6B system

controller; SPD 6AV UV-VIS spectrometric detector and Chromatopac CR 601) equipped with Ultrasphere 5m C18 column (4.6 X 150 mm). The mobile phase was 10% acetonitrile in 0.01 M sodium phosphate buffer pH 6.5 (solvent A) and 60% acetonitrile in 0.01M sodium phosphate buffer (solvent B). Samples were eluted with a 45 min gradient from 10% to 40% of solvent B at a flow rate of 1 $ml.min^{-1}$. Absorbance at 214 nm was monitored.

Organic acids analysis
Organic acids were analyzed by HPLC using a Polypore H column, 220 X 4.6 mm (Applied Biosystems/Brownlee Labs) with a mobile phase of 0.01N H_2SO_4 at a flow rate of 0.3 ml/min and monitored at 210 nm. The system consisted of Waters HPLC model 600 pump, 484 tunable absorbance detector, 712 WISP autoinjector, 600E system controller and 745 data module (Waters, Millford, MA). The identities of the eluted peaks were verified using authentic acid standards.

Other analyses
Culture biomass was routinely determined by measuring absorbance of appropriate diluted cultures at 600 nm in a 1 cm light path. Absorbance values were converted to cell dry weight by comparison to a standard curve. Glucose and protein concentrations were determined using the dinitrosalicylic (Miller, 1959) and Biorad (Bradford, 1976) methods, respectively.

Results

Fermentation time course studies on *E. coli* growth and hEGF production were compared using three different culture media. Final biomass of cultures grown in MBL medium was approximately twice the level observed in the YT and YC media (Figure 1, Table 1). The rate of hEGF production was substantially higher in the MBL medium and its appearance in the medium coincided with a notable reduction in biomass concentrations after a 10 to 12 h of incubation. Final concentrations of hEGF after 48 h was 91.1 $mg.l^{-1}$ in the MBL medium having an initial glucose concentration of 5 $g.l^{-1}$.

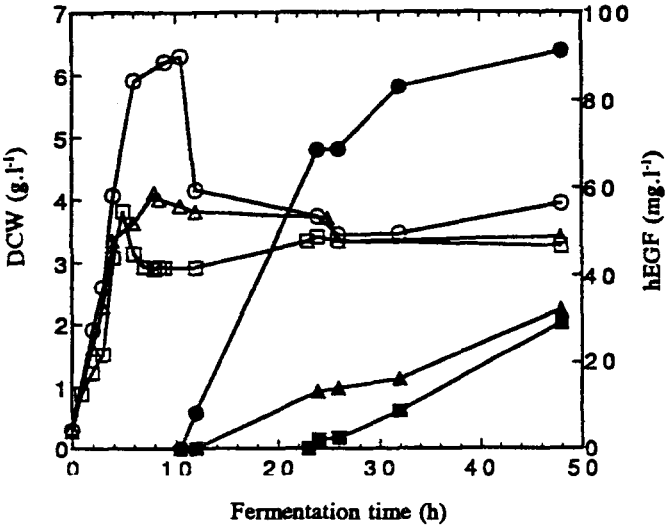

Figure 1 Effect of different media on biomass (DCW $g.l^{-1}$) (open symbol) and hEGF (closed symbol) production by *E. coli*.

MBL (○, ●); YC (△, ▲); YT (□, ■)

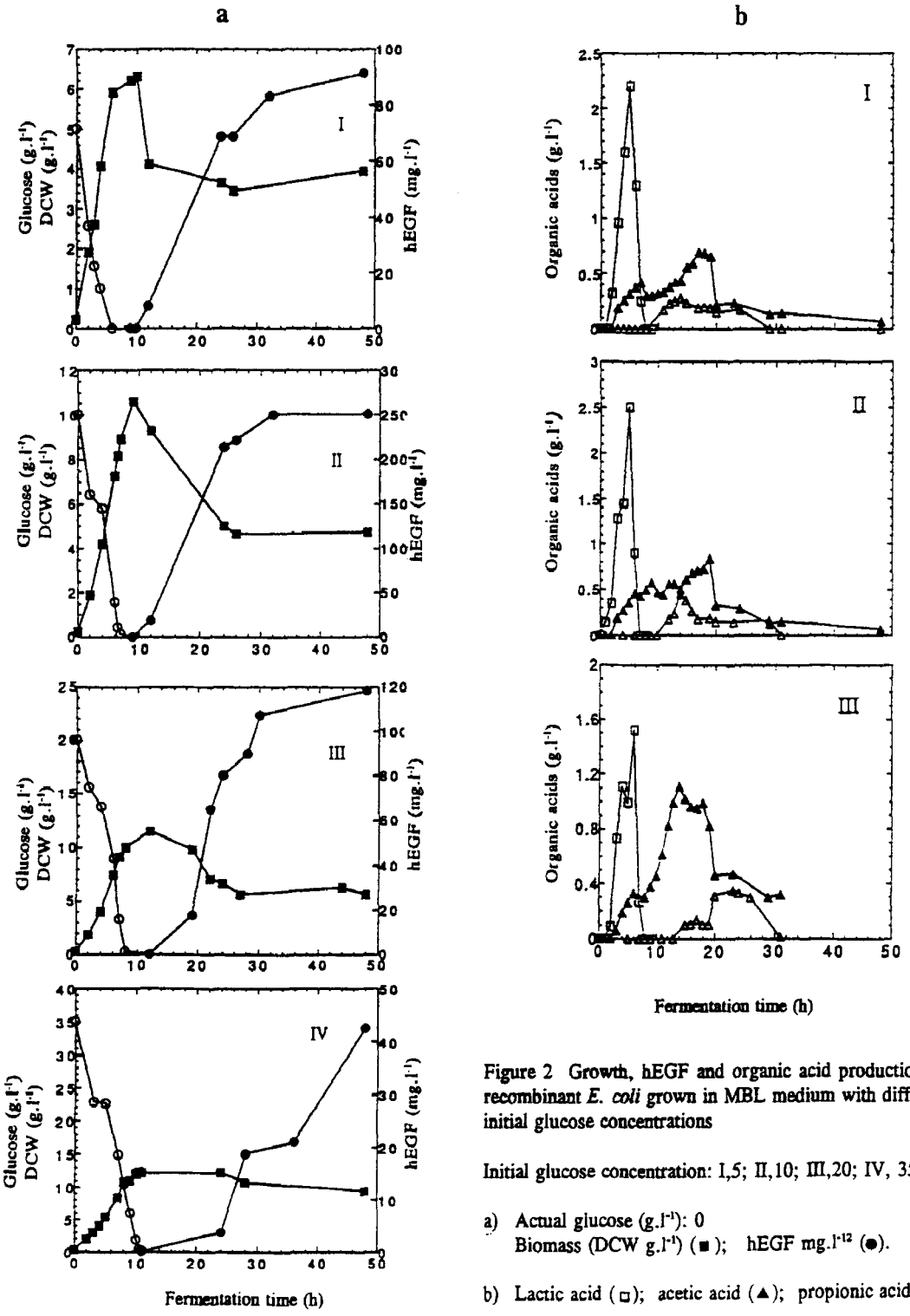

Figure 2 Growth, hEGF and organic acid production by recombinant *E. coli* grown in MBL medium with different initial glucose concentrations

Initial glucose concentration: I,5; II,10; III,20; IV, 35.

a) Actual glucose ($g.l^{-1}$): 0
Biomass (DCW $g.l^{-1}$) (■); hEGF $mg.l^{-12}$ (●).

b) Lactic acid (□); acetic acid (▲); propionic acid (△)

The effect of initial glucose concentration in the MBL medium on growth and hEGF production was investigated. Examples of patterns of growth and hEGF production observed with initial glucose concentrations of 10, 20 and 35 $g.l^{-1}$ are indicated in Figure 2a. Improved biomass production was observed in all three media as compared with the MBL medium containing 5 $g.l^{-1}$. Biomass dry cell weight values with initial glucose concentrations of 10 and 20 $g.l^{-1}$ manifested the characteristic decline observed in Figure 1 with concomitant production of hEGF. The possibility that the appearance of hEGF in the culture broth was due to cell lysis was investigated by comparing intracellular and extracellular hEGF levels during the fermentation. Only 3-5% of total hEGF was found in the cells with the majority (>95%) being secreted to the culture broth at all times during fermentation. Maximum concentrations of hEGF, observed in media with initial glucose concentrations of 10 and 20 $g.l^{-1}$, were 250 and 120 $mg.l^{-1}$, respectively. In contrast, the growth curve in the medium containing an initial glucose level of 35 $g.l^{-1}$ did not manifest the same characteristic decline phase and hEGF production was delayed. Production of hEGF in this medium was also much lower, reaching a concentration of 42.5 $mg.l^{-1}$ after 48 h.

The patterns of glucose depletion and production of acetic, lactic and propionic acid during the fermentation are illustrated in Figure 2b. Glucose was depleted in the three media after 6.5-10 h. In all three cases significant lactic acid production was observed from the start of the fermentation, ranging to maximum levels of 1.6 to 2.5 $g.l^{-1}$ between 5 and 6 h and declining to 0 as glucose was depleted. Propionic acid production was initiated after 9-10 h in cultures having initial glucose concentration of 5 and 10 $g.l^{-1}$ but was delayed for 15 h in the culture containing 20 $g.l^{-1}$ glucose. Maximum propionic acid concentrations were 0.28, 0.45, and 0.35 $g.l^{-1}$ for the 5, 10 and 20 $g.l^{-1}$ glucose-containing cultures, respectively. Maximum acetic acid concentrations of 0.69-0.84 $g.l^{-1}$ were observed in 5 and 10 $g.l^{-1}$ glucose after 17-19 h during a period of high hEGF production, after which levels declined. Time of maximum acetic acid concentration (1.1 $g.l^{-1}$ observed for 20 $g.l^{-1}$ glucose) was 14 h, prior to production of hEGF.

Discussion

Fermentation conditions for production of hEGF by an ampicillin-resistant recombinant *E. coli* strain were established. hEGF yield was influenced by both organic nitrogen and glucose content in the medium. Tsai *et al.* (1987) reported that enrichment of organic nitrogen in fermentation media can enhance synthesis and accumulation of recombinant protein in *E. coli*. Glucose concentration was found to have a profound influence on hEGF production by recombinant *E. coli*. Maximum hEGF production was observed when initial glucose concentration was 10 $g.l^{-1}$. Increase of the initial glucose concentration to 20 $g.l^{-1}$ and 35 $g.l^{-1}$ both suppressed hEGF production. Glucose suppression of recombinant peptide synthesis by *E. coli* cells has been reported previously (Evans *et al.*, 1991; Kracke-Helm *et al.*, 1991).

A maximum hEGF yield of 250 $mg.l^{-1}$ was obtained in pH controlled laboratory fermenters containing MBL medium and 10 $g.l^{-1}$ glucose after 32 h which corresponds to a productivity of 7.8

$mg.l^{-1}.h^{-1}$. *S. cerevisiae* has been reported to produce EGF titres of 3.5 $mg.l^{-1}$ (Clements *et al.*, 1991) while *Pichia pastoris* produced 108 $mg.l^{-1}$ (Siegel, 1992). In contrast, a very high level of hEGF production (1.1 $g.l^{-1}$ in 6 d) was recorded with a recombinant strain of *Bacillus brevis* (Ebisu *et al.*, 1992), which represents a similar productivity (7.6 $mg.l^{-1}.h^{-1}$) to that reported in our studies.

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