Indonesian Journal of Cancer Chemoprevention
ISSN: 2088–0197
e-ISSN: 2355-8989

Expression of Human Erythropoietin Containing 2 Additional N-Link in CHO-K1 Cells under Different Culture Conditions

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Abstract

Human erythropoietin (hEPO) is a glycoprotein that regulates the formation of erythrocytes and mainly used in anemia patients. Previously, we have reported the expression of modified human EPO with 2 additional N-linked in mammalian cell CHO-K1. The aim of this current research was to study the optimum condition for modified recombinant hEPO (rhEPO) production in CHO-K1. To do this, several parameters of culture conditions were applied including antibiotic concentrations, seeding densities, time of incubations, fetal bovine serum (FBS) concentrations and cell culture media. The result showed that the presence of antibiotic G418 improved the expression level with the highest was at 1% of concentration. Meanwhile, seeding density of 2–3x10^5 cells/6 cm dish and seven day of incubation time were the best condition for rhEPO protein expression. From five different combination media used, F12 medium with 10% FBS gave the highest expression of rhEPO protein. From this study was also found that at passage 16 the expression level was still increasing proving that the clone expressing the protein of our interest is promisingly stable.

Keywords : *EPO, erythropoietin, protein expression, CHO-K1, optimation*

INTRODUCTION

As the main protein involved in the maintenance of red blood cell level in the body, the function of erythropoietin (EPO) is highly regulated by the presence of oxygen in the body (Krantz, 1991; Lacombe, *et al*., 1998). The decrease of oxygen in tissues will enhance the production of EPO in kidney (D'Andrea, *et al*., 1989). Thus, the production of red blood cell is influenced by the level of oxygen in the body. Erythropoietin (EPO) is a complex and intensely glycosylayted particle comprising of 165 amino acids. Its molecular mass is 30.4 kDa, but it relocates with an apparent size of 34-40 kDa on SDS-polyacrylamide gels. However, the correct molecular weight profoundly relies upon the level of glycosylation. This molecule contains three N-linked and one O-linked carbohydrate side chains with maximum may contains 14 residues of sialic acid. Approximately, 40% of its molecular weight is contributed by its sugar partition (Egrie, *et al*., 1986). The N-linked sugar side chains give off an impression of being fundamental for the formation of EPO, limit clearance, increase stability, thus can

Submitted: November 2, 2018
Revised: November 26, 2018
Accepted: November 28, 2018

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perform erythropoiesis normally in bone marrow (Fried, *et al*., 1972; Zanjani, *et al*., 1981; Yin, *et al.*, 2000). Precedent study Egrie, *et al.* (2001) had demonstrated that the expansion of sialic acid content of EPO with the integration of 2-N linked can increase serum half-life and biological activity. Insufficiency of EPO becomes one of the main causes of cancer anemia, this makes the use of recombinant therapeutic protein EPO becomes one of the solutions (Kasper, *et al.*, 1997). Present study shows that EPO is very beneficial to alleviate cancer-associated malignant anemia and can improve survival outcomes for patients with cancer (Zhao, *et al*., 2017). Mortality among patients with anemia is twice as high as that among those without anemia at three year post-cancer diagnosis (Rice, *et al*., 1999; Leng, *et al.*, 1999., Chung, *et al*., 2003). This indicates that the use of EPO in treating cancer anemia can be very helpful.

With numerous licenses for the principal biologicals derived from recombinant innovation are terminating. Normally, biosimilars are becoming an increasingly consequential area of interest for the pharmaceutical industry globally and this likewise opens up open doors for creating nations to deliver their own particular biologic items (Katherine, *et al*., 2014; Blakstone, *et al*., 2013; Declerck, *et al*., 2017). With the long haul objective of creating biosimilar EPO, investigation of human EPO molecule containing 2 extra N-linked in mammalian cell CHO-K1 is in progress. We already reported the expression of this modified recombinant human EPO (rhEPO) in CHO-K1 cells and its *in vitro* proliferative activity in TF-1 cells (Santoso, *et al*., 2014).

The objective of this current study is to obtain the optimum conditions of expression of hEPO protein in mammalian cells CHO-K1 using general commercial media. The optimation of culture conditions under study includes antibiotic concentrations, seeding densities, time of incubations, fetal bovine serum (FBS) concentrations and cell culture media.

MATERIALS AND METHODS

Cell culture and reagents

The CHO-K1 cells were obtained from Prof. Masashi Kawaichi, Nara Institute of Science and Technology (NAIST), Japan. The cells were cultured in Nutrient Mixture F-12 Ham (F12) media (Sigma N6658) in the presence of 10% of fetal bovine serum (FBS, Sigma), 100U of benzylpenicillin and 100 µg of streptomycin (Gibco, Invitrogen). CHO-K1 cells were transfected with plasmid J-EPO, (pJ-EPO) containing 2 additional N links and the stable cells expressing rhEPO were used for this study (Santoso, *et al*., 2014). As much as 1% of antibiotic G418 (Sigma, A1720) was added to the media just before use to maintain protein expression. The cell was cultured in an incubator with the condition of 5% CO_2 and 37^{o}C temperature. Unless otherwise stated, the number of cell seeded was $1.5x10^5$ cells/6 cm dish. The cells were seeded in 5 mL media in 6 cm dish.

Optimation study

For the optimation study, the procedure is described as follow: 1) antibiotic G418 concentrations: the cells were cultured with the addition of 0, 0.5, 1, 1.5 and 2% of antibiotic G418 in F12 medium plus 10% FBS, 2) seeding density: the cells were cultured for four days in F12 medium in the presence of 10% FBS, 1% antibiotic G418, and seeded as many as $2x10^5$, $3x10^5$, $4x10^5$ and $5x10^5$ cells/6 cm dish; 3) incubation time: the cells were cultured in F12 medium in the presence of 10% FBS with seeded cells as many as $2x10^5$ cells/6 cm dish and incubated for 2, 3, 4, 5, 6 or 7 days in the presence of 1% antibiotic G418, respectively; and 4) media and its concentrations: the cells were cultured in CD-CHO (100%), CD-CHO/F12 (3:1)/2.5% FBS, SFM (100%), SFM/F12 (3:1)/2.5% FBS and F12/10% FBS media in the presence of 1% antibiotic G418.

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SDS-PAGE and Western blotting.

The purity of the protein analyzed by SDS/PAGE performed as previously described in a 12% separating gel with a 5% stacking gel using the Mini-PROTEAN-3 apparatus (BioRad, Hercules, CA, USA). Following electroporation, proteins were transferred to Amersham Hybond ECL (GE Healthcare) by electroblotting. Western blots were performed using polyclonal anti human EPO antibody (Sigma, St. Louis, MO, USA) as the primary antibody and anti-rabbit IgG alkaline phosphatase linked whole antibody (Promega, Madison, WI, USA) as the secondary antibody. The bands were detected by BCIP/NBT color development substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) (Promega, Madison WI, USA).

RESULTS

Several techniques of transfection strategies have been established to steadily integrate vector DNA into mammalian cells. Previously, we also reported the optimization cationic lipid mediated transfection of pJ-EPO in CHO-K1 cells (Septisetyani, *et al*., 2012). The transfected cells were selected in view of aminoglycoside phosphotransferase (antibiotic G418) selectable. To evaluate the antibiotic G418 concentration in relation to rhEPO expression and cell growth, several concentrations of antibiotic G418 were added to the F12 growth media in the presence of 10% FBS.

Data shows (Figure 1A) that the rhEPO protein bands present around 34-45 kDa corresponded to the theoretical molecular mass for the EPO that the exact molecular weight highly depends on the degree of glycosylation. As shown in Figure 1A the rhEPO protein expression was approximately the same at 0, 0.5 and 1.5% of G418 antibiotic concentration; and the highest is at 1%. However, at highest G418 antibiotic concentration (2%), the expression dropped. To examine the presence of antibiotic G418 on the growth of the cells, cell densities were observed after 4 days of incubation. The data reveals that at 0-0.5% of antibiotic G418, the cell densities are, approximately, the same ($7x10^5$ cells/6 cm dish). However, at higher concentration (1-2% of antibiotics), the cell density dropped, approximately, to 5 to $6x10^5$ cells/6 cm dish, respectively.

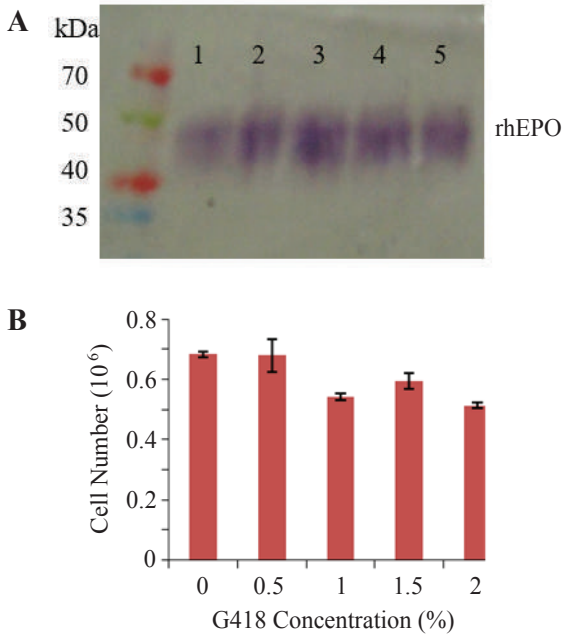

Figure 1. Expression of rhEPO protein in CHO-K1 cell in several antibiotic G418 concentrations. The cells (200,000 cells/6 cm dish) were cultured for 4 days. A: Western blot analysis of CHO-K1 cells expressing rhEPO. Lane 1-5 correspond to 2, 1.5, 1, 0.5 and 0% of antibiotic concentrations, respectively. B: Cell densities after four days of incubation.

To analyze the stability of the cells to express rhEPO protein, the cells were passaged for 5 times in the presence (1%) or absence of antibiotic G418. The results show that both cells were able to express rhEPO; and the expression level increased with more passage numbers (Figure 2A and B). The data showed that at passage 16, the expression level (in the presence of 1% antibiotic G418) was still increasing (Figure 2C).

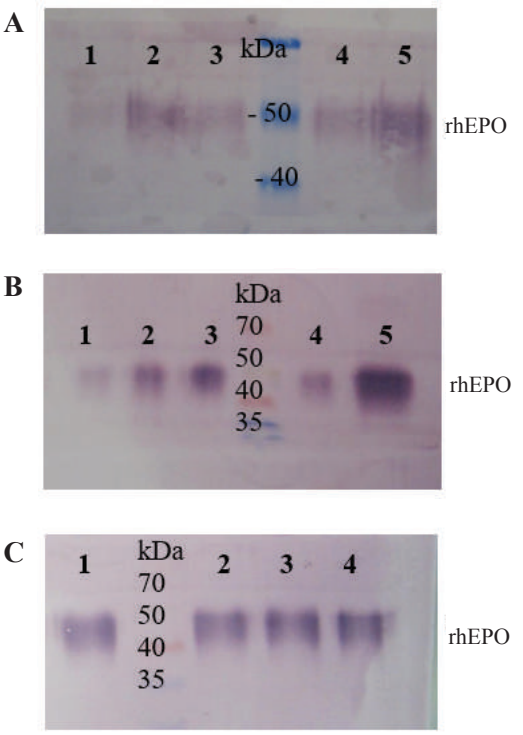

Figure 2. Western blot analysis of CHO-K1 cells expressing rhEPO protein with or without antibiotic G418 in F12 medium in the presence of 10% FBS. A: The cells were cultured without antibiotic G418. Lane 1-5 corresponds to 1-5 passage numbers, respectively. B: The cells were cultured in the presence of 1% antibiotic G418. Lane 1-5 corresponds to 1-5 passage numbers, respectively. C: The cells were cultured in the presence of 1% antibiotic G418. Lane 1-4 corresponds to cells with passage numbers 13-16, respectively.

To further optimize the best condition for the cell to grow, next step for this optimation study is to evaluate the number of cell seeded. To do this, several numbers of cells were seeded in F12 + FBS 10% medium. Following four days of incubation, the numbers of cells were counted and the protein hEPO expression was analyzed using Western blot. The data in Figure 3A reveals that higher expressions were observed at seeding density of $2x10^5$ and $3x10^5$ cells/6 cm dish. While lower expressions were observed at higher seeding density ($4x10^5$ and $5x10^5$ cells/6 cm dish). After 4 days of incubation, the cell densities linearly correlated with the number of cell seeded with the lowest and highest were when the cells were seeded at $2x10^5$ and $5x10^5$ cells/6 cm dish, respectively (Figure 3B).

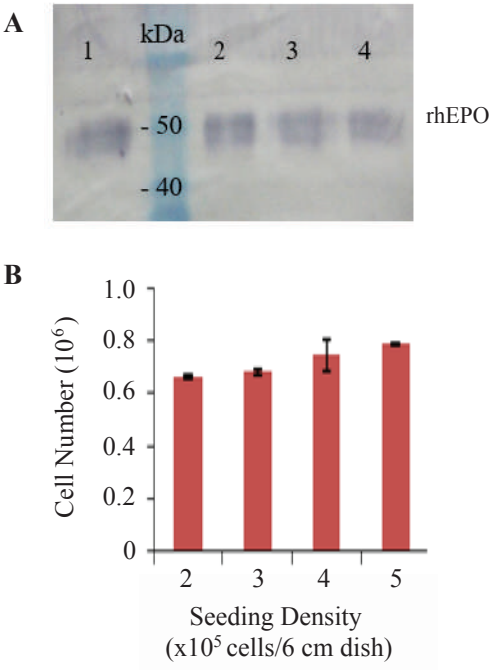

Figure 3. Optimation of CHO-K1 cell seeding density. A: Western blot analysis of CHO-K1 cells expressing rhEPO. Lane 1-4 correspond to cell densities of 200,000; 300,000; 400,000 and 500,000 cells/6 cm dish. B: Cell densities after four days of incubation.

To further evaluate for how long the cell has to be cultured to obtain the highest titer, 200,000 cells/6 cm media was cultured in F12 + FBS 10% medium. The cells were cultured and harvested everyday from day two to seven. As revealed in Figure 4A, optimation of incubation time indicates a linear relationship between time of incubation and level of expression. The data shows that seven days of incubation time gave the highest level of expression. While, the lowest level of expression occurred at two days of incubation. The cell density data showed that cell number increases with the increase of incubation time; and the peak occurred at day four with the total number of cell was, approximately, $6x10^5$ cells/6 cm dish. After four days of incubation, the cell density decreases sharply until at seven days

Indonesian Journal of Cancer Chemoprevention
ISSN: 2088–0197
e-ISSN: 2355-8989

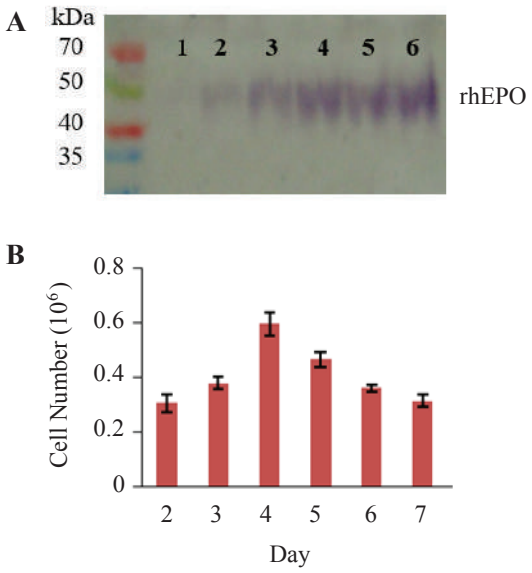

Figure 4. Optimation of incubation time. Cells were seeded at density 200,000 cells/6 cm dish and incubated for 2, 3, 4, 5, 6 or 7 days (1-6 respectively). A: hEPO protein expression. B: Cell density at harvesting day.

of incubation. This result indicated that higher protein expression was obtained at longer incubation time without showing correlation with the cell density.

With the advance of biologic medicines, manufacturing of protein therapeutic products with the use of CHO cells has become the preferred choice. However, one of the main problems in this system is that most of mammalian cells have to grow in the presence of FBS. The presence of FBS may complicate the downstream process of the biologics of interest (Reinhart, *et al*., 2013). Thus, one of the goals of this study is to evaluate how the cells grow in several media including serum free media. To do this, the cells were grown in several media and followed by observation of its expression and cell density (Figure 5). From the five different media used, SFM/F-12(3:1)/2.5% FBS and F12/10% FBS media gave, approximately, the same level of rhEPO protein expressions (Figure 5A), followed by CD-CHO/F12 (3:1)/2.5% FBS and SFM 100%. To have a better understanding of the protein expression in relation to the cell growth, the cells grown in several media were counted. The data shows that the highest cell density was found when the cells grown in SFM/F12 (3:1)/FBS 2.5% medium followed by CD-CHO/F12 (3:1)/FBS 2.5%, F12/10% FBS, CD-CHO (100%), and SFM (100%), respectively.

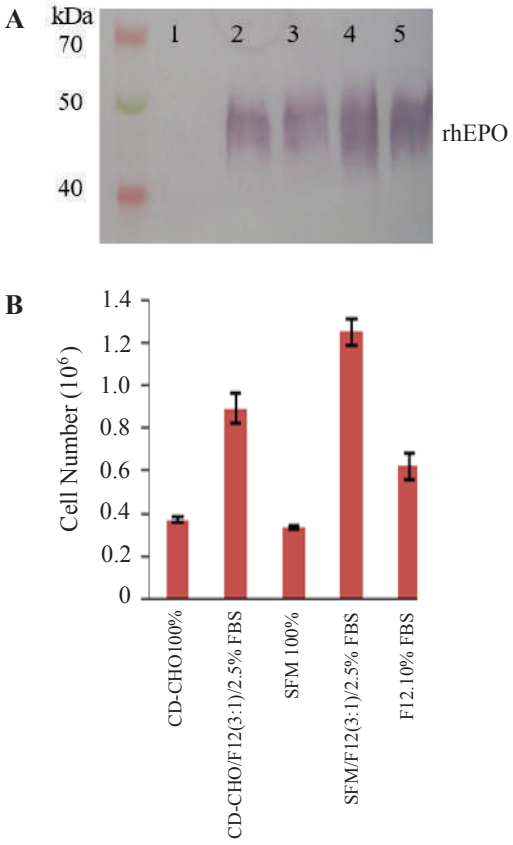

Figure 5. Expression of rhEPO protein in CHO-K1 cell line in several media. A: Western blot analysis of CHO-K1 cell conditioned media. Lane 1-5 correspond to CD-CHO 100%, CD-CHO:F12 (3:1)/FBS 2.5%, SFM 100%, SFM:F12/FBS (3:1)/FBS 2.5% and F12/10%FBS in the presence of 1% antibiotic G418, respectively. B: Cell density after four day incubation.

DISCUSSION

These days, cell culture has transformed into one of chief fields in present day biotechnology, especially in the field of human wellbeing. Advancement of development in interdisiplinary fields, for example, human medication, cell science

and biotechnology has provoked the generation of biologics including vaccines, hormones and monoclonal antibodies (Feng, *et al.*, 2010; Kunert, *et al.*, 2016). Nonetheless, since a large portion of these particles are glycoproteins, post-translational adjustment stages are exceptionally indispensable (Matasci, *et al*., 2008). To oblige this condition the utilization of mammalian cell culture innovation is vital. With the use of limiting dilution technique, our previous work (Septisetyani, *et al*., 2012) was able to clone CHO-K1 cell transformant harboring codon optimized human epo gene containing five N-linked oligosaccharide chains. To optimize the growth and titer of the obtained clone, the cloned cell was observed in several conditions including antibiotic concentrations, type of media, times of incubation and numbers of cell seeded.

One of antibiotic frequently used in mammalian cell line work is antibiotic G418 (Feng, *et al*., 2010). To observe how the titer and growth of our cell transformant performed in the presence of antibiotic G418 concentration, several concentrations of this antibiotic were applied in CHO-K1 cell transformant harboring codon optimized human epo gene. The result showed that the highest expression of rhEPO protein occurred at 1% antibiotic concentration. Interesting to note that at 0% of antibiotic concentration, as seen in Figure 1A, the concentration of rhEPO was relatively high. It is known that when transfected cells were kept under selective pressure (for example antibiotic G418 or methotrexate), the expression level of the gene of interest stably maintained at high level and the reverse occurred in the absence of selective pressure. Thus, the higher protein expression at zero concentration of antibiotic G418 than that of 2% has to be taken caustiously at higher passage numbers. It is possible that with the increasing number of passage, in the absence of selective pressure, the expression of the protein will decline. In another word, the presence of selective pressure may be needed to keep the expression of protein of interest to remain high. In case of cell density, the data shows that the cell number drops at 1–2% of antibiotic G418 concentration. The decrease of the cell density, especially at the highest concentration (2%), was followed by the decrease of the protein expression (Figure 1).

Consistent gene expression for specific period of time is the main indication of stable cell lines. Unfortunately, obtaining stable cell lines can be exceptionally costly and tedious (Feng, *et al*., 2010; Bussow, *et al*., 2015). To analyze the stability of our cloned cells, the cells were cultured in the presence and absence of 1% of antibiotic G418 for 16 passages. The number of passage starts when the cells were treated with experimental treatment. As seen in Figure 2, the cells were still strongly expressing the protein of our interest meaning that the cells were stable. Interesting to note that at 16 passages, the trend of the expression was still increasing indicating that there is a high possibility that our gene of interest was inserted stably into the genome of the cell.

Seeding density in cell culture may play crucial role in subsequent cell division, particularly if that has to do with specific cell line (Zhou, *et al*., 2011). Following seeding, cell development in culture begins from the slack stage to the log stage where the development turns to grow exponentially (Rolfe, *et al*., 2012). In adherent system, normally, cells quickly grow until no room left for expansion. At this point, proliferation greatly reduced and finally, ceases completely. To ensure the cell culture keeps on developing, the cell culture has to be passaged at the right time.

Considering the dynamic growth of cell explained above, optimum seeding density become important. To observe the optimum number of cell seeded, several cell densities (2, 3, 4 and $5x10^5$ cells/6 cm dish) were seeded. The result showed that the highest expression was obtained when the cell was seeded at $2–3x10^5$ cells/6 cm dish (Figure 3). It is natural to expect that the highest seeding density would give the highest expression level.

However, the data obtained showed that the highest seeding density ($4x10^5$ cells/6 cm dish) did not give the highest expression level. The likely

explanation of this data was that lower seeding density (2–3x10^5 cells/6 cm dish) gave the optimum condition for the cells to synthesize the rhEPO protein. The inverse relationship between the protein expression and cell growth may reflect the balance nutrients requirement of cells at particular time.

To some extent time of incubation of cell culture is related to the amount of protein being expressed. To observe for how long the cell has to be cultured in order to obtain the highest expression level, the cells were cultured for seven days. A linear relationship pattern was obtained between degree of expression level and time of incubation with the highest at day seven. Having known the relation between expression and time of incubation, the relationship between time of incubation and cell density was then observed. The data showed that cell density increases with the increase of incubation time. After reaching the peak at day four the cell density decreases sharply with the lowest at seven days of incubation (Figure 4). Thus, the peak of cell density was not followed by the peak of protein expression. This data might show that it takes time for the cell to synthesize the protein of interest (rhEPO) that finally peaked at day seven.

In term of scalability, mammalian cells have historically been considered to be difficult to work due to factors such as low yield, serum requirement and medium complexity (Reinhart, *et al*., 2013; Carrillo, *et al*., 2015). The data (Figure 5) shows that the presence of FBS is significant for the growth and expression of the protein of interest with the highest was at 10% of FBS. Interesting to note that the expression level at FBS 10% (in F12) and 2.5% [in SFM/F12 (3:1)] are, aproximately, the same. However, the protein expression in 2.5% FBS [in CD-CHO/F12 (3:1)] was little lower. The low protein expression in SFM (100%) and almost no expression at all in CD-CHO (100%) were understandable since the attachment of CHO-K1 cell required FBS (Figure 5). While FBS can bolster the development of numerous types of cells, however, the type of its particular components that are critical for every individual cell have not yet been recognized thoroughly (Shahdadfar, *et al*., 2005; Valk, *et al.*, 2017). Mostly in the form of FBS, serum has been utilized in cell culture for a considerable length of time, and its utilization is really expanding. Serum may not be the suitable enhancement to use in cell culture, particularly if the protein was planned for biopharmaceutical items for use in human (Schroder, *et al*., 2004; Zhang, *et al*., 2013). The use of serum free media has picked up acknowledgment and significance, nonetheless, building up a powerful serum free media has been troublesome (Freshney, 2010; Valk, *et al*., 2017), as demonstrated in this work that the utilization of 10% FBS gave the best results.

Expansion and competition of costly biologics have fuelled endeavors to enhance and upgrade cell culture media with the aim of achieving greatest outcomes and bringing down the expense of production (Valk, *et al*., 2017; Tatsuma and Asayama, 2017). Commonly now, due to the need to support high cell densities and efficiency, the media used for biologics production are without serum and have substantially higher supplement fixations than traditional media (Carrillo, *et al*., 2015). Cell growth and viability are paramount for biologics production. However, when serum is basically needed for some cells to grow, the growth and achieving the cells to express the protein of interest becomes very challenging (Fontes, *et al*., 2014). As productivity and growth can have inverse relationship, and the cell limited resources are divided between those two functions, nutrient essential for growth may compete with those for protein production. To face this problem, initial and thorough optimation is extremely important for having the highest protein production possible in mammalian cell culture.

CONCLUSION

This research has shown that to certain extent antibiotic G418 improves the hEPO protein expression level with the highest was at

1% of concentration. Seeding density and time of incubation affect the expression level with the best conditions are at 2–3x10^5 cells/6 cm dish and seven day of incubation time, respectively. Medium F12 with 10% FBS proves to be the best medium for rhEPO expression. However, the use of SFM/F12 (3:1) medium with lower concentration of FBS (2.5%) also gave promising result. Observed for 16 passages, the expression level was still increasing indicating that the clone is stable.

ACKNOWLEDGEMENTS

We would like to thank Indonesian Institute of Sciences for the fund used in this research.

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