0273-2289/92/3435—0161$02.60

Optimal Nutrient Feed Policies for Heterologous Protein Production

S. W. HARCUM, D. M. RAMIREZ, AND W. E. BENTLEY*

Center for Agricultural Biotechnology, Department of Chemical Engineering, University of Maryland, College Park, MD 20742

ABSTRACT

Recombinant *Escherichia coli,* which overproduce heterologous protein, redirect endogenous metabolic activity to that mediated by the recombinant expression vector. Consequently, cells may experience perturbations in the biosynthetic reaction network, including the amino acid biosynthesis pathways. These cells are characterized by decreased growth rate, decreased cell mass yield, and increased heterologous protein degradation. This study investigates the dynamics of chloramphenicol-acetyl-transferase (CAT) synthesis and degradation in *E. coli* JM105 grown on minimal media, and correlates the observed phenomena with induction strength. Coordinated amino acid feeding was shown to increase the heterologous protein yield. Rational design of nutrient feeding possibilities is explored.

Index Entries: Heterologous protein expression; stress response; protease activity; nutrient feed policies.

INTRODUCTION

In order to develop an economically successful process for the production of a recombinant protein, high expression levels are often required. One method of obtaining a high protein content is by amplifying gene copy number, via runaway-replication expression vectors. However, the concomitant increase in protein expression often results in cell death (*1*). A more common method for protein overexpression in procaryotes is to amplify the appropriate messenger RNA by inserting an inducible promoter upstream of the foreign gene and controlling its transcription rate.

*Author to whom all correspondence and reprint requests should be addressed.

Transcription is regulated by the concentration of a gratuitous, nonmetabolized inducer, such as isopropyl-β-D-thiogalactoside (IPTG) for the *lac*-like promoters. The inducer binds to and inactivates the repressor, allowing transcription to occur at a high rate (2). Since the inducer is not metabolized, transcription will continue unabated until the cell dies, nutrients run out, or dilution by cell growth or media transport sufficiently reduces the intracellular inducer concentration. Excessive transcription may impose a "metabolic burden" on the cell because of the increased requirement for raw materials, in particular amino acids and other precursors (3). The amino acid composition of the foreign protein can be drastically different than that of the average *E. coli* protein, so that an additional burden on the cell's amino acid synthesis network may result simultaneously with increased protein expression. Temporary amino acid depletion could occur, even in a nonauxotrophic host. This type of perturbation may elicit a stringent response.

In recombinant *E. coli* that overproduce foreign protein, elevated levels of protease activity specifically directed toward the foreign protein have been observed (4). These proteases are a part of the heat-shock response (4), which is normally induced by elevated culture temperature (5–8). Interestingly, the heat-shock response can be induced by a stringent response as well, which includes proteases that degrade abnormal proteins (9,10). One of the most prevalent heat-shock proteins is the protease La (2,4,6,9,11,12). The protease La is a tetramer of 94,000 Daltons and degrades abnormal proteins at the expense of ATP energy (6,9,12). Thus, cells that have increased La activity expend additional energy for protein degradation that may otherwise have been available for biosynthesis. Further, Goff and Goldberg (4) have suggested that, in some cases, foreign proteins encoded by plasmid DNA might appear "abnormal" to the host cell and, therefore, more vulnerable to degradation.

The objective of this study is to investigate the dynamics of chloramphenicol-acetyl-transferase (CAT) synthesis and degradation in *E. coli* JM105 [pSH101], grown on minimal media, and to correlate cell mass yield, growth rate dynamics, and the dynamics of product synthesis and degradation with induction strength. In addition, amino acid feeding policies were developed to provide cloned-gene-product (cgp) precursors and to circumvent cgp degradation, in order to increase heterologous protein yield.

MATERIALS AND METHODS

Host and Expression Vector

E. coli JM105, a $LacI^q$ strain, obtained from Pharmacia (Pleasant Hill, CA), was transformed with the plasmid pSH101 (Amp^r, Cm^r), which was constructed from the plasmid pKK233-2 and the CAT GenBlock

(HindIII cartridge), both purchased from Pharmacia. The CAT GenBlock (HindIII cartridge) required site-directed mutagenesis at the initiation site, such that an NcoI restriction site on the CAT GenBlock (HindIII cartridge) would ligate with the NcoI restriction site on the plasmid pKK233-2. The resulting plasmid (pSH101) provides for constitutive expression of β-lactamase and, under IPTG-inducible *trc* promoter control, overexpression of CAT. Stop codons located downstream of the CAT gene prevent read-through transcription and translation, so that the amplified protein expression is limited to the CAT gene.

E. coli RR1 [pBR329] was purchased from the American Type Culture Collection (Rockville, MD). CAT and β-lactamase are constitutively expressed from this plasmid, along with the polypeptides for tetracycline resistance, which in turn, can be induced by the addition of tetracycline.

Culture Conditions

Fermentations were run in Applikon fermenters (3 L) with a 2-L working volume. M-9 minimal media was used in all fermentations and prepared as described by Rodriguez and Tait (*13*), except that 25 μg/mL streptomycin (Sigma, St. Louis, MO) and 1 μg/mL thiamine (Sigma) were added. Thiamine is required by JM105 for growth in M-9 minimal media. Casamino acids were omitted. Ampicillin (40 μg/mL) was used for cultures containing the plasmid pSH101 in order to ensure a homogeneous plasmid-bearing population. The temperature was controlled at 37°C ± 1°C, and pH was maintained at 7.0 ± 0.2. The dissolved oxygen concentration was maintained above 60% saturation. Cultures, grown overnight in shake flasks at 37°C and 200 rpm in LB media, were started from a freezer culture and stored at 4°C. Shake flasks containing M-9 minimal media were then inoculated with the LB culture (1% v/v). These M-9 cultures grew for 12 h, at which time they were used to inoculate the fermenters (also 1% v/v).

E. coli RR1 [pBR329] fermentations in M-9 minimal media required supplemental leucine, proline, and thaimine at 41, 164, and 0.166 μg/mL, respectively. *E. coli* RR1 [pBR329], grown overnight in shake flasks at 37°C and 200 rpm in LB media, were inoculated from a freezer culture and later stored at 4°C. Shake flasks containing M-9 minimal media were then inoculated with the LB culture (1% v/v). These M-9 cultures grew overnight, at which time they were used to inoculate the shake flasks for the phenylalanine addition experiments. *E. coli* JM105 [pSH101] cultures were also inoculated from overnight M-9 minimal shake flasks for the phenylalanine addition experiments.

Fermentation Monitoring and Analysis

Growth was monitored by OD (600 nm in the linear range 0.01–0.25 OD) on a Gilford Response Spectrophotometer. Samples above 0.25 OD were diluted with dH_2O until in the linear range. OD measurements were

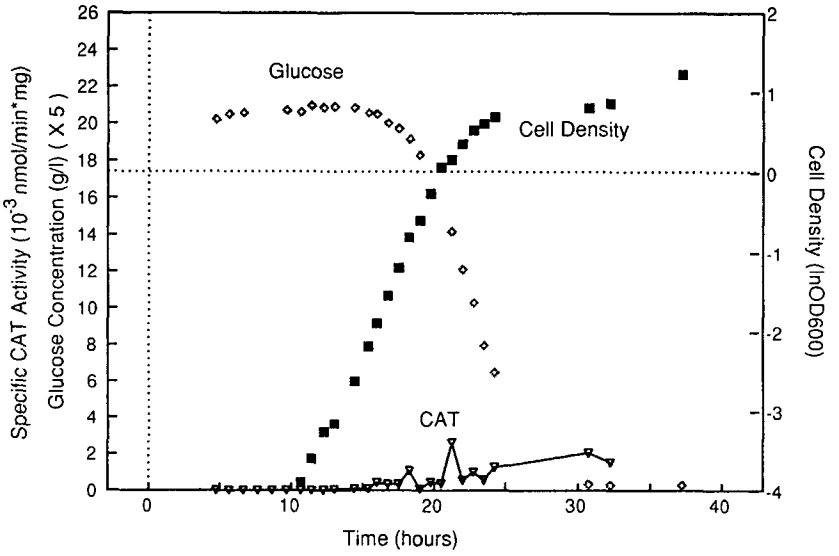

Fig. 1. Cell density, glucose concentration, and specific CAT activity vs time for *E. coli* JM105 [pSH101] in M-9 minimal media. Initial glucose concentration was 4 g/L.

corrected for the background media OD. Cell extract for the CAT activity assay was prepared according to the procedure of Rodriguez and Tait (*13*), except cells were frozen in the TDTT (50 m*M* Tris-HCl, pH 7.8, 30 μM DL-dithiothreitol) buffer prior to sonication after two TDTT washings. All time series samples (approx 600 μL) were sonicated together for 80 s with a 30% pulsed duty cycle using a Sonifier Cell Disruptor 350 with a microtip by Branson Sonic Power, Co. (Danbury, CT). The CAT activity assay was performed as described by Rodriguez and Tait (*13*), except reagent volumes were doubled and samples were diluted up to 1/32 to avoid reagent depletion. Total protein was measured by Bio-Rad (Richmond, CA) protein assay dye reagent concentrate. Specific CAT activity is reported as nmol CAT/min·mg total protein and is the average of duplicates. In samples where cells were removed by centrifugation, the supernatants were frozen and assayed later for glucose concentration on a YSI Model 27 glucose analyzer.

RESULTS AND DISCUSSION

Figures 1, 2, and 3 illustrate the time course of cell density, glucose concentration, and specific CAT activity during an *E. coli* JM105 [pSH101] fermentation on M-9 minimal media. The uninduced fermentation is shown in Fig. 1. The specific CAT activity represents the background expression level of the CAT gene under the control of the repressed *trc* promoter.

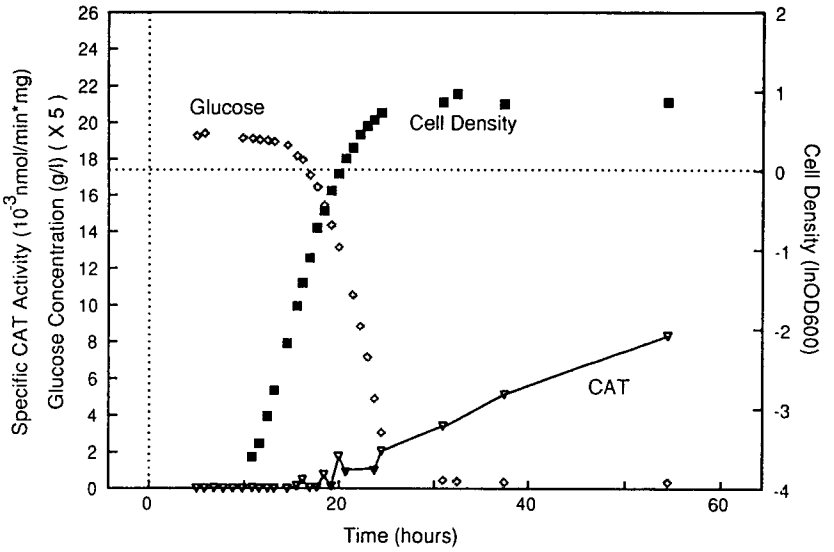

Fig. 2. Cell density, glucose concentration, and specific CAT activity vs time for *E. coli* JM105 [pSH101] in M-9 minimal media. Induction by the addition of 2 m*M* IPTG was at 15.6 h. Initial glucose concentration was 4 g/L.

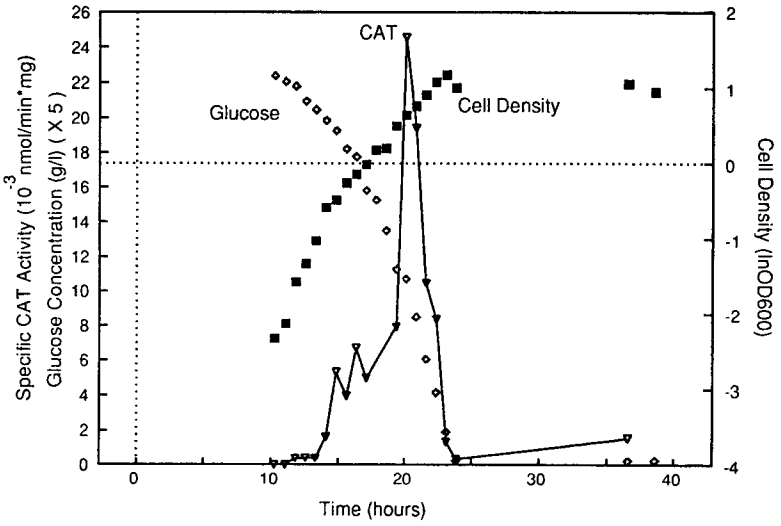

Fig. 3. Cell density, glucose concentration, and specific CAT activity vs time for *E. coli* JM105 [pSH101] in M-9 minimal media. Induction by the addition of 5 m*M* IPTG was at 12.6 h. Initial glucose concentration was 4 g/L.

This level of CAT expression is 2–3× lower than that produced under the native CAT promoter, which is contained on the plasmid pBR329. The uninduced cells stop growing only when the glucose is depleted from the media. The cells are maintained in the stationary phase an additional 10–15 h.

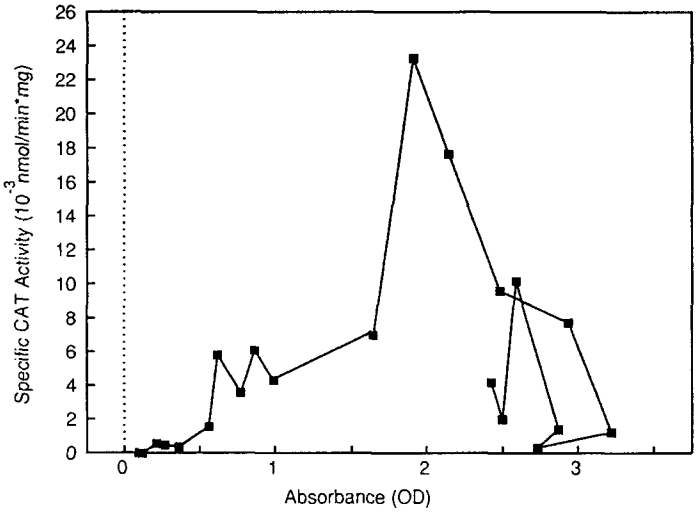

Fig. 4. Specific CAT activity for JM105 [pSH101] in M-9 minimal media vs cell density (OD). Same experiment as depicted in Fig. 3. Specific CAT activity begins to decrease while cell density is still increasing.

A low-level induction (2 m*M* IPTG) fermentation is shown in Fig. 2. Specific CAT activity increases throughout the fermentation, eventually reaching approx 8.0×10^3 nmol/min·mg total protein near the end of the fermentation. The specific CAT activity in this fermentation is at least 4× greater than in the final sample of the uninduced culture and twice that typically found in *E. coli* RR1 [pBR329]. CAT production continues during the stationary growth phase for the 2 m*M* IPTG fermentation. Interestingly, the cells continue to produce CAT, albeit at a slower rate than during cell growth, even when there is no extracellular glucose to supply energy. Therefore, the cells are exploiting endogenous metabolic energy reserves to produce CAT.

The highest specific CAT activity is observed at an intermediate induction level (5 m*M* IPTG), as shown in Fig. 3. The maximum specific CAT activity occurs prior to substrate depletion and the stationary growth phase of the fermentation. In this case, the rapid increase in CAT productivity upon induction is followed by an even more rapid decrease in specific CAT activity. Perhaps, the expression rate or intracellular concentration of CAT triggers a cellular response that enhances its degradation, as described previously. Subsequent transmission electron microscopy revealed no inclusion bodies (*14*). In Fig. 4, the specific CAT activity is plotted as a function of OD, which clearly illustrates that the specific CAT activity decreases while the cell density continues to increase. More commonly, protein degradation occurs during the stationary phase, after nutrient supplies have been depleted (*15*). Thus, our results indicate that the elicitation of cloned-protein degradation is dependent on its synthesis, and not the presence or absence of abiotic nutrients. A brief window of

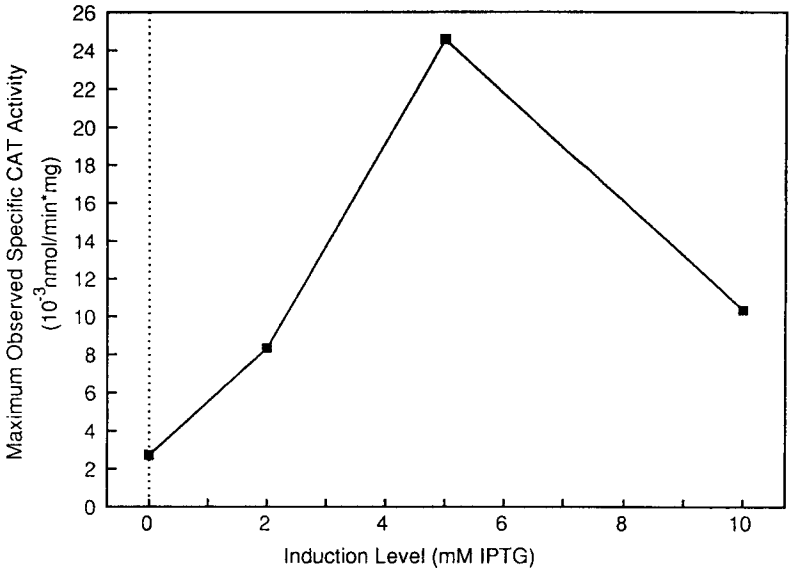

Fig. 5. Maximum observed specific CAT activity vs induction level. Maximum observed specific CAT activity occurs at an intermediate induction level.

high activity apparently exists at high heterologous protein expression rates, which could be very important when considering cell harvest and separation times for bioprocess design. Further increases in inducer concentration did not increase the specific CAT activity. Instead, the maximum specific CAT activity obtained decreased to 1.0×10^4 nmol/min·mg for a high induction level fermentation (10 m*M* IPTG, Fig. 5). This might be due to increased protease activity, such that a high expression rate is accompanied by a high degradation rate. This is a topic of further study. Consequently, an induction level for *E. coli* JM105 [pSH101] between 2–10 m*M* IPTG results in the maximum peak specific CAT activity. An induction level that corresponds to a fully derepressed repressor would have the maximum transcription rate for the host/expression vector system and, potentially, the maximum peak specific CAT activity. However, this may be short-lived, so that the induction level with the maximum peak specific CAT activity is not always optimal for recombinant protein production.

Cellular Biosynthetic Efficiency

The growth rate of *E. coli* JM105 [pSH101] prior to induction was approx 0.4 h^{-1}, and after induction with IPTG at 2, 5, and 10 m*M*, the growth rate decreased by approx 0.1 h^{-1}. The addition of an inducer, which stimulates cloned-protein expression, imposes an additional metabolic burden on the recombinant cell, because the metabolic activity is redirected to that mediated by the rDNA expression vector. As a result, the cell growth rate decreases (*16*). Our results also indicate that the cell

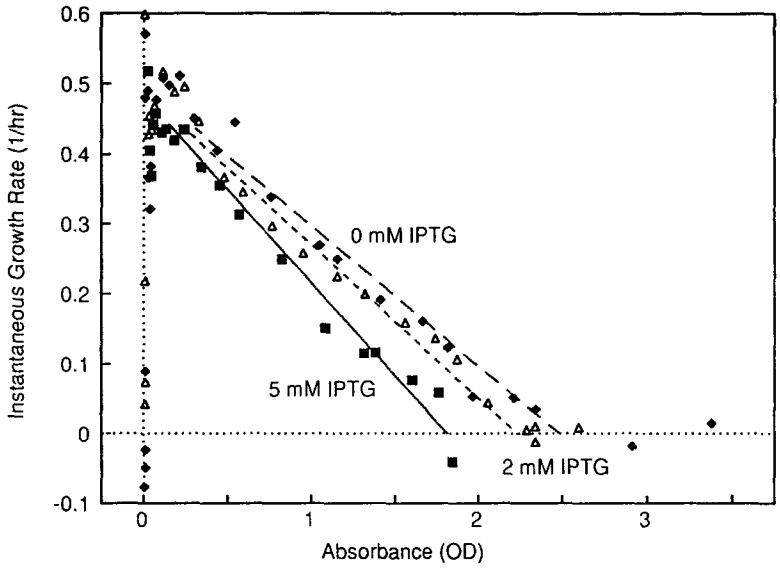

Fig. 6. Instantaneous growth rate for JM105 [pSH101] vs cell density (OD). Indicates reduced efficiency for biomass synthesis.

produces cell mass less efficiently. The cell yield coefficient from glucose ($Y_{x/s}$) decreases from approx 0.36 to 0.30, 0.27, and 0.27 (g cell/g glucose) for the induction levels 2, 5, and 10 m*M* IPTG, respectively (data not shown). An exponentially growing cell that is induced to overproduce a foreign protein reduces the efficiency of its cellular biosynthetic network. Moreover, reduced biosynthetic efficiency is a dynamic problem and is correlated with the expression rate of the recombinant protein. In Fig. 6, the instantaneous specific growth rate of the cultures, calculated from its definition:

$$\mu \equiv (1/x)^*(dx/dt) \quad (1)$$

where x and dx/dt denote cell mass concentration and its time derivative, respectively, continually decreases as the fermentations proceed and the cells adapt to increased CAT production. During ideal exponential growth, this value remains constant. The growth rate approaches zero near the time at which the glucose is exhausted (not shown). Interestingly, the *rate of decrease* in growth rate appears to be a function of induction strength. This is made evident by noting that the slopes of the lines indicated become more negative with increased induction strength. This analysis indicates that, in the most strongly induced culture (5 m*M* IPTG), the cells are burdened more significantly than in the other cultures and *progressively* lose the ability to continue synthesizing biomass. Thus, the cells become increasingly less efficient as they produce more recombinant product, and the decrease in efficiency is directly related to the induction strength.

Table 1
Amino Acid Composition
by Family for CAT and Average *E. coli* Protein

Amino acid families	CAT, % by wt	Average *E. coli* protein, % by wt
Aromatic	28.5	10.4
Aspartate	29.5	32.2
Glutamate	18.5	24.6
Pyruvate	16.0	22.2
Serine	7.5	10.6

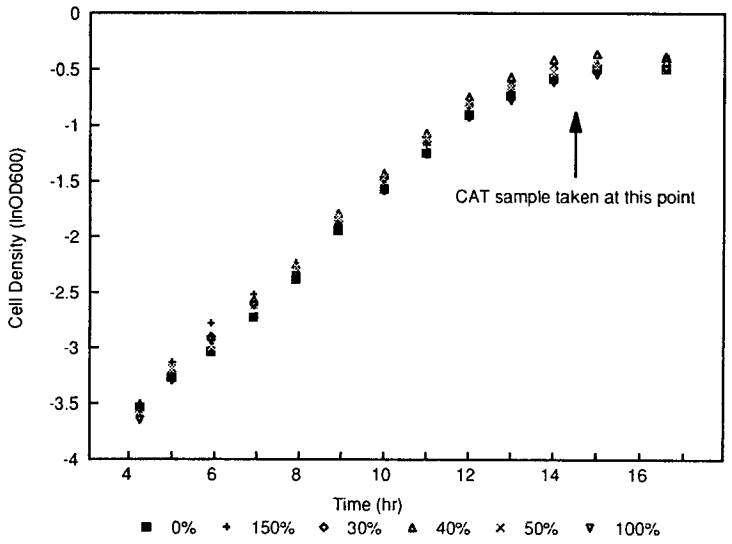

Fig. 7. Growth of *Escherichia coli* JM105 [pSH101] for various phenylalanine concentrations (100% is 1.838×10^{-3} g/L).

Nutrient Feeding Strategies

Since CAT does not have the same amino acid composition as the average *E. coli* protein (*see* Table 1), a rapid increase in CAT expression could temporarily deplete the intracellular pool of an amino acid that *E. coli* does not normally synthesize in large quantities. More specifically, phenylalanine is 11.5% by weight of the CAT protein, and 4.4% by weight of the average *E. coli* protein. Potentially, a rapid increase in CAT expression could differentially deplete phenylalanine from the intracellular amino acid pool. Hence, phenylalanine was added at various concentrations to determine its effect on the production of CAT. In Fig. 7, the growth of *E. coli* JM105 [pSH101] is depicted with several addition levels of phenylalanine. Samples were taken at 14.5 h (indicated by an arrow) and ana-

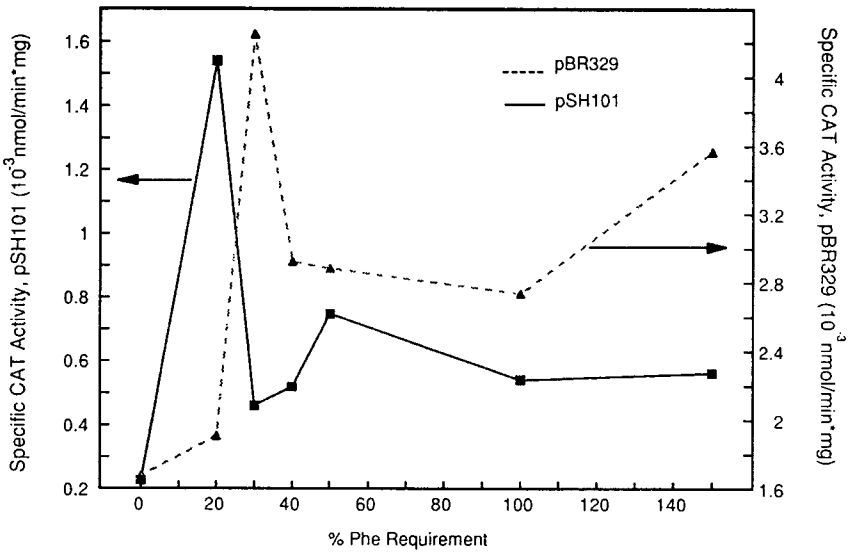

Fig. 8. Specific CAT activity for *E. coli* RR1 [pBR329] and *E. coli* [pSH101] vs phenylalanine added.

lyzed for CAT. The OD and growth phase of the cultures were similar in all cases. In this way, specific CAT activity variations, which have been shown to be time dependent (Figs. 1-3), are minimized, and a comparison between cultures can be made. The amount of phenylalanine added was calculated based on the anticipated final yield in a 100-mL shake flask culture. The added amount of phenylalanine is indicated as the abscissa and is expressed as a percentage of that required given no endogenous phenylalanine synthesis. Results of phenylalanine addition on specific CAT activity are depicted in Fig. 8.

When phenylalanine was fed to *E. coli* to supplement the amount endogenously synthesized for CAT production, increased CAT activity was found in all cases for both *E. coli* JM105 [pSH101] and *E. coli* RR1 [pBR329]. The observed specific CAT activity was lower for all *E. coli* JM105 [pSH101] cultures since the overexpression of the *lac* repressor in this $LaqI^q$ *E. coli* strain when not induced. Neither strain is an auxotrophic mutant. Therefore, we have attempted to regulate the existing capabilities of the host cell by minimizing any temporary amino acid depletion. The increase in CAT observed does not, however, validate the hypothesis that phenylalanine was depleted or low in these cells. The additional phenylalanine could have been used for other cellular purposes. For example, the growth rate was higher for all phenylalanine-fed cultures (Tables 2 and 3), which could be the result of its consumption as an additional carbon and/or nitrogen source. A suitable control experiment has recently been developed and will be included in a subsequent communication.

Table 2
Growth Rate and Specific CAT Activity
as a Function of Phenylalanine Added *E. coli* RR1 [pBR329]

Phenylalanine, %	Phenylalanine, μM	Growth rate, (h^{-1})	Specific CAT activity, nmol/min·mg
0	0	0.38	1675
20	2.23	0.39	1906
30	3.34	0.42	4249
40	4.45	0.42	2924
50	5.56	0.43	2884
100	11.13	0.43	2737
150	16.69	0.43	3566

Table 3
Growth Rate and Specific CAT Activity
as a Function of Phenylalanine Added *E. coli* JM105 [pSH101]
(no IPTG Addition)

Phenylalanine, %	Phenylalanine, μM	Growth rate, (h^{-1})	Specific CAT activity, nmol/min·mg
0	0	0.34	227
20	2.23	0.36	1541
30	3.34	0.35	460
40	4.45	0.36	520
50	4.45	0.37	748
100	11.13	0.35	542
150	16.69	0.35	562

The trends illustrated in Fig. 8 for *E. coli* JM105 [pSH101] and RR1 [pBR329] are strikingly similar. At a low phenylalanine level (20–40%), a sharp maximum in specific CAT activity is obtained. Further phenylalanine addition actually decreases the CAT yield in both strains. Clearly the maximum benefit obtained from phenylalanine addition is at a lower level than the calculated maximum phenylalanine requirement. Thus, the cells must continue to synthesize phenylalanine in the optimal case. Whitney et al. (*15*) noted that indiscriminate amino acid addition during the production phase of a batch culture increased yield by 50%. Our results are consistent, but further demonstrate that indiscriminate addition of amino acids is not optimal. Instead, a further increase in recombinant protein yield can result from a regulated or planned addition strategy. Decreased specific CAT activity at higher phenylalanine concentrations (40–150%, Fig. 8) might be due to feedback inhibition by phenylalanine on the

synthesis of the other aromatic amino acids, which are coordinately controlled. The production of CAT, therefore, is more likely affected by this regulation than the production of *E. coli* cell mass (*see* Table 1). It is noteworthy that these phenomena occur in both strains, despite different promoters controlling transcription (the native constitutive CAT promoter for pBR329, and the inducible *trc* promoter from pKK233-2 in pSH101). IPTG was not added to these fermentations, because we were interested in the background effects of nutrient feeding without interference from the dynamics associated with inducer/repressor interactions. This suggests that the effect of phenylalanine addition is the result of an inherent characteristic of *E. coli* amino acid regulation and not the genetic control sequences of cgp expression. In other words, amino acid addition influences the protein translation rate and not the DNA transcription process, which is consistent with our amino acid depletion hypothesis.

SUMMARY

CAT productivity is increased by the addition of an inducer for the JM105 system. However, maximum productivity does not coincide with the highest induction level. An intermediate induction level has the highest observed specific CAT activity. Also, the maximum specific CAT activity is maintained only briefly before rapid degradation occurs. Low induction levels are accompanied by CAT production throughout the fermentation. High foreign protein production may burden the amino acid synthesis network, resulting in a stress response in which the stress protease activity increases. This decreases the observed foreign protein productivity. Coordinated precursor addition, such as phenylalanine addition for CAT synthesis, can increase dramatically the heterologous protein yield, possibly by reducing the abnormalities in the amino acid synthesis network. The degradation of proteins, including identification of specific stress-induced proteases, and perturbations in the amino acid regulation of recombinant systems are presently being investigated in our laboratory.

ACKNOWLEDGMENTS

The authors would like to acknowledge the generous assistance of Dr. Prasad Reddy of the Center for Advanced Research in Biotechnology (MBI/NIST) in constructing pSH101. Also, we acknowledge the support of the Engineering Research Center's BioProcess Scaleup Facility at the University of Maryland. Financial support for this work from the National Science Foundation (Award No. BCS-9010756) is also greatly appreciated.

REFERENCES

1. Bentley, W. E. and Kompala, D. S. (1990), *Annals of the New York Academy of Sciences* **389,** 121–138.
2. Neidhardt, F. C., Ingraham, J. L., and Schaechter, M. (1990), *Physiology of the Bacterial Cell: A Molecular Approach* Sinauer Associates, Sunderland, MA.
3. Bentley, W. E. and Kompala, D. S. (1990), *Biotechnology Letters* **12,** 329–334.
4. Goff, S. A. and Goldberg, A. L. (1985), *Cell* **41,** 587–595.
5. Neidhardt, F. C., VanBogelen, R. A., and Vaughn, V. (1984), *Annual Review of Genetics* **18,** 295–329.
6. Phillilps, T. A., VanBogelen, R. A., and Neidhardt, F. C. (1984), *Journal of Bacteriology* **159,** 283–287.
7. Lindquist, S. and Craig, E. A. (1988), *Annual Review of Genetics* **22,** 631–677.
8. Patrusky, B. (1990), *Mosaic* **21,** 2–11.
9. Larimore, F. S., Waxman, L., and Goldberg, A. L. (1982), *The Journal of Biological Chemistry* **257,** 4187–4195..
10. Grossman, A. D., Taylor, W. E., Burton, Z. F., Burgress, R. R., and Gross, C. A. (1985), *Journal of Molecular Biology* **186,** 357–365.
11. Mizusawa, S. and Gottesman, S. (1983), *Proc. Natl. Acad. Sci. USA* **80,** 358–362.
12. Chung, C. H. and Goldberg, A. L. (1981), *Proc. Natl. Acad. Sci. USA* **78,** 4931–4935.
13. Rodriguez, R. L. and Tait, R. E. (1983), *Recombinant DNA Techniques: An Introduction,* Benjamin/Cummings, Menlo Park, CA.
14. Ramirez, D. M. (1991), Optimal Feeding Strategies for the Production of Foreign Proteins, MS Thesis, University of Maryland, College Park, MD.
15. Whitney, G. K., Glick, B. R., and Robinson, C. W. (1989), *Biotechnology and Bioengineering* **33,** 991–998.
16. Bentley, W.E., Mirjalili, N., Anderson, D. C., Davis, R. H., and Kompala, D. S. (1990), *Biotechnology and Bioengineering* **35,** 668–681.