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Dissolved Oxygen Concentration Affects the Accumulation of HIV-1 Recombinant Proteins in *Escherichia coli*

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Abstract

A central problem in aerobic growth of any culture is the maintenance of dissolved oxygen concentration (DOC) above growth-limiting levels especially in high-cell density fermentations that are usually of the fed-batch type. Fermentor studies have been conducted to determine the influence of DOC on the production of heterologous proteins in *Escherichia coli*. The results demonstrated that there is a significant degree of product-to-product variation in the response of heterologous protein accumulation to DOC. For translational fusions of the human immunodeficiency virus-1 (HIV-1) proteins p24Gag and Env41, the imposition of a dissolved oxygen (DO) limitation resulted in 100 and 15% increases in the respective product yields. On the other hand, the imposition of a DO limitation had no effect on the production of a similar translational fusion of the HIV-1 protein p55Gag, and a large negative effect on the production of an influenza protein (C13). The stimulatory effects of DOC on p24Gag production were investigated further. The results of my studies suggested that the stimulatory effect observed at reduced agitation rates on p24Gag accumulation was owing to an oxygen effect and not a shear effect. Furthermore, the results of my investigations indicated that the effect a DOC had on the production of p24Gag was strongly influenced by the cell density at which the culture was induced.

Index Entries: Dissolved oxygen; HIV-1 proteins; recombinant proteins; *Escherichia coli*; heterologous gene expression.

Introduction

Recombinant DNA technology has enabled us to produce various proteins of therapeutic importance with microorganisms. A variety of host/vector systems has been developed for use in the production of

heterologous proteins and peptides. Without a doubt, one of the most popular hosts and widely utilized prokaryotic expression systems for the synthesis of heterologous proteins is *Escherichia coli (1)*. Numerous vectors have been constructed for use with *E. coli*. Of particular use are plasmids that employ tightly regulated promoters for the control of heterologous gene expression *(1,2)*. Once an optimal expression system has been selected, protein production can be enhanced by increasing the production of protein per cell per time (specific productivity), or by increasing the cell concentration per unit time (cell productivity) *(3)*. Yields of *E. coli* dry cell mass in shaker flask culture range from 1 to 2 g/L, whereas in fermentors up to 100 g/L can be achieved *(4)*. Fermentations utilizing a tightly regulated expression system are conducted in two phases: a growth phase and a production phase. During the growth phase, the environmental conditions are controlled to allow for rapid growth without heterologous gene expression and minimal plasmid loss. Once a suitable cell density has been achieved, the production phase of the fermentation is initiated by giving the appropriate signal. During the production phase, the environmental conditions are controlled to achieve both maximal gene expression and cell mass *(5)*.

Regardless of the phase, nutrient limitations are typically detrimental to the fermentation. As a result, considerable attention is given to the design of the fermentation medium. Sufficient major and minor nutrients are added to the medium to ensure that the desired levels of growth and product accumulation are achieved *(6)*. The optimal rate of nutrient addition and, consequently, productivity are ultimately limited by the rate at which cells can aerobically catabolize the carbon source without generating growth-inhibitory metabolites such as acetate and lactate. The volumetric yield of recombinant products can be improved by controlling the specific growth rate and the substrate concentration *(7)*. In fermentations in which very high cell densities are desired, nutrient feeding regimes must be used to avoid problems associated with substrate inhibition or inhibitory by-product formation *(3,7)*. A major nutrient that can have a marked effect on both rate of cell growth and cell yield is oxygen *(8)*. During the growth phase of the fermentation, an oxygen limitation will result in major decreases in both growth rate and cell yield. All approaches thus far have focused on improving the O_2 mass transfer rates by manipulating various environmental parameters; therefore, the dissolved oxygen concentration (DOC) in fermentors is typically controlled at or above 20% of air saturation via control strategies in which the dissolved oxygen (DO) level is maintained at a set level by the agitation and/or aeration rate *(5,8)*.

Although the impact of DOC on cell growth is well known, its impact on the expression of heterologous proteins has been the topic of only a limited number of studies *(9,10)*. My investigation describes the initial results of studies aimed at exploring the influences of DOC on the expression of heterologous proteins in *E. coli*. The recombinant proteins chosen for this study are derived from HIV-1 (p24, p55, and env41) and influenza virus C13 protein, which is a hybrid protein consisting of the first 81 amino

acids of viral nonstructural protein (NS1) and the HA2 subunit of viral hemagglutinin *(11)*.

Materials and Methods

Cultures

The cultures used in these studies are all derivatives of *E. coli* K-12 strain N99, also known as ATCC 33956 (F^-, galK2, *lacZ*, thr, bio, *rpsL*, λ^-). All of these strains carry a cryptic lambda cI^{857} lysogen *(1,2)*. The plasmids pGK41, pGK24, and pGK55 produce translational fusions of the human immunodeficiency virus-1 (HIV-1) proteins Env41, p24Gag, and p55Gag, respectively. These translational fusions were generated by fusing the first 56 codons of galactokinase (EC 2.7.1.6) in-frame to the N-terminus of respective protein coding regions *(12,13)*. The parent of these plasmids is pSKF and is heat inducible *(1)*. Similarly, the pGKc13 construct produces the influenza protein C13 *(11)*. Expression in all cases is under the control of the lambda promoter p_L *(1,2)*. At 32°C the cI^{857} repressor binds to the p_L promoter on the plasmid and blocks transcription of the expression cassette. At 42°C the repressor is inactivated and the desired gene product is expressed. The specific growth rates of the HIV recombinant proteins producing a host/vector system and the host either with plasmid but no gene insert or without the plasmid at 32°C were 0.71 ± 0.01 h^{-1}. Specific growth rate prior to induction had no significant effect on product expression levels *(14)* (data not shown).

Media

Complex media were used in the current study. Medium 1xSB contained the following: 12 g/L tryptone; 24 g/L yeast extract; 13 g/L glycerol; 15.3 g/L K_2HPO_4; 1.7 g/L KH_2PO_4; 0.05 g/L ampicillin. Medium 2xSB was double the strength of 1xSB. The pH of all media was adjusted to 7.0 prior to sterilization. In this medium, glycerol was used as a C-source because it is not a fermentative substrate, so that the accumulation of potentially harmful catabolites such as acetate or ethanol would not be a significant problem. In addition, the medium is very strongly buffered, containing a high concentration of potassium phosphates. Fermentations were conducted in 2xSB medium. The purpose of the 2xSB formulation is severalfold:

1. The carbon source is not limiting during the course of growth/induction *(14)*.
2. Amino acid availability increases the pool size of charged tRNAs and guanosine 5'-triphosphate, thereby reducing/eliminating amino acid misincorporation, i.e., protein synthesis errors, during high-level expression *(15)*.
3. A complex medium composition is particularly desired considering the effects of heat shock (induction temperature) on the production of recombinant proteins *(16–18)*.

Seed Development

A 75-µL aliquot of thawed cells was added to a 2.8-L Fernbach flask containing 1 L of 1xSB. This culture was then incubated at 32°C and agitated at 200 rpm overnight. Cell growth was monitored by following the increase in optical density (OD) at A_{650} nm. Under these conditions the cells were in late exponential/early stationary phase when used to inoculate the fermentor, which allowed rapid adaptation and fast growth in the fermentor.

Production Fermentations

Fermentors containing 9.5 L of 2xSB were inoculated with an aliquot of the overnight seed sufficient to bring the postinoculation absorbance to 0.5 OD. Growth from an inoculation density of $A_{650\,nm}$ 0.5 to OD_{650} 5.0 or 10 was achieved in about 3 to 4 h. The temperature, airflow rate, back pressure, and initial agitation rate were set at 32°C, 5 standard liter per minute (SLPM), 0.5 bar, and 300 rpm, respectively. The pH was not controlled in these studies. During the course of the fermentations, the pH typically dropped 0.05–0.10 units and acetic acid concentration was <0.2 g/L *(16,18)* (data not shown). Prior to induction, the DO was maintained at or above 20% by coupling the DOC output to the agitator control. The fermentors were operated in this manner until a suitable induction point was achieved. Once the induction OD was reached, the temperature set point was shifted to 42°C (a 10°C change in temperature was typically achieved within 15 min), and the agitator control was placed in local. The fermentors were then operated at constant agitation for the duration of the experiment. Generally, the induction phase was about 4 h. When the fermentation experiments were terminated, the residual glycerol concentration was typically about 9 g/L.

Analysis

During the course of induction, samples were removed from the fermentors and aliquots centrifuged at 15,000*g* for 2 min at room temperature. The supernatants were discarded and the pellets frozen at –20°C. Samples were then lysed by boiling in phosphate-buffered saline supplemented with 0.5% sodium dodecyl sulfate (SDS). Duplicate aliquots of each sample were then analyzed for total protein by the Lowry method. Separate pellets were then lysed by boiling in Laemmli sample buffer supplemented with 0.5% SDS, and duplicate aliquots from each sample were analyzed by polyacrylamide gel electrophoresis *(19)*. Each lane on the gel was loaded with the same amount of protein. The gels were stained with Coomassie blue, and the amount of the protein of interest was quantified by scanning laser densitometry. Calculated values for product concentration represented four data points obtained from three different fermentation runs and fell within an experimental error of ±1.5%. Note that previous protein studies as well as my experiments on the recombinant HIV GalK fusion proteins showed that the expressed proteins are soluble, resistant to proteolysis, and stable during analysis; i.e., insignificant product loss occurs

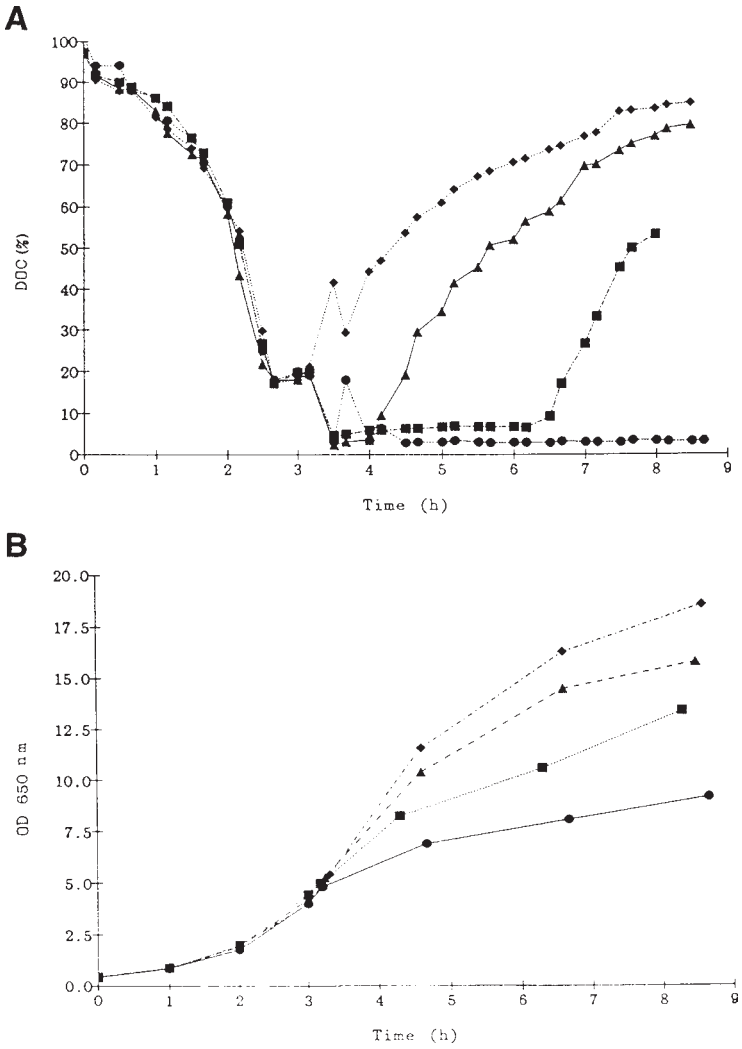

Fig. 1. The influence of induction phase agitation rate on **(A)** DOC and **(B)** cell growth. OD 10 induction in glycerol containing complex medium: 200 (●), 300 (■), 400 (▲), and 500 rpm (◆).

using the method described above *(13,20)*. Additionally, based on amino acid analysis of the recombinant proteins, there is no evidence for missense errors, i.e., misincorporation of amino acids during high-level expression, data not shown *(15)*.

Results

Influence of Agitation Rate and Cell Density at Induction on the Production of p24

As expected, decreasing agitation rates during the induction phase resulted in both decreased DOC (Figs. 1A and 2A) and decreased growth

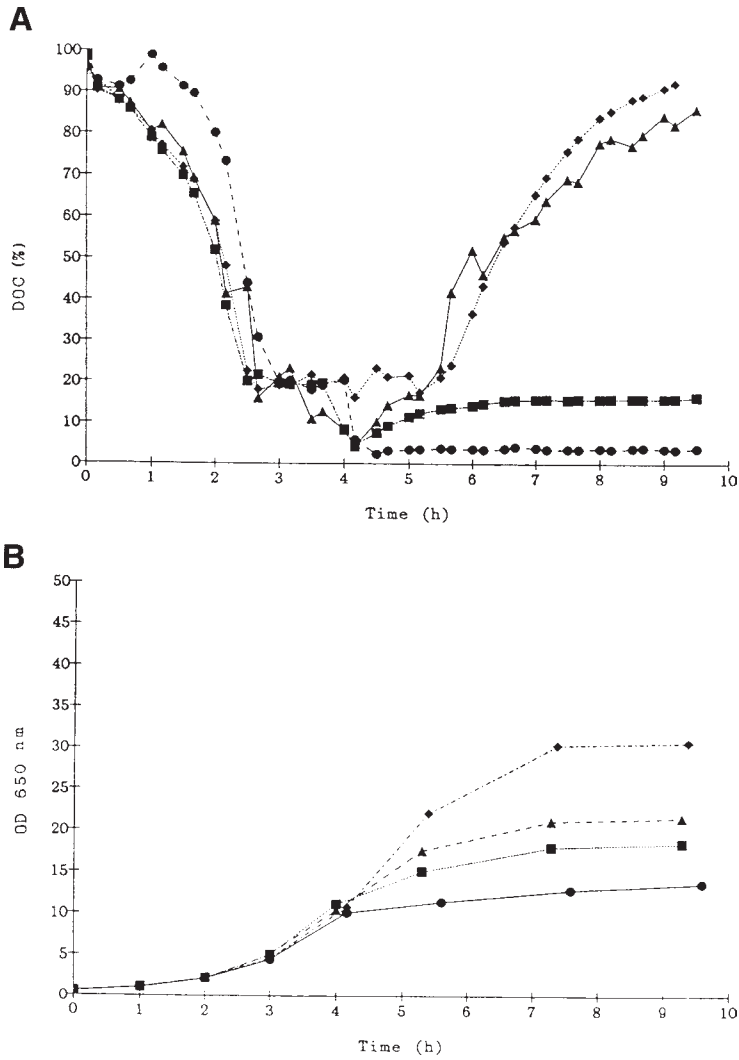

Fig. 2. The influence of induction phase agitation rate on **(A)** DOC and **(B)** cell growth. OD 5 induction in glycerol containing complex medium: 200 (●), 300 (■), 400 (▲), and 500 rpm (◆).

rates and cell yields (Figs. 1B and 2B) regardless of the OD at which induction occurred. On the other hand, the influence that agitation rate had on p24 yield (*yield* is defined here as specific product concentration) and p24 volumetric productivity (*volumetric productivity* is defined here as product concentration) were strongly influenced by other operational parameters. At an OD induction of 10, decreasing induction phase agitation rates resulted in both decreasing p24 yields (Fig. 3A) and decreasing p24 volumetric productivities (Fig. 3B). This direct relationship between agitation rate and p24 yield and volumetric productivity was not observed in OD 5 inductions. At this OD, p24 yield increased as the agitation rate was

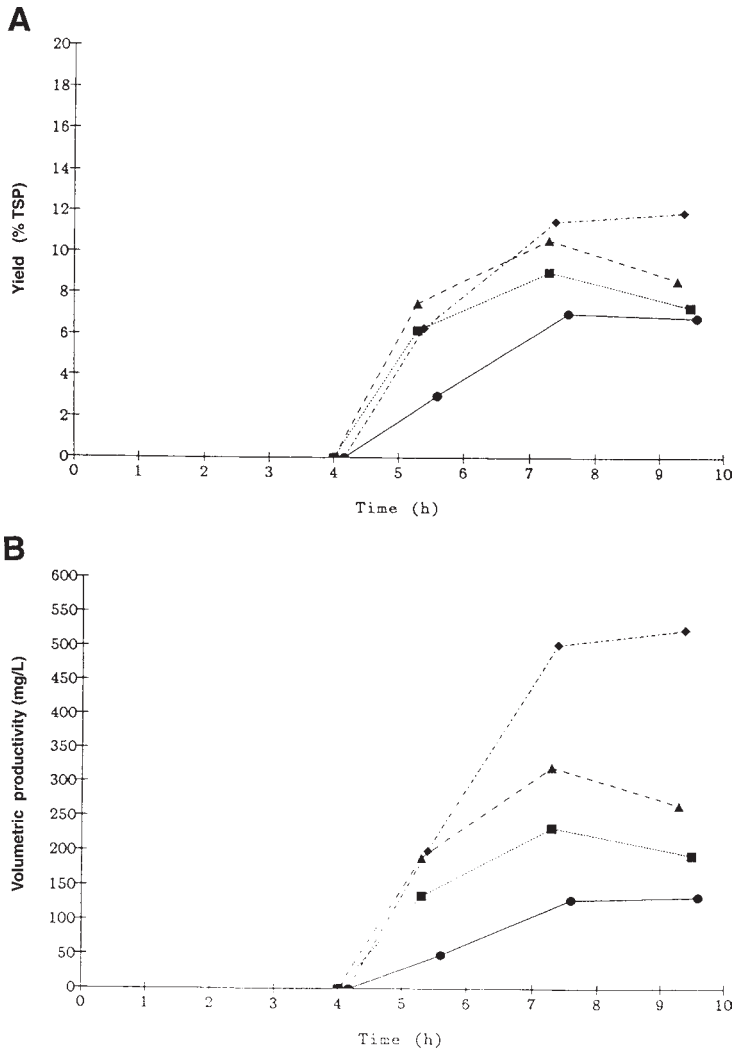

Fig. 3. The influence of induction phase agitation rate on **(A)** p24 yield and **(B)** volumetric productivity. OD 10 induction in glycerol containing complex medium: 200 (●), 300 (■), 400 (▲), and 500 rpm (◆).

decreased to 300 rpm (Fig. 4A). A further decrease in agitation rate resulted in a lower specific rate of p24 production. However, the maximal yield observed at 200 rpm was comparable to that observed at 300 rpm, and the yield was still increasing when the experiment was terminated. In OD 5 inductions the influence of decreasing agitation rate on the p24 volumetric productivity (Fig. 4B) was analogous to that observed on p24 yield (Fig. 3A).

Influence of DOC on Production of p24 in OD 5 Induction

The enhancement in the p24 yield observed in OD 5 inductions at decreased induction agitation rates was quite surprising. To determine

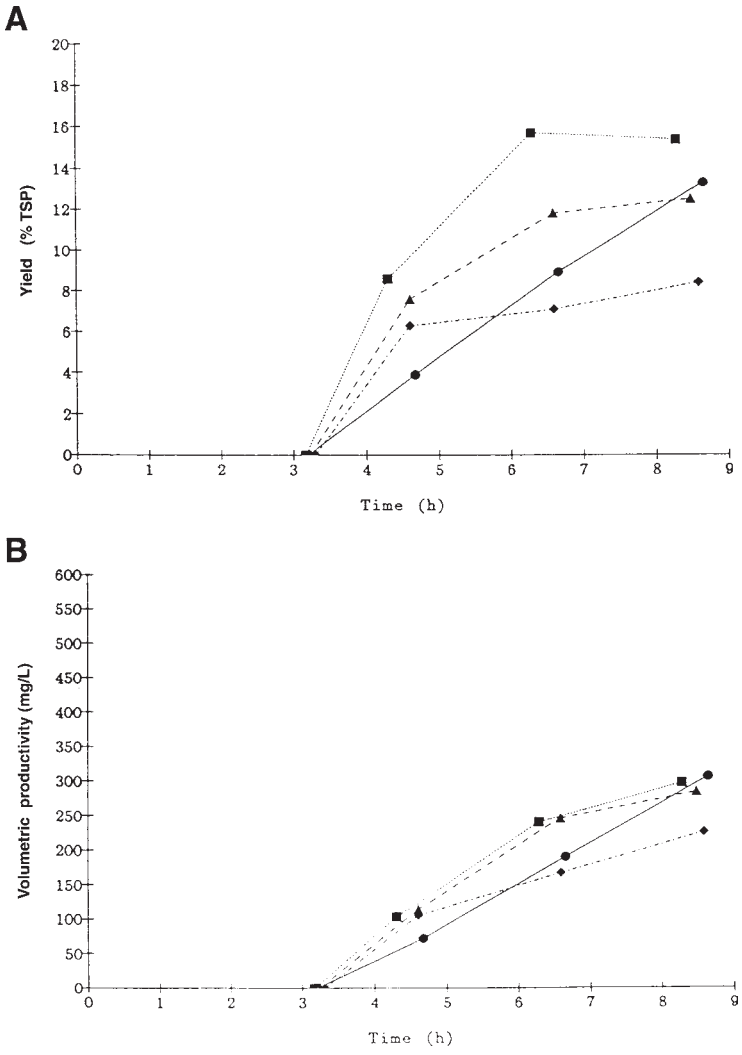

Fig. 4. The influence of induction phase agitation rate on **(A)** p24 yield and **(B)** volumetric productivity. OD 5 induction in glycerol containing complex medium: 200 (●), 300 (■), 400 (▲), and 500 rpm (◆).

whether the enhancement observed was owing to a DO or a shear effect, studies were conducted in which the DOC was controlled on induction by a combination of increased aeration rate and the use of enriched air (40% oxygen). In these studies the induction phase agitation rate was maintained at 300 rpm. The cell growth and DOC levels observed when either enriched air or air was used for sparging are given in Fig. 5A. It can be seen that the use of enriched air resulted in no DO limitation and that the cell growth observed with enriched air was essentially the same as that observed with air and an induction phase agitation rate of 500 rpm (Fig. 2B). The DO profile and cell growth observed with house air (Fig. 5A) was

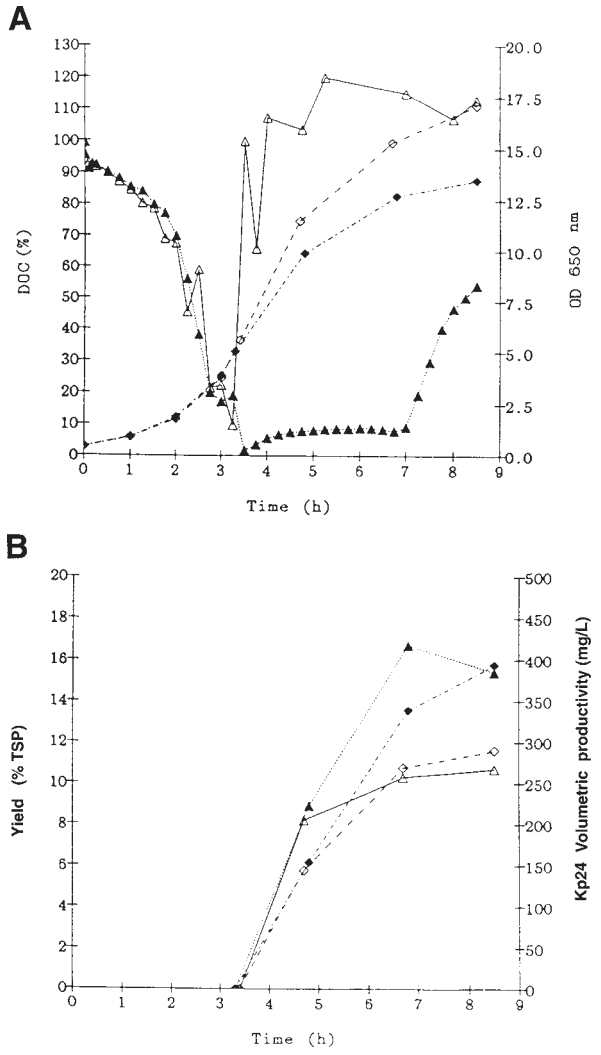

Fig. 5. The influence of enriched air (40% oxygen) on **(A)** DOC and cell growth and **(B)** p24 yield and p24 volumetric productivity. At OD 5 induction in glycerol containing complex medium, the agitation rate in both fermentors was lowered to 300 rpm: (A) cell growth in OD 650 nm–enriched air (◇), percentage of DOC-enriched air (△), percentage of cell growth in OD 650 nm house air (◆), percentage of DOC in house air (▲); (B) yield-enriched air (△), yield air (▲), volume productivity–enriched air (◇), volume productivity air (◆). Kp24 = GalKp24 fusion protein.

essentially the same as that observed initially (Fig. 2A and 2B, respectively). The p24 yields and p24 volumetric productivities, i.e., specific product concentration and product concentration, respectively, observed with or without enriched air are given in Fig. 5B. In the presence of enriched air, the p24 yield falls in between those observed when house air was used at induction phase agitation rates of 400 and 500 rpm (Fig. 4A). The p24 volumetric productivity observed when enriched air was used (Fig. 5B) was

Table 1
Influence of Agitation Rate on Production of Heterologous Viral r-Proteins in *E. coli*

	Percentage of expression observed at 500 rpm agitation rate[a,b]	
Protein	300 rpm	500 rpm
HIV-1 p24	200 (0)	100 (>20)
HIV-1 p55	98 (0)	100 (>20)
HIV-1 p41	115 (0)	100 (>20)
C13	10 (0)	100 (>20)

[a]Agitation rate during induction phase in which OD of induction = 5.
[b]Percentage of DO during induction phase is given in parentheses.

approximately the same as that observed when house air was used at an induction phase agitation rate of 400 rpm (Fig. 4B). The enhancement in both yield and volumetric productivity under DO-limiting conditions was again observed (Fig. 5B). The results clearly indicated that the enhancement observed is related to oxygen concentration/availability and not to a shear effect.

Influence of Induction Phase Agitation on Production of p55, Env41, and C13

Table 1 presents the influence of induction phase agitation rate on the yield of several heterologous proteins. It can be seen that the influence agitation rate/DOC has is highly protein dependent. In the case of Env41, an enhancement in product yield is again observed at low DOC, i.e., at 300 rpm, but the magnitude of enhancement is much less than that observed for p24 (15 vs 100%). In the case of p55, product yield was not affected by agitation rate/DOC. Finally, a marked decrease in product yield at low agitation rate/DOC was observed for C13.

Discussion

Oxygen is essential for the aerobic growth of *E. coli*, yet it is the most difficult to supply because of its low solubility. The concentration of DO is usually determined by its transfer rate. The DO level can be maintained by either an increase in the rate of O_2 transfer or by a reduction in the O_2 uptake rate in the culture *(21,22)*. The dependence of the growth rate on DOC follows a Monod saturation dependence *(23)*. The DO level may affect not only the formation and maintenance of microbial biomass, but also the replication of plasmids *(24,25)* and the expression of plasmid-born genes *(10,26)*. The effect of DO level on productivity of r-proteins is not well

documented. Most studies have examined r-proteins from *E. coli* such as β-lactamase, chloramphenicol acetyltransferase, or β-galactosidase *(10)*, or other bacterially derived r-proteins *(27–29)*. Few reports mention the requirement for O_2 enrichment during the fermentation process for production of heterologous r-protein *(30–33)*.

A report by Arcuri et al. *(9)* found that the response to a DO limitation among heterologous r-proteins was variable depending on the cell age and concentration, the growth medium, and the degree of the DO limitation imposed. Since the existent evidence in the literature is somewhat fragmentary and inconclusive with regard to this important parameter, I have investigated the affects of DO limitation on the production of heterologous r-proteins derived from HIV-1. These viral r-proteins were chosen for this study because of their clinical importance.

Decreased agitation rates during the induction phase of the fermentations resulted in decreased cell yields regardless of the cell density at induction (Figs. 1B and 2B). The decreases observed in cell yields are a direct result of the oxygen limitations imposed at the decreased agitation rates (Figs. 1A and 2A). However, the influence that decreased agitation rates had on the p24 yield (specific product concentration) at different induction cell densities was not expected. In OD 10 inductions, a direct relationship between product yield and agitation rate was observed (Fig. 3A), whereas in OD 5 inductions an inverse relationship between product yield and agitation rate was observed (Fig. 4A). The trends in product yield observed in OD 5 and OD 10 inductions were roughly reflected in the respective p24 volumetric productivities, i.e., product concentrations (Fig. 4B and 3B, respectively). However, the absolute volumetric productivities were quite different. The maximum productivity observed in an OD 10 induction was 550 mg/L, and this productivity was obtained at an agitation rate of 500 rpm. In the OD 5 inductions the maximum productivity was 325 mg/L. Productivities of this approximate magnitude were achieved in the 200, 300, and 400 rpm inductions. It appears that the maximum p24 volumetric productivity was achieved under oxygen-sufficient conditions. However, this productivity is achieved at the expense of product yield.

Although it was clear that agitation rate had a significant effect on product yield, it was not clear whether the observed effect was an oxygen concentration or a shear effect. Despite the fact that *E. coli* is not typically thought of as a shear-sensitive organism, these cells do elongate and swell noticeably on induction, and the sensitivity of the cells to shear does increase. Therefore, the possibility of a shear effect existed and it was necessary to delineate this phenomenon. Evidence is provided that the enhancement in the p24 yield seen in OD 5 inductions was truly an oxygen effect and not a shear effect and is demonstrated in the studies in which DO was controlled via sparging instead of agitation (Fig. 5A,B).

In an attempt to understand the mechanism(s) underlying the observed oxygen effect on the p24 production, the production of a variety of heterologous proteins under DO-sufficient and DO-limiting conditions

was investigated (Table 1). The results observed varied greatly. In certain cases, DOC led to enhancement in product yield, i.e., p24 and Env41, whereas in certain cases of p55 and C13, DOC resulted in no effect and a significant negative effect on product yields was found. In analyzing these results, it is important to bear in mind that for all the proteins tested the same host strain of *E. coli* was used. Thus, the difference observed cannot be attributed to differences in response of the host strain to DO availability.

It is well documented that the levels of a number of proteins in *E. coli* are strongly affected by oxygen *(34)*. However, the role of protein induction and repression in the adaptation of *E. coli* to changes in the supply of O_2 and other electron acceptors is only poorly understood *(35)*. Cellular protein components and levels change for more than 170 individual polypeptides during aerobic and anaerobic growth. Several of these proteins belong to the TCA cycle enzymes. These aerobiosis-induced proteins display several different patterns of regulation in response to medium composition *(34)*. In some instances, it has been demonstrated that oxygen exerts its effect at the level of promoter activity *(36)*. The mechanism by which it exerts this effect is subject to speculation. Possible mechanisms include both a direct effect on the promoter itself or a transacting regulatory element *(37,38)* and an indirect effect on the degree of DNA coiling *(39–41)*. It seems likely that the activity of p_L promoter employed in my studies was affected either directly or indirectly by oxygen availability. However, if oxygen was exerting its effect at this level, a genetic response to oxygen level would be expected. The product-to-product variability observed (Table 1) argues against oxygen exerting its effect at the level of transcription. Likewise, the variability observed argues against a generic effect on translation. At present my efforts are focused on looking at the influence of oxygen level on the stability of the heterologous proteins expressed in *E. coli*.

The difference in the effect of oxygen level on the accumulation of p24 observed in OD 5 and OD 10 inductions is quite dramatic and difficult to explain. One plausible interpretation is variability in specific growth rates of the two cultures. However, on further investigation, specific growth rate prior to induction has been found not to affect product expression level to any significant degree *(14)* (data not shown). The explanation perhaps resides in the differences between the two cell populations *(42,43)*. Since the inocula for these experiments were prepared in identical manner and the cells had been grown exponentially for a minimum of 3 h (for OD 5 inductions), the differences observed should not be owing to an inoculum effect. However, a seed effect has not been completely ruled out at this time. The obvious differences between these two conditions are the cell density and the time in fermentation. There is a twofold difference in cell density and approximately a 45-min difference in time of fermentation, i.e., one doubling time. Again, it is difficult to see how this relatively minor difference in growth could account for such a dramatic difference in the effect of DOC on p24 yield. This is especially true in light of the fact that the medium and preinduction operational strategy used are capable of supporting exponen-

tial growth of this strain up to an OD of 35. However, it must be remembered that all of the studies performed were batch studies in which the composition of the growth medium was constantly changing. As a result, it is possible that the change in environmental conditions that occurs between OD 5 and 10, although insufficient to affect growth rate, is sufficient, in some manner, to alter the cells' response to oxygen *(34,35)*, or that cell density affects the cellular content of the stringent response signal substance, ppGpp *(44)*, thus affecting host cell physiology and in turn influencing product formation/stability. The unraveling of this mystery awaits further investigation.

I have examined the effect of DOC on the accumulation of HIV-1 r-proteins in *E. coli*. The imposition of DO limitation could have stimulatory effects on r-protein production. The production of r-protein in response to DOC has proven to be product dependent. The variability observed to this response was found to be contingent on cell type, age, and density; composition of growth medium; and the degree of DO limitation. It is hoped that these results will help guide future optimization studies of therapeutic r-protein fermentation processes for production purposes.

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