Indian Journal of Biotechnology
Vol 7, April 2008, pp 225-229

Oxygen transfer conditions in the production of rainbow trout growth hormone (rtGH) by *Escherichia coli*

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Received 16 April 2007; revised 23 July 2007; accepted 25 September 2007

Batch cultures of *Escherichia coli* pRE1-rtGH 121/MZ1 were carried out at different oxygen transfer rates (OTR) enhanced by the increase of agitation and/or aeration rates. The growth kinetics of *E. coli* was investigated in complex medium under batch cultivation in 3 L fermenters. The agitation rates influenced both cell growth and rainbow trout growth hormone (rtGH) production in the bioreactor. It was found that increasing the agitation speed had a positive effect on the production of rtGH. Moreover, varying the airflow rate (vvm) in the fermentor under constant drive speed, cell growth and rtGH production were greatly influenced. In the production of rtGH by *E. coli*, high aeration rates were found to be essential for good yields of recombinant protein. Agitation speed and aeration rate could affect dissolved oxygen concentration, which in turn affected cell growth and rtGH production. Increased aeration rates induced higher rtGH production, with the highest concentration of 0.957 g/L obtained at 4.0 vvm, within 23 h. The highest volumetric productivity for rtGH of 0.042 g/L/h was obtained at both 1000 rpm and 4.0 vvm.

Keywords: aeration rate, agitation speed, *Escherichia coli*, fermentation, fish growth hormone

Introduction

Recombinant DNA technology allows faster large-scale production of important bio-molecules at higher concentrations than they could be found in natural sources. Industrial success of this technological application depends on the cloning techniques of development, as well as on optimization of culture conditions. Dissolved oxygen (DO) is one of the factors that dramatically affects the cell growth and recombinant protein production of aerobic cultures. Oxygen is both essential for the aerobic growth of *Escherichia coli*, at the same time, the most difficult to supply, because of its low solubility. Due to the rate of oxygen utilization sufficiently great in cultures of even moderate density the concentration of DO may be determined by its transfer rate. The DO level can either be maintained by an increase in the rate of oxygen transfer or increased vessel pressure, agitation and sparge rate[1,2]. For *E. coli*, as one of the most popular host, it is well known that oxygen availability affects its cellular yield[3]. This is particularly important because almost all of the recombinant proteins expressed in this microorganism remain inside the cell, in spite of all the efforts being made in heterologous protein secretion research[4]. Therefore, one of the goals of cultivation conditions optimization is to reach high cell densities. It is crucial to ensure an adequate oxygen supply to the media.

There are several methods of enhancing oxygen transfer rate to a culture: increasing stirrer speed and/or air sparging rate, enriching air inlet with pure oxygen[5], increasing oxygen partial pressure by raising the total pressure of the bioreactor[6]. The adequate method for improving oxygen transfer rate for each case depends on the effects of the factors manipulated to ensure a certain OTR on cell growth, as well as on recombinant protein expression. The effects of oxygen on the production of recombinant proteins are strongly dependent of the strain/plasmid used[7]. The aim of this work was to study the dependence of rtGH production by *E. coli* on oxygen supply during cultivation in a bioreactor. The present study describes the influence of aeration and agitation rate on cell growth and rtGH production.

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Materials and Methods

Microorganism and Media

Escherichia coli pRE1-rtGH 121/MZ1 cells, genetically modified with the plasmid co-expressing a fusion protein of rainbow trout growth hormone (rtGH) and conferring ampicillin resistance, were used in the fermentation experiments[8]. The bacterium was kept frozen in an LB-medium containing 20% (w/v) glycerol solution. Complex medium (*p*H 7.0) for fermentation study was prepared according to the modified protocol published elsewhere[9].

Inoculum Preparation

An Erlenmeyer shake-flask of 250 mL, containing 50 mL of complex medium was inoculated with 1.0 mL glycerol culture solution having 100 mg L^{-1} ampicillin. The inoculated culture was incubated overnight using an incubator shaker operating at 37°C with a rotational speed of 250 rpm, which was then used to inoculate the fermenters used in the fermentation studies.

Fermentation Runs

A 3 L fermenter Bioflow III (New Brunswick Scientific Co., New Brunswick, NJ, USA) containing 2 L complex medium was used to study the optimum aeration and agitation rate. The fermenter was inoculated with 10% (v/v) of inoculum described earlier. Cultures were induced with 5.0 m*M* IPTG when biomass attained a cell dry weight (CDW) of 1.5 g L^{-1}. Agitation was provided with a pair of six-bladed Rushton impellers. Four side-walled equidistant baffle plates were used to prevent vortex formation. The *p*H was measured using a glass electrode immersed in the fermentation broth. DO was measured using a polarographic electrode (Ingold, Germany). Foam control was achieved by the addition of silicone antifoaming agent (Fluka, Switzerland). Experiments were conducted at agitation speeds of 250, 500, 750 and 1000 rpm. The corresponding aeration rate was adjusted to 1.0, 2.0, 3.0 and 4.0 vvm (volume of air/volume of medium/min), respectively. Samples were drawn from the fermenter at regular intervals and analyzed for biomass, rtGH, and glycerol.

Analysis

Biomass Concentration

Optical density was measured spectrophotometrically at 600 nm, and then converted to CDW.

SDS-PAGE

Protein samples were analyzed by electrophoresis in a 12% (w/v) sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) as described by Laemmli[10].

Quantification of Expressed rtGH

The amount of the recombinant rtGH in each sample was determined by measuring the intensity of protein bands after SDS-PAGE using the previously presented method published elsewhere[9].

Glycerol Determination

Glycerol determination was carried out with an enzymatic assay (FG0100) according to the technical bulletin of the manufacturer (Sigma, St Louis, Missouri, USA).

Results and Discussion

Effect of Agitation Speed

The influence of agitation directly on the growth rate and product yield is complicated by its other effects on mixing and oxygen transfer. Different fermentation runs were carried out at a constant aeration rate of 1.0 vvm but with different agitation speeds, 250, 500, 750 and 1000 rpm, respectively. As can be seen from the growth curves (Fig. 1a), the increase in stirring from 250 to 1000 rpm had a significant effect on biomass in general. At higher agitation rates, cell growth increased with a specific growth rate of 0.643 h^{-1} for 750 rpm and 0.885 h^{-1} for 1000 rpm, respectively. Local oxygen limitation is thought to be responsible for a relatively lower specific growth rate of 0.161 h^{-1} at 250 rpm.

The rate of product formation also can be affected by mixing and oxygen transfer. Fig. 1b illustrates the time profile of rtGH production at different agitation speeds. There were substantial differences in rtGH production during the batch fermentations. At low agitation speed (250 rpm), rtGH production curves started to increase after 6 h, while at higher agitation speeds (500, 750 and 1000 rpm) after 3 h. Maximum rtGH concentration (0.585 g L^{-1}) was obtained after 22.5 h at 1000 rpm.

The DO concentration profiles were different for the four levels of agitation speeds tested (Fig. 2a). DO concentration could be consistently maintained at above 20% saturation over the entire fermentation process at 1000 rpm. However, DO concentration was notably lower at 750 rpm and even reached 0% saturation at the beginning of the log-growth phase at 250 and 500 rpm. The oxygen limitation resulted in a

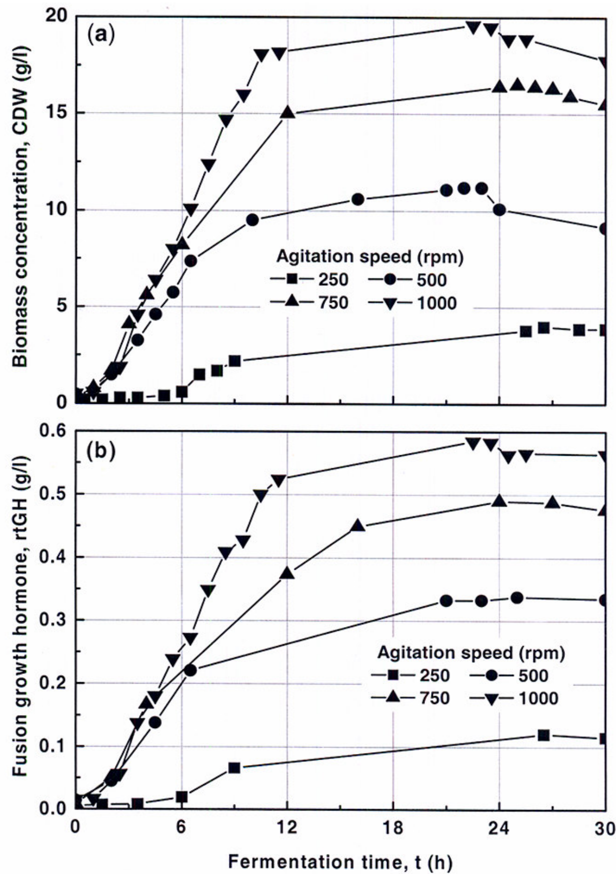

Fig. 1—(a) Growth curves for *E. coli* pRE1-rtGH 121/MZ1, (b) levels of rtGH production for batch cultures at increased stirring rates (250, 500, 750 and 1000 rpm).

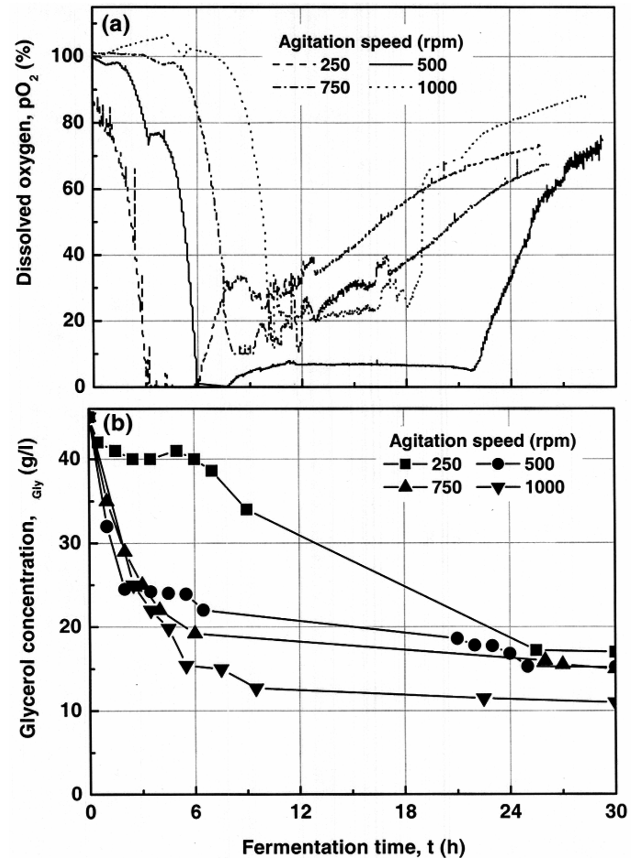

Fig. 2—Time profiles of (a) dissolved oxygen DO in *E. coli* pRE1-rtGH 121/MZ1 using stirred tank bioreactor, (b) residual glycerol concentration of different batch cultures at various agitation speeds (250, 500, 750 and 1000 rpm).

lower cell density and shorter log-growth phase at 250 and 500 rpm. Correspondingly, rtGH concentration associated with 500 rpm plateaued earlier and the maximum rtGH concentration was, therefore, much lower at 250 rpm. A higher agitation speed increased the amount of DO and dispersion of macromolecules in the medium. It might, therefore, have contributed to the greater growth and better rtGH production at 1000 rpm noted in this study. Similar observations were also reported by Manolov[11] and Shioya *et al*[12]. As outlined earlier, oxygen limitation is thought to have contributed to the lower growth and rtGH production observed at lower agitation intensity applied. Glycerol concentration was measured throughout different fermentation runs in order to follow up its consumption rate. As shown in Fig. 2b, glycerol uptake could be correlated with the growth curves shown in Fig. 1a. In general, glycerol was consumed and reached its final value of 11 g L^{-1} at 1000 rpm. This indicates that glycerol was not a limiting substrate even under the highest agitation speed applied in the present study; 1000 rpm was best for *E. coli* to produce rtGH. The growth kinetics of *E. coli* on complex medium with different agitation rates is given in Table 1.

Effect of Aeration Rate on Fermentation Kinetics

Batch fermentations were performed using four different aeration rates (1.0, 2.0, 3.0 and 4.0 vvm) to determine the effect of aeration on rtGH. All the cultures were inoculated identically, and as shown in Fig. 3a, the cell mass increase depended on the aeration rate. The highest biomass concentration (30.4 and 32 g L^{-1}) was obtained in culture grown at an aeration rate of 3.0 and 4.0 vvm, respectively. Similarly, the production of rtGH was markedly increased with aeration rate (Fig. 3b). The highest rtGH concentration of 0.909 and 0.957 g L^{-1} was produced after 23 h at 3.0 and 4.0 vvm, respectively. This indicates that increasing the aeration rate from 1.0 to 4.0 vvm led to the production of almost 2-fold rtGH higher than that obtained at lower aeration rate.

The DO concentration profiles were significantly different under different aeration rates (Fig. 4a). At aeration rate of 2.0 vvm, dissolved oxygen concentration was between 20 and 30% saturation for most of the time. In contrast, DO concentrations were above 35 and 50% saturation at the higher aeration rates of 3.0 and 4.0 vvm, respectively. Oxygen limitation was probably the reason for the lower cell mass (19.6 g/L) and rtGH production (0.585 g L^{-1}) at the lower aeration rate of 1.0 vvm in comparison with the results obtained at higher aeration rates.

These results indicated that aeration could influence DO concentration significantly, which in turn would affect cell growth, rtGH production and substrate utilization. The maximum rtGH concentration had almost no significant difference at aeration rates of 3.0 and 4.0 vvm. Since more power would be needed for a higher level of aeration, 3.0 vvm was considered to be the optimum aeration rate in this study. Aeration obviously results in better mixing of the production medium. Thus, maintaining a concentration gradient between the interior and exterior of the cell and allowing better diffusion at a high aeration rate, which is responsible for the high cell concentration and the enhanced rtGH production.

The concentration of residual glycerol sharply decreased from the beginning of the fermentation with corresponding increase in biomass and rtGH concentration. Remarkable glycerol depletion was observed for the culture grown at the aeration rate of 3.0 and 4.0 vvm, whereas 11 g L^{-1} glycerol remained in the fermentation broth at a low aeration rate of 1.0 vvm during the same fermentation period instead of 7 and 3.2 g L^{-1}.

In general, increased sparge rate simply increases the oxygen mass transfer coefficient and thus allow the cells to uptake more oxygen. The growth kinetics data of *E. coli* with different aeration rates are given in Table 1. The yield of rtGH production based on glycerol consumption ($Y_{P/S}$) and the specific growth rate of the cells (μ) at 4.0 vvm were 0.023 g/g and 1.43 h^{-1}, respectively.

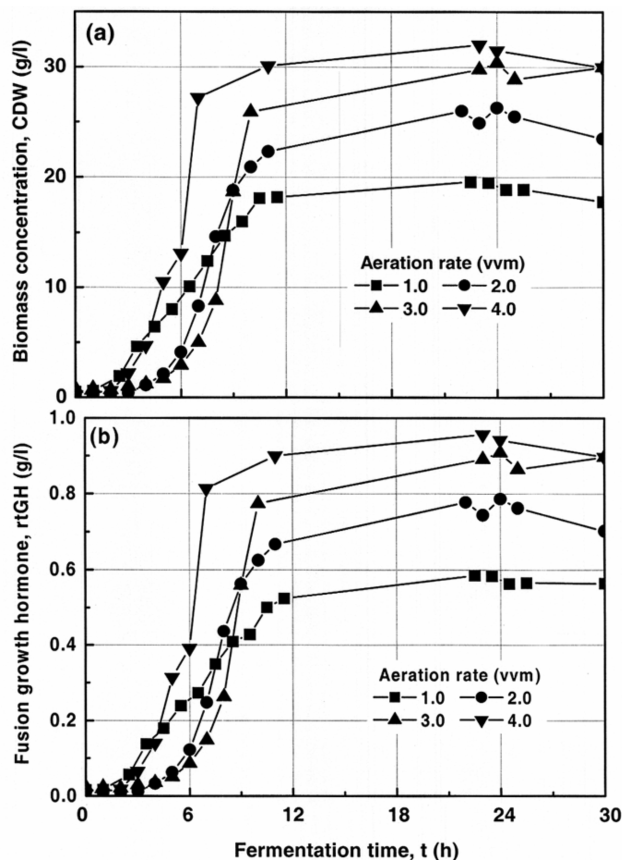

Fig. 3—Time course of (a) cell growth, (b) rtGH produced by *E. coli* pRE1-rtGH 121/MZ1 in batch cultures at different aeration rates (1.0, 2.0, 3.0 and 4.0 vvm).

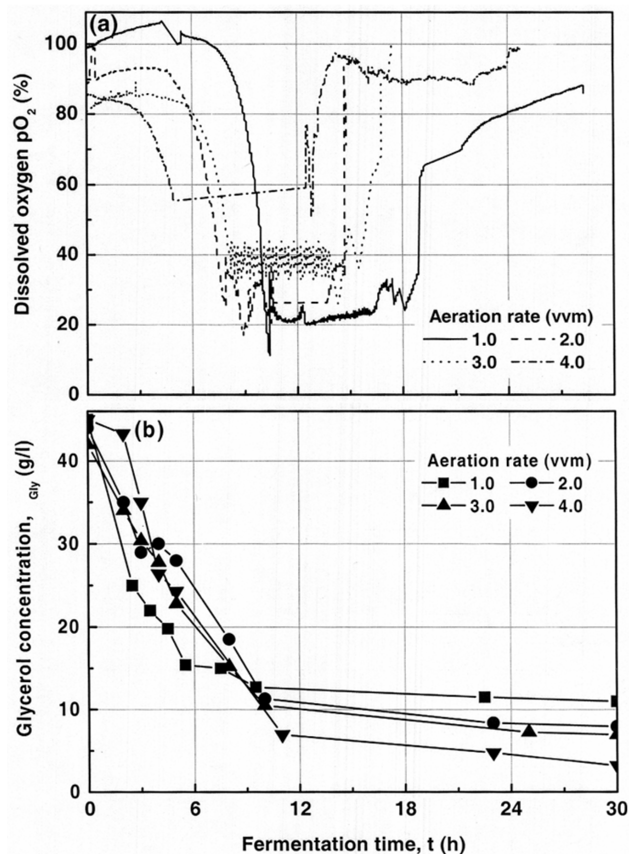

Fig. 4—Time course of (a) DO saturation, (b) residual glycerol concentration during batch cultivation of *E. coli* pRE1-rtGH 121/MZ1 at different aeration rates (1.0, 2.0, 3.0 and 4.0 vvm).

Table 1—Fermentation kinetics of *E. coli* pRE1-rtGH 121/MZ1 on different agitation and aeration rates

Kinetic parameters	Agitation speed, rpm (aeration 1.0 vvm)				Aeration rate, vvm (agitation 1000 rpm)			
	250	500	750	1000	1.0	2.0	3.0	4.0
Maximum biomass concentration, X_{max} (g/L)	4.00	11.2	16.50	19.60	19.60	26.30	30.40	32.00
Maximum rtGH concentration, P_{rtGH} (g/L)	0.12	0.338	0.489	0.585	0.585	0.786	0.909	0.957
Time for maximum rtGH production, t (h)	26.5	25.0	24.00	22.50	22.50	24.00	24.00	23.00
Volumetric rtGH productivity, P (g/L/h)	0.004	0.013	0.020	0.026	0.026	0.033	0.038	0.042
Specific growth rate, μ (h^{-1})	0.161	0.485	0.643	0.885	0.880	1.260	1.510	1.430
Glycerol consumption rate, Q_S (g/L/h)	1.056	1.192	1.250	1.511	1.510	1.540	1.580	1.820
Biomass yield coefficient, $Y_{X/S}$ (g cell dry weight/g glycerol consumed)	0.143	0.376	0.550	0.576	0.576	0.711	0.800	0.765
Product yield coefficient based on substrate consumption, $Y_{P/S}$ (g rtGH/g glycerol consumed)	0.004	0.011	0.016	0.017	0.017	0.021	0.024	0.023

In conclusion, DO concentration was found to have a significant effect on the growth rate as noticeable changes in biomass formation were observed in the experimental studies. By changing agitation and aeration, oxygen supply in the fermenter could be improved, and thus leading to enhanced bacterial growth and rtGH production.

Acknowledgement

The author thanks Prof T Chen, University of Connecticut, USA, for kindly providing the *E. coli* strain.

List of Symbols and Abbreviations

X_{max} maximum biomass concentration (g L^{-1})
P_{rtGH} maximum rainbow trout growth hormone concentration (g L^{-1})
t fermentation time or time required for maximum rtGH production (h)
P volumetric rtGH productivity (g L^{-1} h^{-1})
μ specific growth rate (h^{-1})
Q_s glycerol consumption rate (g L^{-1} h^{-1})
$Y_{x/s}$ biomass yield coefficient (g/g)
$Y_{p/s}$ product yield coefficient (g/g)
OTR oxygen transfer rate (mg-O_2 L^{-1} h^{-1})
CDW cell dry weight (g L^{-1})
ρ_{Gly} glycerol concentration (g L^{-1})
Indices
Gly glycerol
max maximal-, maximum-

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