한국생물공학회지 제4권 제3호
Korean J. Biotechnol. Bioeng.
Vol. 4. No. 3. 208-214(1989)

On-Line Induction of Fermentation with Recombinant Cells: Part III. Condition Optimization and Computer Control

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유전자 재조합 세포 발효의 온·라인 유도 : 제 3부. 조건 최적화 및 콤퓨터 자동 제어

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ABSTRACT

The computer interfaced by the necessary hardwares and the sofware developed in PARTs I and II were used to control the fermentation with recombinant cells in an on-line feedback-feed-forward manner. The recombinant cells were induced for expression either thermally or chemically. Very accurate controls with fast response and no oscillation could be performed.

INTRODUCTION

Developments in recombinant DNA technology offer an opportunity to express heterologous foreign genes in microbial hosts and conditions to overproduce desired proteins can be determined to a certain extent. The relationships between host and vector systems (1), microbial physiology and plasmid copy number(2, 3), and / or promoter strength(4) etc. have been examined.

The aims of recombinant cell fermentations are generally to obtain high volumetric productivity, high product concentration, high yield and low cost. Although some successes were reported on constitutive expression system(5, 6), induction system by which growth phase and production phase can be regulated separately has been introduced to achieve these goals. Each expression system has its own strong points and weak points. Constitutive systems are simple because no chemical additions or temperature shifts need be made. However, the constitutive production of recombinant proteins can interfere with cell growth, resulting in a decreased specific growth rate(7) or even cell death and in enhanced segregational instability(8, 9). These problems can be reduced by utilizing a regulated expression system. In such systems the plasmids are typically more stable, and differences in growth rates between recombinant cells and plasmid free cells are minimal(10).

The purpose of this experiment is to examine the potentiality of on-line optimal induction via low-cost self-made computer controlled fermentation system-(PARTS I and II).

MATERIALS AND METHODS

Bacterial Strains

E. coli strains N5151 and AR120 are used as host strains. AR120 is a cryptic N99 derivative that is cI^+, -Δ-*gal*, Ind^+ and nadA : : Tn10. This cell line is used for doing nalidixic acid inductions with plasmid pAS1-EH801 and can be grown at 37 ℃ and induced at this temperature as well. N5151 is Δ *Bam* Δ H1 bio-defective, cryptic λ lysogen which has temperature sensitive λ repressor, cIts 857, and a derivative of *E.*

coli SA500. It will not lyse at 42℃ but should be grown at 32℃ at all times except when doing thermal inductions.

Plasmid

The schematic diagram of the plasmid, pAS1^EH801, used in this study is shown in Figure 1. This is a chimeric plasmid from pAS1 which is a derivative of pKC30 and phage λ sequences(thickened box)inserted between the *Hind* III and *Bam*HI sites of pBR322(solid line)and form EH801(open box)which is a derivative of pAPR801 coding one of the eight single stranded influenza A virus RNA, NS1. That is, EH801 is inserted into a *Bam*HI site of *E. coli* plasmid expression vector, pASl, carrying strong regulatable phage λ promoter, P_L, and cII ribosome binding site, and giving enhanced transcription efficiency as a result.

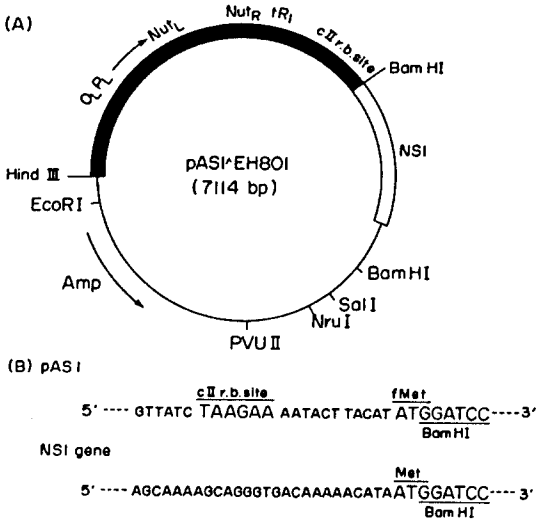

Fig. 1. The scheme of plasmid pAS1^EH801
(A) The restriction map of pAS1^EH801
(B) The sequence of the cII gene ribosome binding site and NS1 gene. The coding sequence of the NS1 gene downstream to the *Bam* HI site were inserted into the unique *Bam* HI site of pAS1.

Plasmid DNA Isolation and Agarose Gel Electrophoresis

Plasmid DNA was prepared by the procedure of Kiser(11) with some modifications. 0.7% agrose type I (Sigma) in TAE buffer was used. Photographs were taken after staining with EtBr followed by destaining with excessive amount of water. Polaroid film (Type 667, Polaroid Corporation) was exposed to UV of 600nm by UV transilluminator(UV Products, Inc.) through a wratten gelatin filter(Kodak).

Expression of NS1 gene

According to hosts two different induction methods were used. For thermal induction, a 50mL culture of strain N5151 carrying pAS1^EH801 was grown at 32℃ in LB broth to an OD_{600} of 2.0. This seed culture was inoculated to a fermentor of 2L working volume(MD-300, L.E. Marubishi)and the inductions were made at different O.D.'s(0.3-2.0) and to various temperatures(36-42℃). For chemical induction, nalidixic acid solution was added to 2L culture of strain AR120 harbouring the same plasmid, to final concentration of 40 μg/mL at 37℃. Also this induced expression was done at different O.D.'s(0.3-2.0).

Purification of NS1 Protein and Its Analysis

The NS 1 protein was purified using the method of Shatzman and Rosenberg *et. al.*(2). SDS-polyacrylamide gel electrophoresis was performed following Laemmli's method(3). Protein bands were analyzed quantitatively with UV spectrophotometer(UV-200S, Shimadzu) after staining with Coomassie Brilliant Blue R250(Sigma) followed by destaining with proper solution. Detailed quantification method can be found elsewhere (14). By independent assay, calibration curve between absolute protein quantity of BSA (Sigma) and optical density at 585nm which was determined from scanning of Coomassie Blue-stained electrophorogram with UV spectrophotometer, could be plotted as in Figure 2 after correcting for the effect caused by the partially remaining background color.

Culture Conditions

Most of experiments were done batch-wise, at pH 7.0, with 2 liter of LB broth containing 50mg / L ampicillin. Aeration rate and guage pressure inside a fermentor were 1 vvm and 0.1 kg / m^2, respectively. Foam was eliminated by adding Antifoam A (Sigma) and an agitation rate between 125 and 1000 rpm was applied as necessary for DO control.

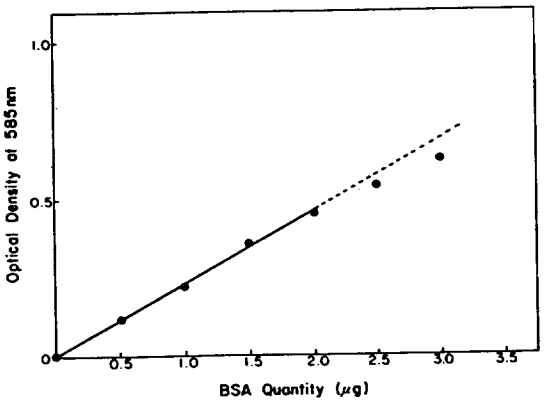

Fig. 2. The Calibration Curve of Protein (BSA) Concentration vs. 1:3 Diluted Optical Density at 585nm.

Determination of Induction Point

For on-line induction of foreign gene expression at desired point of physiological stage reproducibly, measurement of cell concentration is essential. Although numerous ideas to monitor cell concentration were conceived but no method was perfect. These methods were reviewed by Atkinson *et. al.*(15). For the current study cell concentration was estimated from CER. When the cell concentration was below 0.6 mg dry cell weight per mL broth, good linearity between CER and OD_{600} was obtained. With this correlation it was possible to start induction at desired cell concentration in the linear range. For cell concentration outside this range, induction point was determined by time course data from previous experiments.

Determination of Plasmid Stability

Isolated colonies in non-selective LB plate which had been streaked previously were replicated by sterile tooth picks to LB plate containing drug. From the ratio of number of growing colonies to those of nogrowth, plasmid stability was determined. To keep track of plamid copy number or plasmid content and to make clear whether the reason for plasmid instability was structural or segregational, agarose gel electrophoresis was done after plasmid isolation.

Measurement of Dry Cell Weight

Dry cell weight was obtained from an average of three measurements of dried 50mL culture broths which had been dried 24hours at 100℃ by a digital balance(MP-2006, Sartorius) accurate to 10^{-5}g.

RESULTS AND DISCUSSION

Plasmid Stability

For the strain N5151 harbouring plasmid pAS1ΔEH801, rapid decrease in plasmid stability is observed when expression is induced in contrast with fairly good plasmid stability for non-induced culture(Figre 3.). To know whether the instability is structural or segregational, electrophoresis of isolated plasmid samples were done. In the electrophorogram the band for plasmid pAS1ΔEH801(7.1kbp) disappeared, suggesting that the instability is of segregational nature. Because better stabilities were observed when induction temperature was low or when DO was controlled, major reasons for plasmid instability are presumed to be heat shock and DO shock(18) (when the broth temperature was elevated without DO control, DO dropped to 0% within a few minutes.). At lower induction temperature, however, the productivity decreased substantially and thermal induction runs in the following experiments were performed at 42℃.

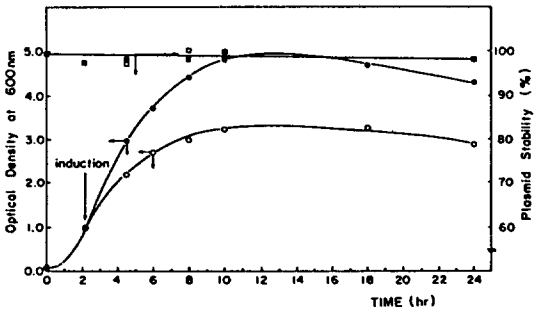

Fig. 3. Plasmid stability change by induction. (N5151)
[●: 32°C, ○: 42°C, ■: 32°C (stability), □: 42°C (stability)]

Strain AR120 carrying the same plasmid exhibits different aspects with regard to plasmid stability(Figure 4.). Both plasmid stability from replica plating and plasmid content from plasmid isolation show no significant difference regardless of doing induction or not. The only difference was observed in the specific growth rate measurements and no effect of DO control on plasmid stability is observed. This shows the inherent stability of this particular host-vector pair even under the induction environment.

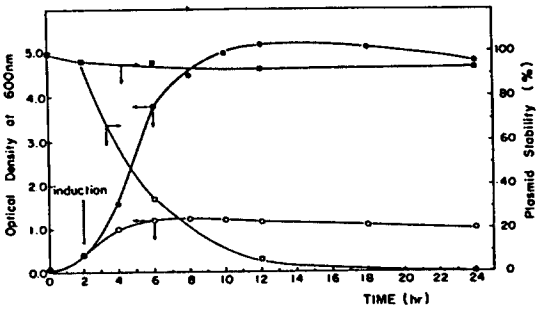

Fig. 4. Plasmid stability change by induction (AR120)
[●: OD (no induction),
○: OD (induction)
■: Stability (no induction),
□: Stability (induction)]

It can be anticipated that pAS1ˆEH801 / AR120 will give a better yield and productivity for this host-vector pair and has high plasmid stability and higher growth rate even when foreign gene expression is induced. Its higher growth rate may originate from its normal growth temperature(37 ℃) being used during induction as compared with the induction temperature(42 ℃) for pAS1ˆEH801 / N5151. The higher induction temperature, not to speak of the physiological stress on cells by heat, may cause faster decomposition of antibiotics giving less selective pressure for strain N5151 harboring plasmid.

Effects of Induction Temperature

Overproduction of foreign gene loads a host cell with various burdens, resulting in slow growth rate or plamid instability(7). In the case of pAS1ˆEH801 / N5151, growth is inhibited at temperatures beyond 35 ℃ and even cell death occurs when the temperature exceeds 43 ℃ . To measure how serious the effect is, average specific growth rates during the 3 hours after induction at OD_{600} 0.64 at different temperatures are compared with mean specific growth rate of the same period without induction. The change in growth rates is plotted in Figure 5. As can be expected, the higher the induction temperature is, the more substantially the growth rate decreases. But as induction temperature goes higher, both product quality and product quantity increased(open circle in Figure 5.). That is, the desired band is thickened in SDS-PAGE, although total protein quantities after purification remains same at temperatures beyond 40 ℃ .

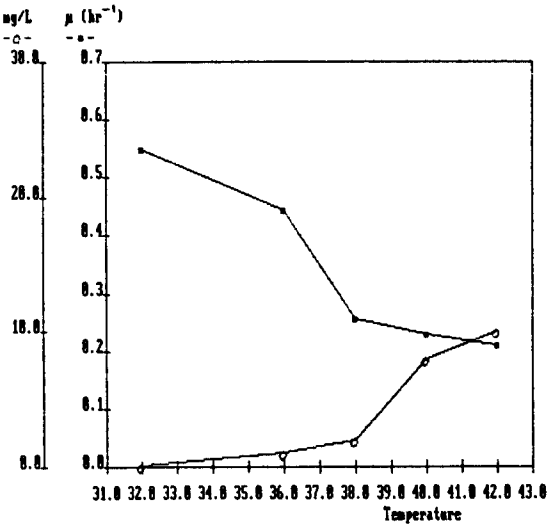

Fig. 5. The effect of induction temperature on growth rate and product formation of pAS1ˆEH801/N5151. (—■—: specific growth rate, —○— : product concentration)

Effects of Switching Time

The time when induced expression is started during the culture also has a considerable effect on cell growth. In Table 1, the comparison of average growth rates during 1 hr after starting induction at 42 ℃ at different O.D.'s with the standard growth rates, μ o, from O.D. measurements at 20 minutes intervals in parallel culture without induction is made. These average growth rates are a little higher than those of Figure 5. because the period taken for average is shorter : 1 hour vs. 3 hours. The data in Table 1 indicates that there exists an optimal time point where induction of culture gives the least effects on cell growth. It may be that cells are more labile in late phase, resulting in fast inactivation at high temperature and also in early phase cells are too immature to resist high temperature. For product formation, similar tendency can be easily deduced and the results confirms such anticipation(Figure 6.). In this case the remaining substrate concentration plus the state of cells as described above, may affect final product concentration.

Experiments with AR120 together with chemical in-

Table 1. The Effect of Switching Time on pAS1^EH801/N5151 Growth Rate

Optical Density at Induction	$\mu(hr^{-1})$	$\mu_0(hr^{-1})$	relative ratio
0.34	0.501	0.925	0.542
0.48	0.560	0.891	0.629
0.59	0.632	0.856	0.738
0.71	0.790	0.850	0.929
0.99	0.662	0.774	0.855
1.11	0.543	0.703	0.772
1.20	0.564	0.699	0.807
1.30	0.426	0.696	0.575
1.40	0.389	0.676	0.612
1.52	0.337	0.636	0.530
1.68	0.270	0.580	0.466
1.71	0.296	0.577	0.513
1.86	0.266	0.528	0.503

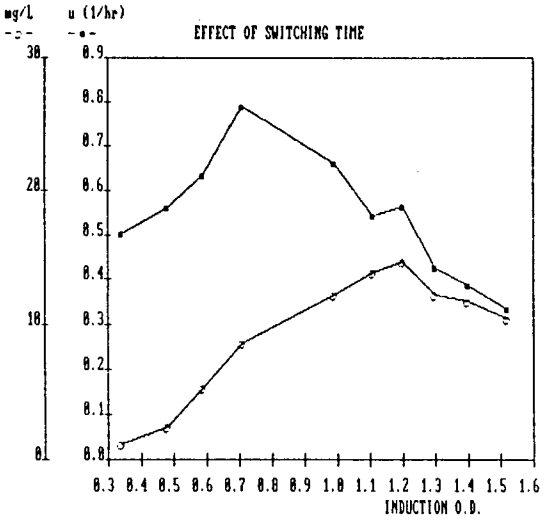

Fig. 6. The effect of switching time on growth rate and product formation of pAS1^EH801/N5151.
(—■—: specific growth rate,
—○—: product concentration)

duction exhibits different behaviour in that no such optimal point exists. The comparison of growth rates of induced culture to growth rates of noninduced, standard culture is displayed in Table 2. The concentration level of the chemical inducing agent used, i.e. nalidixic acid, is low enough that there may appear a kind of dilution effect as the population density increases or it may be that there occurs some environmental changes in the culture medium as the fermentation progresses.

Table 2. The Effect of Switching Time on pASI^EH801/AR120 Growth Rate

Optical Density at Induction	$\mu(hr^{-1})$	$\mu_0(hr^{-1})$	relative ratio
0.13	0.401	0.915	0.438
0.51	0.435	0.791	0.549
0.81	0.454	0.620	0.732
2.48	0.213	0.239	0.891
4.10	0.064	0.066	0.970
4.95	0.022	0.022	1

The product concentration profile of pAS1^EH801 / N5151. Early induction gives good quality but low overall amount because of the suppression of cell growth and late induction reveals electrophorogram results similar to non-induced run(Figure 7.). The effects of switching time on growth rates and product formation are summarized in Figure. 8.

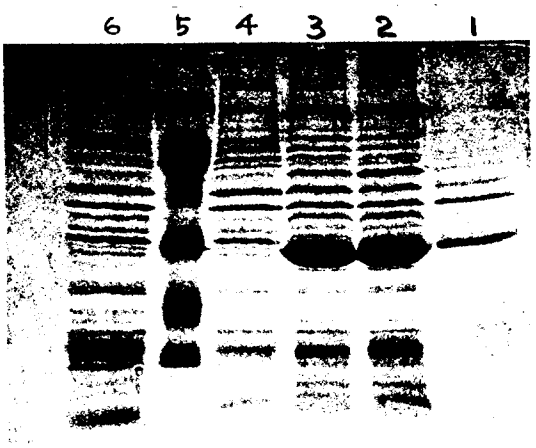

Fig. 7. SDS-Polyacrylamide gel electrophoresis of pAS1^EH801/AR120 extracts. Lanel, induction at OD_{600} 0.13; Lane 2, induction at OD_{600} 1.20 without DO control; Lane 3, induction at OD_{600} 1.20 with DO control; Lane 4, induction at OD_{600} 4, 10; Lane 5, Molecular Weight standard (SDS-6, Sigma): Lane 6, extract sample without induction.

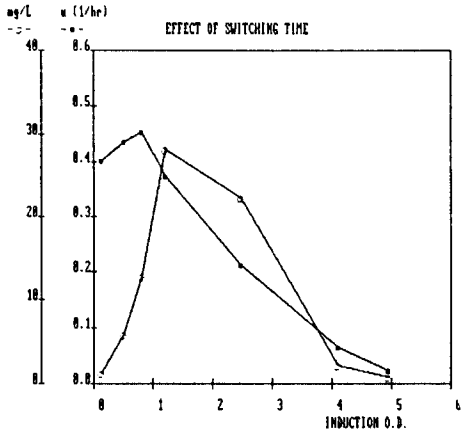

Fig. 8. The effect of switching time on growth rate and product formation of pAS1^ EH801/AR120.
(—■—: specific growth rate,
—○—: product concentration)

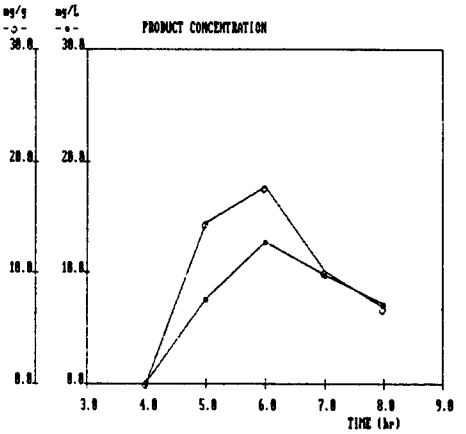

Fig. 9. The effect of harvesting time on product formation of pAS1^EH801/N5151. Thermal induction was started at 4 hour after inoculation.
(—■—: mg product/L media
—○—: mg product/g dry cell weight)

Effects of Harvesting Time

In batch fermentation, substrate concentration will gradually decrease as cells grow, and this decrease will eventually lead to stationary phase and death phase. Thus the concentration of NS1 gene product as an intracellular product will depend on the point of harvest. Concentration levels of products formed from pAS1^ EH801 / N5151 and pAS1^EH801 / AR120 at various harvesting times are shown in Figures 9. and 10, respectively. Maximum product concentrations are reached 2 hours after thermal induction or 4 hours after chemical induction.

The reasons for the different optimal harvesting times of the two cases may include the relatively more rapid inactivation of λ repressor(cIts 857) and relatively high rate of cell death at 42℃. The cIts857 gene product inactivates relatively quickly as the temperature is elevated and maximum product concentration per cell has an Arrhenius type temperature dependence(16). Thus rapid inactivation of cI product at 42℃ makes rapid activation of λ P_L promoter resulting in fast increment of foreign gene product. In contrast the high temperature used for induction will certainly be unfavorable for the survival of the product-containing cells as well as the stable maintenance of the intracellular gene product.

In the case of pAS1^EH801 / AR120, nalidixic acid

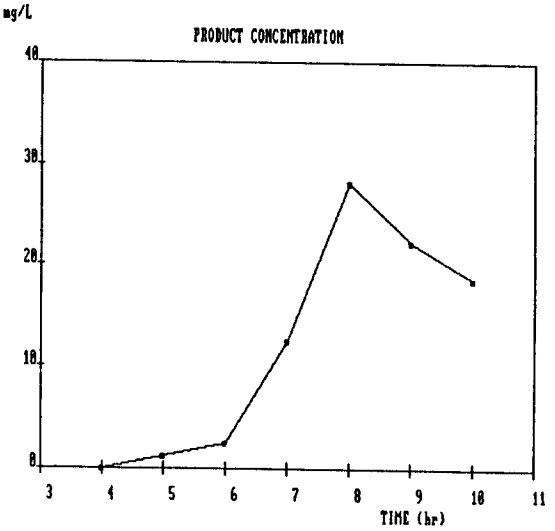

Fig. 10. The effect of harvesting time on product formation of pASI^EH801/AR120. Thermal induction was started at 4 hour after inoculation.

induces SOS repair, resulting in recA protease synthesis which in turn inactivates cI gene product. This multi-step inactivation increases the activity of λ P_L promoter relatively slowly. Also as described earlier, this strain maintains fairly good plasmid stability and the growth is

not inhibited seriously when induced to express foreign gene. Consequently, maximum product concentration should be achieved relatively later than thermally induced case. The fact that maximum product concentration of pAS1ΔEH801 / AR120 is higher than that of N5151 may have much to do with the fundamental difference in inducing mechanism thus the chemical induction giving rise to less damage to the host cell.

CONCLUSION

The on-line feedback computer control system developed was applied to the induction of recombinant strains to produce the foreign protein. Even for the same recombinant plasmid, depending on the host strains, it required different induction methods. The different induction mechanisms were the cause of the differences in plasmid stability, specific growth rate, and the product level. In the case of thermal induction there appears optimal points in the time course of cell growth where the induction causes the least detrimental effect on relative cell growth as well as product formation. For the two mechanisms of induction used, i.e. thermal and chemical, optimal time points exist for both maximum specific growth rate and maximum product concentration level following induction. The time point for induction for maximum product concentration level lags behind that for induction for maximum specific growth rate following induction.

요 약

필요 하드웨어와 소프트웨어를 자체 개발하고 콤퓨터 인터페이싱을 수행하여 유전 재조합세포 발효 공정을 온·라인으로 휘드·백 및 휘드·포워드 식 자동제어 할 수 있었다. 유전자 재조합 세포들이 발현을 위하여 온도 유도 및 화학약품 유도 방법을 사용하였다. 빠르고 안정된 반응을 보이고 정확한 자동 제어를 수행할 수 있었다.

ACKNOWLEDGMENT

The authors are very much obliged to Allan R. Shatzman and Martin Rosenberg of Smith, Kline, and French Laboratories for kindly supplying the plasmid and host systems. This research was financially supported by grants from Ministry of Education and KOSEF, for which authors feel deeply grateful.

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(Received September 22, 1989)