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Process optimization for large-scale production of TGF-α-PE40 in recombinant *Escherichia coli*: effect of medium composition and induction timing on protein expression

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The effects of medium composition and induction timing on expression of a chimeric fusion protein TGF-α-PE40 (TP-40) in *Escherichia coli* strain RR1 were examined using a complex medium at several fermentor scales. Two distinctive phases in *E. coli* catabolism were identified during fermentation based on preferential utilization between protein hydrolysate and glycerol. Maximum specific and volumetric productivities were achieved by inducing the culture when the cells were switching substrate utilization from protein hydrolysate to glycerol. By increasing the yeast extract concentration in the production medium, initiation of the catabolic switch was delayed until high cell mass was achieved. The final titer of TP-40 at the 15-L fermentation scale was doubled from 400 mg L^{-1} to 850 mg L^{-1} by increasing the yeast extract concentration from 1% to 4% (w/v) and delaying the time of induction. This fermentation process was rapidly scaled up in 180-L and 800-L fermentors, achieving TP-40 titers of 740 and 950 mg L^{-1}, respectively.

Keywords: *E. coli*; fermentation; induction; recombinant; expression; scale-up

Introduction

There is no single universal fermentation process that is optimized for expression of all recombinant proteins in *Escherichia coli*. Levels of foreign gene expression in *E. coli* are highly system-specific depending on the biochemical characteristics of the recombinant protein, induction mechanism of gene expression, host strains, and cultivation conditions. Understanding the relationship between microbial physiology, host/vector system, and gene expression during fermentation is essential to optimize the production of recombinant proteins. The optimization process often involves improvement of the fermentation conditions focusing on medium composition [13,19], growth conditions [3,6,16], induction method [11,15], and minimum metabolite inhibition [1,10,12] as well as its genetic systems. Fermentation parameters for optimal expression of a gene of interest are frequently determined empirically since the combined effects of these various conditions are not easily predictable [2,21].

An important approach to optimize production of a recombinant protein during an *E. coli* fermentation has been to achieve maximum specific productivity and cell mass simultaneously using an appropriate induction method [4,22]. Often times, induction of a culture at an early stage of exponential growth yields a high specific productivity, but results in unsatisfactory volumetric productivity due to the low final cell mass. In contrast, induction at a higher cell mass produces a low specific productivity [19]. The use of isopropylthio-β-d-galactoside (IPTG) to induce the expression of foreign genes under control of the tac promoter has been widely used for various recombinant proteins [5,9]. Nevertheless, timing of IPTG induction was often determined by off-line optical density measurements of the culture as an indicator of a certain growth phase with little emphasis on physiology of the host cell. Therefore, understanding the relationship between induction timing and the metabolic state of the host cell during the fermentation cycle is critical for optimization of recombinant protein production.

TGF-α-PE40 (TP-40) is a chimeric fusion protein composed of two independent domains of human transforming growth factor-alpha and a portion of *Pseudomonas* exotoxin A [17]. This protein is a candidate anti-tumor agent due to its selective binding to epidermal growth factor receptors on certain types of cancer cells and its ability to inactivate eukaryotic translation elongation factor 2 by ADP ribosylation [8,18]. Expression of this recombinant protein is tightly regulated under control of the tac promoter.

Our efforts have been focused on improving production of TP-40 by optimizing fermentation conditions. The physiological effect of TP-40 expression on the cellular metabolism of the *E. coli* host cell was reported earlier [7]. In the present study, we demonstrated that two distinctive phases of *E. coli* host cell catabolism based on preferential utilization of the substrates exist during the fermentation cycle. The relationship between the catabolic state of the host cell and induction, and its effect on productivity of TP-40 were investigated at both the laboratory (15 L) and the pilot plant (180 and 800 L) scales. Optimization of medium composition and timing of induction for enhanced productivity are reported.

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Materials and methods

Bacterial strains and plasmid DNA

E. coli strain RR1[supE44 hsdS20(rB-mB-) ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1] containing plasmid pTP40NAIq was obtained from the Department of Virus and Cell Biology of Merck Research Laboratories (West Point, PA, USA). The plasmid pTP40NAIq was derived from the pTP40N by inserting the lacIq gene for tighter regulation of tac promoter and the ampicillin-resistant gene as an additional selective marker [8]. The *E. coli* strain containing the plasmids was designed as RR1/pTP40NAIq.

Growth procedures

Medium formulation: *E. coli* strains were grown in the complex medium M101 containing per liter: 3.5 g KH_2PO_4, 3.5 g K_2HPO_4, 3 g $(NH_4)_2SO_4$, 0.24 g $MgSO_4$, 2 mg thiamine·HCl, 50 mg neomycin, 10 g glycerol or 10 g L^{-1} glucose, 10 g yeast extract (Difco Laboratories, Detroit, MI, USA) and 50 g Hy-soy peptone (Sheffield Products, Norwich, NY, USA). An antifoaming agent (Ucon LB625, 0.3 ml L^{-1}, Union Carbide, Danbury, CT, USA) was used in all stirred reactors. The medium was adjusted to pH 7.2 with NaOH prior to sterilization (25 min at 122°C). Glucose and $MgSO_4$ were sterilized separately and added as post-sterile additions to the fermentors. Thiamine·HCl and neomycin sulfate were dissolved in water and filtered through a 0.2-μm filtering device (Corning, Corning, NY, USA), then added to the fermentors after the medium was sterilized.

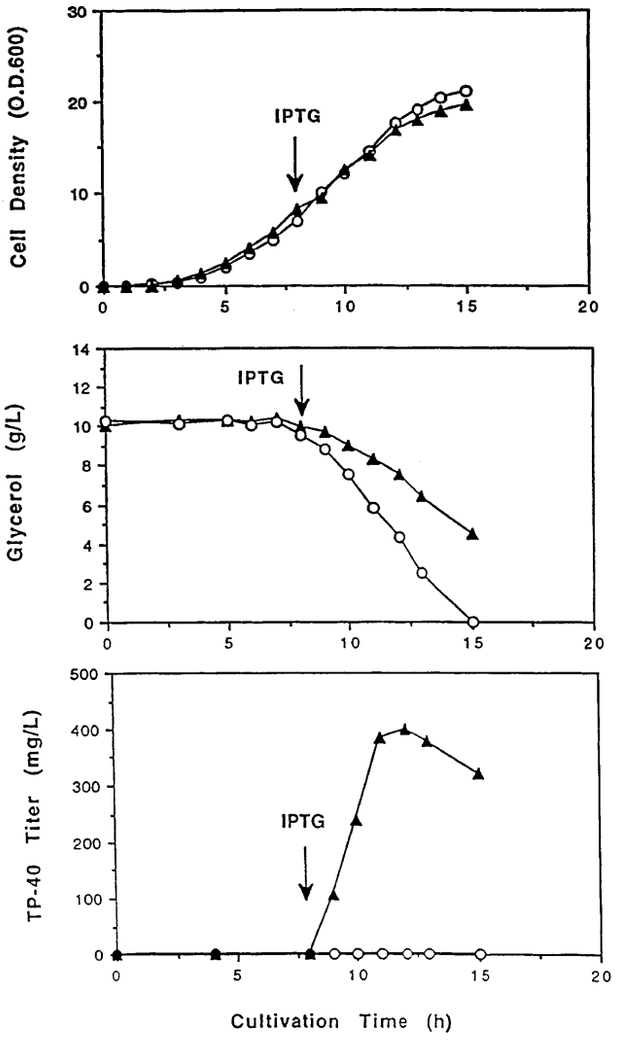

Figure 1 Effect of TP-40 expression on physiology of the *E. coli* construct RR1/pTP40NAIq during fermentation in M101 medium containing 1% (w/v) glycerol and 1% (w/v) yeast extract. Profiles of culture growth, glycerol consumption and TP-40 production were compared between the batches with (–▲–) and without (–○–) IPTG induction (arrow).

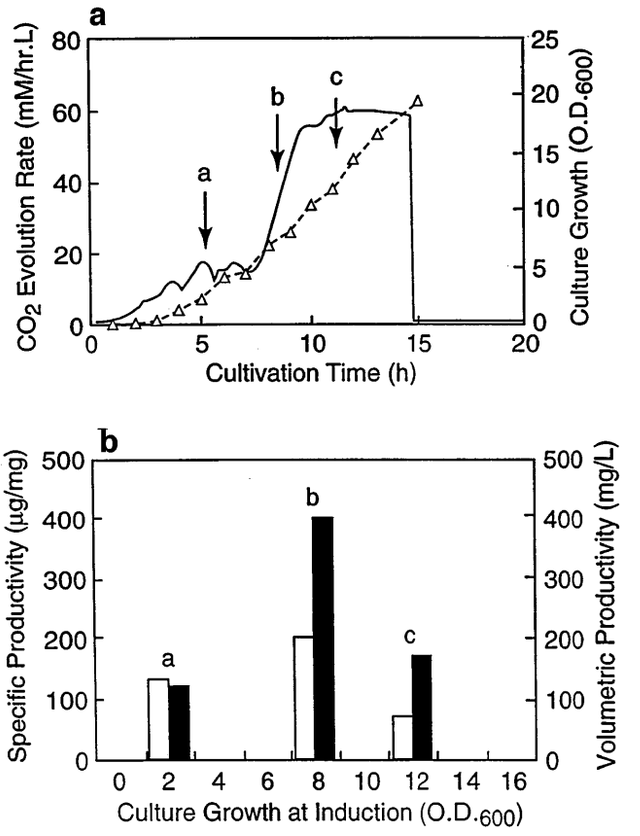

Figure 2 Effect of induction timing during fermentation on productivity of TP-40 at the 15-L scale. The profiles of culture growth (---△---) and CER (——) were obtained from uninduced culture in M101 containing 1% (w/v) yeast extract (a). Maximal levels of TP-40 with different induction timings (a, b, c) were compared for both specific (□) and volumetric (■) productivity in the same medium (b).

Stock maintenance and inoculum preparation: Frozen glycerol (15%) seed stock culture grown in M101 neomycin medium was maintained at –70°C. For seed development, the frozen stock culture was added directly to a 2-L shake flask containing M101/neomycin and grown for 8 h at 37°C with vigorous shaking (200 rpm). Eight milliliters of this seed culture (a 0.05% inoculum) were transferred to a 23-L fermentor containing 15 L of production medium. Seed development for the large scale fermentors was similar to that of the laboratory scale with the inoculum volume adjusted to maintain a 0.05% inoculation. Seed flasks were transferred directly to the

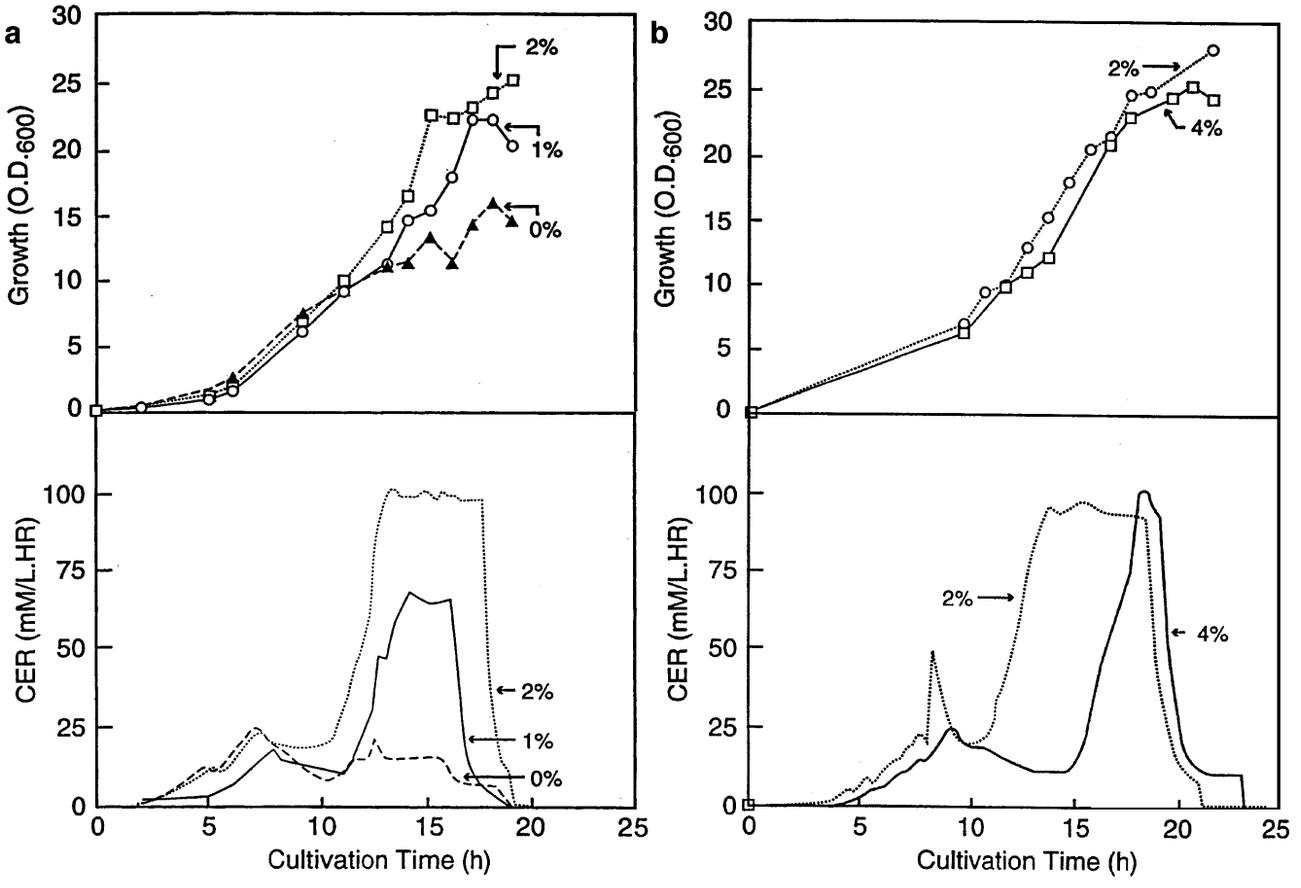

Figure 3 Comparison of carbon evolution rate (CER) and growth profiles of RR1/pTP40NAIq without induction in M101 media containing different concentrations of glycerol (a) or yeast extract (b). M101 media of the batches in the panel (a) contained 2% (w/v) yeast extract with various concentrations of glycerol (0, 1, 2%, w/v). Those in the panel (b) contained 2% (w/v) glycerol with two different yeast extract concentrations (2, 4%, w/v).

large scale fermentors without an additional seed tank stage.

Cultivation conditions: Twenty-three liter fermentors (Chemap, Basel, Switzerland) with a 15-L medium volume were employed for the laboratory scale batch fermentations. Pilot-scale fermentors of 280-L and 1900-L volumes were used with 180-L and 800-L medium volumes respectively. Cultivation conditions including induction method were similar among the different scale fermentations. Temperature, vessel pressure, and air flow were maintained at 37°C, 0.3 bar, and 0.5 vvm, respectively. The culture pH was controlled at 7.2 by automatic addition of 20% NaOH or 30% H_3PO_4 solutions. The agitation rate was increased as required to maintain the dissolved oxygen concentration greater than 50% of air saturation. Expression of TP-40 was induced by adding IPTG (Dioxane-free, Chem Impex International, Wood Dale, IL, USA) to the culture broth at a final concentration of 1 mM.

Analytical procedures

Cell growth was determined by measuring optical density (OD) at 600 nm. Off-gas analysis for carbon dioxide evolution and oxygen uptake rates during fermentation was performed by a Perkin-Elmer model 1200 mass spectrometer (Perkin-Elmer, Pomona, CA, USA) connected to a HP1000 computer (Hewlett Packard, Cupertino, CA,USA) for automatic data processing. Glycerol and TP-40 were measured by HPLC (Spectra Physics, San Jose, CA, USA) equipped with a model 8780 autosampler, 8490 UV detector, 8800 pumps, and 8430 refractive index detector. An Aminex HPX87-H column (300 × 7.8 mm, Bio-Rad, Richmond, CA, USA) at 60°C with a 0.01 N H_2SO_4 mobile phase (0.6 ml min^{-1}) connected to a refractive index detector at 210 nm was used for glycerol analyses. Samples for HPLC analysis were prepared by diluting culture supernatant with the mobile phase (1:5). The TP-40 level in the culture was estimated at 280 nm by reverse phase chromatography using Hy-Tack C-18 column (Glycotech, Hamden, CT, USA) at 60°C with gradient elution using acetonitrile and water. Purified TP-40 (Merck Research Laboratories) was used as the standard. To prepare samples for TP-40 assay by HPLC, cell pellets were recovered by centrifugation of a 1-ml aliquot of culture broth and washed with CM80 buffer (6 mM sodium phosphate and 0.12 M sodium chloride, pH 7.2). Cell pellets were resuspended in a solution (1.8 ml) of 8 M guanidine hydrochloride containing 10 mM EDTA and extracted overnight at 25°C. The next morning, 0.2 ml acetonitrile containing 0.1% trifluoracetic acid was added to each tube and the suspension was centrifuged at 1310 × *g* for 5 min using an Eppendorf microfuge (Brinkmann, Westbury, NY, USA). The supernatant (0.6 ml) was then

filtered through a 0.2-μm cellulose acetate microcentrifuge filter (Costar, Cambridge, MA, USA) prior to HPLC analysis. Protein concentrations in the samples were determined by the BCA method (Pierce, Rockford, IL, USA) with a BSA as a standard. The unit of specific productivity was expressed as 1.0 μg of TP-40 per mg of extracted total cell protein. For free amino acid analysis, broth samples were centrifuged and the supernatants were assayed without hydrolysis using Waters Picotag equipment and methodology. Samples were derivatized with phenylisothiocyanate and the resulting phenylthiocarbamyl derivatives were analyzed via a reverse phase HPLC column with detection at 254 nm. Quantitation of individual amino acids was performed with a hydrolysate standard which did not contain asparagine, glutamine, or tryptophan. Therefore, they were quantitated using lysine, and phenylalanine standards, respectively.

Results and discussion

Growth conditions and expression of TP-40 in fermentor

The previous process for TP-40 production by recombinant *E. coli* employed a complex fermentation medium (M101) containing glucose as a major carbon source. Acetate accumulation in the glucose-containing medium after induction was one of the reasons for cessation of *E. coli* cell growth and low TP-40 production [7,20]. To minimize the acetate inhibition effect on TP-40 expression, the carbon substrate (10 g L^{-1}) in M101 medium was changed from glucose to glycerol. Specific productivity of TP-40 of the glycerol-grown culture was approximately two-fold higher than that of the glucose-grown culture (results not shown). In addition, glycerol substitution simplified the medium preparation for large-scale fermentations by *in situ* sterilization of the carbon source. Therefore, glycerol was chosen for the rest of experiments in this work in lieu of glucose.

The fermentation profiles for culture growth, glycerol consumption, and TP-40 production with or without induction in M101 medium containing 1% (w/v) glycerol and 1% (w/v) yeast extract were compared (Figure 1). The growth rate and final cell concentration of both induced and uninduced cultures were similar, whereas the consumption rate of glycerol was significantly different. For the induced culture, the rate of glycerol consumption was slow, resulting in the residual glycerol concentration of 4.5 g L^{-1} at the end of fermentation. Also, no detectable peak of carbon dioxide evolution rate (CER) appeared throughout this fermentation cycle (results not shown). In contrast, glycerol was completely metabolized by the non-induced cells, and a major CER peak surged when the culture growth reached an optical density at 600 nm at 7.0 (Figure 2a). The plasmid pTP40NAIq carrying the TP-40 gene contained a lac Iq repressor gene to regulate TP-40 expression strictly under the tac promoter to minimize the detrimental effect of leaky expression. The time course of TP-40 production during fermentation indicated that no detectable amount of TP-40 was found until IPTG was added to the medium and that maximum specific and volumetric productivities were reached within 3–4 h after induction. Maximum productivity of TP-40 after each induction event was measured after a 4-h post-induction period.

Effect of induction timing on productivity of TP-40

The effect of induction timing on TP-40 productivity was investigated by adding IPTG to the M101 production medium containing 1% (w/v) yeast extract in different catabolic states of the host cell. Three different cultures were induced when the cell growth (OD_{600}) reached 2.0, 8.0, and 12.0, representing a basal level (less than 20 mM L^{-1} h^{-1}), a transition period, and a plateaued peak of the CER profile when compared to an uninduced culture, respectively (Figure 2a). The maximum specific (195 μg mg^{-1}) and volumetric (400 mg L^{-1}) productivities were achieved by inducing the culture when the cell growth (OD_{600}) was 7.0 and the CER rapidly increased (catabolic transition period). Lower specific productivities of TP-40 were obtained when the culture was induced before or after the surge of CER (Figure 2b).

Two distinctive catabolic phases of the cell during fermentation

The effects of varying concentrations of yeast extract on cell growth and cell physiology were examined at the 15-L fermentor scale. Profiles of CER and cell growth during batch fermentations in M101 were compared (Figure 3). Major CER peaks were attributed to the utilization of glycerol during the fermentation since the peak appeared only after glycerol in the medium began to be consumed (Figure 1). The maximum level of the CER peaks increased proportionally to the higher initial concentrations of glycerol in the medium (Figure 3a). The culture grown without glycerol only maintained a basal level of CER without a noticeable peak despite its comparable growth profile with others (Figure 3a). The lag time period until initiation of the CER peak from inoculation was significantly affected by initial yeast extract concentrations, but not by glycerol concentrations. The higher yeast extract concentration (4%, w/v) in the enriched medium than in the control medium (2%, w/v) delayed the CER peak by approximately 4 h (Figure 3b). The apparent growth profiles and the maximum levels of CER peaks were very similar between these two batches.

The amount of free amino acids in M101 medium during fermentation was monitored by HPLC, and the result indicated that certain amino acids were utilized faster than others. Serine and threonine were depleted from the medium before glycerol began to be utilized. This suggested that serine and threonine might be readily metabolized for culture growth by direct deamination yielding pyruvate and 2-oxobutyrate, respectively. Levels of free glycine, proline, and asparagine in the medium became very low within 2 h after induction. Arginine, tyrosine, cystein, isoleucine, leucine, phenylalanine, and lysine were rarely utilized by the end of fermentation, whereas most other amino acids were slowly depleted from the medium by the time of harvest. Calculated values of the respiratory quotient (RQ) during fermentation were between 1.4 and 1.2 at the early growth stage and dropped to 0.87 when the CER peaked during the later growth phase (Figure 4). The RQ values greater than 1.0 suggested that constituents of

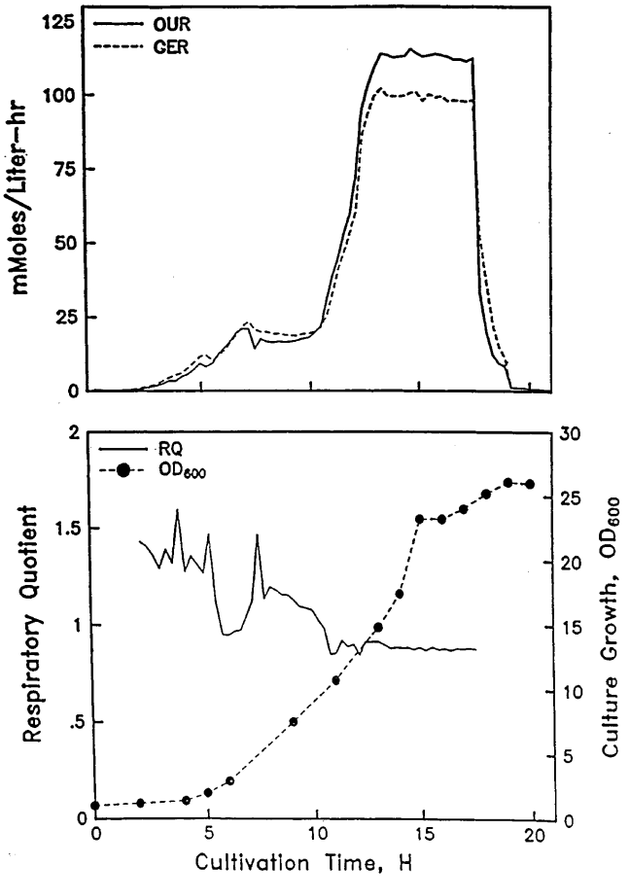

Figure 4 Time curves of oxygen uptake rate (OUR), CER, RQ, and culture growth without induction in M101 medium containing 2% (w/v) yeast extract and 2% (w/v) glycerol at the 15-L scale. The respiratory quotient was calculated as the ratio of CER over OUR.

protein hydrolysate (presumably amino acids and small peptides) which are more oxidized than glucose were catabolized during the initial growth stage. RQ values less than1.0 during the later growth stage indicated that the primary substrate for the culture growth had been switched from protein hydrolysate to glycerol which is a more reduced form than glucose. An increase in culture pH during the initial phase of cell growth prior to the surge of CER indicated amino acid catabolism by *E. coli*, accumulating ammonium ions as a result of deamination of amino acids. During the growth phase generating high levels of CER, the culture pH decreased, indicating acid formation as a result of glycerol consumption. Acid and base were automatically added to the fermentors in response to these pH changes to maintain the pH at 7.2 throughout the fermentation.

Two distinctive catabolic phases were found based on preferential utilization of protein hydrolysate to glycerol during the fermentation in M101 medium. The initial growth of the culture was achieved by utilizing the protein lysate as a carbon and energy source without noticeable consumption of glycerol. When key nutrient(s) in the protein hydrolysate were depleted from the medium after achieving a certain cell mass, the catabolism of *E. coli* was switched to glycerol as indicated by a high CER peak. The timing of the transition of catabolic phases was dependent upon the initial concentration of yeast extract in the medium regardless of the initial glycerol concentration. In addition, use of various amounts or sources (lots) of yeast extract in M101 medium caused significant differences in final cell mass and productivity of TP-40, whereas Hy-soy peptone at various concentrations did not affect the fermentation performance [14; unpublished data]. This result suggested that the initial concentration of yeast extract in the production medium was the limiting factor for higher cell growth and TP-40 production under the current fermentation conditions.

Development of fermentation process for enhanced productivity and scale-up

To enhance the volumetric productivity of TP-40, our efforts focused on increasing the final cell concentration while maintaining maximum specific productivity. An experiment was conducted at the 15-L scale to examine the changes in the growth profiles and catabolic phases of *E. coli* using various yeast extract concentrations without induction. By increasing yeast extract concentration in the medium from 1% to 2% and 4% (w/v), the length of the protein hydrolysate catabolic phase, measured from inoculation to the initiation of CER peaks, increased from 7.5 to 11 and 15 h respectively (Figure 2a and 3b), while the cells continued to grow. This approach allowed *E. coli* to attain a higher cell concentration prior to induction of the culture at the transition of catabolism from protein hydrolysate to glycerol. By employing different induction timing, the final cell concentration (OD_{600}) at harvest after 4 h post-induction period increased from 19 to 26 and 38, respectively (results not shown).

Modified M101 medium with 2% (w/v) glycerol containing either 1% or 4% (w/v) yeast extract were compared for TP-40 productivity after inducing the culture at various stages of the catabolic phase (Figure 5). The highest volumetric productivities (400 and 850 mg L^{-1} of TP-40) were

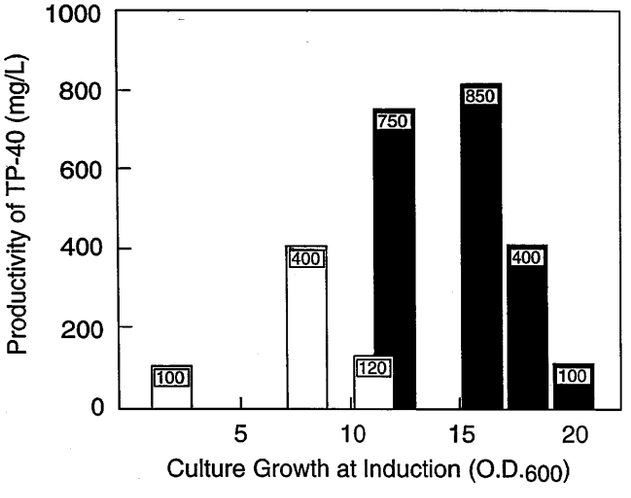

Figure 5 TP-40 productivities with different induction timings during fermentation at the 15-L scale. Maximum TP-40 productivities were compared between the previous process with M101 medium containing 1% (w/v) yeast extract (□) and the modified process employing an enriched yeast extract concentration (4%, w/v) and a delayed induction timing (■).

Table 1 Comparison of TP-40 productivity at three fermentation scales

Medium[a] volume (L)	Cell density at induction (OD_{600})	Cell density at harvest (OD_{600})	Post-induction period (h)	Specific productivity ($\mu g\ mg^{-1}$ protein)	Titer (mg L^{-1})
15	16	28	4	175	850
180	19	31	4	141	740
800	17	33	4	168	950

[a]M101 medium containing 2% (w/v) glycerol and 4% (w/v) yeast extract.

achieved when the cultures were induced at OD_{600} = 8.0 and 16.0 for the batches with 1% and 4% (w/v) yeast extract, respectively. The specific productivities of TP-40 from these batches were comparable at 195 and 175 mg mg^{-1}. Based on growth profiles of the uninduced cells, both cultures containing 1% and 4% (w/v) yeast extract were in the transition phase immediately before the surge of CER peaks at OD_{600} = 8.0 and 16.0, respectively. Similar fermentation performance including culture growth rate, lag time, and relationship between timing of induction and TP-40 productivity was observed upon scale-up from the 15-L to the 180-L and 800-L scales. Carbon dioxide evolution profiles also followed the two-phase behavior observed for the laboratory-scale fermentations. Specific productivities and titers of TP-40 at the 180- and 800-L scales ranged from 141–168 mg mg^{-1} and 740–950 mg L^{-1}, respectively, which were comparable with values obtained at the 15-L scale (Table 1).

Conclusion

Identifying two distinctive catabolic phases during *E. coli* fermentation in the M101 complex medium and understanding the relationship between induction timing and metabolic state of host cells were critical to improve production of TP-40. The volumetric productivity of TP-40 was doubled by increasing the yeast extract concentration in M101 medium and by delaying the induction timing (Figure 5). This productivity increase was maintained upon both 12- and 50-fold scale-up to 180-L and 800-L batch volumes respectively. Attempt with higher concentration of yeast extract up to 6% did not increase the final cell mass or TP-40 productivity. Investigation of other growth parameters and medium ingredients including dissolved oxygen control, substrate feeding, and plasmid stability will be needed for further improvement of the fermentation.

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