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Enhancement of Human γ-Interferon Production in Recombinant *E. coli* Using Batch Cultivation

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Abstract Development of inexpensive and simple culture media and appropriate induction conditions are always favorable for industry. In this research, chemical composition and stoichiometric data for γ-interferon production and recombinant *Escherichia coli* growth were used in order to achieve a simple medium and favorable induction conditions. To achieve this goal, the effects of medium composition and induction conditions on the production of γ-interferon were investigated in batch culture of *E. coli* BL21 (DE3) [pET3a-*ifn*γ]. These conditions were considered as suitable conditions for the production of γ-interferon: 2.5× M9 medium, supplemented with a mixture of amino acids (milligram per liter), including glutamic acid 215, aspartic acid 250, lysine 160, and phenylalanine 90, and induction at late-log phase (OD_{600}=4.5). Under these conditions, dry cell weight of 6±0.2 g/l and γ-interferon concentration of 2.15±0.1 g/l were obtained. Later, without changing the concentration ratio of amino acids and glucose, the effect of increase in the primary glucose concentration on productivity of γ-interferon was investigated. It was found that 25 g/l glucose will result in maximum attainable biomass and recombinant human γ-interferon. At improved conditions, a dry cell weight of 14±0.2 g/l, concentration and overall productivity of γ-interferon 4.2±0.1 g/l and 420±10 mg/l h, respectively, were obtained.

Keywords γ-Interferon · Induction condition · Productivity · *E. coli* · Batch culture

Introduction

Human γ-interferon (hIFN-γ) is a glycosylated protein with a total molecular weight of 25 kDa and is composed of 143 amino acid residues [1, 2]. IFN-γ (originally called macrophage-

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activating factor) is among the most important macrophage stimulants. Macrophage stimulation with IFN-γ induces direct antimicrobial and antitumor mechanisms as well as up-regulating antigen processing and presentation pathways [3]. The recombinant hIFN-γ (rhIFN-γ) expressed in *Escherichia coli* is not glycosylated and has a molecular weight of 17 kDa, but it is still physiologically active [4]. *E. coli* is one of the most widely used hosts for the production of heterologous proteins [5–8] because of its simple nutrient requirement, high growth rate, and its well-known molecular genetics and physiology.

The goal of optimizing the production of recombinant proteins is to produce the highest amount of functional product per unit volume per unit time. For intracellularly accumulated recombinant protein, overall productivity is dependent on final cell density and the specific yield of the protein [milligram protein per gram dry cell weight (DCW)]. Four strategies have been used so far to optimize the production of recombinant proteins, namely, choice of culture media, mode of cultivation, strain development, and expression system control [5–7].

Induction conditions can significantly affect the specific cellular product yield [9–13]. The amount of inducer, the strategy of its addition, culture conditions at the time of induction, and post-induction duration have been reported to have important effects on the efficiency of induction [10–16].

The amount of inducer required to titrate the repressor molecules is a function of total cell mass, and the optimal concentration of the inducer needs to be determined for maximizing the recombinant protein synthesis at different cell concentrations [11–13, 16]. Induction time is another important parameter because maximum yield of foreign protein in fermentation depends on the point in the growth phase at which expression is induced [14, 15, 17, 18]. For strains whose growth and/or viability is drastically reduced following the induction, induction in late-log or stationary phase does not hamper reaching high cell densities and increased product formation [11, 17].

The composition of the growth medium at or during the induction phase can significantly affect heterologous protein expression. The overexpression of a protein places an added metabolic burden on cell's energy, carbon and amino acid pools, which may result in reduced cell growth rates and a stress response characterized by enhanced protease, and heat shock protein synthesis. It has been shown that providing additional amino acids by supplementing the medium with casamino acids and/or peptone or yeast extract during induction can increase productivity and stability [15, 19]. In many cases, when the amino acid composition of a recombinant protein is significantly different from the native proteins of the host cell, the cell's attempt to replenish amino acids, which are depleted by the onset of the foreign protein expression, will lead to an additional burden. Hence, it can be expected that supplementing the medium with particular amino acids increases the productivity of the recombinant protein.

The amino acids can be grouped into six biosynthetic pathways depending on the intermediate from which they are derived, including serine, pyruvate, glutamic acid, aspartic acid, and two groups of cyclic amino acid families [20]. The aspartic acid group content is around 39.5% of the total amino acid in rhIFN-γ. Lysine, aspartic acid, and asparagine have the highest percentage values of total amino acid composition among the aspartic acid group. However, asparagine is derived from aspartic acid in intracellular biosynthetic pathways by a single reaction. Therefore, aspartic acid and lysine were selected to be added to the medium at the induction time. The glutamic acid family composition in total amino acids of rhIFN-γ is 19.6%. Proline, arginine, and glutamine are synthesized from glutamic acid; consequently, glutamic acid is chosen to be added to the medium at the induction time. Phenylalanine was selected to be added to the medium during the induction time from two groups of cyclic amino acids. Amino acids from pyruvate and serine have shorter biosynthetic pathways. In addition, they are derived from

intermediates that are produced in glycolysis pathways. Due to these reasons, none of these amino acids were added at the induction time.

Much of the effort aimed at increasing recombinant protein production in bacterial strains have been directed at maximizing the biomass production, and little is known about the effects of media composition on the expression of recombinant proteins. However, it is also known that the production of recombinant proteins in microbial strains depends on the composition of the medium in which the organism is grown [10, 12, 21, 22].

The present study intends to evaluate the effects of induction condition, medium composition, and the coordinated addition of amino acids on increasing the production of rhIFN-γ in batch culture. Next, keeping the concentration ratio of amino acids and glucose unchanged, the effect of increase in the primary glucose concentration on productivity of γ-interferon was also studied.

Materials and Methods

Microorganism and Vector System

E. coli strain BL21 (DE3) (Novagen, Inc.) was used as the host for rhINF-γ expression. This strain was transformed with a commonly available plasmid, pET3a-inducible expression vector (Novagen, Inc.), in which the hINF-γ gene (Noor Research and Educational Institute, Tehran, I.R. Iran) was inserted into the *Not*I and *Nde*I sites. Host cells were transformed with the plasmid using the calcium chloride procedure. Transformed cells were spread on several Luria–Bertani (LB) agar plates containing 100 mg/l ampicilin.

Media and Solutions

LB agar medium was used for plate cultivation of *E. coli* strain BL21 (DE3) and M9-modified medium was used for preparation of seed culture. M9-modified medium consisted of 10 g glucose, 15 g K_2HPO_4, 7.5 g KH_2PO_4, 2 g citric acid, 2.5 g $(NH_4)_2SO_4$, 2 g $MgSO_4{\cdot}7H_2O$, and 1 ml trace element solution per liter. The trace element solution contained (in g/l) 2.8 $FeSO_4{\cdot}7H_2O$, 2 $MnCl_2{\cdot}4H_2O$, 2.8 $CoSO_4{\cdot}7H_2O$, 1.5 $CaCl_2{\cdot}2H_2O$, 0.2 $CuCl_2{\cdot}2H_2O$, and 0.3 $ZnSO_4{\cdot}7H_2O$ in 1 M HCl. Batch cultivations were simultaneously carried out in two 2-l bench-top bioreactors (Infors AG Switzerland) with the working volume of 1 l.

Analytical Procedures

Cell growth was monitored by measuring culture turbidity and dry cell weight (DCW). Turbidity was determined by measuring optical density (OD) at 600 nm. Samples were diluted with NaCl solution (9 g/l) to obtain an OD_{600} between 0.2 and 0.5. In order to determine DCW, 5 ml of broth was centrifuged at 4,000×*g* for 10 min, washed twice with deionized water, and dried at 105°C to constant weight.

Samples from cultivation were directly chilled on ice with further centrifugation (3 min, 5,700×*g*, 4°C), and the supernatant was collected by direct filtration through a 0.2-μm disk filter. Afterwards, the samples were stored at −20°C for further analysis. Glucose was analyzed enzymatically using kits (ChemEnzyme CO., I.R. Iran).

The expression level of rhIFN-γ was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis using poly-acrylamid 12.5% (*w*/*v*). Gels were stained with Coomassie brilliant blue R250 and then quantified by gel densitometer. Total soluble protein was

analyzed by Bradford method with bovine serum albumin as a standard [23] and rhIFN-γ was measured by ELISA assay.

Batch Cultivation

Batch culture was inoculated by adding 100 ml of an overnight-incubated seed culture (OD_{600}=0.7–1) into the bioreactor containing 900 ml of medium. Cultivation temperature was controlled at 37±0.5°C. The pH was controlled at 7±0.05 by the addition of 25% (*w*/*v*) NH_4OH or 3 M H_3PO_4. Dissolved oxygen was controlled at 30–40% of air saturation by controlling both the inlet air and agitation rate. Foam was controlled by adding silicon-antifoaming reagent. The expression of rhIFN-γ was performed by the addition of 1 mmol/l of isopropyl-β-D-thiogalactopyranoside (IPTG).

Experimental Procedure

The experiments were designed for three purposes:

1. Selection of a simple medium: Three different media, which are 2.5× M9 medium (M9 medium with 2.5-fold concentration of components except glucose) with 1 ml trace element solution per liter, 2.5× M9 medium without trace elements, and modified M9 medium were used for cultivating recombinant *E. coli*. The expression of rhIFN-γ were induced at a DCW of 1.2 g/l (OD_{600}=2.5) with 1 mmol/l IPTG. Sampling continued until the growth ceased.
2. Investigation of production conditions in selected medium: The effects of induction time at three levels (OD_{600}=1, 2.5, and 4.5) and the addition of several amino acids (glutamic acid, aspartic acid, lysine, and phenylalanine) at three levels (0, A, and B) at the time of induction on the concentration of rhIFN-γ in batch fermentation of recombinant *E. coli* were investigated. In order to determine the quantities of selected amino acids, we assumed the following: (1) cell growth will end up to a predicted value based on $Y_{x/s}$=0.5 g DCW/g glucose, (2) total cell protein is 65% of DCW and expression level of rhIFN-γ is 55% of the total cellular protein; therefore, specific yield of rhIFN-γ is 357.5 mg/g DCW; (3) recombinant *E. coli* requires half of selected amino acids for production of assumed level of rhIFN-γ, i.e. glutamic acid 215 mg/l, aspartic acid 250 mg/l, lysine 160 mg/l, and phenylalanine 90 mg/l (level A); and (4) recombinant *E. coli* requires twofold of selected amino acids for production of assumed level of rhIFN-γ, i.e. glutamic acid 875 mg/l, aspartic acid 1,000 mg/l, lysine 640 mg/l, and phenylalanine 360 mg/l (level B).
3. Evaluating the initial glucose concentration on the productivity of rhIFN-γ: The set of batch fermentations with different concentrations of initial glucose (10, 15, 20, 25, and 30 g/l) were performed twice, and the effect of initial glucose concentration on cell density, specific growth rate, changes in glucose concentration, and concentration and specific yield of rhIFN-γ in batch culture of *E. coli* BL21 (DE3) [pET3a-*ifnγ*] were investigated.

Results and Discussion

Selection of a Simple Medium

The effect of medium concentration on DCW, specific growth rate, rhIFN-γ concentration, and its specific yield are depicted in Fig. 1a.

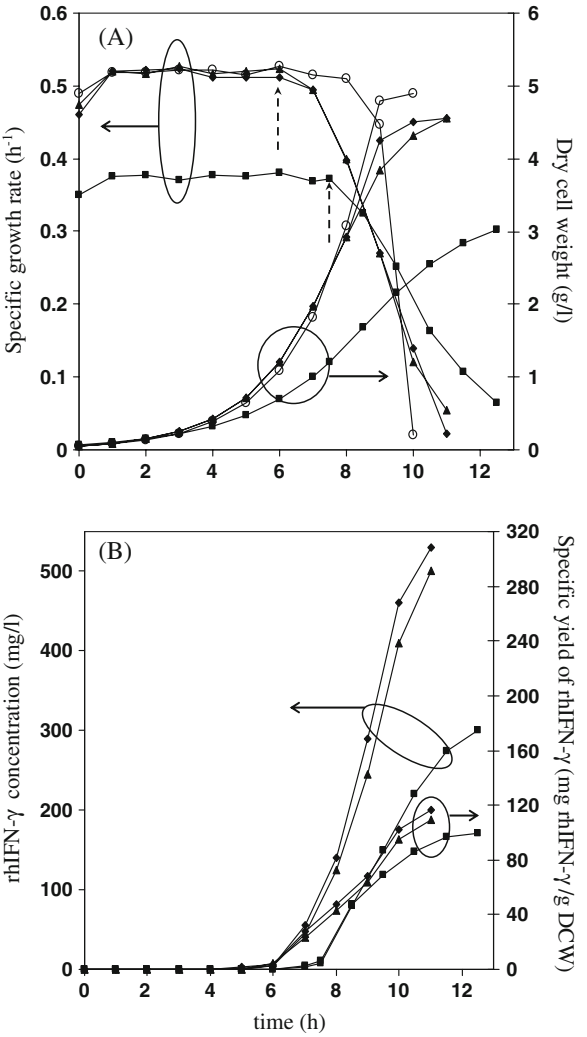

Fig. 1 The effects of media composition on cell density, specific growth rate (**a**), and concentration and specific yield of rhIFN-γ (**b**) in batch cultures of *E. coli* BL21 (DE3) [pET3a-*ifn*γ]. Specific growth rate (h^{-1}), cell density (g DCW/l), concentration (mg/l), and specific yield (mg/g DCW) of rhIFN-γ and in modified M9 medium (*filled diamond*), 2.5× M9 medium with trace elements (*filled triangle*), 2.5× M9 medium without trace elements (*filled square*) and modified M9 medium without induction (*empty circle*). The *dashed arrow* indicates induction time

The maximum specific growth rate, as shown in Fig. 1a, is 0.52 h^{-1}, which is equal to 2.5× M9 with trace elements and modified M9 media. However, in 2.5× M9 medium without the trace elements, the maximum specific growth rate is reduced to 0.38 h^{-1}. After the induction, the specific growth rate decreases in all media until it approaches zero and the growth stops. Of course, decrease of specific growth rate after induction is more severe compared with when no induction is applied due to metabolic burden caused by the overexpression of heterologous protein on recombinant cell [1, 15, 24]. In addition, the DCW in 2.5× M9 with trace elements and modified M9 media reaches 4.5 g/l after 10 h, but this value is reduced to

3 g/l in 2.5× M9 medium without trace elements. These results show the substantial role of trace elements in increasing the specific growth rate and the DCW. The effective role of trace elements is caused by the metallic ions involved, which can be used as co-factors in enzymatic reactions and in transfer of nutrients into the cell [21, 25].

The changes in glucose concentrations during cultivation in three different media show almost the same trend in 2.5× M9 with trace elements and in modified M9 media. In both of these media, glucose concentration approaches zero toward the end of the process where growth and production halt. In other words, glucose depletion coincides with the growth halt and production. However, in 2.5× M9 medium without trace elements, glucose depletion occurs at a much lower pace, and even at the end of the process, a considerable amount of glucose still remains in the medium.

The effect of medium components on the concentration and the specific yield of rhIFN-γ (mg rhIFN-γ per gram DCW) has been shown in Fig. 1b. It can be inferred that the concentration (520±10 mg/l) and the specific yield of rhIFN-γ (115±10 mg/g DCW) are almost equal for both 2.5× M9 with trace elements and modified M9 media. However, in 2.5× M9 medium lacking trace elements, rhIFN-γ concentration reduces to 300±10 mg/l. Therefore, 2.5× M9 with trace elements and modified M9 media remain as possible choices for further experiments. Considering that experimental results have been almost identical for both media, we chose the modified M9 medium because of its simplicity.

Improvement of Production Conditions in Modified M9 Medium

The time of induction—because of its considerable effect on final cell density—is one of the most important factors that affect the productivity. The result was showed in Table 1, that time of induction has been the more suitable in its third level (OD_{600}=4.5) at which the cell is induced when it is in late log-phase.

Addition of amino acids has a substantial effect on growth and expression level. However, there are two important points that should be taken into account when using this kind of medium supplementation: (1) Considering the concentration threshold for the addition of amino acids above which the amino acids may have detrimental effects on the host and (2) maintaining a balance between the additional costs caused by adding amino acids and the additional value of the product [5, 17, 21, 26]. Under the more suitable

Table 1 Experimental results of improvement of production conditions in batch cultures of *E. coli* BL21 (DE3) [pET3a-*ifnγ*] in modified M9 medium.

Factor	Level	rhIFN-γ concentration (mg/l)
Induction time (OD_{600})[a]	1	480±10
	2.5	520±10
	4.5	1,100±100
Amino acid mixture[b]	0	1,100±100
	A[c]	2,150±100
	B[d]	2,100±100

[a] Induction with IPTG 1 mmol/l

[b] Induction in cell density 2.2 g/l (OD_{600}=4.5) with IPTG 1 mmol/l

[c] Level A (mg/l): glutamic acid 215, aspartic acid 250, lysine 160, and phenylalanine 90

[d] Level B (mg/l): glutamic acid 860, Aspartic acid 1,000, lysine 640, and phenylalanine 360

induction conditions (induction at OD_{600}=4.5 and amino acids in level A), 2.15±0.1 g/l of rhIFN-γ and 6.0±0.2 g/l of DCW were achieved (see Table 1).

The effect of the addition of amino acids at the time of induction on cell density and changes of specific growth rate are depicted in Fig. 2a. It can be seen that the addition of amino acids has caused the growth rate enhancement after the induction. It should be born in mind that, under usual conditions, the specific growth rate decreases after the induction, which is a result of the metabolic burden mounted on cell during the overexpression of the recombinant protein. Here, however, the growth rate has increased from 0.52 h^{-1} to 0.7 h^{-1} after the induction and has then decelerated due to the depletion of carbon source.

The effect of medium supplementation with amino acids on concentration and specific yield of rhIFN-γ is shown in Fig. 2b. Evidently, the concentration and productivity have

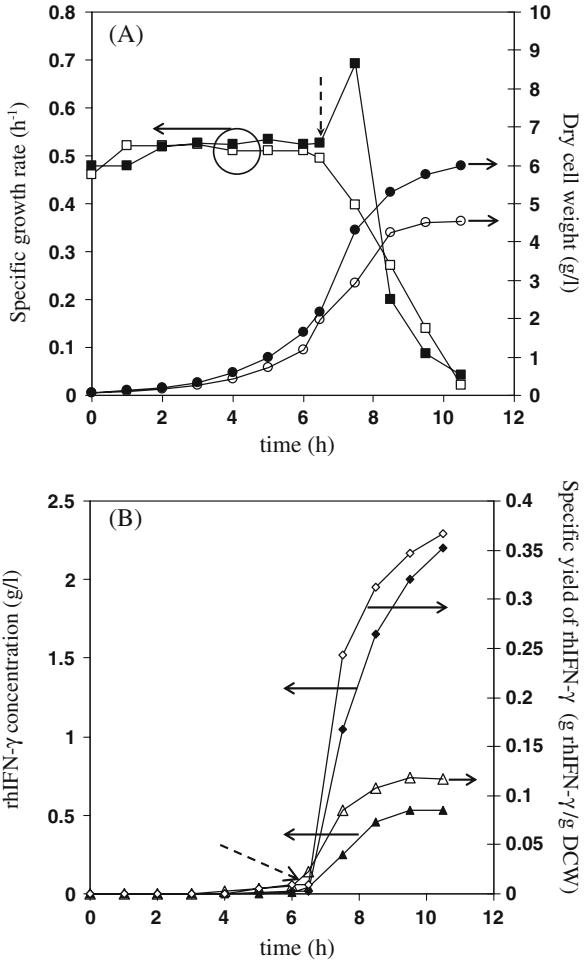

Fig. 2 The effects of media enrichment in induction time with amino acid on cell density and specific growth rate (**a**) and concentration and yield of rhIFN-γ (**b**) in batch cultures of *E. coli* BL21 (DE3) [pET3a-*ifnγ*] in modified M9 medium. Cell density (*empty circle*, *filled circle*), specific growth rate (*empty square*, *filled square*), concentration (*empty diamond*, *filled diamond*), and specific yield of rhIFN-γ (*empty triangle*, *filled triangle*) in lack of enrichment and in media supplemented with an amino acid mixture (glutamic acid 215 mg/l, aspartic acid 250 mg/l, lysine 160 mg/l, and phenylalanine 90 mg/l), respectively

both experienced an increase after the addition of amino acids. Metabolic burden caused by the overexpression of heterologous protein can be so high in cases where the amino acid content of the recombinant protein and the host cell are highly different. The cell may react to this burden by increasing the synthesis of heat shock proteins and proteases, both of which can decrease cell growth rate and productivity [12, 24, 27, 28]. Provision of amino acids in post-induction phase can compensate for the depletion of amino acids and ameliorate the synthesis of housekeeping and especially cell wall proteins [17, 24–26]. However, the metabolic consequences of amino acid supplementation must be carefully considered when developing a feeding strategy, since increasing the concentration of some amino acids (e.g., phenylalanine and histidine) can significantly repress or inhibit the biosynthesize of other amino acids and/or essential metabolites [11, 20, 24, 27].

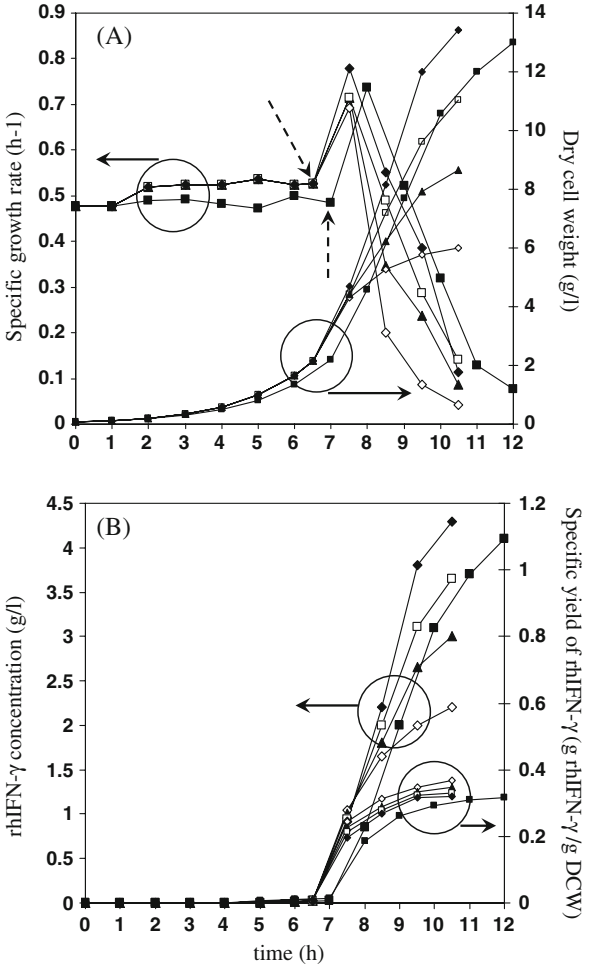

Fig. 3 The effects of the initial glucose concentration on specific growth rate, cell density (**a**), and concentration and specific yield of rhIFN-γ (**b**) in batch cultures of *E. coli* BL21 (DE3) [pET3a-*ifn*γ]. Specific growth rate (h^{-1}), cell density (g/l), concentration (g/l), and specific yield (g/g DCW) of rhIFN-γ for media include (g/l glucose): 10 (*empty diamond*), 15 (*filled triangle*), 20 (*empty square*), 25 (*filled diamond*), 30 (*filled square*). The *dashed arrow* indicates induction time

Improving the Production in Batch Culture

Considering the promising results obtained from the addition of amino acids into the culture, another set of experiments were planned: increasing the initial glucose concentration of the medium (while maintaining the relative concentrations of glucose and amino acids) and investigating the effect of this change on cell growth and concentration of rhIFN-γ.

The effect of initial glucose concentration on specific growth rate in batch culture is shown in Fig. 3a. Further increments of the initial glucose concentration of 25 g/l have an inhibitory effect on growth and cause a reduction in specific growth rate. It has been reported elsewhere [22, 27, 29–31] that increasing the initial glucose concentration to 40–50 g/l will cause the cessation of growth. The specific growth rate increases after the induction, which is a consequence of amino acid supplementation, but it starts to decrease 1 h after the induction as a result of the metabolic burden caused by the overexpression of the recombinant protein, and the growth comes to a halt 4 to 5 h after the induction.

The impact of initial glucose concentration on cell density in batch culture shows the same trend as the specific growth rate (Fig. 3a). Final cell density naturally increase when the initial glucose concentration from 10 to 25 g/l but reduces when initial glucose concentration exceeds this value. Accordingly, the cell density increases after the induction as a result of amino acid supplementation, but it then decreases and the growth halts 4 to 5 h after the induction.

The effect of the initial glucose concentration on concentration and specific yield of rhIFN-γ in batch culture was illustrated in Fig. 3b. The concentration of rhIFN-γ does not change with the initial glucose concentration during the pre-induction phase, but they show an increase from 2.2 to 4.3 g/l after the induction, which is in accordance with previous researches [11, 19, 27, 30, 31]. Increasing the initial glucose concentration from 10 to 25 g/l will increase the concentration of rhIFN-γ from 2.2 to 4.3 g/l, but further enhancements of glucose concentration have adverse effects on the concentration of rhIFN-γ as a result of the inhibitory effects of glucose. The specific yield of rhIFN-γ does not affect considerably with the increase of initial glucose concentration. Considering the final concentration of rhIFN-γ, the value of 25 g/l was chosen as the better value for the initial glucose concentration.

Table 2 Effect of production conditions in batch cultivation of *E. coli* BL21 (DE3) [pET3a-*hifnγ*] on the rhIFN-γ production in M9 modified medium.

Factors	Control cultivation[1]	Initial improvment[2]	Final improvment[3]
Initial glucose concentration (g/l)	10±0.5	10±0.5	25±0.5
Cultivation time (h)	10.5±0.5	10.5±0.5	10.5±0.5
Final cell density (g/l DCW)	4.4±0.1	6.0±0.2	14±0.2
Final concentration of rhIFN-γ (mg/l)	520±10	2,150±100	4,200±100
Specific yield of rhIFN-γ (mg/g DCW)	115±10	350±10	300±10
Overall productivity of rhIFN-γ (mg/l.h)	50±5	210±10	420±10

[a] Induction in cell density 1.2 g/l (OD_{600}=2.5) with IPTG 1 mmol/l

[b] Induction in cell density 2.2 g/l (OD_{600}=4.5) with IPTG 1 mmol/l and amino acid in level A (mg/1), glutamic acid 215, aspartic acid 250, lysine 160, and phenylalanine 90

[c] Induction in cell density 2.2 g/l (OD_{600}=4.5) with IPTG 1 mmol/l and amino acid in level A (mg/1), glutamic acid 215, aspartic acid 250, lysine 160, and phenylalanine 90

The effect of improvement on cell growth and production of rhIFN-γ is shown in Table 2, juxtaposing the improved and control cultivations. As it is evident, the final concentration and productivity of rhIFN-γ have increased fourfold after the initial improvement. Furthermore, the results show that the final cell density and concentration of rhIFN-γ enhance more than twofold by increasing the initial glucose concentration from 10 to 25 g/l. Reported values for concentration of rhIFN-γ (4.2 g/l) and the productivity of the process (420 mg/l h) are the highest values reported for a recombinant protein to date.

Conclusion

Modified M9 was shown to be superior in terms of DCW and concentration of rhIFN-γ and chosen because of its simplicity. This medium has been improved in terms of time of induction and the addition of amino acids. It is concluded that inducting the culture at cell density of 2.2 g/l (OD_{600}=4.5) and supplying the medium with 215 mg/l glutamic acid, 250 mg/l aspartic acid, 160 mg/l lysine, and 90 mg/l phenylalanine for 10 g/l of initial glucose (at the time of induction) will result in the best values for the final concentration of rhIFN-γ (2.15±0.1 g/l) and DCW (6±0.2 g/l). The most promising results are obtained when the initial glucose concentration of medium is 25 g/l. At this concentration, maintaining the improved condition achieved in the previous experiments, the final concentration of rhIFN-γ reached the value of 4.2±0.1 g/l and the productivity reached the value of 420±10 mg/l h, which are the highest values reported to date for any kind of recombinant protein.

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