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OPTIMIZATION OF PROCESS OF PRODUCTION OF TPA RECOMBINANT PROTEIN IN PERSPECTIVE OF SCALEUP

ALIREZA EHGHAGHI[1*], FARNAZ EGHBALPOUR[1], FATEMEH DAVAMI[1], FEREIDOUN MAHBOUDI[1] AND MAHBOUBEH BAHROUDI[2]

1: Biotechnology Research Center Pasteur Institute of Iran

2: Department of Microbiology Tehran University of Medical science Iran

***Corresponding Author: E Mail: ehghaghis@yahoo.com**

ABSTRACT

The extent of growth and increase of production of protein under influence of peptones rely on nutrient composition in medium. This study examines Optimization of process of production of tPA recombinant protein in perspective of scaleup. Results of culture in proCHO5, CDDG44 and BRC mediums indicated that production of tPA in recombinant CHO cells in proCHO5 medium compared to CDDG44 and BRC mediums have been seen with more extent of density and PCV. This result was convincing enough for us, because proCHO5 medium is an optimized medium for production of recombinant proteins in suspended cells.

Keywords- tPA Recombinant Protein; proCHO5; CDDG44; BRC

INTRODUCTION

Proteins are macromolecules that embed at any areas in cells. They play an important role in cell growth, metabolism, organizing, transfer and mutation. In multicellular organisms, proteins control immune system, nerve signals, growth and division of whole organism. Abnormal mutations or transcription of proteins can be the starting point for most of diseases. As human genome contains over 30000 genes, and the number of proteins which are produced goes beyond, opportunities are acquired for pharmaceutical companies in order to develop newer drugs. Hence, during the last decades, proteins were transformed to major aims for development of new treatments **(Tanswell P, Tebbe U, ,**

Neuhaus KL *et al*., 1992). When proteins were used for the first time for treatment purposes, they were purified from human and animal resources, such as cadaveric human growth hormone, beef and pork insulin and stimulating hormone in human secretory glands of urea. Anyhow, use of these resources will be followed by major concerns. Fortunately, today, fewer products from animal resources are used. Indeed, since the late of 1970s, growth in biotechnology has paved the way for the possibility of production of protein from "DNA containing the genetic traits" using prokaryote and eukaryote host systems. These proteins enjoy inherited traits, and can be differentiated from internal proteins, mentioned that they are produced by means of transcription of the genes which enjoy new inherited traits **(Coleman, Benach, 1992)**. Insulin has been the first recombinant protein which has been used for human, whereby *E.coli* was produced by Eli Lili in 1984. The first recombinant protein produced in eukaryotic host has been Tissue-type plasminogen activator (t-PA) from chinese hamster ovary cells which was produced in 1986. In 2007, there existed over 170 biopharmaceutical products, confirmed by U.S. food and drug administration, but it was expected that this amount increases in upcoming years. In 2006, about 5000 protein products were reported in discovery and pre-clinical and clinical trials. Escherichia coli with cultured mammalian cells among host transcription systems had the most use. About half of therapeutic protein products in market were being produced from mammalian cells. Even if mammalian cells and tissues have been more complicated and expensive than microbial culture, these cells sustained in priority, because mammalian cells enable to modify proteins after transfer of PTM especially Glycosylation. Other hosts enable to protein Glycosylation such as cultivated plants and insects and yeasts **(Ouriel *et al*., 1995)**. This process in these hosts was different from what seen in mammalian cells. Yet, efforts sustained on so far as Glycosylation in these non-mammalian hosts to be conducted in form of mammalian hosts. In 2007, FDA put an emphasis on the first b biopharmaceutical product which had been produced in insect cells for human use. Alternative hosts include transgenic animals and plants, but these hosts have still remained in development stage.

Common Hosts in Production of Recombinant Proteins

To express recombinant proteins due to rapid growth in environment, low cost, ease in genetic manipulation, specified genetic and physiological characteristics, protein's ability of high expression can be a suitable host, but proteins produced in this host generally lack appropriate post- translational modifications and desirable folding, appearing in insoluble cumulative forms. Despite prokaryotes, eukaryotic hosts will have the ability for appropriate post- translational modifications like development of Disulfide bonds, glycosylation and proteolytic cut, which result in production of active proteins **(Bode C, Kohler B, 1995)**. Recombinant proteins produced in yeast are generally produced in Pichia Pastoris and/or S.cerevisiae. A rapid growth is seen in fungi, where easier genetic manipulation is seen in fungi rather than other eukaryotes. Fungi have also the ability for some translational modifications like simple glycosylations. Lacks of ability of these hosts for complicated translational modifications constrain use of them **(Sandra J, Degen F,Rajput B**1986**)**. Insect cells culture is also a suitable method for production of recombinant proteins especially recombinant vectors. These cells can grow easier in large extent. The major problem in these culture systems can be their limited capacity in changes in proteins that are produced in form of precursor proteins, and extent of expression in these hosts due to probability of accumulation of proteins in insoluble forms is limited. Furthermore, differences in glycosylation patterns are seen between this host and mammalian cells **(Pavlou, A. K. and Reichert, J. M. 2004)**. In suspended cultures, herbal cells especially tobacco and manipulated plants are used to express tPA recombinant protein. The most important advantage to use in-vitro plant cell culture systems can be high production speed and ability for changing growth environment for higher control of producing protein.

The weak point in these producing systems can be low growth speed of plant cells and difference in Glycosylation pattern rather than mammalian cells **(Rijken DC.Wijngaards G.Zaa-de JongM. Welbergen J, 1979)**. Production of protein in transgenic plants has been widely discussed in various studies **(Dawson, S and A. Henney** 1992**)**. Ease of use and low cost of culture are two major advantages of this expression system. Yet, protein purification is much more difficult than plant cells culture **(Dawson,S and A.Henney، 1992)**. Mammalian cells enable to produce highest extent of protein for treatment purpose, because these changes fulfill an essential transcription with higher acuracy than all the defined hosts. Unfortunately, mammalian cells for maintenance during culture are high-cost, having a slower growth rather than what mentioned above. Further, these cells subject to viral infection that raises pollution in final product.

Thrombolytic Therapy

Thrombus is a semi-solid mass developed from fibrin fibers (elastic protein responsible for clotting), that can block blood flow. Anticoagulants products such as aspirin, anti-vitamin K, and heparin for a long time can be used for treatment and prevention fromclotting. In this sense, these products will able to reduce the size of the clot, yet rapid and complete resolution of the clot results in improvement of signs and patient's survival. For this, drugs such as thrombolytic drugs were made. Nevertheless, still these new drugs are prescribed with anticoagulant as a complementary therapy for avoiding recurrence **(Wurm, F. and Bernard, A. 1999)**.

Checklist of thrombolytic drugs prescribed for using in UK, how to use and their prices has been represented in **Table 1**. Two other drugs which are not available in UK have been also defined. These drugs activate transforming blood plasminogen to Plasmine, resulting in failure of fibrin. Fibrin is a key protein in thrombosis, using in clot-busting drugs. These drugs are prescribed in form of intravenous vein.

At the early stages, prescription of drugs which solve clots formed in the coronary arteries can be healed. Pulmonary embolism, transient schemic attack, and myocardial infarction are fatal disasters which without emergency treatment might result in complicated complications such as failure in organs and death. At this period, prescription of thrombolytic such as tissue plasminogen activator by unblocking results in minimizing tissue damage and ultimately survival of patient. Prescription of thrombolytic such as tissue plasminogen activator in an acute thrombotic event causes clot dissolution and reperfusion of the tissue with oxygenated blood. Since followed by treatment with thrombolytic drugs, the risk of bleeding associated with treatment will raise, whereby an effective and safe drug must be selected for each patient.

Table 1: Comparison of thrombolytic drugs

	SK	SAK	VPA	UK	r- ProU	rt-PA	r-PA	nPA	TNK-t-
Mol. Wt (KD)	**48**	**16.5**	**52**	**32/54**	**49**	**68**	**39**	**53.5**	**65**
Fibrin specificity	+	+++	+	+	++	++	+	+	+++
Half-life (min)	**10**	**6**	**168**	**2**	**9**	**5**	**15**	**57**	**25**
PAI - 1 resistance	**NA**	**NA**	**Yes**	**NO**	**No**	**NO**	**NO**	**No**	**YES**
Elimination	**Kidney**	**Liver**	**Liver**	**Kidney**	**Liver**	**Liver**	**Kidney**	**Liver**	**Kidney**
Immunogenicity	**Yes**	**Yes**	**Yes**	**No**	**No**	**No**	**No**	**No**	**No**
Cost	+	**NA**	**NA**	++	**NA**	+++	+++	**NA**	++++

An Overview on Studies

In 1973, Staley Cohen and Herbert Boyer were recognized as pioneers in use of recombinant DNA technology for cloning and expression of foreign genes in organisms. Production of the first recombinant protein was reported some years later the date Genentech (Biotechnology Company) announced somatostatin gene expression in the *E.coli*. Extent of hormone activity obtained equivalents to extraction of somatostatin from 500,000 sheep brains. Followed by this success, in 1982, Genetch supplied recombinant insulin obtained from *E.coli* as the first recombinant DNA technology by America Food and Drug Administration (FDA) to the market **(Krause J, 1988)**. They cloned RSF1010 plasmid DNA streptomycin resistance in Salmonella typhimurium to escherichia coli plasmid pSC101, and observed resistance to streptomycin in transformed cells.

Formation of a recombinant protein is a controversial stage in process of discovery and production of drug. Institutions developed from pharmaceutical biotechnology industry are mainly based on production of monoclonal antibodies (mAbs) and its pieces. Today, needing to recombinant proteins in basic studies and clinical applications keeps increasing. During recent years, biotechnology techniques by rise of changes in the context of health and food have been largely increased, where recombinant products with genetic engineering techniques, gene manipulation and recombinant DNA produced in various organisms, have raised a huge development in type and diversity of drug products **(Bergsdorf N.,Pohl G. and Wallen Ranby M. 1982)**. Drugs and vaccines which have been made of biotechnology help millions of people who suffer from heart disease, Alzheimers, Parkinsons, diabetes, spinal cord injury and cancer per year. Today, drug recombinant products with high molecular weight have been substituted with small molecules. Today, production of recombinant proteins has been transformed to a big global industry with annual volume over 50 billion dollar. In industrial sector, CHO cell in production of clotting factors 7, 8, 9,calcitonin, DNAse disease, cystic fibrosis,erythropoietin, FSH, LH, gonadotropin,stem cell factor, t-PA, interferon beta and Platelet-derived growth factor (PDGF) are used. Many monoclonal antibodies such as Anti-Human IL-4 and MAbs against molecules CD106, CD29, CD90 have been in cell CHO.

METHODOLOGY

Necessary Conditions for Cell Culture

To do cell culture tests, a sterile environment and culture room equipped with ventilation and filter systems is required. Working with cell, all surfaces, shelves and laminar flow hood must be cleansed and sterilized with ethanol 70% or another alternative detergent.

After ending the tests, disinfectants and disinfection of culture room and materials and devices to avoid expansion of microbial pollution are required. In general, agents and disinfectants including hypochlorite such as chlorinated water and bleach, alcohols such as ethanol and isopropanol, as well as aldehydes such as formaldehyde and glutaraldehyde are used in culture room.

Culture Mediums for Optimization Process

Culture mediums which are used are divided into two mediums: 1-mediums prepared with uncertain compositions, 2- mediums with certain compositions. Culture mediums CDDG44 and Procho5 are prepared from Lonza and Gibco companies. Culture medium BRC has been designed and prepared by biotechnology sector in Pasteur Institute of Iran.

Reverse osmosis systems: to prepare culture mediums, preparation of a reverse osmosis is required; further, to prepare culture medium BRC, autoclaved double distilled water at 121 ° C for 20 min with adjusted pH is used.

Storage of Cells and Culture Mediums

After preparation of culture medium, firstly the sterilization must be fulfilled accurately. The used culture mediums due to enjoying enzymes, hormones, cofactors and bicarbonate buffers have not the capability for autoclave, sterilized by passing through a membrane filter with diameter 0.2 micrometer by using vacuum pump for acceleration in suction in culture medium and avoidance from pollution. To measure the required solutions, disposable micropipettes were used.

Preparation of Cells

Studying the effect of peptones on extent of growth and expression of recombinant protein in transfected CHO cells as well as TGE requires the cells adapted with considered culture mediums.

De-Freezing Cells

- Culture medium was heated till 37°C.
- Vial containing frozen cells has been removed from nitrogen tank and submerged into a container containing water at 37°C.
- -When contents of vial were melted, the lid was sterilized and opened with ethanol (70%).
- Contents of vials containing cells CHODG44 were transferred to sterile falcons
- 2 times the size of the cells were added to culture medium.
- Then, the culture medium was centrifuged at 1100 rpm during 5 min at temperature 18°C, to obtain cell pellet
- The liquid on the surface of culture medium was removed, because this liquid contains DMSO which is toxic for the cell, and it must be removed from the cells.
- Little falcon was added to culture medium, then the cells were uniformed with sterile Pasteur pipettes.
- Cells were cultured for growth in a suitable concentration.

Incubation of Cells

An incubator to keep cells at temperature 30 to 40°C is required in a cell culture laboratory. Temperature of incubation depends on type of cultured cells. Temperature of incubation for considered cells is about 37°C.

Sterilization of Incubator

For Sterilization of incubator, alcohol (70%) can be the best substance.

During sterilization, pouring water or use of sodium chloride solution or halogenated solutions which cause paint corrosion or strong alkaline or acidic solution must be avoided.

In using alcohol for cleansing inside of incubator especially in cleansing incubator with high heaths, it must take essential considerations, because under such conditions alcohol will be evaporated, spanning all the space of incubator, whereby there will be the risk for explosion; hence, all the remained alcohol must be properly cleansed.

Instruction

Cells were counted by neobar lam as follows:

- Neobar lam (Hemocytometer) has been cleansed with 70% ethanol in order not to scratch the silver surface of neobar.
- Lamella was placed on middle part of Hemocytometer.

- The cell sample which has been totally mixed was carefully separated in order that cell masses do not remain in cell sample.
- About 20 µl of cell suspension sample was taken with sampler.
- The cell suspension was slowly discharged at the edge of Hemocytometer container, such that the sample was slowly directed from sampler tip under the lamella.
- The lamella was placed in bottom of microscope and focused with 20x lens in order to observe grid lines at each container
- By moving lamella, field of vision at central area of grid is regulated in a way that the largest area under vision has been enclosed via three parallel lines; the whole field of vision at this area with area of $1mm^2$is filled with 20x lens.
- Using square areas in smaller figure, the cells of this area was counted.
- To count the cells, it requires counting the cells and calculating the mean.

The formula for calculation of number of cells in volume:

$$c = n/v$$

C=the number of cell in volume

n=the number of counted cells

V=the sample size of cell suspension

Depth of container is 0.1mm in a Neobar lam.

Since, counting has been fulfilled at the central area with area 1 mm^2 with three enclosed parallel lines; sample size equals to 0.1 mm^3 which it can be written as 1 $x10^{-4}$ml.

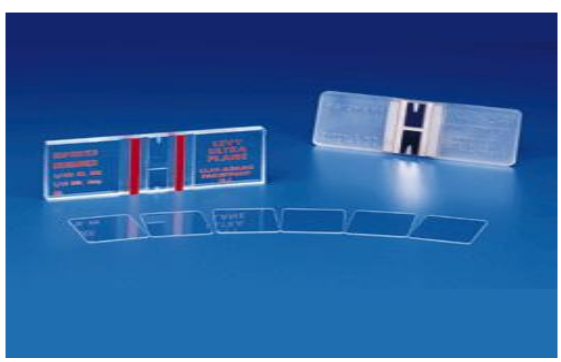


Figure 1: Neobar lam

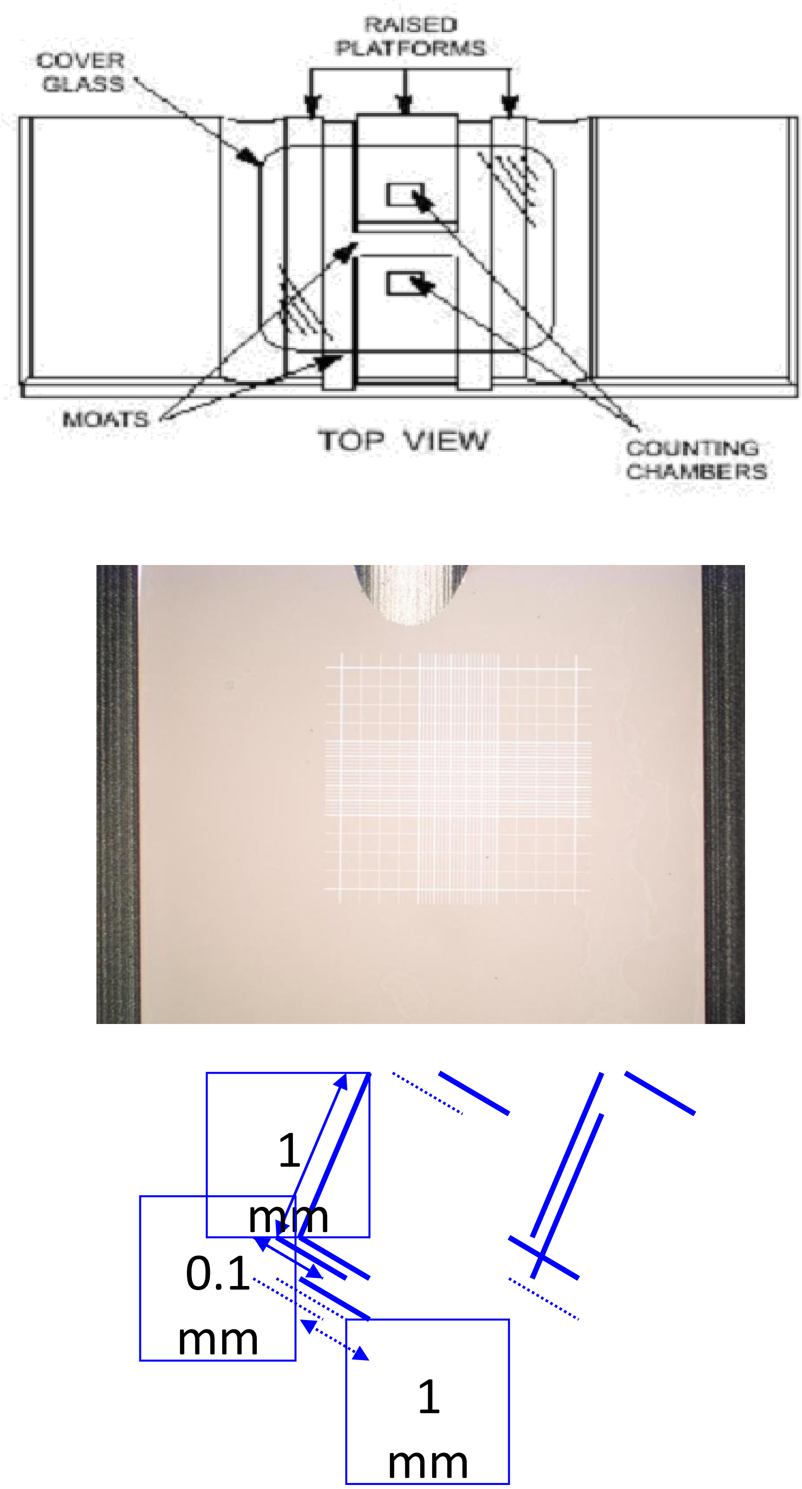

RAISED
PLATFORMS
COVER
GLASS
MOATS
TOP VIEW
COUNTING
CHAMBERS
1
mm
0.1
mm
1
mm

Figure 2

Evaluation of the status of culture medium in terms of PH

Most of cells grow properly in range of PH=7.4. Changing the color of medium during culture process serves as a pH indicator.

If the color at culture medium changes of yellow color, the reason can be as follows:

- Overgrowth of cells
- Bacterial Contamination
- High percent of CO_2 in incubator

Change of medium color to purple can be for the reasons as follows:

little growth of cells, Fungal infection, little concentration of CO_2 in incubator.

Packed cell volume method was fulfilled using Valupac® tubes and a suitable measurer after centrifuging for 1 minute in 2650g PCV.

Table 1: Color of culture medium at various PHs

PH	Under 65	6.5	7.0	7.4	7.6	7.8
Color	lemon color	Yellow	Orange	Red	Pink	Purple

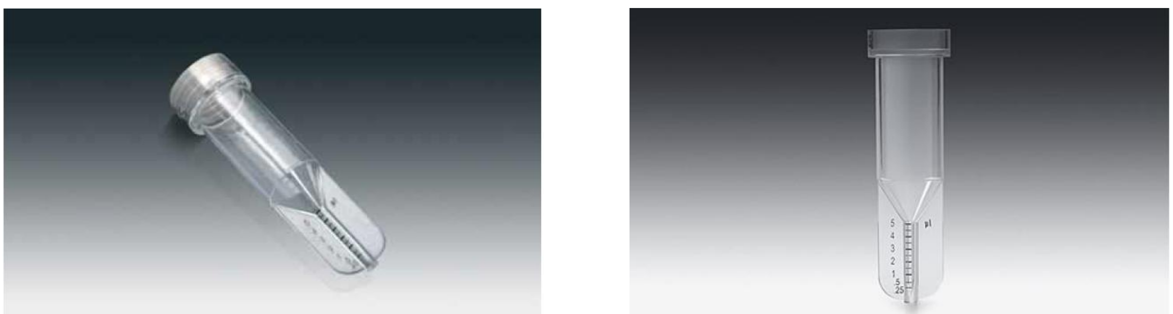


Figure 3: pH Indicator

Measurement of the percentage of transfection

ELISA

TPA proteins due to having natural biological activity cause rise of tissue plasminogen activator and transforming it to Plasmin, where the Plasmin causes hydrolysis of amide bond in polypeptides, and this is attributed as amylolytic activity. Thereby, measurement of amylolytic activity indirectly indicates biological activities of tPA. To examine amylolytic activity of tPA obtained in culture medium of transfected rCHODG44, the kit for the measurement of tPA activity produced from Biopool company with high sensitivity of measurement (0.5 IU/ml) was used. The basis of this kit is based on joint basis of t-PA to monoclonal antibody SP-322 coated in specific wells. After the proposed stages, protocol by presence of chromogenic substrate S-2251 and substrate plasminogen, absorption rate via Elisa reader were measured. The color formed equivalent to t-PA activity extent can be seen in the samples.

Figure 4: Elisa Reader

The existing materials in kit and other substances are as follows:

- Lyophilized.8well strips coated with monoclonal antibody SP-322
- PET buffer
- Nacl
- EDTA
- Tween 20
- Phosphate
- t-PA activity standard :Lyophilized human plasma containing 2IU/ml human melanoma t-PA
- Plasminogen reagent:Lyophilized plasminogen and FDP
- Glacial acetic
- HEPES buffer pH: 8.5
- HEPES
- Tween 20
- 0.2% Sodium azide
- EDTA
- Citrate buffer pH : 5.9
- Substrate reagent:Lyophilized H-D-But-CHT-Lys-Pna and Poly D-Lysin

Preparation of Buffers

1. Formation of buffers and stages of test were fulfilled based on protocol manufacturer's kit.
2. To depict standard curve, 500µl deionized distilled water was added to vial t-PA, and it was mixed for 5 minutes.

Various amounts of standard t-PA and citrate buffer were added to wells as follows:

Citrate buffer(µl)	(µl)	Concentration(IU/ml)
150	0	0
150	50	0.5
100	100	1.0
50	150	1.5
0	150	2.5

- 6ml HEPES buffer solution was added to plasminogen vial and mixed for 5 minutes.
- 6ml HEPES buffer solution was added to substrate vial and mixed for 5 minutes.

- Stop solution was prepared by mixing 1 ml glacial acetic acid into 9 ml distilled water. 1.7 m acetic acid was obtained, where it is used for doing any test.

Instruction

- 100µl of standard t-PA samples (0, 0.5, 1.0, 1.5, 2.0IU/ml) was added to each of sink s.
- 2- 100µl of dilution 1:100 Supernatant was added to culture mediums of transfected cells.
- 3-Sink strip was shook for 30 minutes at room temperature in 600 rpm.
- 4-contents of sink strips were removed and omitted by reversing sinks and building four hits on the absorption surface.
- 5-the sinks were washed with PET buffer solution for four times given the stage 4.
- 6-50µl of substrate and plasminogen was added to each sink, and the strips were placed on shaker 600 rpm for 80 minutes at room temperature.
- 7-to stop reaction, 50µl of stop solution was added to each sink, and the strips were placed on shaker 600rpm for 1 minute.
- Optical absorbance in test at wavelength 402 and 405 were read

Determine Glucose concentration

- Firstly, 6gr Glucose powder was solved in 10 ml distilled water. Then, dilutions (1:100, 1:10, 1.0) were prepared. Concentration of each of dilutions was read with NANODROP-1000device.

Cell freezing

Cell lines which are reproduced as well as cell lines which are finally removed, a storage must be kept in freezer so as to avoid cell mutation and protect cell line against contamination and other unfavorable disasters. The process of Cell freezing almost fulfills in all cell lines in a similar way. When the cells keep growing, this time will be the best time for storing them. DMSO is a suitable maintenance composition for freezing cells, which barricades formation of crystal in cells membrane. Process of Cell freezing is fulfilled in several cooling stages. This process in which the cells become gradually cold is called gradual freezing. The considered cells must not have any microbial contamination. The cells must be healthy, growing logarithmic phase. For this, the cells must be of the cells which are in the stage before reaching to cell congestion stage.

Instructions

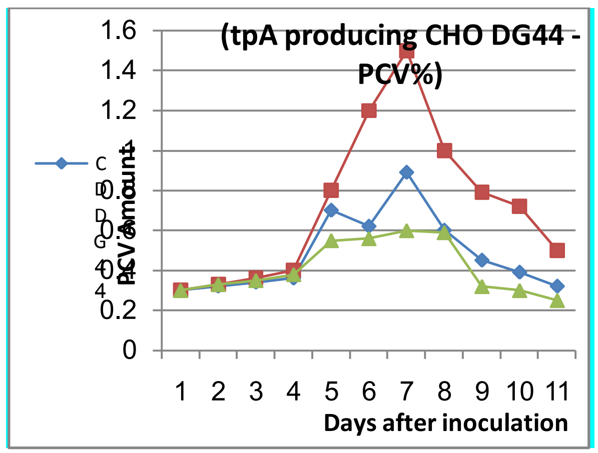
- Confluency of cells must reach to over 90% (90% confluence). Growth of cell beyond or under allowed level will reduce the lifetime of cells in freezing process.

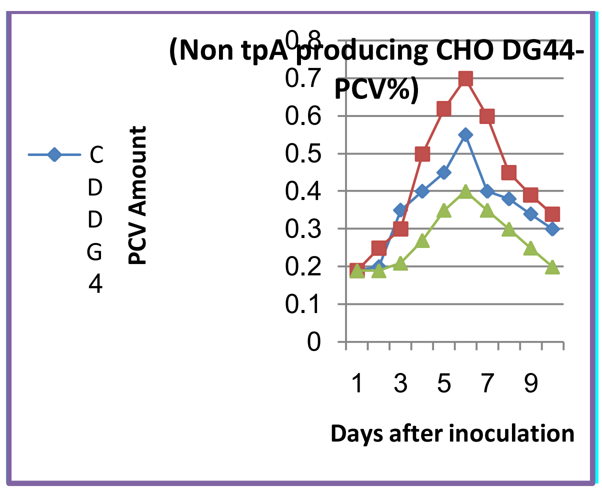
- Capacity of each vial for storing cells ranges from 4 x 10 ^{6} cell/ml to cell/ml2 x 10 ^{7}.
- -cells in culture medium for freezing at volume 1 ml are transferred to each vial.
- Vials containing the cell at temperature 70°C must be kept for 12 hours.
- Finally, vials are placed in freezing containers inside nitrogen tank at -196 °C.
- For long term maintenance, the culture medium must encompass 30% FBS + 10% DMS

DISCUSSION AND CONCLUSION

Determination of growth pattern of cells CHO DG44 in various culture mediums

CHO clone was similarly cultured in three culture mediums CDDG44, proCHO5 and BRC with various chemical compositions to examine growth pattern. As the results **(Figure 5)** indicate, the highest rate of cell density and cell growth has been obtained in proCHO5 medium. Furthermore, the obtained results do not indicate a significant difference in terms of growth of cell CHO in extent of cell density and PVC in culture mediums CDDG44 and BRC. However, extent of these factors in medium CDDG44 was a little more. The highest extent of cell density was in a culture period in 11 days that the extent of living cells started to decrease from the sixth day. This comparison was made on transfected CHO cells at three mediums CDDG44, proCHO5 and BRC with various chemical composition, yet the highest rate of cell density was seen in ninth day, while this decrease was observed intransfected cells at medium proCHO5 at seventh day.





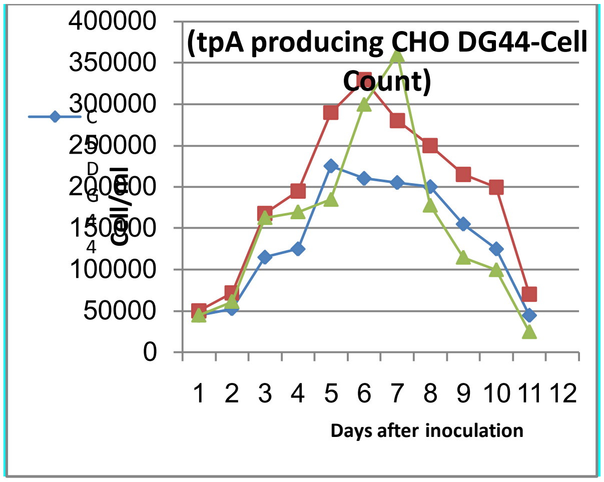


Figure 5: Determination of growth pattern of cells CHO DG44 in various culture mediums

Concentration of peptone relying on type of culture medium

CHO cells producing t-PA at three culture mediums including CDDG44, proCHO5 and BRC were cultured with various chemical compositions. Peptones with concentrations 1g/L and 2g/L with plant origin were added to these mediums. Adding peptone in medium proCHO5 caused increasing cell mass and PCV. Peptone with concentration 1g/L of peptones 6, 2, 5, 3 and 4 caused increasing cell density.

Amount of PCV was seen in both concentrations of peptones except for peptone 5 in concentration 2g/L. adding peptone in medium proCHO5 causes increasing cell density, where decrease has been only seen by presence of peptone 1 by increasing amount of PCV.

Peptones with concentration 1g/L of peptones 6, 5, 2, 4 and 3 were seen with highest amount of cell density for about 115%, 59%, 54%, 45% and 20%.

Peptones with concentration 2g/L of peptones 6, 2, 5, 4 and 3 were seen with highest amount of cell density for about 156%, 70%, 65%, 55% and 18%.

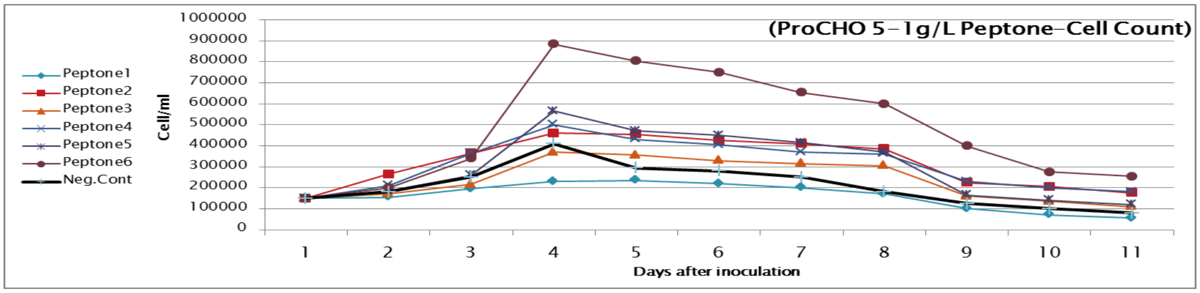
Amount of PCV for all the peptones except for peptone 5 which had a decrease in concentration 2g/L, indicated a significant increase in both concentrations 1g/L and 2g/L.

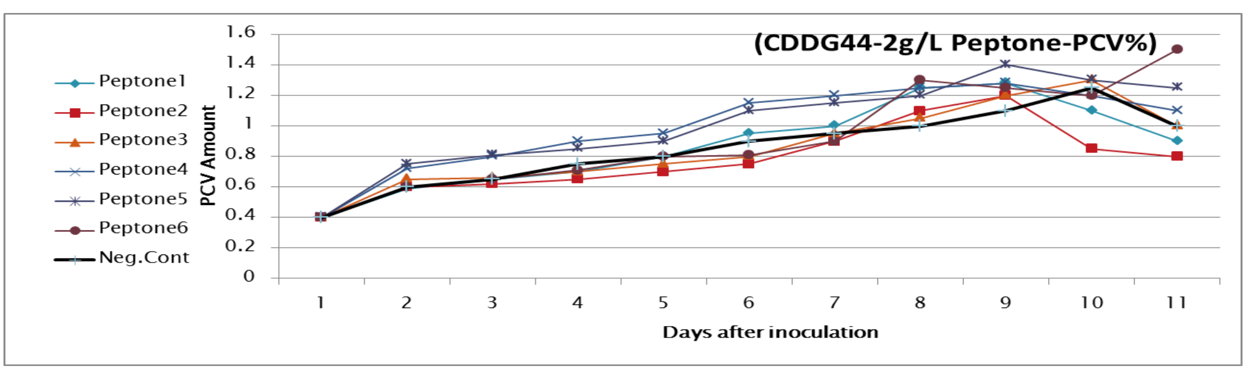
In medium CDDG44, cell density and biopsy have increased in peptones 1, 2 and 6 after adding concentrations 1g/L and 2g/L. the highest increase of cell density in peptone 1 was observed about 43%.

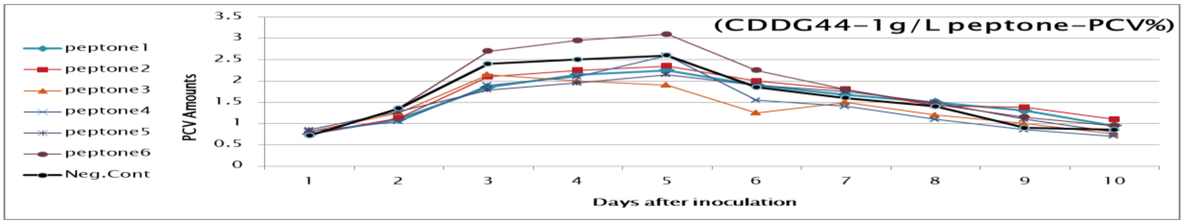
The highest amount of PCV in concentration 1g/L in peptone 6 was about 54%, and the amount of PCV in concentration 1g/L in peptone 2 and 3 was in turn about 27% and 23.3%. Thereby, feeding process with concentration 2g/L caused an increase for about 72%, 63% and 21% in PCV mediums containing peptones 2, 6 and 1.

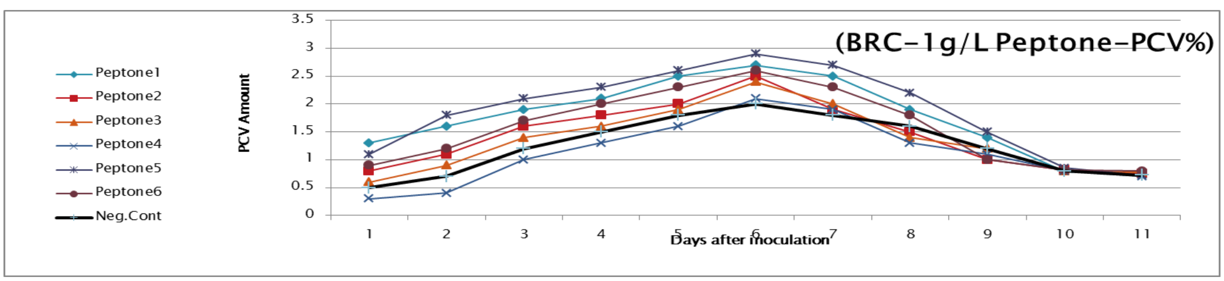
All these investigations were conducted in medium BRC with concentration 1g/L and 2g/L, whereby all the peptones except for peptone 4 caused an increase in cell mass and PCV, where the amount of PCV for peptones 5, 1, 6, 2 and 3 indicated as increase for about 45%, 35%, 30%, 25% and 20%, respectively.

However density of living cells did not indicate a significant increase, peptone 4 was just an alternative which had no significant effect on medium CDDG44. Further, results of peptone with concentration 2g/L had no significant effect on cell density.





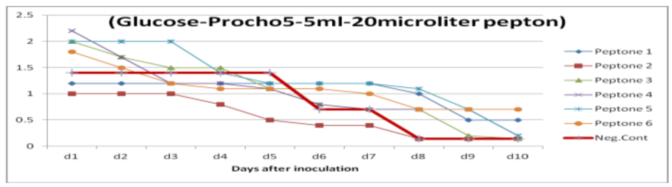


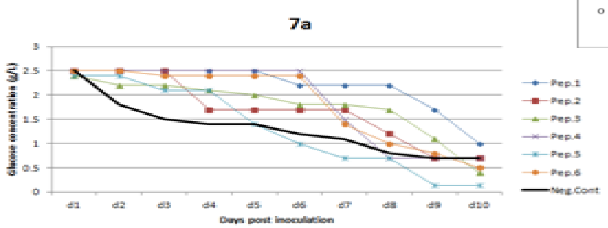


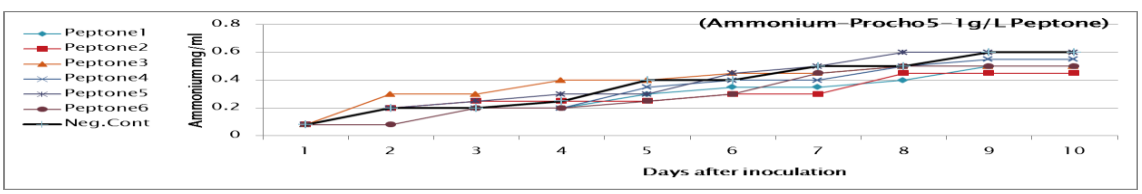
Investigation of metabolic changes in cells CHO DG44 producing t-PA

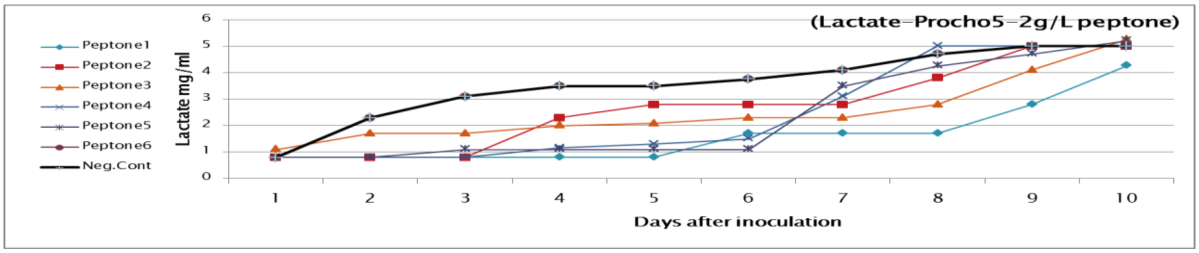
Investigation of metabolic changes in medium proCHO5

CellsCHO DG44 were cultured in medium proCHO5 with concentration 2g/L, where lower amount of glucose was consumed in culture period. Conversely, glucose in concentration 1g/L of peptones 1, 2 and 6 has been less consumed. Production amount of ammonia in samples fed with peptone compared to control sample in 4-7 days of culture was lower, but kept increasing in 8-10 days of culture. Mean of produced ammonia during 10 days of culture was determined in concentrations 1g/L and 2g/L of peptone. Amount of produced lactate at the end of culture by presence of peptones 4, 5 and 6 has kept increasing. Interestingly, this amount in both concentrations 1g/L and 2g/L of peptones has been about 5.03mM and 5.3 mM.





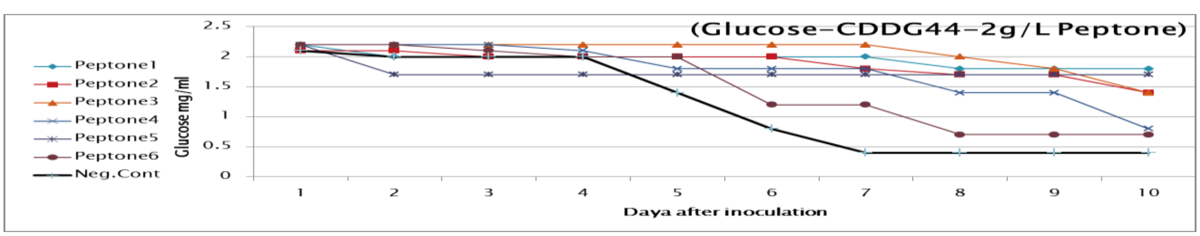


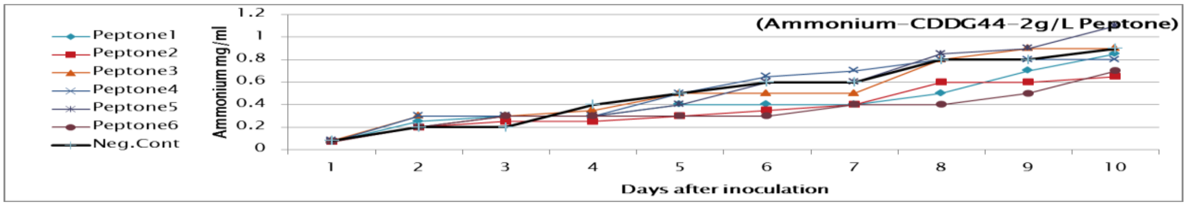


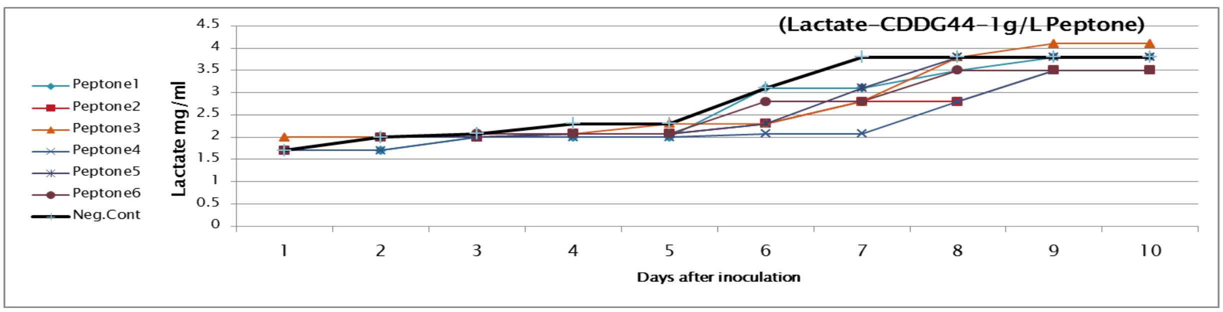
Metabolic changes at medium CDDG44

As shown in figure above, it can observe that consumption patterns of supplements at medium CDDG44 with concentrations 1g/L and 2g/L of peptone indicates increasing consumption of glucose in peptone.

Further, amount of ammonium production by presence of peptone has kept increasing by day 6, and then this amount remained fixed from day 6 to 10. Mean of concentration of ammonia in concentrations 1g/L and 2g/L in turn equals to 0.58 and 0.83mM. figures above indicate concentration of lactate during culture. All the peptones except for peptone 3 have produced lower lactate at concentration1g/L, where the final amount of lactate produced in day 10 for concentrations 1g/L and 2g/L has been in turn equal to 4.18 and 5.46.



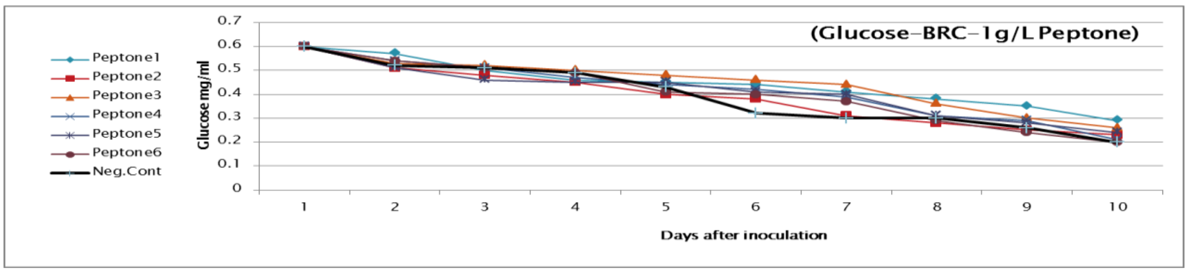


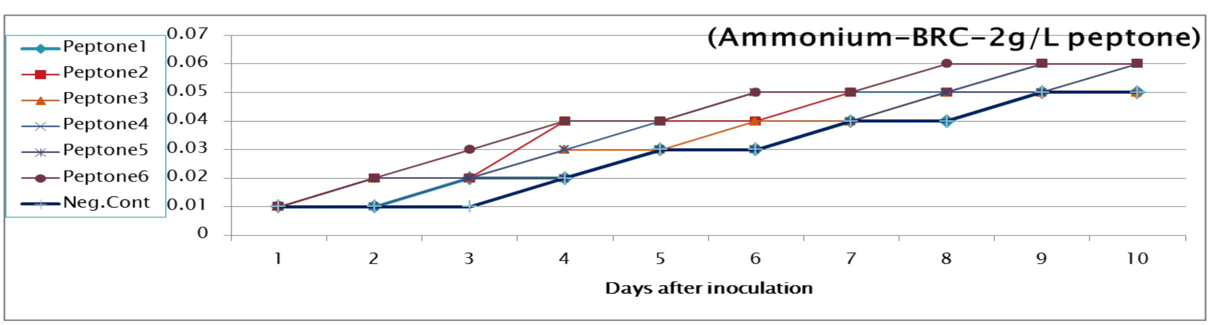


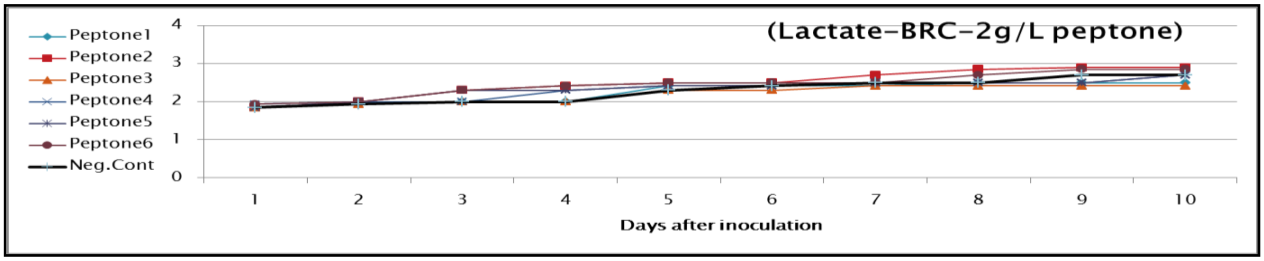
Investigation of Metabolic changes in medium BRC CD

Metabolic pattern in cells Cho in medium BRC CD indicates that feeding strategy with peptone supplements causes an improvement in conditons of growth of cells at this medium. Thereby, no significant change was seen in consumption amount of glucose.

On amount of ammonia produced during culture, mean of ammonia production was measured about 0.43 g/L by increasing ammonia production by presence of peptone 4 and 6 in day 10, where this amount is lower than amount of ammonia for about 0.5g/L. further, production of lactate in medium BRC fed with peptones does no indicated a significant increase. Nevertheless, final amount of production of lactate by presence of peptone has been equal to 6.03mM, where this is a little more than amount of lactate(5.6 mM) produced in control sample.



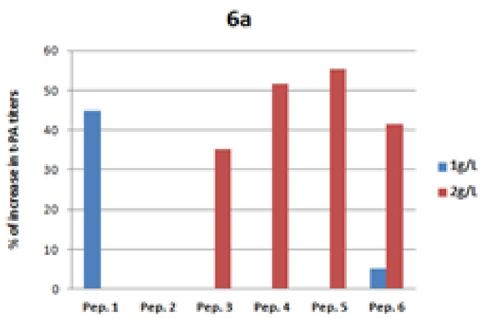


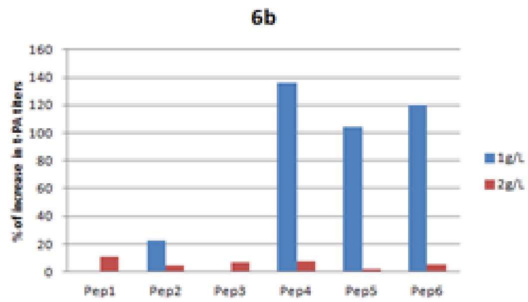


Investigation of effect of peptones on expression of recombinant protein

As the results indicate, an increase in expression amount of protein for about 55%, 51%, 41% and 35% was seen in medium proCHO5 with concentration 2g/L by presence of peptones 5, 4, 6 and 3, respectively. Further, an increase for about 44% in expression amount was seen in peptone with concentration 1 g/L. average amount of ammonia in day 9 for concentration 1 g/L and 2 g/L has been in turn equal to 0.85 mM and 0.51 mM. Lactate accumulation as a byproduct by presence of peptones 4, 5 and 6 has been seen with a significant increase.

Interestingly, the same pattern in amount of lactate accumulation was observed by presence of concentrations 1 g/L and 2 g/L of these two peptones. Further, amount of 5.03 and 5.3 lactate has been produced by presence of these two peptones. On the other hand, an increase of lactate accumulation for about 136%, 120%, 104% and 22% was observed in peptones 4, 5, 6 and 2 with concentration 1g/L in medium CDDG 44, where no significant increase was observed in lactate accumulation with concentration 2g/L of peptone. In medium BRC, Increase of expression for about 139%, 60.36% and 33.64% was seen in day 9 with concentration 2g/L in peptones 1, 6 and 5. The same as medium CDDG44, no significant difference in amount of expression was seen in control group with concentration 2g/L in peptone.





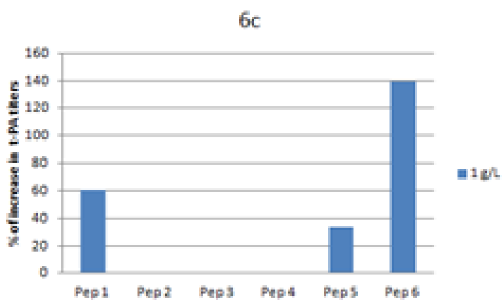


Fig.6: Percentage of increase in t-PA titers in day 9 with 1 and 2g l^{-1} peptone concentrations. **Fig. 6a**: ProCHO5 medium supplemented with peptones, **Fig. 6b**: CD DG44 medium supplemented with peptones, **Fig. 6c**: BRC CD medium supplemented with peptones.

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