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The Effect of Cellular Energetics on Foreign Protein Production

YUN-FEI KO,[1,3]
WILLIAM E. BENTLEY,[1,2] AND WILLIAM A. WEIGAND*,[1,3]

[1]Department of Chemical Engineering; [2]Center for Agricultural Biotechnology; and [3]Center for Biotechnology Manufacturing, University of Maryland, College Park, MD 20742

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ABSTRACT

Escherichia coli strain F-122 was used to determine if there are additional physiological effects, other than decreasing energetic efficiency accompanied by the excretion of the acetate, on foreign protein production. This organism was the host for expressing HIV_{582}-β-galactosidase fusion protein under the control of the *trp* promoter, with ampicillin resistance. By comparing parallel batch cultures with and without acetate addition, it was found that the presence of acetate in the media did not influence β-galactosidase activity. In these experiments, it appears that the low protein productivity often observed during acetate formation is the result of inefficient cell metabolism, rather than acetate acting as a specific inhibitor of protein production.

Index Entries: *Escherichia coli;* acetate excretion; cellular energetics; foreign protein.

Nomenclature: F_c, the carbon factor; F_e, the energy factor; G_s, the scaling factor; *HAC*, acetate concentration; P_f, cell mass fraction of foreign protein, g/g; Y_s, cell yield; Z, fraction of carbon flux flow into the EMP pathway at time t; n, total glucose flux; η, normalized efficiency coefficient.

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

INTRODUCTION

Acetate production is an overflow phenomenon where acetyl CoA is diverted from the tricarboxylic acid cycle to acetyl phosphate and then acetate. This results in the production of 1 mol of substrate level ATP/mol of acetate. The excretion and accumulation of acetate have been shown to be a major factor in determining process performance. It is recognized as an undesired byproduct for three reasons.

1. Acetate inhibited cell growth. When its concentration was above 14 g/L cells growth stopped (*1*),
2. Acetate is essentially a metabolically useless compound whose excretion decreases the process yield (*2,3*), and
3. The accumulation of acetate in the growth medium has been suggested to have deleterious effects on the expression of products by recombinant *E. coli* (*4–9*). For example, high levels of acetate excretion and accumulation in recombinant *E. coli* cultures reduced product formation for interferon (*7*), human insulin-like growth factor (*4*), and penicillin acylases (*2*).

Therefore, it is of practical importance to define a policy that reduces acetic acid excretion and the unfavorable consequences of its presence in the culture medium. Different operating policies have been proposed to supply the substrates, nutrients, and oxygen to the fermentation, such as dialysis culture (*10,11*), DO stat (*12*), the modified DO stat (*13*), and the balanced DO stat (*14*). In general, operating strategies were developed based on either oxygen tension (DOC), carbon source concentration (glucose), or both (*10–16*). However, these control rules, although important for improvement, may not be optimal operating policies, since they are based on the experience of researchers or experimentally observable phenomena, rather than on a detailed analysis of the key physiological phenomena.

The objectives of this work are: (1) to determine if there are additional physiological effects, other than the decreasing energetic efficiency accompanied by secretion of acetate, on foreign protein production during recombinant *E. coli* fermentation processes, and (2) to analyze the effect of the decreasing energetic efficiency on foreign protein production based on the metabolic modeling approach introduced in previous papers (*17,18*).

The Effect of Acetate Excretion on Foreign Protein Production

In earlier papers (*17,18*), we have pointed out that the dissolved oxygen and glucose concentrations exert influence on the balance between the fermentative and oxidative carbon flows, provided that none of the medium components are limiting. More specifically:

1. At high glucose uptake rates, the TCA cycle may become saturated; hence substrate-level phosphorylation may produce more ATP resulting in acetate overproduction;
2. In addition, if oxygen is limited, the rate of NAD(P)H oxidation will become slower than under fully aerobic conditions. Hence the cells will produce more acetate in order to reduce the generation of NAD(P)H in the TCA cycle while maintaining sufficient ATP production; and
3. At high specific growth rate, usually energy (ATP) is overproduced, and the cells will also produce more acetate in order to balance the catabolic and anabolic reactions.

In brief, oxygen deficiency will force glucose through the fermentative pathways. Similarly, glucose overfeeding will force glucose through the fermentative pathways. In addition, when the reducing equivalents generated by the carbon flow exceed the capacity of the oxidative respiratory pathway, glucose is diverted to the fermentative pathway, and acetate is formed. It is clear that these discussions that the excretion and accumulation of acetate are a good indication of *E. coli* cellular energetics, i.e., the decreasing energetic efficiency is related to the secretion of acetate. Also, acetate is a utilizable carbon and energy source for *E. coli*. Also, there is no direct evidence that acetate acts as a specific inhibitor or repressor during foreign protein production. After considering these points, our hypothesis is that the observed deleterious effects on the expression of foreign protein during recombinant *E. coli* cultures are caused by the lower energetic efficiency rather than the secretion and accumulation of acetate. The experiments performed to test this hypothesis are described below.

MATERIALS AND METHODS

Bacterial Strain

Escherichia coli strain F-122 obtained from Univax Biologics, Inc. was used as a host strain for expressing HIV_{582}-β-galactosidase fusion protein under the control of the *trp* promoter with ampicillin resistance.

Media

The medium was M9 minimum supplemented with L-tryptophan, L-leucine, L-proline, and vitamin B1 at levels of 90, 41, 164, and 0.166 μg/mL, respectively, according to Rodriguez and Tait (*8*). In order to avoid the lag period caused by secreting the fusion protein in the early stage of cell cultivation, the tryptophan concentration used in this study is five times higher than the standard tryptophan concentration, 18 μg/mL, in the Rodriguez receipe. All chemicals (Sigma) were reagent-grade.

Cultivation

Experiments were performed in 250-mL Erlenmeyer flasks, at 37°C and 250 rpm in a reciprocating water bath shaker (New Brunswick Scientific). Inoculum was prepared by first adding 1 mL of *E. coli* frozen stock to 100 mL medium with 40 μg/mL of ampicillin. This was grown overnight until the optical density (OD) was approx 0.9 U OD (600 nm), and then 2 mL of this culture were used to inoculate the flask containing 100 mL of the same medium as that employed in the overnight culture. One hour before inoculation, these flasks were put in the bath shaker to heat them to 37°C.

Growth Rate Measurement

OD was measured on a Milton Roy Spectronic 21 spectrophotometer. All measurements were made at 600 nm in the linear range (0.05–0.25 OD units). Samples with higher concentrations of cells were diluted with sterile deionized water to obtain an OD in the linear range. All the readings of OD were corrected with the OD of the respective sterile media.

Assay of β-Galactosidase

The enzyme activity, β-galactosidase, was determined according to the protocol proposed by Miller et al. (*19*).

RESULTS AND DISCUSSION

Effect of Glucose Concentration on Cell Growth

This experiment was undertaken to see whether high glucose concentration resulted in substrate inhibition and to make sure no acetate formation occurred during the exponential growth phase. Cultures were run in M9 minimal media with glucose concentrations between 2 and 20 g/L. No significant change in specific growth rate was observed. A slight increase in the lag period was noticed. However, it is not likely to be a contributing factor that affects foreign protein production. Also, no significant amount of acetate is formed during the exponential growth period within the range of glucose concentrations tested. The experimental results are listed in Table 1.

Effect of Extracellular Acetate Concentration on Cell Growth

Cultures were grown in media with identical glucose concentrations, but varying acetate contents. Osmolarity was kept constant by varying the NaCl concentration, which was present in excess. In this way, any effect on cell growth could be attributed to acetate concentration. No

Table 1
Shaking Flask Experiment:
Cell Growth vs Glucose Concentration

Glucose conc., g/L	Specific growth rate, 1/h	Lag time, h
2	0.61	3
4	0.62	3
8	0.64	3
12	0.65	3.5
16	0.66	4
20	0.65	4

Table 2
Shaking Flask Experiment:
Cell Growth vs Extracellular Acetate Concentration

Glucose conc., g/L	Specific growth rate, 1/h	Acetate Conc., g/L
4	0.63	0
4	0.62	1
4	0.62	2
4	0.61	2

growth rate effect was observed in the range of 0–3 g/L acetate. The experimental data are shown in Table 2.

Effect of Extracellular Acetate Concentration on Specific Enzyme Activity

In cultures similar to the second experiment performed, the effect of acetate on β-galactosidase specific activity was quantified. The experimental error can be minimized by not only utilizing the growth media and reagents prepared at the same batch, but also running two sets of flasks at the same time. Each set of flasks consisted of two flasks, one with acetate added and the other without acetate added. Cultures were sampled for β-galactosidase activity at the OD values indicated. By comparing parallel cultures with and without acetate, it is apparent that the presence of acetate in the media does not influence the β-galactosidase activity as shown in Table 3. Reports in the literature have suggested that acetate is a significant factor negatively influencing protein yield. Our hypothesis, which is consistent with the experimental result, is that acetate formation is more a result of inefficient cell metabolism and low protein productivity than a specific inhibitor of protein production.

Table 3
Shaking Flask Experiment:
Specific Enzyme Activity vs Extracellular Acetate Concentration

Glucose, g/L	Acetate, g/l	OD_{600}	Specific enzyme activity × 10^3
Set 1			
4	0	0.700	4.341
4	0	0.828	4.462
4	0	1.040	6.319
4	3	0.718	4.301
4	3	0.854	4.508
4	3	1.068	6.245
Set 2			
4	0	0.692	4.482
4	0	0.808	4.597
4	0	0.972	6.591
4	3	0.682	4.509
4	3	0.792	4.665
4	3	0.962	6.491

Cell Yield and Foreign Protein Production

In previous papers dealing with our metabolic model of cell energetics (*17,18*), the cell yield, Y_s, was defined as:

$$Y_s = Y_0 F_c F_e \tag{1}$$

Y_0, the carbon cell yield, should be determined by experiments based on the wild-type cells growing on the same carbon source that will be used for later fermentations. For instance, it is generally assumed that the value of Y_0 is 0.5 and 0.45 for wild-type and recombinant *E. coli* utilizing glucose as the sole carbon source under aerobic conditions, respectively (*20,21*). The carbon correlation factor, F_c, was defined as:

$$F_c = 1 - (\text{HAC}) / [(\text{Glucose})_i - (\text{Glucose})_{\text{HAC}}] \tag{2}$$

where $(\text{Glucose})_i$, $(\text{Glucose})_{\text{HAC}}$, and (HAC) are the initial glucose concentration, the glucose concentration at the sampling time of a batch culture, and the acetate concentration at the same sampling time, respectively. The energy factor can be written as (*18*):

$$F_e = (1.34\eta / Z * G_s) \tag{3}$$

where η, Z, and G_s are the normalized energy efficiency, the fraction of carbon flux that flows into the EMP pathway, and the scaling factor as defined previously (*17,18*). It should be understood that the excretion of acetate by *E. coli* does not mean that the cellular energetic efficiency is

low. There are four different conditions that cause increased acetate production. These are:

1. High glucose uptake rates;
2. High specific growth rates;
3. Low dissolved oxygen concentrations; and
4. A combination of these conditions.

Further, these conditions can be classified according to their metabolic responses as: Type I, the F_c-dependent response, which relates to the balance between the catabolic and anabolic reactions; Type II, the F_e-dependent response, which relates to the maintenance of proper redox ratio of reducing power, i.e., NAD:NADH; and Type III, the mixed-type response, which is both F_c- and F_e-dependent. This involves the complicated interactions among the fueling, precursor metabolite generating, and biosynthetic reactions. The excretion of acetate caused by condition 1 or 2, or the combination of these two conditions only decreases the substrate carbon yield and does not affect the cellular energy efficiency. Thus, these conditions are classified as Type I, and the effect of acetate excretion on the substrate carbon yield can be estimated by the carbon balance or F_c, Eq. (2). The excretion of acetate caused by condition 3 is always accompanied by low energy efficiency, which decreases the protein yield. This condition is Type II, and the effect of acetate excretion on the substrate yield can be estimated by the normalized dimensionless energy factor, F_e, Eq. (3). The excretion of acetate caused by Type III, the combination of condition 3 and the others, is always accompanied by both low energy efficiency and poor carbon source utilization. Here there is a decrease of the substrate yield and a reduction of protein production. Also, the effect of acetate excretion on substrate yield can be estimated by Eq. (1). The effect of acetate excretion on substrate yield is well represented by this dimensionless factorization approach as shown in previous work (*18*).

Engineering Considerations

Expression of heterologous protein is a rather complicated process. Many factors, including the host-vector system, plasmid copy number, promoter strength, growth and production media, and so on, affect the protein yield. In particular, the inducer strength, the induction time, and the reactor configuration should be optimized by production engineers for higher profits or higher protein titers. These effects have been examined by using the structured model by Bentley and Kompala (*20,21*). When the rate-limiting step is dominated by the inefficient cellular energetics, we have found that the effects of growth rate, induction time, and inducer strength vary in direct proportion to the cell yield for these simulated batch runs by using this model. The equations developed above can be used to estimate the cell yield.

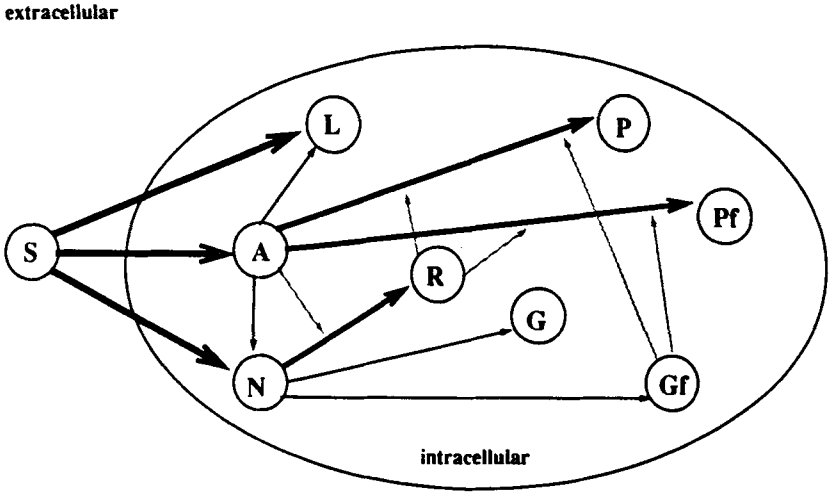

Fig. 1. Constituent pool mass flow diagram—the schematic representation of the Bentley and Kompala model. Arrow width indicates relative quantity of material; dotted lines indicate flow of information. S, substrate; A, amino acids; N, nucleotides; L, lipids; R, ribosomes; G, chromosomal DNA; P, protein; P_f, foreign protein; G_f, plasmid DNA.

Simulation Study

The dynamic and steady-state simulations of the Bentley and Kompala model equations (*20,21*) show very good agreement with widely observed experimental trends for both plasmid copy number and foreign protein content. Their model structure was developed to accommodate and characterize the dynamics of the key macromolecular events occurring within the cell as shown in Fig. 1. The width of the mass flow vectors on this figure indicates the relative magnitude of the mass flow between pools, and the flow of information is indicated by dotted lines. This lumped metabolic model of recombinant cells includes eight intracellular constituent pools. These are: protein, P; foreign protein, P_f; chromosomal DNA, G; plasmid DNA, G_f; ribosomes, R; lipids, L; nucleotides, N; and amino acids, A. The model consists of a differential equation for each pooled constituent. Also, each differential equation consists of a synthesis rate term and the appropriate terms for depletion or turnover. The synthesis rate expressions are comprised of saturation kinetic terms relating intrinsic cellular components, as previously described by Shuler and Domach (*22*).

Computer simulations of batch cultures with the Bentley and Kompala model, with specific growth rate varied from 0.45 to 1.3 (1/h), induction time changed from 1 to 15 (h), and inducer concentrations from 1.0 to 2.1 (μg/mL), show that the ratio of foreign protein production for two different operating conditions is the same as the ratio of cellular yields. The conditions used in this simulation study are listed in Table 4. Details of the simulations, cases I–VI, are described as follows:

Table 4
Conditions Used in the Simulation Study[a]

Case	Preinduction sp. growth rate, 1/h	Induction time, h	Inducer strength, μ g/mL
I	0.95	1.0	2.1
II	0.63	1.0	2.1
III	0.63	3.0	2.1
IV	0.57	3.0	2.1
V	0.45	15.0	2.1
VI	0.45	15.0	1.0

[a]For each simulated case, cases I–VI, the value of the cell yield is varied from 0.5 to 0.2 as a result of acetate excretion or low cellular energetic efficiency.

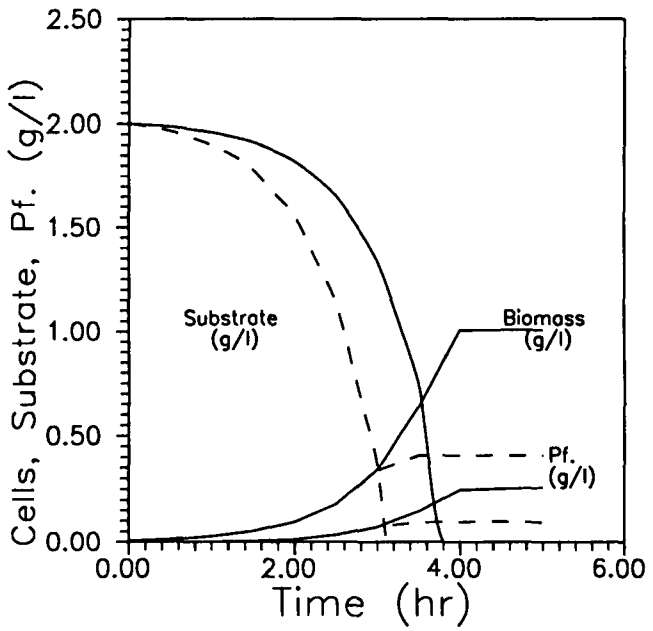

Fig. 2. The transient response of biomass, glucose, and foreign protein concentrations, g/L, in a typical batch simulated study, case I. The solid and dashed lines represent the value of cell yield at 0.5 and 0.2, respectively.

- Figures 2 and 3A illustrate case I, the base case. Both of these figures have the same preinduction specific growth rate, 0.95 (1/h), induction time 1 h, and inducer concentration, 2.1 μg/mL, but with different cellular yields, 0.2 and 0.5, respectively. The transient relationships among the biomass, substrate, and foreign protein production are shown in Fig. 2 where the solid and dashed lines represent the value of cell yield at 0.5 and 0.2, respectively. Figure 3A shows an expanded view of the induction response in the early exponential growth phase (induction

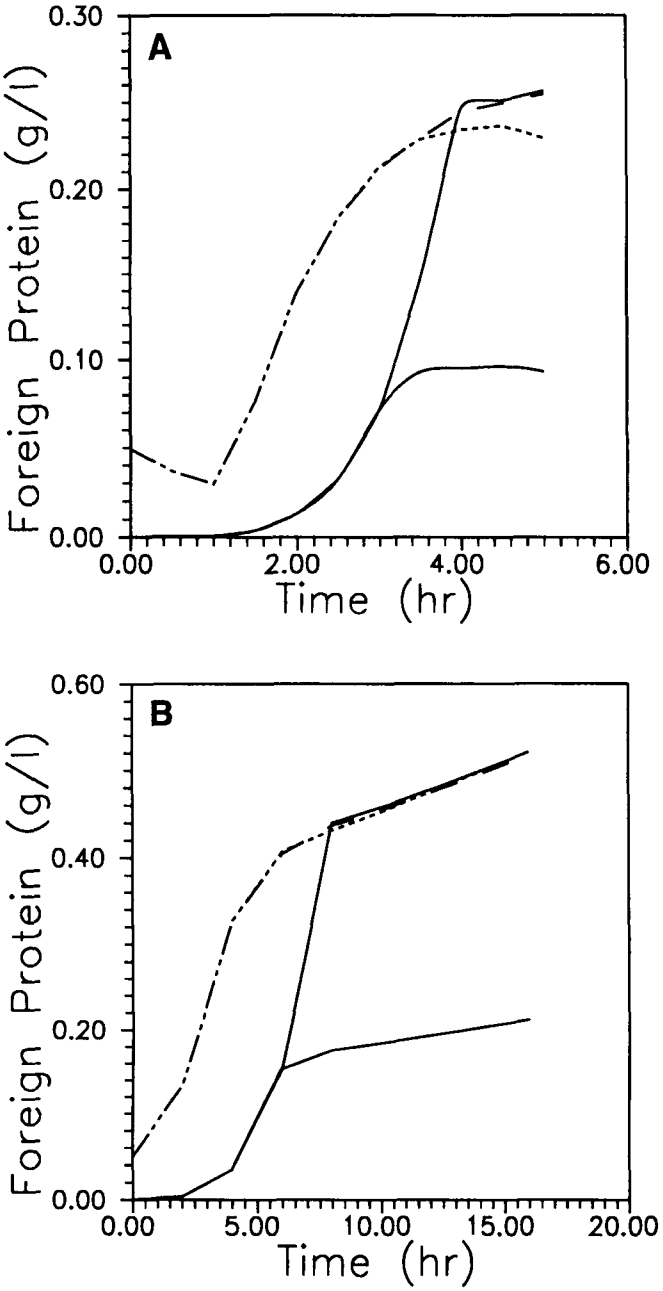

Fig. 3A–F. The relationship of foreign protein production at different cell yield values for cases I–VI, respectively. Solid lines are foreign protein concentrations in gram per liter, g/L. Dashed and dotted lines are fraction of foreign protein produced, i.e., foreign protein concentration divided by biomass concentration, at the yield values of 0.5 and 0.2, respectively.

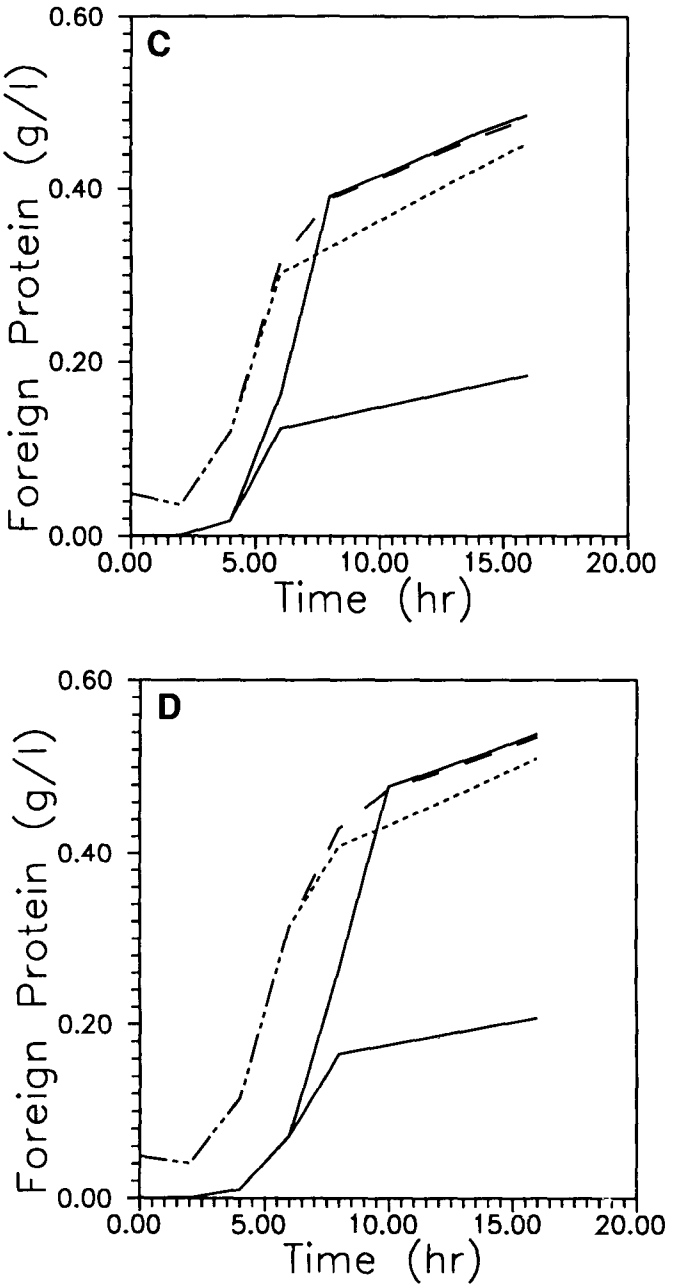

Fig. 3 (*continued*).

time = 1 h), and the relationship between the amount of foreign protein and the cell mass fraction of the foreign protein. The dashed and dotted lines are the cell mass fractions, g/g, of foreign proteins at different cell yield values, 0.5 and 0.2, respectively. In addition, the foreign protein concentrations, g/L, produced at different cell yield values, 0.2 and 0.5, are represented by the lower and upper solid lines, respectively.

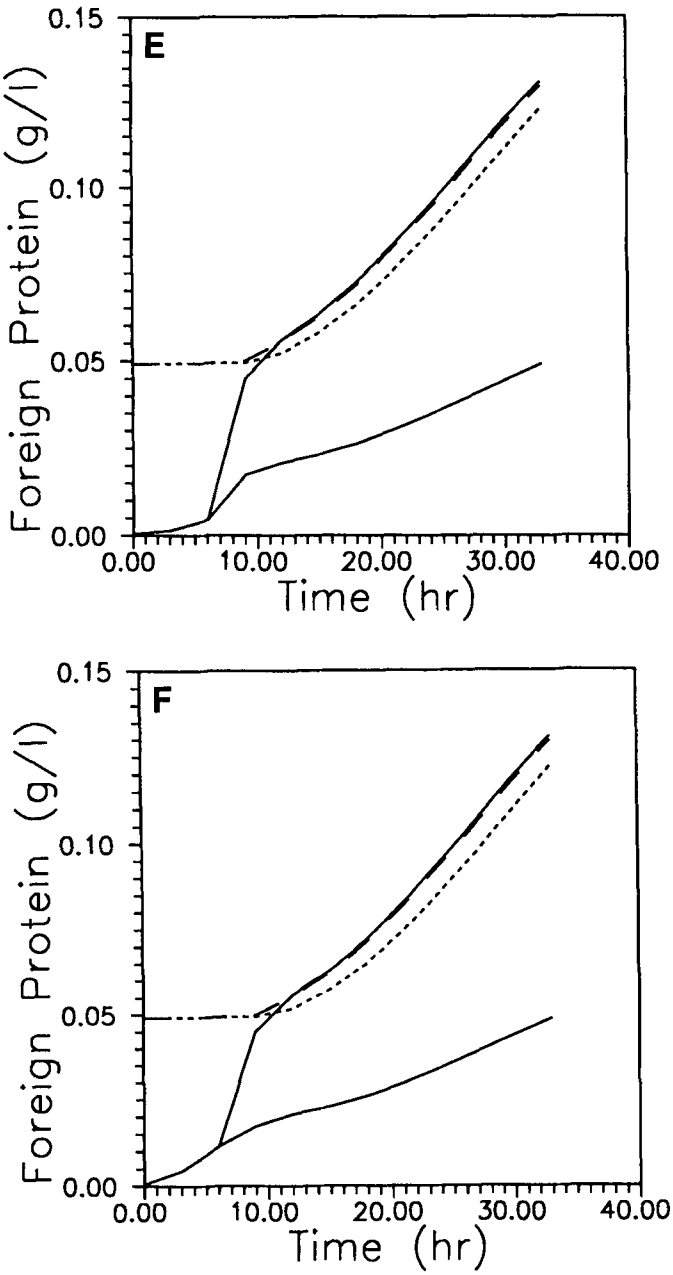

Fig. 3 (*continued*).

- Case II: The conditions are the same as for case I, except the preinduction specific growth rate is 0.63 (1/h). Figure 3B shows the induction response in the early exponential growth phase.
- Case III: The conditions are the same as for case II, except the induction time is 3 h, which simulates the induction response in the late exponential growth phase as shown in Fig. 3C.

- Case IV: The conditions are the same as for the case III, except the preinduction specific growth rate is slightly lower at 0.57 (1/h). Figure 3D shows the induction response in the late exponential growth phase.
- Case V: The conditions are the same as for case IV, except the preinduction specific growth rate is lowered to 0.45 (1/h), whereas the induction time is increased to 15 h. This shows the induction response in the extended stationary phase (Fig. 3E).
- Case VI: The conditions are the same as for case V, except the inducer concentration is reduced to 1.0 μg/mL. This shows the induction response in the extended stationary phase with a different inducer strength (Fig. 3F).

It can be clearly seen from the simulations illustrated on Fig. 3A–F that the ratio of foreign protein production between these batch cultures is nearly the same as the ratio of their respective cellular yields. The changes in protein fraction, P_f (g/g), shown in Fig. 3A–F, were caused by a change in cell yield from 0.2 to 0.5. These changes are shown for different values of growth rate, inducer strength, and induction time. In each case, the protein fraction change was small compared to the cell mass change caused by the cell yield. These results indicate that, over this range of simulated batch culture conditions, the best way to increase protein yield is to maximize the cell yield. This result occurs because the Bentley and Kompala model implicitly accounts for cellular energetics by the value of the cellular yield. This simplifying approach to cellular energetics is also used with other structured models (23,24). The validity of this implicit relationship between cell energetics and cell yield is reinforced by our experimental findings. That is, our experiments indicate that acetate formation is an event that is concomitant with inefficient metabolism and the resulting lowered protein expression, rather than acetate acting as a specific inhibitor of protein formation. In other words, in the experimental region examined in this study, the major effect of acetate formation is a reduction of cell yield. The comparisons between the mass fraction, g/g, of foreign protein and the amount, g/L, of foreign protein shown by Fig. 3A–F, also indicate that inefficient cell metabolism is the major factor causing lowered foreign protein production.

A detail structured model provides important intracellular information. However, these highly interrelated nonlinear differential equations are mathematically difficult to use for optimization. In addition, this lumped metabolic model of recombinant cells does not provide information desired for process control, for example, acetate, dissolved oxygen, and glucose concentrations. Thus, for engineering purposes, it is desirable to have a correlation between the extracellular measurable variables and the cellular energetics for facilitating culture optimization.

If maximizing foreign protein production is the ultimate objective, then this goal can be approached in a simplified way by combining the cellular yield equations and detailed structured model. This will be addressed in detail in another paper.

CONCLUSIONS

By comparing parallel cultures with and without acetate, it was found that the presence of acetate in the media does not influence the β-galactosidase activity for concentrations up to 3 g/L. Reports in the literature have suggested that this is a significant factor negatively influencing protein yield. It appears that acetate formatioan is more a result of inefficient cell metabolism and low protein productivity than a specific inhibitor of protein production, at least for the limited concentration range tested. Also, a series of simulations for batch cultures with a structured model indicate that a change in cell yield had a minor effect on protein fraction, and so protein production is best improved by maximizing the cell production. The result is consistent with the two-phase, i.e., the separated cell growth and protein production, production strategy.

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REFERENCES

1. Lee, Y. L. and Chang, H. N. (1990), *Biotechnol. Bioeng.* **36,** 330–337.
2. Freschko, J. and Ritch, T. (1986), *Chem. Eng. Commun.* **45,** 229–241.
3. Meyer, D. J. and Jones, C. W. (1973), *Eur. J. Biochem.* **36,** 144–151.
4. Forberg, C. and Haggstrom, L. (1988), *J. Biotechnol.* **8,** 291–300.
5. Jensen, E. B. and Carlsen, S. (1990), *Biotechnol. Bioeng.* **36,** 1–11.
6. Meyer, H. P., Leist, C., and Fiechter, A. (1984), *J. Biotechnol.* **1,** 355–358.
7. Poole, R. (1983), *Biochim. Biophys. Acta* **726,** 205–243.
8. Rodriguez, R. L. and Tait, R. C. (1983), *Recombinant DNA Techniques: An Introduction,* The Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA.
9. Shimizu, N., Fukuzono, S., Fujimori, K., Nishimura, N., and Odawara, Y. (1988), *J. Ferment. Technol.* **66,** 187–191.
10. Landwall, P. and Holme, T. (1977), *J. Gen. Microbiol.* **103,** 345–352.
11. Landwall, P. and Holme, T. (1977), *J. Gen. Microbiol.* **103,** 353–358.
12. Miller, J. H. (1972), *Experiments in Molecular Genetics,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

13. Reiling, H. E., Laurila, H., and Fiechter, A. (1985), *J. Biotechnol.* **2,** 191–206.
14. Konstantinov, K., Kishimoto, M., Seki, T., and Yoshida, T. (1990), *Biotechnol. Bioeng.* **36,** 750–758.
15. Cutayar, J. and Pollon, D. (1989), *Biotechnol. Lett.* **11,** 155–160.
16. Lundstrom, H., Brobjer, M., Osterlof, B., and Moks, T. (1990), *Biotechnol. Bioeng.* **36,** 1056–1062.
17. Ko, Y. F., Bentley, W. E., and Weigand, W. A. (1993), *Biotechnol. Bioeng.* **42,** 843–853.
18. Ko, Y. F., Bentley, W. E., and Weigand, W. A. (1994), *Biotechnol. Bioeng.* **43,** 847–855.
19. Miller, J. E., Backman, K. C., and Hatch, R. T. (1987), *J. Ind. Microbiol.* **2,** 143–149.
20. Bentley, W. E. and Kompala, D. (1989), *Biotech. Bioeng.* **33,** 46–61.
21. Bentley, W. E., Mirjalili, N., Andersen, D. C., Davis, R. H., and Kompala, D. (1990), *Biotechnol. Bioeng.* **35,** 668–681.
22. Shuler, M. L. and Domach, M. M. (1983), *Am. Chem. Soc.* ACS Symp. Series, **207,** 93–115.
23. Mori, H., Yano, T., Kobayashi, T., and Shimizu, S. (1979), *J. Chem. Eng. Jpn.* **12,** 313–319.
24. Ryu, D. D. Y. (1980), *Biotech. Bioeng.* **22,** 289–298.