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ERYTHROPOIETIN PRODUCTION IN CHINESE HAMSTER OVARY: THE THREADS AND KNOTS OF PROPER CULTURE ENVIRONMENTAL FACTORS IMPROVED GLYCOSYLATION, FUNCTION AND PRODUCTIVITY

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ABSTRACT

Erythropoietin (EPO) is a glycoprotein that regulates erythropoiesis under hypoxic conditions through the proliferation and differentiation of red blood cell precursors. It is used in the treatment of anemia caused by chronic renal failure and cancer therapies. EPO is commercially produced in Chinese Hamster Ovary (CHO) cells in industrial scale bioreactors. Limitations in EPO production protocols include low yields, high costs and insufficient glycosylation. Adjustments in mechanical parameters such as temperature reduction and carbon dioxide concentrations have been shown to increase both quantity and quality of EPO production. As cells use up glucose, acidifying lactate is produced as waste which adversely affects glycosylation and quantity of EPO. The accumulation of lactate shortens the culture span which affects the viability of production systems. This has shown to be remedied through the addition of lactate salts such as sodium lactate which neutralize acidity and decrease specific growth rate. Additionally, medium protecting additives in stir-tank bioreactors can have an effect on EPO quality and quantity. The use of environmental friendly, non-toxic additives like poly-γ-glutamic acid can help protect cells during culture by minimizing sheer stress resulting in better quality EPO than that produced using Pluronics that have been shown to inhibit DNA synthesis. Not only the correct choice of the combination profile- temperature, pH, lactate salts and sheer protecting additives- is helpful; but also the appropriate alternating time cycle for each factor in the correct profile is also as much important.

KEYWORDS: Erythopoietin, EPO, CHO, Temperature, Poly-gamma-glutamic, Lactate

INTRODUCTION

Erythropoietin (EPO) is a clinically important glycoprotein that allows for the process of erythropoiesis to take place under hypoxic conditions. It acts as a cytokine for erythrocyte precursors' maturation and differentiation. EPO is produced in abundance by hepatocytes in the fetal stage, while in later stages of life, EPO production is directed to kidneys [1] . EPO was the first hematopoietic growth factor to be industrially produced and is used to treat anemia caused by cancer therapies, acquired immune deficiency syndrome (AIDS) and renal failure [2].

It is 30kDa in size and consists of a 165 amino acid peptide core attached with four carbohydrate chains- three N-linked oligosaccharides that are present on asparagine sites and one O-linked oligosaccharide on a serine site. The process by which these glycan structures get attached to the protein is known as glycosylation and is paramount to the efficacy and function of EPO as well as its secretion, receptor recognition and bioactivity[2][3].

Large scale production of recombinant EPO is done through the use of mammalian cell cultures such as Chinese Hamster Ovary (CHO) due to their ability to offer appropriate post translational modifications –namely glycosylation-in a consistent manner. Moreover, CHO can grow in a suspension culture which makes their scaling-up possible with a steady and high production rate and yield [4][5]. Currently, about 70% of commercial recombinant proteins are grown and produced using CHO cells [6].

The common bio-technique used to produce recombinant EPO in CHO cells is done by transfecting the EPO gene's cDNA into competent CHO cells through methods such as liposomes or calcium phosphate aided transfection. The EPO gene is a 5.4 kb gene that lies within *HindIII* and *BamH1* restriction sites. The corresponding EPO fragment is inserted into a pDSVL expression vector which contains a dihydrofolate reductase gene. The vectors are then transfected into CHO cells using calcium phosphate and transformants are selected [7]. Improvements to this method were found to lie through the co-expression of epidermal growth factor receptor (EGFR) along with EPO in CHO cells. The EGF growth factor can exist in a membrane anchored form and can activate membrane receptors-

the process of juxtacrine stimulation. This has been shown to significantly enhance CHO cell growth and subsequently have higher EPO expressions [8]. More recently, the utilization of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats associated RNA guided nucleases) have been shown to efficiently integrate genes into CHO cells in a site-specific manner which will in turn allow rapid, relatively simple and economic manner of EPO production [9].

An ideal industrial scale production culture of EPO should be able to provide a high yield with steady productivity. It should also be able to offer consistent glycosylation in an economical cost-effective manner while ideally allowing the culture time span to be prolonged [3]. CHO cells that produce EPO industrially are typically cultured in stir tank bioreactors. This method is efficient as these genetically modified CHO cells are capable of being grown in serum free media and the parameters can be feasibly adjusted to provide optimum growth. However limitations such as accumulation of waste and alteration of pH are the main challenges [3][10]. In addition the use of protective additives such as Pluronic F68 in such cultures has been linked with decreased productivity overall [4]. Other mechanical factors such as temperature and carbon dioxide concentrations utilized in the bioreactors have been shown to be hindering to the improvement of EPO accumulation and quality [11][12]. This review paper describes different mechanical based methods and alterations that can potentially increase EPO production yields and improve their glycosylation.

REDUCING LACTATE ACCUMULATION

Two factors that limit cell growth in cultures are predominantly nutrient depletion and waste accumulation- this will typically translate to a short culture span with low cell density. One of the main toxic waste products that significantly inhibit growth is lactate which is produced as a result of the acidification it inflict on the medium. As mammalian cells are very sensitive to pH and only grow over a narrow pH range, the control of lactate is essential and its removal is necessary for enhanced production. Traditionally, as media is replenished periodically, this incurs higher costs and has been shown to adversely affect cell growth [13][10]. Moreover, pH changes can also alter protein processing, secretion and glycosylation [5].

Sodium lactate is a sodium salt which can help to neutralize the acidity caused by lactate production and help to restore appropriate pH. Moreover, the addition of sodium lactate has been shown to decrease specific growth rate of CHO cells which is beneficial to extend culture time span. The decreased specific growth rate of cells also helps to direct cellular energy towards protein synthesis of EPO while a faster growth rate allows for energy to be diverted more towards material synthesis for CHO growth instead of EPO production [10].

Concentration of 80mM and higher were not found to improve EPO production and decreased specific growth to a degree that makes efficient growth unfeasible. A concentration of 40mM was found to be most appropriate and increased final EPO accumulation by 172% overall and culture time was increased by an additional 50- 100 hours. In addition, sodium lactate also helped to decrease glucose consumption of cells by approximately 23% and subsequently reduced the over all of lactate production by more than 50% [10].

TEMPERATURE REGULATION

The optimal temperature described in a large body of research for the successful and efficient growth of EPO in CHO is 37ºC. However, cell cultures with a temperature of 37ºC exhibited a decrease of cell viability over time. Alternatively, cell viability in cells grown at lower culture temperatures remained significantly higher. Lowering the culture temperatures that are used can have favorable effects on overall quality and quantity of produced EPO. Through lowering culture temperatures, glucose consumptions were shown also to be relatively reduced which in turn will decrease the production of lactate by cells and so help prolong culture span by maintaining pH and preventing the fast accumulation of toxic, acidifying lactate in cultures. The increased mRNA level of EPO in decreased culture temperature suggests it's better for higher protein accumulation at lower temperature cultures. In addition, an increase in EPO transcription levels was observed at low temperatures [12].

Despite the positive effects of lowering temperature, the temperature cannot be lowered very much as it will decrease the rate at which CHO cells grow which is uneconomical as it decreases yield rate. The testing of different temperatures revealed that at a temperature of 32ºC showed much higher EPO production than the traditionally used 37ºC. The EPO produced in this condition was also of a superior quality and was characterized by having better glycosylation patterns as well. An additional advantage of reducing temperature is a reduction in cost due to reduced glucose consumption rates and heating costs [12]. We suggest alternating temperature during culture conditions using automatic timers. Alternating the temperature intervals between 37ºC and 32ºC will accommodate both the health, growth rate and erythropoietin better production. We suggest initiating a culture at 37ºC and lowering it to 32ºC in later culture stages.

CARBON DIOXIDE GAS SWITCHING

Carbon dioxide is an important parameter that is employed to culture mammalian cells. It has an important role of maintaining pH at an optimal range of 6.9 – 7.4 and controlling cell activity. Traditionally, 5% carbon dioxide levels are supplied to mimic the carbon dioxide conditions typically present in living organisms [11]. However, despite the importance of carbon dioxide for cell growth, applying a 5% carbon dioxide percentage to cells being cultured in vitro can cause a reduction in polysialylation and N-glycolyneuraminic acid which has an adverse influence on glycosylation of cells [11]. In addition, carbon dioxide leads to partial pressure buildup which is not favorable for cell growth as it has been attributed with cell growth inhibition and decrease in recombinant protein production in CHO cells [14]. Theoretically, an intense reduction of carbon dioxide can show favorable effects on glycosylation of cells- however this is not feasible due to its importance on the growth of cells [11].

In order to optimize carbon dioxide conditions, varying its concentration throughout the culture cycle can induce positive effects on the levels of glycosylation and production rates of recombinant EPO being produced from CHO. Upon growing cells in the typical 5% carbon dioxide concentration, the culture pH appeared to be stable at first and then proceeded to decrease due to the accumulation of lactate. Alternatively, cells that were culture under no carbon dioxide supplies secreted EPO at a rate that was approximately three times higher than the preceding carbon dioxide condition [11]. However, the cell growth itself was very slow and the cells perished more quickly and this was paired by a fluctuation in pH levels which is highly undesirable for mammalian cell growth. In contrast, the supply of 0.05% - 0.5% carbon dioxide allowed stable cell growth and maximum cell density. Based on the results obtained, the switching of carbon dioxide concentration can provide an economical method to enhance EPO production. Through initiating the culture at 5% carbon dioxide and following it 0.03% carbon dioxide (the same amount present in atmospheric air) the EPO production rate was five times higher than cells incubated with air alone and had the same production rate as those culture at 5- however, the notable and favorable difference is that the EPO was of a better quality and the viable count of CHO cells was also higher [11] .

MECHANICAL PROTECTION VIA POLY-γ-GLUTAMIC ACID

Mammalian cells are very fragile and can be easily damaged in large bioreactors. Shear rate has a direct influence on mixing characteristics in stir tank bioreactors. Shear stress that results from agitation within the bioreactors can negatively affect cells. To help combat this, protective additives such as Pluronic F68 (PF-68) are typically added to the cultures to protect the cells from sheer stress and support high cell density. Pluronics are nonionic surfactants that are characterized as being hydrophobic and function by affecting the characteristics exhibited by cell culture medium. PF-68 stabilizes the foam layer that forms in bioreactors and prevents cells from interacting with it which effectively decreases the speed of the bubbles and thus minimizes the damage they can inflict on the cells [15]. However issues that include a decrease in protein productivity and DNA synthesis which lead to a decrease in growth rates have been exhibited under the use of PF-68 [4].

The use of alternative additives to protect cell cultures is a possible mechanism to help improve productivity and quality of EPO produced by CHO in stir-tank bioreactors. Poly-γ-glutamic acid (γPGA) is an alternative protectant that can be used. It is a biodegradable polymer that is derived from the gram positive bacteria *Bacillus subtilis*. It is nontoxic in nature and safe for human use, water soluble, edible and biodegradable. CHO cells that were grown with the addition of 50 kDA γPGA had a higher final concentration than those grown under the presence of PF-68 and those grown without any culture additives [4].

DISCUSSION

The successful and efficient production of EPO in CHO cells requires an understanding of the different conditions and parameters which affect productivity and quality of the EPO being produced by the cells. Mammalian cell cultures such as CHO are fragile and are thus easily affected by minor alterations to their surrounding environments. pH is a major determinant of the success and viability of CHO cells *in vitro*. Since these cells are grown over a very narrow pH range of 0.2-0.4 variation from the optimal condition, maintenance of pH range is paramount. Approximately 97% of utilized glucose by CHO cells is converted to lactate. Lactate is a toxic waste product which has acidifying properties and cause pH levels to drop in an unfavorable manner that is not conducive to cell growth and EPO secretion. Through the addition of neutralizing agents such as sodium lactate, pH levels can be maintained at an optimum range and production of EPO was found to be increased by 2.7 fold and culture time span was elongated. In addition to neutralizing the culture medium, the addition of sodium lactate causes a decrease in the specific growth rate of CHO cells. When this decrease occurs, cellular energy is diverted towards the production of EPO primarily as opposed to the production of other materials that allow cell longevity. As a result, a concentration

of 40mM of sodium lactate can neutralize the media and reduce specific growth rate enough to allow higher EPO production without sacrificing the economic cost benefit ration required for industrial processes [5][10].

Temperature is also a parameter that can have an indirect effect on pH levels. CHO cells are typically cultured at 37ºC in order to simulate the temperature of the bodies from which they are derived. However, at 37ºC, cell viability showed a decrease to 80% after 18 days of initiating the culture. Alternatively, lower temperatures allowed cell viability to remain at a range of 90% which translate to the ability to maintain a longer culture time span. This can be attributed to the indirect effect temperature has on pH of culture. When temperature is lower, the glucose consumption by CHO cells is regulated and reduced by approximately 76% at temperature ranges between 30ºC and 32ºC. This decrease in glucose consumption translates to less lactate being produced which prolongs cell culture time span and limits acidification of the medium which will allow proper glycosylation and high yields [12].

Another parameter that can easily be regulated and merits combining with reduced temperature and sodium lactate addition is gas switching of carbon dioxide. While 5% carbon dioxide is typically used in cell culture bioreactors due to it being the concentration present in mammals and it pH regulating properties, the effects of its build up in the bioreactors and reduction of polysialylation and N-glycolyneuraminic acid is unfavorable for cell growth. Alternatively, 0.03% carbon dioxide use resulted in 3 time's higher secretion rate followed by rapid cessation of the cells. However, since reduction or removal of carbon dioxide is not feasible and is necessary for cell growth, alternating carbon dioxide concentrations between the extremes of 0.03% (air) and 5% can significantly improve glycosylation as well as enhance the production and accumulation of EPO. This method of gas switching resulted in the production of EPO that was superior in quality and had far better glycosylation than that produced under traditional 5% carbon dioxide conditions [11]. In addition to alterations of parameters, the addition of surface protectants such as the novel *Bacillus* derived biodegradable polymer poly-γ-glutamic acid can be beneficial. Traditionally used Pluronic F68 is useful in the reduction of shear stress. However, it has been linked with the reduction of EPO synthesis as a result of lowering DNA synthesis in cultures.

In addition, its chemical makeup necessitates the need for expensive purification. In contrast poly-γ-glutamic acid can provide protectant properties without inhibiting cell growth and it remains nontoxic to humans and edible as well. In addition, EPO from cultures with poly-γ-glutamic acid were found to have superior EPO quality[4]. Finding the magic formula of such parameters is valuable. Sub-divisions of the same recipe of various temp, pH, surfactant, CO2 level should be used at different times along the period of cultures. From our analysis and review we suggest a promising scenario using mentioned parameters as shown in figure 1.

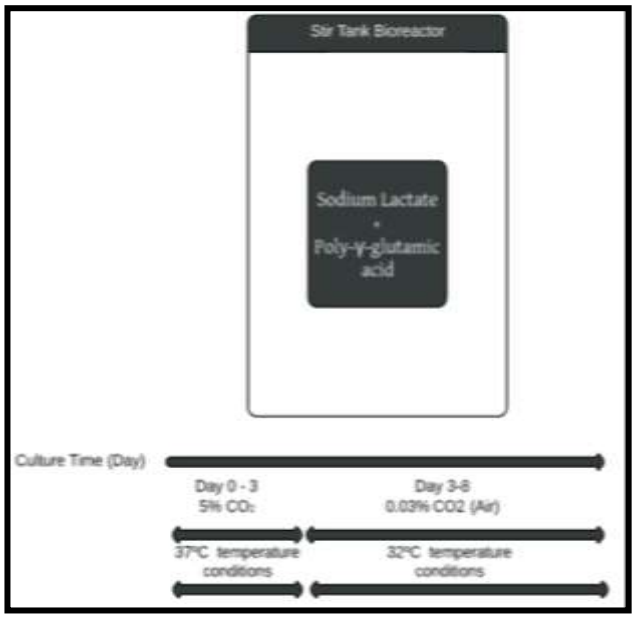

Figure 1: Suggested bioreactor setup with described parameters.

CONCLUSION

EPO is an important kidney and liver derived glycoprotein that mediates the process of erythropoiesis under hypoxic conditions in the human body. Recombinant EPO is a medically valuable therapeutic protein due to its role in the treatment of anemia due to cancer therapies and chronic renal failure. It is primarily produced in mammalian cell lines such as CHO due to their ability to perform the glycosylation that is necessary for the proper function of the

glycoprotein. Enhancing the production and quality of therapeutic glycoproteins like EPO is desirable to produce superior quality with high yields while maintaining an economical cost. Through the optimization of external parameters such as gas supplies, temperatures and the addition of useful additives this goal can be achieved. The set of parameters, alterations and optimizations discussed provide valuable insight into the effect environmental influences can have on EPO production in growing CHO cells- particularly the influences of altering pH. Through combining lower culture temperatures with carbon dioxide gas switching and the addition of sodium lactate – or other pH neutralizing agents- and biodegradable, nontoxic cell culture protectants an optimized mammalian culture system for the production of EPO can possibly be achieved.

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