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On-line Monitoring of IPTG Induction for Recombinant Protein Production Using an Automatic pH Control Signal

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Abstract The response of IPTG induction was investigated through the monitoring of the alkali consumption rate and buffer capacity during the cultivation of recombinant *E. coli* BL21(DE3) harboring the plasmid pRSET-LacZ under the control of *lac* promoter. The rate of alkali consumption increased along with cell growth, but declined suddenly after approximately 0.2 h of IPTG induction. The buffer capacity also declined after 0.9 h of IPTG induction. The profile of buffer capacity seems to correlate with the level of acetate production. The IPTG response was monitored only when introduced into the mid-exponential phase of bacterial cell growth. The minimum concentration of IPTG for induction, which was found out to be 0.1 mM, can also be monitored on-line and *in-situ*. Therefore, the on-line monitoring of alkali consumption rate and buffer capacity can be an indicator of the metabolic shift initiated by IPTG supplement, as well as for the physiological state of cell growth.

Keywords: on-line monitoring, IPTG induction, alkali consumption, buffer capacity, IPTG response

INTRODUCTION

Most methods of industrial fermentation use automatic pH control to maintain the pH of culture media within a specified range [1]. Beyond its capability of pH control, it can be applied to automatically supply substrate for fed-batch culture in a pH-stat model [2]. Furthermore, it has been reported that the control signal may supply the alkali consumption rate and the buffer capacity of the culture medium [3]. Utilization of pH measurement has been studied to estimate the product level [4], as well as the level of biomass in aerobic yeast fermentation [5]. As previously described, pH control signals enable us to estimate key parameters during microbial growth.

The rate of alkali consumption may be related to the level of metabolic activity of microorganisms. From previous studies, the pH variation during microbial growth has been attributed to the production of organic acid and the consumption of ammonium ions, especially in *E. coli* [6]. The ammonia consumption is reported to be proportional to biomass. Acetate production, however, depends largely on cultivation conditions, as well as on the specific growth rate [7]. *E. coli* produces a significant amount of acetate under anaerobic conditions. It even produces significant amounts of acetate under aerobic conditions when in the presence of high level of glucose in the medium [8]. A high level of acetate accumulation was reported to inhibit the expression of recombinant protein and cell growth [9]. Thus, the rate of alkali consumption can give us a mixed signal comprised of metabolic activity and acetate production. Therefore, we suggest that the rate of alkali consumption is an indication for the monitoring of a sudden metabolic shift that happens during microbial growth. Using a model recombinant *E. coli* carrying an inducible β-galactosidase on its plasmid, we investigated whether the metabolic shift followed by IPTG induction influences the rate of alkali consumption.

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MATERIALS AND METHODS

Materials

The strain used in this study was *Escherichia coli* BL21(DE3) carrying a plasmid pRSET-LacZ, purchased from Invitrogen (Carlsbad, CA, USA). The recombinant plasmid, pRSET-LacZ, carries the complete sequence of the *lacZ* gene under the T7 promoter, which enables the expression of β-galactosidase by IPTG (isopropyl-γ-d-thiogalactopyranoside). A transformation of competent *E. coli* BL21(DE3) cells by the pRSET-LacZ plasmid was performed. Blue colonies on an X-Gal LB plate with IPTG were isolated and further purified. One of the colonies was chosen and used for batch cultivations.

Culture Conditions

The *E. coli* cells were maintained as 20% (v/v) glycerol stock at −80°C, after growth in Luria-Bertani(LB) medium (10 g/L yeast extract, 5 g/L tryptone, and 10 g/L

NaCl). The culture medium for batch cultivation and expression monitoring contained the following compositions per liter: 0.5 g of KH_2PO_4, 5 g of NH_4Cl, 5 g of NaCl, 0.4 g $MgSO_4$, 3 g of yeast extract, 10 g of casamino acid, and 20 g of glucose. The cultivations were performed in a 5-L jar fermentor (BK5L, Boksung Eng., Korea) modified to monitor the rate of alkali consumption and the buffer capacity of culture broth. Alkali consumption rate is mol of NaOH supplied to control pH of the culture medium for a given period of time and buffer capacity was derived from the pH shift just after the addition of alkali [3].

Analytical Methods

Cell growth was monitored by measuring the absorbance at 680 nm for conversion to dry cell weight. The conversion table was prepared from several samples by measuring the absorbance, as well as by weighing the saline-washed cells after drying in an oven for 24 h at 105°C. The residual glucose level in the culture medium was analyzed by the DNS method [10]. The acetic acid concentration was determined by gas chromatography (3400cx, Varian, Palo Alto, CA, USA) equipped with a Gaschrom 220 column and an FID detector.

The synthesis of β-galactosidase by *E. coli* BL(21) [pRSET-LacZ] was measured using the Miller's protocol [11]. One unit(U) of β-galactosidase was defined as hydrolyzing one micromole of ONPG to *o*-nitrophenol and galactose per minute by the crude extracts prepared from the cells harvested from the fermentor at pH 7.5 at 37°C.

RESULTS AND DISCUSSION

IPTG Induction Distorted the Rate of Alkali Consumption and Buffer Capacity

The recombinant *E. coli* BL21(DE3) harboring the pRSET-LacZ plasmid was cultured in the 5-L jar fermentor. The rate of alkali consumption and buffer capacity were subsequently monitored. Fig. 1B shows that the rate of alkali consumption increased up to 0.022 $molh^{-1}L^{-1}$ along with cell growth and declined with the retardation of growth. However, the buffer capacity of the culture medium was completely increased. An additional batch cultivation was carried out under identical conditions and IPTG supplementation was made after 9 h of cultivation. As shown in Fig. 1A, the rate of alkali consumption increased up to 0.017 $molh^{-1}L^{-1}$, but suddenly decreased after 0.2 h of IPTG induction. The rate of alkali consumption dropped to less than half of its maximum value in a short period of time. The buffer capacity also began to decline approximately 1 h after IPTG induction.

The data suggest that IPTG caused a series of metabolic changes. Such a metabolic shift can be explained by the incorporation of IPTG into *E. coli* BL21(DE3), which triggers *T7/lac* promoter to synthesize T7 RNA polymerase and to subsequently transcribe the cloned *lacZ* gene on the plasmid by the strong T7 polymerase

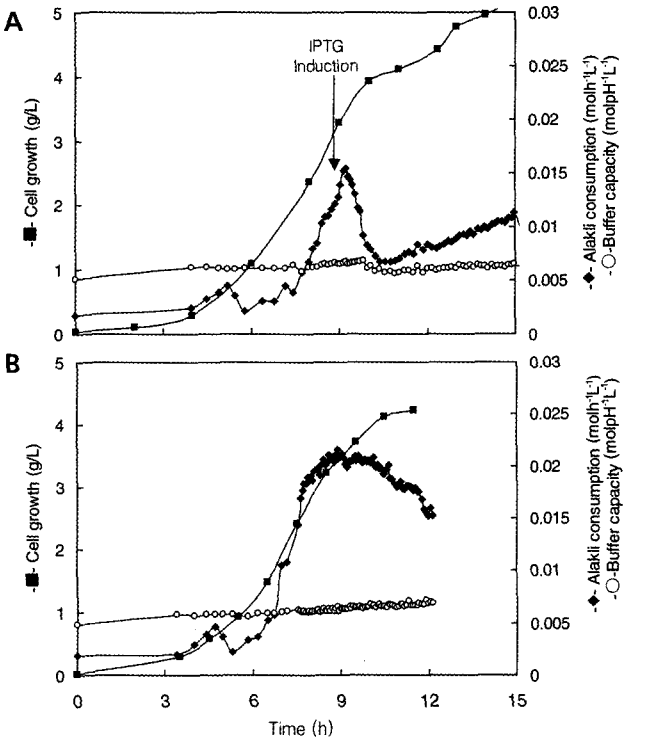

Fig. 1. The profiles of alkali consumption and buffer capacity during cultivation of *E. coli* BL21 [pRSET-lacZ] with IPTG induction (A), without IPTG induction (B).

[11]. It was also reported that the synthesis of plasmid-encoded proteins and plasmid-DNA replication often places a metabolic burden on the biochemical capacities of the cell which usually reduces the growth rate of the producing culture [12]. To the best of our knowledge, the metabolic burden caused by the strong expression of recombinant protein was hardly monitored on-line, except in the case of the measurement of biomass. However, monitoring of the alkali consumption rate clearly shows the metabolic burden caused by IPTG induction in the experiment. Therefore, monitoring of alkali consumption is the only method of observing the metabolic changes caused by IPTG induction.

Accounting for the time required to synthesize T7 RNA polymerase was the time lag of 0.2 h between IPTG induction and the drop in alkali consumption. This can be supported by the response time for *lacZYA* gene promoter, which is reported to be approximately 70 mins [13]. It has also been reported that the expression of *DnaK*, *GroEL*, and *GroES* genes, which code heat shock proteins, increased after 0.5 h of IPTG post-induction [14]. Therefore, it may take time for the IPTG induction to initiate gene expression and to cause metabolic changes. However, we are unsure how the alkali consumption rate began its decline. If there was a sudden change of ion components in the culture medium, both buffer capacity and alkali consumption should change simultaneously. The sudden change of alkali consumption only suggests that a rapid alteration of the metabo-

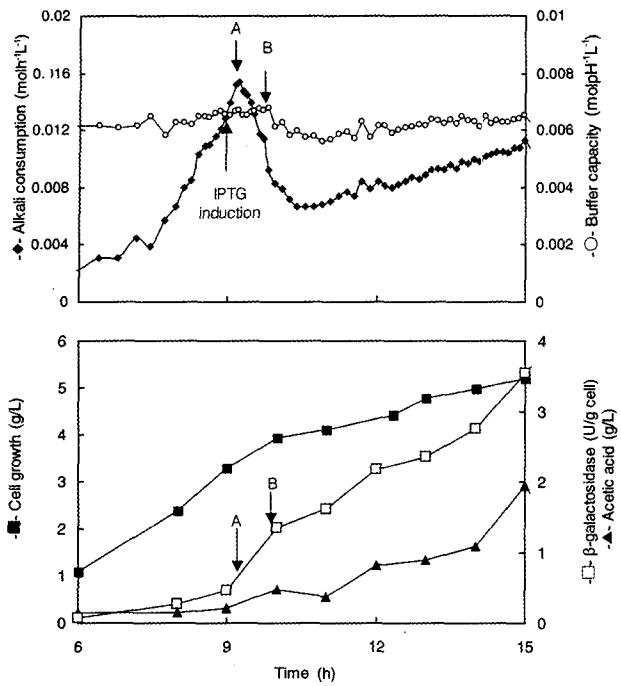

Fig. 2. The response of IPTG induction showing rapid decline of alkali consumption and change of buffer capacity, with the profiles of β-galactosidase expression and acetic acid production.

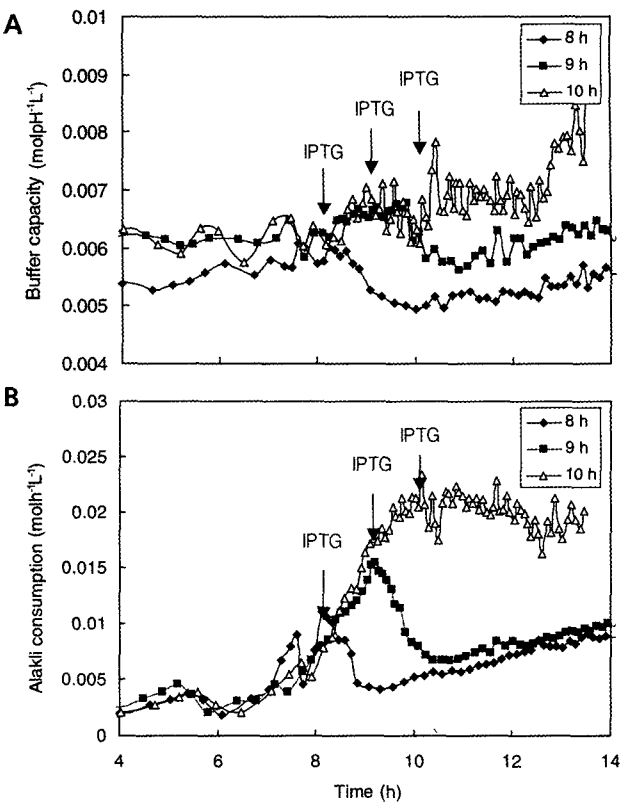

Fig. 3. The responses of buffer capacity and alkali consumption by IPTG supplementation at 8, 9, and 10 h of cultivation.

lism of cells occurred, which did not influence the buffer capacity at neutral pH. However, no further investigation was carried out reporting this study to address this issue.

The buffer capacity also dropped suddenly after 0.9 h of post-induction, which implies a decrease in the ion components possessing buffer capacity by the cells in the culture medium. Buffer capacity can solely be determined by the concentrations and specific buffer capacities of ion components in the culture medium. In Fig. 2, the concentrations of acetic acid were also measured and were plotted together and the arrows indicate the moments of IPTG induction, the sudden decline of alkali consumption rate and the dropping of buffer capacity. With the drop in buffer capacity subsequent to the post-induction period, a slight decrease in the acetate level, 0.2 g/L, was observed. The rapid production of galactosidase also began at this time. The buffer capacity, as well as acetic acid, started to increase again after approximately 1 h. These results suggested that IPTG induction initiated a series of sophisticated metabolic changes, one of which is the temporary cessation of acetate production.

The Dependency of the Responses by Induction Time

In a series of the cultivation of *E. coli*, varying the IPTG induction time from the mid-exponential phase to the stationary phase was performed. Thus, IPTG was supplemented at 8, 9, and 10 h of cultivation and the rate of alkali consumption and buffer capacity were monitored for the batch runs (Fig. 3). The induction times correspond to alkali consumption rates of 0.008, 0.013, and 0.021 $molh^{-1}L^{-1}$, respectively. When the induction was made at 8 or 9 h of cultivation, the rate of alkali consumption continued to increase up to 0.012 and 0.017 $molh^{-1}L^{-1}$ and decreased to 0.004 and 0.007 $molh^{-1}L^{-1}$, respectively. Time lags of around 0.2~0.3 h occurred between the induction and the sudden drop in alkali consumption. However, the IPTG induction at 10 h did not alter the profiles of alkali consumption or the buffer capacity, as happened in the batch run without induction. The levels of β-galactosidase expression were 1024, 931, and 351 U/g dry cell when the induction was made at 8, 9, and 10 h of cultivation, respectively.

The results correspond well with the optimum timing of IPTG induction observed by other researchers. In general, the maximum yield of a recombinant protein was obtained in the middle or late exponential phases [15-18]. It has been reported that the yeast extract concentration has changed the optimum time of IPTG induction, though the profiles of cell growth did not show any significant difference [17]. Nevertheless, the time of IPTG induction was often determined by off-line optical density measurement of the culture as an indicator of a certain growth phase with little emphasis on physiology of the host cell. Therefore, an understanding of 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. Thus, the

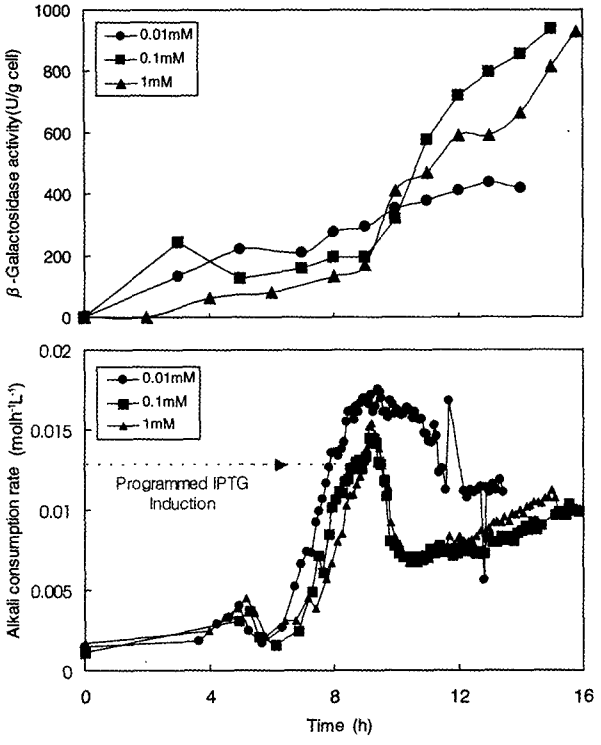

Fig. 4. The dependency of *lac-Z* gene expression and the response of alkali consumption by varying levels of IPTG, supplemented at 0.01 $molh^{-1}L^{-1}$ of alkali consumption rate.

rate of alkali consumption can be an indicator for identification of the physiological state of cell growth. It also informs us whether or not the IPTG induction has caused a metabolic change within the cell.

The Response of the Alkali Consumption Rate by the Concentration of IPTG

In order to investigate the dependency of β-galactosidase expression by IPTG concentration, batch runs were carried out with 0.01, 0.1, and 1 mM of IPTG. The supplementation of IPTG was programmed to automatically operate when the alkali consumption rate reached approximately 0.013 $molh^{-1}L^{-1}$. The production of β-galactosidase reached at 937 and 931 U/g dry cell when IPTG levels were 1 and 0.1 mM, respectively. However, when 0.01 mM of IPTG was used, the level decreased to 418 U/g dry cell. In this case, the rapid decline in alkali consumption was not monitored. Even prior to induction, the expression levels of β-galactosidase slowly increased up to approximately 200 U/g dry cell. This can be explained by the background ("leaky") expression of T7 promoter found in the hosts containing a source of T7 polymerase, such as (DE3) lysogenic strains[19]. When 1 and 0.1 mM of IPTG were used, the rate of alkali consumption increased to a maximum level of approximately 0.015 $molh^{-1}L^{-1}$ after 0.2 h of induction. In a relatively short period of time, both of the rates dropped to around 0.006 $molh^{-1}L^{-1}$. The response profiles are almost identical when IPTG supplied more than 0.1 mM. This suggests that 0.1 mM of IPTG is enough to initiate recombinant T7 RNA polymerase in the host cell.

The effect of inducer concentration on expression of recombinant production has been studied by many researchers. The optimum IPTG concentrations were usually reported between 0.1 mM to 0.2 mM [18,20,21]. It has also been reported that an IPTG concentration of 0.05 mM was sufficient for achieving maximal expression of partial fragments of immunoglobulin [22]. It has been seen that effective IPTG concentrations, as determined by on-line monitoring of the alkali consumption rate, are very consistent with the previous results. Furthermore, monitoring the response of IPTG informs us whether the induction successfully initiated the biosynthesis of a target recombinant protein. This can be applied to confirm the unit operation of induction by lactose. Since IPTG is both expensive and toxic, it might be unsuitable for the mass production of therapeutic proteins for use by humans. Because it is cheaper and safer than IPTG, lactose has been considered as an alternative inducer and has been applied in the production of several recombinant proteins [18]. However, lactose can be metabolized and its internal concentration may decrease. It would be safer to confirm whether the supplemented lactose successfully initiated the promoter by the application of the induction monitoring method presented in this study. The induction monitoring can be applied to another recombinant protein production system using Gal promoter with multiple stepwise feedings of galactose during fed-batch cultivation [23,24]. Since the step-wise feeding was designed to induce the promoter, the induction monitoring might had provided the responses for the successive feedings.

The on-line monitoring of alkali consumption presented in this report is a non-invasive method of monitoring the initiation of recombinant protein production. The non-invasive method of on-line monitoring of recombinant protein expression usually requires a reporter protein, such as green fluorescent protein [25,26] or a sophisticated instrumentation, such as flow injection analysis system [27]. Therefore, the response of alkali consumption can be applied to confirm the unit operation of induction in recombinant protein production on an industrial scale. To the best of our knowledge, there has been no report for *in-situ* and on-line monitoring of critical state variables relevant to metabolic load caused by the initiation of a strong promoter. Furthermore, the optimum time for IPTG induction can also be determined by monitoring the rate of alkali consumption so as to reduce batch-to-batch variation.

CONCLUSION

The monitored alkali consumption rate and buffer capacity appeared as on-line indicators for the metabolic changes initiated by IPTG induction. The response at varying IPTG levels was found to be correlated with the amount of recombinant protein production. Although the mechanisms of the drop in alkali consumption and the

fluctuation of buffer capacity were not clearly understood, it was demonstrated that the on-line monitoring of alkali consumption rate and buffer capacity can be an indicator for the metabolic shift initiated by IPTG supplementation, as well as for the physiological state of cell growth.

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