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ORIGINAL PAPER

Impact of dissolved oxygen concentration on some key parameters and production of rhG-CSF in batch fermentation

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Abstract The impact of different levels of agitation speed, carbondioxide and dissolved oxygen concentration on the key parameters and production of rhG-CSF in *Escherichia coli* BL21(DE3)PLysS were studied. Lower carbondioxide concentrations as well as higher agitation speeds and dissolved oxygen concentrations led to reduction in the acetate concentrations, and enhanced the cell growth, but inhibited plasmid stability and rhG-CSF expression. Similarly, higher carbondioxide concentrations and lower agitation speeds as well as dissolved oxygen concentrations led to enhanced acetate concentrations, but inhibited the cell growth and protein expression. To address the bottlenecks, a two-stage agitation control strategy (strategy-1) and two-stage dissolved oxygen control strategy (strategy-2) were employed to establish the physiological and metabolic conditions, so as to improve the expression of rhG-CSF. By adopting strategy-1 the yields were improved 1.4-fold over constant speed of 550 rpm, 1.1-fold over constant dissolved oxygen of 45%, respectively. Similarly, using strategy-2 the yields were improved 1.6-fold over constant speed of 550 rpm, 1.3-fold over constant dissolved oxygen of 45%, respectively.

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Keywords Acetate concentration · Agitation speed · Dissolved oxygen · High level expression · rhG-CSF · Specific product yield

Introduction

The commercial production of recombinant proteins for industrial and medical use has increased significantly in recent years [1, 2]. Because of low manufacturing costs, processes using *Escherichia coli* remain the production system of choice for production of recombinant industrial enzymes and therapeutic proteins. rhG-CSF is a therapeutically useful glycosylated protein for neutropenia and related disorders [3]. The non-glycosylated form of rhG-CSF is also biologically active as its glycosylated form [4]. It can be produced by recombinant DNA technology using *E. coli* as host [5–7]. This method could result in profitable mass productivity due to fast cell growth and product formation. Since, fermentation facilities for the production of recombinant proteins might be near capacity [8], there is an increasing interest in technologies that maximize the production of recombinant proteins in *E. coli*. Significant improvements have focused on protein expression to increase the production of recombinant proteins in *E. coli* [9]: these include improvements in DNA transcription, RNA translation, protein folding and stability.

Insufficient/restricted oxygen supply leads to the excretion of several metabolites from the mixed acid metabolism, i.e., succinate, formate, acetate, lactate, ethanol and hydrogen gas. Excretion of such metabolic by-products is undesirable, since the overall productivity of the bioreactor [10, 11] and the production of recombinant protein might be severely affected [12, 13]. The oxygen transfer rate is usually controlled by the rate of agitation

and gas flow. It is also possible to increase the driving force of oxygen transfer by increasing the pressure in the bioreactor [14]. However, this method has the drawback that the partial pressure of CO_2 is increased as well, which leads to growth inhibition and toxic effects by acetate production [15].

Accumulation of acetate under aerobic conditions is bacterial strain-specific [16] and generally occurs during high growth rates and/or low oxygen concentrations [17]. Over loading the tricarboxylic acid (TCA) cycle by rapid metabolic flux through glycolysis is considered to be the primary cause of acetate accumulation. The formation of acetate during growth phase in aerobic fermentation of *E. coli* is a well-known phenomenon with studies indicating that above 40 mM, acetate concentrations showed negative affect on growth and recombinant protein production [18]. The electron transport system and TCA cycle are believed to be the rate-limiting steps that affect high acetate production.

Acetate concentration can also be affected by gluconeogenesis. This can be done by the conversion of oxaloacetate directly to phosphoenolpyruvate through phosphoenolpyruvate carboxykinase and by the conversion of pyruvate to phosphoenolpyruvate through phosphoenolpyruvate synthase. Both reactions will cause the reduction of pyruvate and Acetyl-CoA concentration [19, 20] and to decrease in acetate accumulation. The pathway for the conversion of pyruvate to acetyl-CoA depends on growth conditions. At aerobic conditions, pyruvate conversion is executed by pyruvate dehydrogenase, and at anaerobic conditions, the conversion is done by pyruvate formate lyase [21]. The latter enzyme is repressed and inhibited by oxygen [22, 23]. Some research has been performed investigating the effect of dissolved oxygen and carbondioxide concentrations on cell growth and recombinant protein production [24, 25]. However, the information on the impact of dissolved oxygen (DO) and dissolved carbondioxide on rhG-CSF production is not described in the available literature. Based on the therapeutic importance of rhG-CSF, the impact of DO and CO_2 concentrations on *E. coli* metabolism for production of biomass and expression of protein need to be investigated.

In this work, the impact of agitation speed, carbondioxide and dissolved oxygen concentration on cell growth, plasmid stability, acetate accumulation and expression of rhG-CSF in batch fermentation were studied. Based on the outcome of the results, a two stage DO control strategy was proposed to improve the efficiency of rhG-CSF expression. Since, there is no information available so far and since this is the first attempt made to study the key parameters, which affect the rhG-CSF expression in *E. coli* BL21 (DE3) PLysS.

Materials and methods

Materials

Escherichia coli BL21 (DE3) PLysS was used as a host to insert the plasmid pET-3a (expression plasmid with T7 RNA polymerase) bearing the human G-CSF mutant (A + T content optimized) gene [26]. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was procured from Bangalore Genei Pvt Ltd, Bangalore, India. All the other chemicals were purchased from local companies and are of molecular biology grade.

Processing methods

Composition of media and seed development

The inoculum was developed by transferring a single colony of transformed *E. coli* into 10 ml of Luria Bertani (LB) medium containing antibiotics in a 250-ml shake flask and incubated on orbit shaker at 37 °C for 10 h at 150 rpm. In the second step, 6 × 0.5 ml of first step inoculum was used to inoculate into 6 × 50 ml of a medium [27] containing glucose 10 g, yeast extract 1 g, K_2HPO_4 10 g, KH_2PO_4 13 g, $(NH_4)_2HPO_4$ 3 g, NaH_2PO_4 4.6 g, $MgSO_4 \cdot 7H_2O$ 0.46 g and trace salt solution 10 ml [$CuSO_4 \cdot 5H_2O$ 2 mg, $Al_2(SO_4)_3$ 10 mg, $MgCl_2 \cdot 4H_2O$ 20 mg, $Na_2MoO_4 \cdot 2H_2O$ 50 mg, H_3BO_3 1 mg, $COCl_2$ 2.5 g, $ZnSO_4$ 5 mg, Fe(II)SO_4 50 mg and $NiCl_2 \cdot 6H_2O$ 1 mg (per litre) along with antibiotics (ampicillin 100 μg/ml, chloramphenicol 34 μg/ml). This second step inoculum in 6 × 500 ml shake flasks was incubated on orbit shaker at 37 °C for 6 h at 220 rpm and used for seeding the bioreactor.

rhG-CSF expression in Bioreactor

rhG-CSF fermentation was carried out in a 5-l Biostat-B-fermenter (B. Braun International, Melsungen, Germany) with a working volume of 3 l. Ten percent of seed culture was used to inoculate the fermenter medium. The temperature was kept at 37 °C, pH was monitored by a mettler toledo electrode and controlled at 6.9 ± 0.2 by the addition of 28% (w/v) ammonia solution. The fermenter was equipped with 2 six-bladed Ruston turbine impeller. The dissolved oxygen was measured by polarographic probe (Mettler–Toledo, process analytical, MA, USA). To investigate the agitation effect on cell growth, plasmid stability, acetate accumulation and rhG-CSF expression, the agitation speed was constantly controlled at 150, 350, 550 and 750 rpm, respectively, at aeration rate of 1.0 volume of air per unit volume of mass per minute (VVM). On the other hand, to asses the carbondioxide effect on the above-mentioned parameters, different concentrations of CO_2 like 5, 10, 15

and 20% were purged by mixing with air flow (DO 25%). DCO_2 was monitored and measured continuously during the process using a reliable steam sterilizable in situ DCO_2 probe (YSI 8500, YSI, Inc). Also on the other hand, to study the significance of dissolved oxygen on fermentation process of rhG-CSF, the DO was controlled at 5, 25, 45 and 65% by adjusting aeration rate of 0.4–1.4 VVM and agitation speed of 550 rpm. The oxygen uptake rate (OUR) was measured by dynamic method [28]. The culture was induced with 2.0 mM IPTG at 3 h of propagation. Samples were collected at regular intervals and centrifuged (Biofuge, USA) at 6,000 rpm for 30 min at 4 °C. The supernatant was stored at −20 °C for acetate analysis, and the cell pellets were analyzed for plasmid stability and protein expression.

Analytical methods

Cell growth was monitored by measuring the culture optical density (OD) at A_{600} nm and wet cell weight (WCW). In order to determine WCW, 5 ml of culture medium was centrifuged at 6,000 rpm for 30 min, washed twice with 9 g/l (w/v) NaCl isotonic solution. The water drops sticking to the walls of the tube were removed by paper towels. Acetate concentration was analyzed enzymatically by using a Boehringer–Mannheim Kit; cat No: 148621, which is based on NADH formation when acetate is converted into citrate and acetyl-CoA by acetyl-CoA synthetase [29]. Glucose concentration in the culture supernatant was determined using the YSI glucose analyzer (YSI Inc., Yellow Spring, OH, USA). The relative viscosity was defined as the ratio between the fermented broth flow time and the initial culture broth. Flow time of the culture broth was measured with an Ubbelohde viscometer at 35 °C. The capillary diameter of the viscometer is 0.64 mm. The stability of the plasmid in the recombinant *E. coli* strain was determined by sampling aseptically from the bioreactor at different cell densities, diluting with 9 g/l (w/v) NaCl, and by pouring it on to LB agar plates with and without antibiotics (ampicillin and chloramphenicol, three replicates for each case). The fraction of plasmid-containing cells was calculated as the average ratio between viable colonies on LB with antibiotics and to those on LB without the antibiotics [30, 31]. The cell density was measured by turbidometry and the protein expression by SDS-PAGE. The volumetric yield of rhG-CSF was determined by gel densitometry method.

Results and discussion

Impact of agitation speed

Transformed *E. coli* BL21 (DE3) PLysS harbouring pET-3a–G-CSF was grown in batch fermentation and the culture induced with 2.0 mM IPTG at 3 h of propagation. The effect of agitation speed on cell growth, plasmid stability, acetate accumulation, specific and volumetric product yield were investigated as described under Sect. "Materials and methods." The biomass accumulation was proportional to the agitation speed. When all the glucose was consumed, the biomass concentration at 750 rpm was 15.0 OD_{600} (1.5 OD/g glucose), at 550 rpm it was13.28 OD_{600} (1.32 OD/g glucose), at 350 rpm it was 11.8 OD_{600} (1.18 OD/g glucose) and at 150 rpm it was 10 OD_{600} (1.0 OD/g glucose). This may be due to unbalanced cellular metabolism by increasing acetate accumulation. The acetate accumulation was inversely proportional to the agitation speed when all the glucose was consumed and the cells entered stationary phase, reaching 0 at 750 rpm, 0.5 g/l at 550 rpm, 2 g/l at 350 rpm and 9 g/l at 150 rpm. Glucose was consumed in 7 h for 750 rpm, 7 h for 550 rpm, 8 h for 350 rpm and 8 h for 150 rpm (Fig. 1a–d).

The plasmid stability was more than 95% stable upto pre-induction phase at all speeds of agitation; thereafter, a slight decrease was observed in all samples, but highest plasmid loss (15%) was observed at high agitation speed of 750 rpm. This may due to alteration in cellular metabolism and increase in the metabolic burden of cells arising from recombinant gene expression. The highest specific product yield of 64 mg/OD/l was obtained at the agitation speed of 550 rpm. When agitation speed was greater or lesser than 550 rpm, the maximal specific product yield was decreased as shown in Figs. 1e and 2 (50 mg/OD/l at 750 rpm, 38 mg/OD/l at 350 rpm and 25 mg/OD/l at 150 rpm). It is obvious that too low an agitation speed was not beneficial to cell growth and rhG-CSF production. The decrease in cell growth is proportional to the consumption of glucose from 750 to 150 rpm. Although the highest cell growth was obtained at an agitation speed of 750 rpm, the highest specific and volumetric yield was obtained at an agitation speed of 550 rpm. Our results coincided with the the results of others investigations [32, 33]. But, no earlier reports have discussed about the impact of agitation speed on cell growth, plasmid stability, acetate accumulation and rhG-CSF expression [25, 26]

In the production of rhG-CSF by *E. coli* BL21 (DE3) PLysS, the relative viscosity of culture broth became steep declined slope (Fig. 3a) with an increase of agitation speed, which coincided with the glucose consumption. The glucose consumption is proportional to the agitation speed, which is inversely proportional to the relative viscosity of culture broth. The relative visosity of the culture broth was about 8–16% at stationary phase of culture. Figure 3b and c reveal the kinetics of OUR and DO at various agitation speed during the cultivation. The DO reached its lowest value of 55, 39, 22 and 12% of air saturation at the agitation speed of 150, 350, 550 and 750 rpm, respectively. The

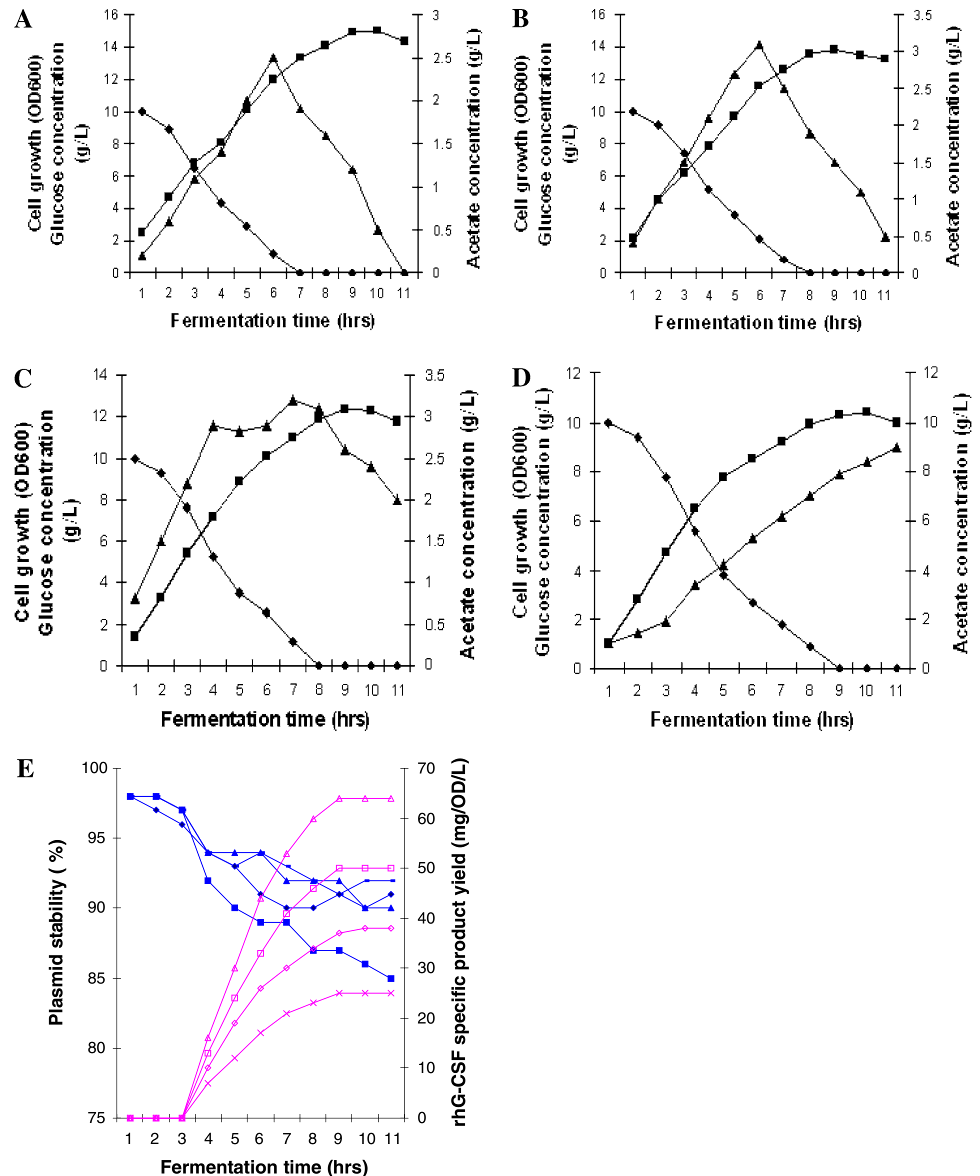

Fig. 1 Cell growth and acetate concentration at four different agitation speeds during batch fermentation of rhG-CSF. **a** 750 rpm, **b** 550 rpm, **c** 350 rpm, **d** 150 rpm. The *curve* represents (!.) Cell growth, (%) Acetate concentration, (∀) Glucose consumption. **e** Plasmid stability and specific product yield of rhG-CSF in *E. coli* BL21(DE3)PLysS. The *thick black curves* represent the stability of plasmid (! 750 rpm, % 550 rpm, ∀ 350 rpm, ■150 rpm). The *light black curves* represent the specific product yield (∋ 750 rpm, + 550 rpm, (350, 3,150 rpm)

corresponding maximal OUR value was 0.412, 0.642, 0.779 and 1.092 mM/L/h. It is interesting to find that, except for in the case of 150 rpm, the final values of DO at the end of cultivation were higher than its initial value. It may be probably due to the reduced viscosity of culture broth at the end of cultivation that oxygen transfer improved in the fermenter which coincided with the results of other studies [33]. Thus, the higher oxygen supply in the postinduction phase of cultivation at constant agitation speed may inhibit the increase in expression of rhG-CSF.

Impact of CO_2 concentration

In order to investigate the impact of carbondioxide concentration on plasmid stability, cell growth, acetate accumulation and rhG-CSF production, experiments were performed as described under Sect. "Materials and methods." The plasmid stability 98% in control sample, 98% at 5% CO_2, 98% at 10% CO_2, 88% at 15% CO_2 and 78% at 20% CO_2 was observed. When all the glucose was consumed and cells attained a stationary phase, the acetate accumulation was proportional to the concentration of CO_2, and reaching 0.6 g/l for control sample, 0.9 g/l for 5% CO_2, 1.5 g/l for 10% CO_2, 2.2% for 15% CO_2 and 2.9 g/l for 20% CO_2. The cell growth (OD) at A_{600} 11.5 for control sample, 11.8 for 5% CO_2, 11.9 for 10% CO_2, 8.5 for 15% CO_2 and 7.2 for 20% CO_2 was observed.

The results clearly indicate that the plasmid stability was constant till CO_2 concentration of 10%, and afterwards it declined. This may be due to altering the cell physiology by accumulation of acetate during the cell metabolism with increased concentration of CO_2. The host cells may be

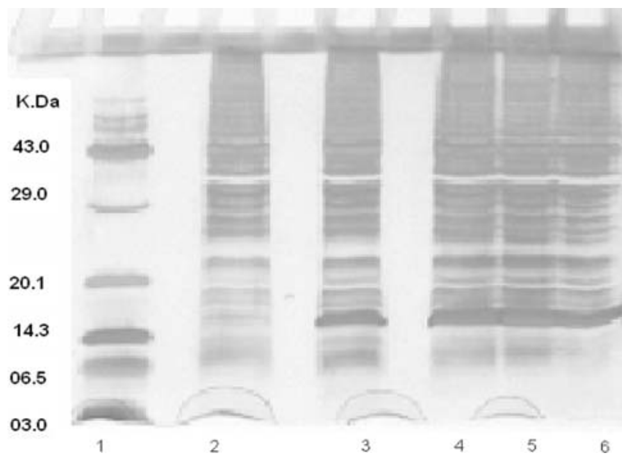

Fig. 2 SDS-PAGE analysis of expression levels of rhG-CSF in *E.coli* BL21 (DE3) PLysS. The cell pellet 50 μg from culture harvest equivalent to was electrophoresed on 15% gel. *Lane 1* molecular weight markers, *lane 2* control (without induction), *lanes 3–6* corresponds to the agitation speed of 750, 550, 350 and 150 rpm, respectively

struggling to cope with high concentrations of CO_2, and this in turn affects the processes in terms of growth and achievement of maximal biomass. These findings coincided with the other research reports [34, 35]. Eventhough the *E. coli* can cope with the stress exerted by CO_2 up to 10% concentrations, the growth was slowed somewhat. Thus, the cell growth at moderate elevated CO_2 levels was similar to that of air sparged processes, whereas at high levels of CO_2 the growth was markedly different, and may represent the outcome of a disruption of normal metabolism [34, 35].

The specific product yield was 59 mg/OD/l for control sample, 61 mg/OD/l for 5%CO_2, 48 mg/OD/l for 10%CO_2, 40 mg/OD/l for 15%CO_2 and 25 mg/OD/l for 20%CO_2, respectively (Fig. 4). Expression levels were highest at the CO_2 concentration of 5% and thereafter it declined. This maybe due to the fact that when *E. coli* cultures were exposed to gradually increasing concentration of CO_2, mixed acid fermentation products appear [10]. Of these products, only acetate is produced when *E. coli* is grown aerobically in the presence of glucose [36]. The efficiency of expression was different at all CO_2 concentrations in terms of soluble protein fraction. The soluble protein fraction was 14 mg/OD/l in the control sample, 27 mg/OD/l in 5% CO_2, 29 mg/OD/l in 10% CO_2, 31 mg/OD/l in 15% CO_2, 23.6 mg/OD/l in 20% CO_2. The variation observed was between 45 and 95% of total rhG-CSF expression. After induction, protein inclusion bodies formation was observed at 3 h (27 mg/OD/l of soluble fraction) for culture grown at 5% CO_2, at 2 h (29 mg/OD/l) for 10% CO_2, at 3 h (31 mg/OD/l) for 15% CO_2 and at 4 h (23.6 mg/OD/l) for 20% CO_2. The ratio between soluble and insoluble inclusion bodies were 1:4.2 for control sample, 1:2.2 for 5% CO_2, 1:1.7 for 10%CO_2, 1:1.3 for 15% CO_2 and 1:1.1 for 20% CO_2. The disparity in inclusion body formation

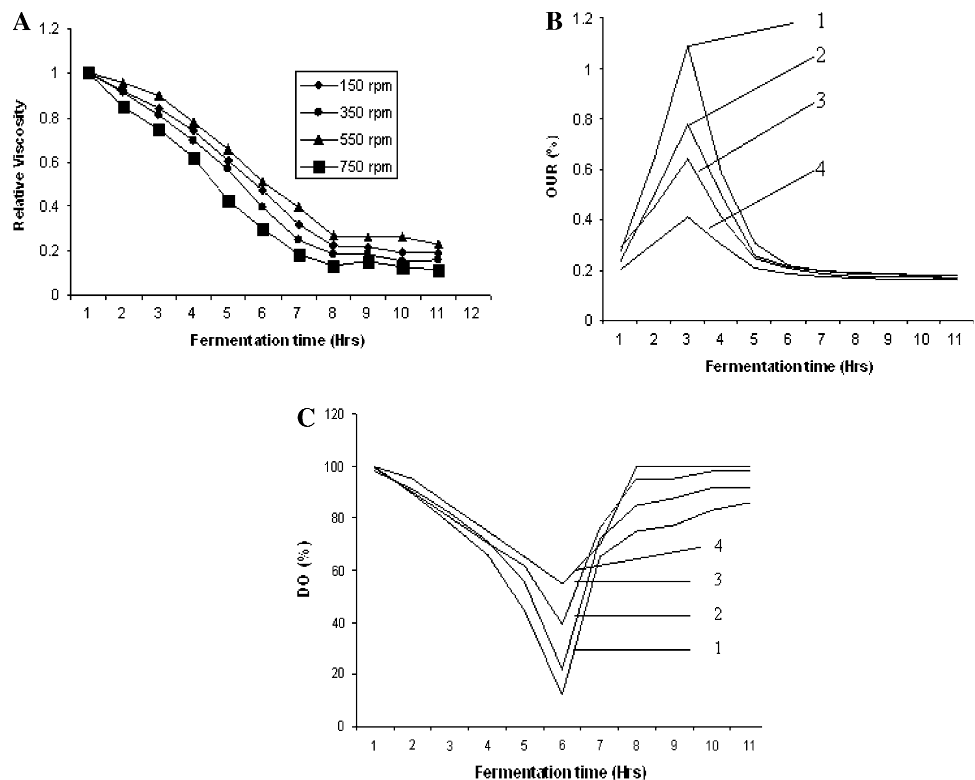

Fig. 3 Time courses of **a** relative viscosity, **b** Oxygen uptake rate, **c** Dissolved oxygen during the fermentation of *E.coli* BL21(DE3)PLysS at different agitation speed. *Curve 1* 750 rpm, *curve 2* 550 rpm, *curve 3* 350 rpm, *curve 4* 150 rpm

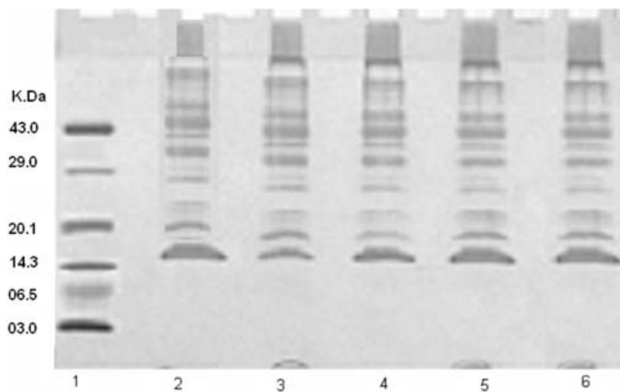

Fig. 4 SDS-PAGE analysis of expression levels of rhG-CSF in *E.coli* BL21 (DE3) PLysS. The cell pellet 50 µg from culture harvest equivalent to was electrophoresed on 15% gel. *Lane 1* molecular weight markers, *lane 2* control (without CO_2 sparging), *lanes 3–6* corresponds to the concentration of 20%, 15, 10 and 5% CO_2, respectively

may be attributed to the low levels of product formation due to metabolic burden associated with increasing the acetate accumulation [15]. No earlier studies have described the impact of CO_2 [25, 26] for rhG-CSF.

Impact of DO concentration

In order to investigate the impact of dissolved oxygen on cell growth, glucose consumption, acetate concentration, plasmid stability and rhG-CSF production experiments were performed as described under Sect. "Materials and methods." Glucose was consumed in 6 h when the cells grew at 65% DO, 7 h at 45% DO, 8 h at 25% DO and 9 h at 5% dissolved oxygen concentration. The acetate accumulation was inversely proportional to the dissolved oxygen concentration, when all the glucose was consumed and cells attained a stationary phase, reaching 0 for 65% DO, 0.2 g/l for 45% DO, 1.0 g/l for 25% DO and 4.0 g/l for 5% of DO concentration. The cell growth at 65% was 16.4 OD_{600} (1.64 OD/g glucose), at 45% it was 16 OD_{600} (1.6 OD/g glucose), at 25% it was 14 OD_{600} (1.4 OD/g glucose) and at 5% it was 11 OD_{600} (1.1 OD/g glucose) (Fig. 5a–d). The plasmid stability was increased till DO concentration of 45% and afterwards it decreased.

The result reveals that the growth of cells was much faster at a DO of 65% and corresponding higher cell weight obtained. However, a significant decrease in plasmid stability observed due to physiological burden associated with high levels of recombinant gene expression at higher DO levels. The efficiency of expression was different at all DO concentrations in terms of soluble protein fraction. The soluble protein fraction was 8 mg/OD/l in 65% DO, 28 mg/OD/l in 45% DO, 35 mg/OD/l in 25% DO, 23 mg/OD/l in 5% DO. The variation observed was between 15 and 65% of total rhG-CSF expression. After induction, protein inclusion bodies formation observed at 1 h (9 mg/OD/l soluble fraction) for culture grown at 65% DO, at 2 h (28 mg/OD/l) for 45%DO, at 3 h (35 mg/OD/l) for 25% DO and at 4 h (23 mg/OD/l) for 5% DO. The ratio between soluble and insoluble inclusion bodies were 1:6.1 for 65% DO, 1:2.4 for 45%DO, 1:1.7 for 25% DO and 1:1.4 for 5% DO. The early inclusion body formation may be attributed to the high level expression of recombinant protein and insufficient molecular chaproens present in cytoplasm of *E. coli* host [15]. No earlier studies have described the impact of DO [25, 26] for rhG-CSF.

Based on the results obtained, it was concluded that the enhanced acetate accumulation at lower DO was not only due to the lower TCA cycle activity, but also due to the altered transcription level of rhG-CSF gene associated with glucose and acetate metabolism. *E. coli* (BL21DE3)PLysS, unlike *E. coli K* (JM109), is known to be a low acetate producing strain even it grows at high glucose concentrations [14, 15]. But in this case, the strain *E. coli* (BL21DE3) PLysS was unable to control the acetate accumulation when oxygen concentration was at 5% of air saturation. Acetate is produced from acetyl-CoA by the phosphotransacetylase acetate kinase pathway and from pyruvate metabolism. Conversion of pyruvate "down stream" to acetyl-CoA, when the TCA cycle is not operating at full capacity, is accompanied by accumulation of acetyl-CoA, thus creating conditions that favour acetate production [37–39].

The consumption of glucose and the relative viscosity of culture both at different DO levels varied in the same way as observed in the experiments of constant agitation speeds (data not shown). At 65% DO of air saturation, the rhG-CSF yield (49 mg/OD/l) decreased along with the decreased plasmid stability. At the DO of 45%, the highest specific rhG-CSF yield was 67 mg/OD/l and highest volumetric yield 1.07 g/l. But at 25% of DO concentration, owing to the slower cell growth, the rhG-CSF expression increased steadily up to 4 h and later it declined. In this case, the final rhG-CSF specific product yield reached 59 mg/OD/L, which was still higher than that obtained at 65% of DO (Fig. 5e).

The highest specific product yield was observed in the first 4 h of postinduction period at 45% of DO; afterwards, it was seen similar to 25% DO. Combining this result with that obtained in the experiments of constant agitation speed experiments it was concluded that the lower oxygen supply was beneficial to express relatively higher rhG-CSF production after 4 h of induction.

rhG-CSF production with two-stage oxygen control strategy

Based on the cell growth and the specific production yield of rhG-CSF at constant agitation speeds and DO

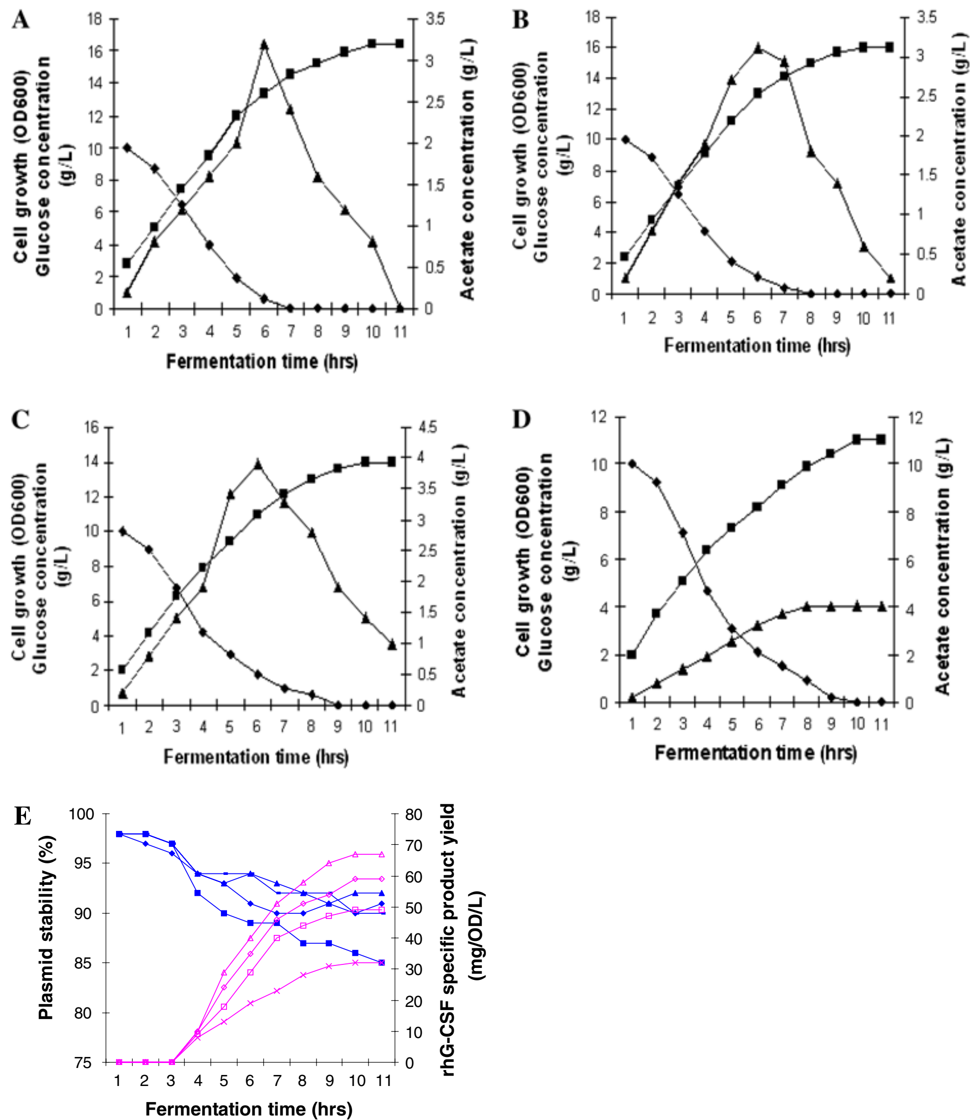

Fig. 5 Cell growth and acetate concentration at four different dissolved oxygen concentrations during batch fermentation of rhG-CSF. **a** 65% DO, **b** 45% DO, **c** 25% DO, **d** 5% DO. The *curve* represents (!.) Cell growth, (%) Acetate concentration, (%) Glucose consumption. **e** Plasmid stability and specific product yield of rhG-CSF expressed in *E. coli* BL21(DE3)PLysS. The *thick black curves* represent the stability of plasmid (! 65%DO, % 45% DO, %25% DO, ■5% DO). The *light black curves* represent the specific product yield (∋65% DO, +45% DO, (25% DO, 35% DO)

levels, it is suggested that a properly higher oxygen supply (45% DO) may be necessary for inhibition of acetate accumulation, better cell growth and high level expression up to 4 h of postinduction; thereafter, relatively low level of the oxygen supply (25% DO) is essential for maintaining plasmid in stable manner and to obtain relatively high specific product yield. For this purpose, two kinds of two-stage oxygen supply strategy were employed. In strategy 1, the agitation speed was set at 550-rpm upto 4 h of postinduction and thereafter was switched to 350 rpm at constant 1 VVM air. In strategy 2, DO concentration was maintained at 45% with agitation speed set at 550 rpm up to 4 h of postinduction and thereafter, was switched to 25% DO/350 rpm. Figure 6 shows the time profiles of rhG-CSF fermentation by using these two-stage oxygen supply control strategies. The fermentation parameters are listed in Table 1 and Fig. 7.

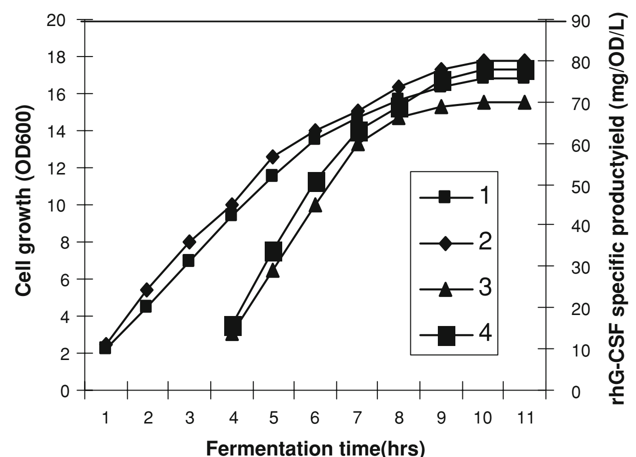

Fig. 6 Time course profiles of rhG-CSF production by *E.coli* BL21(DE3)PLysS under two stage oxygen supply strategy. *Curve 1 and 3* represents the cell growth and specific yield of rhG-CSF in two stage agitation control strategy, *curve 2 and 4* represents cell growth and specific yield of rhG-CSF in two stage DO control strategy

Table 1 Comparison of parameters in rhG-CSF batch fermentation under different oxygen supply strategy

	Constant agitation speed (rpm)		Constant DO control (%)		Two stage oxygen supply strategy	
	350	550	25	45	1[a]	2[b]
Maximum cell growth (OD 600)	11.8	13.28	14.0	16.0	16.8	17.8
Plasmid stability (%)	91.0	90.0	91.0	90.0	92.0	92.0
Cell yield to glucose (g/l)	1.18	1.32	1.4	1.6	1.68	1.78
Acetate concentration (g/l)	2.0	0.5	1.0	0.2	0.4	0.35
rhG-CSF specific product yield (mg/OD/l)	38.0	64.0	59.0	67.0	70.0	78.0
rhG-CSF volumetric product yield (mg/l)	449	850	826	1,072	1,176	1,388

[a] Agitation speed was controlled at 550 rpm till 4 h postinduction and thereafter reduced to 350 rpm

[b] DO was maintained at 45% of air saturation till 4 h postinduction and thereafter shifted to 25%

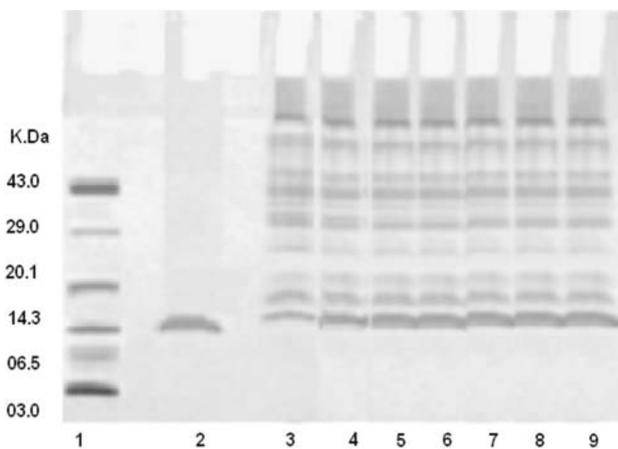

Fig. 7 SDS-PAGE analysis of expression levels of rhG-CSF in *E.coli* BL21 (DE3) PLysS. The cell pellet 50 µg from culture harvest equivalent to was electrophoresed on 15% gel. *Lane 1* molecular weight markers, *lane 2* Standard G-CSF (Neupogen), *lanes 3* control (without induction), *lanes 4–9* corresponds to the samples represented in the Table 1, respectively

Compared to the best results obtained during the cultivations at constant agitation speeds of 550 and 350 rpm, the maximal rhG-CSF volumetric yields were increased by 1.4- and 2.6-folds and specific product yields were improved by 1.1- and 1.8-folds, respectively, by using strategy 1. Similarly, compared to the best results obtained in the cultivations at constant DO of 45 and 25%, the maximal rhG-CSF volumetric yields were increased by 1.3- and 1.7-folds, and specific product yields were improved by 1.1- and 1.3-folds, respectively, by using strategy 2. rhG-CSF yield resulted 1,388 mg/l under two-stage DO control strategy, which was higher in comparison to two-stage agitation speed control strategy of 1,176 mg/l. Other researchers also used the two-stage oxygen supply strategy for betterment of yields [40–42]. But, so far, no reports explored on the relationship between the changed demand of oxygen, cell growth and rhG-CSF production [5, 25].

The stratagies applied for fermentation process described here in this report is simple, highly successful and reproducible. The overall specific product yield of rhG-CSF is higher than those reported by other researchers [5, 25]. The increase in the overall specific product yield could be due to enhanced cell growth, plasmid stability and low acetate accumulation by employing suitable physiological and metabolic conditions through two kinds of two stage DO supply during batch cultivation.

Conclusions

In this study, we employed different agitation speeds, CO_2 concentrations and oxygen supply, which had shown distinct effects on cell physiological parameters and expression of rhG-CSF in batch fermentation. Lower carbondioxide concentration, higher agitation speed and dissolved oxygen had led to reduction in the acetate concentrations, and enhanced the cell growth, but inhibited plasmid stability and rhG-CSF expression. Similarly, higher carbondioxide concentrations and lower agitation speeds as well as dissolved oxygen concentrations led to enhanced acetate concentrations but inhibited the cell growth and protein expression. To address the bottlenecks described above by providing suitable physiological and metabolic conditions, we established two kinds of two-stage oxygen supply. The two stage agitation control strategy and two stage DO control strategy had resulted in enhanced plasmid stability, high cell growth, low acetate accumulation and high level protein expression during batch cultivation of rhG-CSF.

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