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14. ABSTRACT

This report describes the design of a CGMP facility for yeast and bacteria on the first floor and basement of Othmer Hall, the home for the University of Nebraska-Lincoln Biological Process Development Facility. Design and a preliminary cost estimate is completed and the Program statement for the project is being submitted to the University of Nebraska Board of Reagents for approval. This report also describes research on the expression of antibodies in Chinese Hamster Ovary (CHO) cells against serotype A botulinum neurotoxin. We have completed construction of a simplified and easy to use CHO expression plasmid for the light and heavy chain, a system for selection of higher expression clones using dfr and zeocin for selection and GFP (green fluorescence protein) and flow cytometery for selection of higher expressing clones, and finally a development of a defined media for fed-batch production of mAb in CHO.

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Table of Contents

Introduction

The funding for this project is comprised of two components. The first component, which represents 95% of the funding, is dedicated to filling out shell space in Othmer Hall, the home for the Biological Process Development Facility. The other 5% is dedicated to research focused on the expression of antibodies against the botulinum neurotoxins in Chinese Hamster Ovary (CHO) cells. This report will provide a summary of May 2004-August 2006 activities, which includes the design of a scaled-down Current Good Manufacturing Practice (CGMP) facility for yeast and bacteria at the 150 L working volume bioreactor scale and mammalian cell culture research of a chimeric full-length antibody against serotype A and methods to screen transformed mammalian clones. The report is structured with the first part describing the construction and design activities followed by three separate reports the describe cell culture research activities.

Body

Construction Project

cGMP Construction Project

On December 2003 the University Of Nebraska Board Of Regents (BOR) approved the CGMP basement project, which included conceptual design to bid documents. During this process it became apparent that building the processing suites in the basement was not the preferred option. The design was changed to include the processing space of the CGMP facility on the first floor of Othmer Hall and the raw material storage and the CGMP utilities in the basement of Othmer Hall. A sketch of the first floor (Figure 1) and basement (Figure 2) are presented below. The intent is to use modular clean rooms for the CGMP processing space and a majority of our existing CGMP processing equipment in this facility. As per University requirements, this new design proposal must be submitted to the University of Nebraska Board of Reagents for approval and is scheduled for the November 2006 BOR meeting.

The CGMP facility will have the following capabilities:

Fermentation Suite: 150 L working volume fermentor for yeast and bacteria, harvesting and clarifying capabilities using either continuous centrifugation or cross flow membrane filtration and cell disruption.

Purification Suite: 0.1 to 6 L/min chromatography skid and two ultrafiltration systems.

Asceptic Processing Suite: Isolator system with a VHP (vaporized hydrogen peroxide) unit for sterilization capable of producing Master and Working Cell Banks and manual filling of upto 1500 liquid vials.

First Floor Support Space: Dirty staging, clean staging, buffer prep, and gowning.

Basement Support Space: Pure steam generator, pure steam condenser to produce water for injection (WFI), WFI storage tank, water treatment system (softener and reverse osmosis system) for the pure steam generator, Clean-In-Place system, and WFI distribution system. Included are room temperature and cold room storage for released and quarantined raw materials, final product release and quarantine storage ($4^{o}C$ and $-80^{o}C$).

It is anticipated that upon approval it will take approximately 12 months to build and an additional 3 to 5 months to validate. If fully approved in November 2006, the project will start first quarter 2007.

Figure 1. First Floor Layout of CGMP Facility

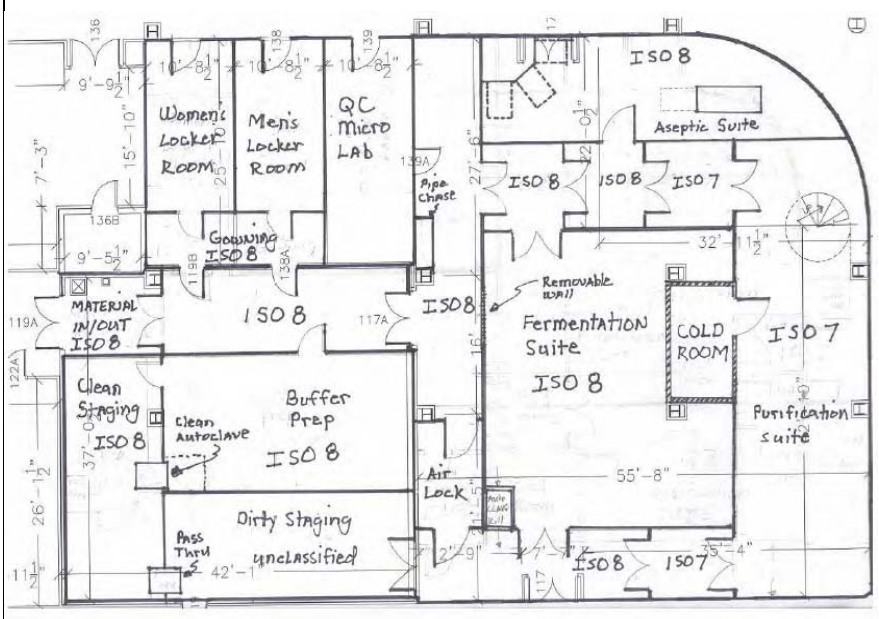
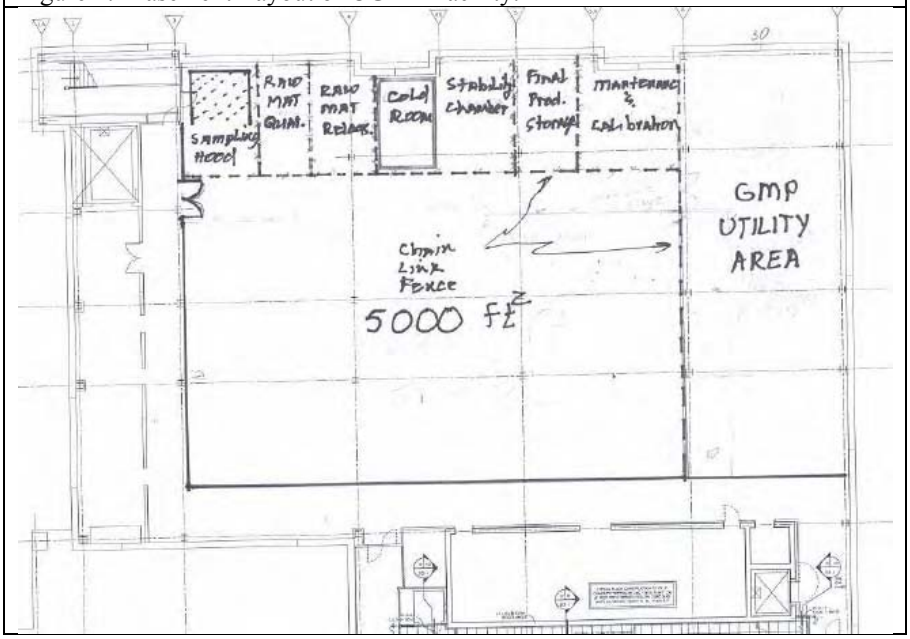


Figure 2. Basement Layout of CGMP Facility.



Monoclonal Antibody Research Project

Construction of pG6-GFP-Zeo for expression of light and heavy chains of S25 Antibody.

Introduction

Botulinum neurotoxins (BoNTs) are listed as one of the 6 highest risk threats for bioterrorism by the Centers for Disease Control (CDC) due to their potency, lethality, and ease of production. Botulinum neurotoxin is expressed in the bacteria *Clostridium botulinum* and is one of the most poisonous substances known. The large scale production of humanized monoclonal antibodies (huMabs) with neutralizing activity against toxins (i.e. botulinum neurotoxin) has been identified as one of the highest priorities to counter bioterrorism as drugs for the treatment of botulism are currently unavailable. Recently, potent neutralizing monoclonal antibodies against botulinum neurotoxin serotype A (BoNT/A) were identified, characterized and further cloned to yield humanized Mabs against BoNT/A. In our previous published study, we produced S25 antibody against botulinum neurotoxin serotype A (BoNT/A). However, procedures to obtain stable cell lines are lengthy and the plasmids are not easily applicable for any antibody production. Our overall goal is to develop a mammalian expression system that can be used for production of humanized antibodies and to develop and optimize a production strategy for the large scale production of the antibody based therapeutics for countering BoNT and other agents of biowarfare and bioterrorism. To achieve these objectives, we constructed an expression vector that the heavy and light chains of any antibody could be sub-cloned in two steps. Selection with a dual selectable marker and subsequent screening based on Green Fluorescence Protein (GFP) decreased the number of clonal cell lines that needed to be screened, and allowed for isolation of an optimized production cell lines. Once this strategy was proven to work for S25 antibody production, we have then constructed general plasmids that can be utilized for any antibody production in CHO cell lines. Outline of these experiments and resulting plasmids are given in the experimental procedures section of this report.

Experimental Procedures

Starting with the original plasmid of pcDNA3.1 (+) (Invitrogen) (Fig. 1), the experimental steps to construct the general expression vector pG6-GFP-Zeo and the cloning of the heavy and light chains of S25 antibody in this plasmid to obtain pG9-GFP-Zeo is given below. All PCR performed with Hot Star Pfu Turbo (Stratagene) DNA polymerase. All ligations were performed using Quick Ligation kit (Bio-lab laboratories).

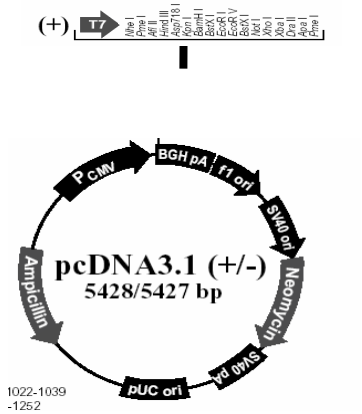


Fig. 1 Diagram of plasmid of pcDNA3.1 (+)

1. PcDNA3.1 (+)/Neo (-) (pG1)

Cut pcDNA 3.1(+) with Pvu II
And removed PvuII fragment from pcDNA3.1 (+),
Relegate

2. PcDNA3.1 (+)/Neo (-)/MCS_t (pG2)

Cut pG1 with Hind III and EcoR V, Klenov
And truncate MCS in 1, relegate

3. PcDNA3.1 (+)/Neo (-)/MCS_t/β-globin.dhfr (pG3)

(See Fig. 2)

PCR amplification of fragment β-globin .dhfr
From plasmid ps25 and cut plasmid pG2 with Mfe
I and Nru I and inserted β-globin, dhfr unto this
Plasmid in front of CMV promoter

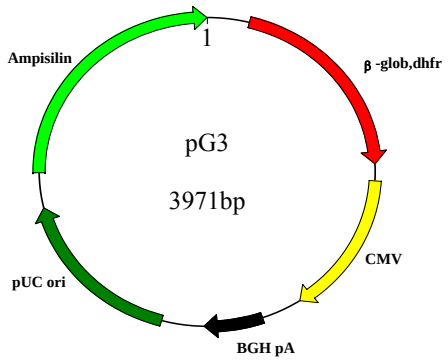


Fig. 2. pcDNA3.1 (+)/Neo(-)/MCS_t/β-globin.dhfr (pG3) construct

4. pG3/TKpA (pG4)

PCR amplification of fragment TKPA from
PREP4- and cut of pG3 with *Nru I* and *Mlu I*
and insert TKpA after dhfr into this plasmid

5. pG3/TKpA/MCS.SV40pA (pG5)

(See Fig. 3)

PCR amplification of fragment of SV40, MCS
From PREP4 and cut pG4 with *Bgl II* and *Mfe I*
and inserted SV40, MCS fragment into this
Plasmid, upstream of β-globin promoter in pG4

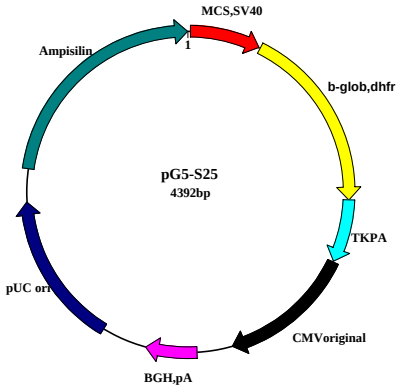


Fig. 3. pG3/TKpA/MCS.SV40pA (pG5) construct

6. pG3/TKpA/MCS.SV40pA/CMV (pG6)

(see Fig. 4)

PCR amplification of fragment CMV from pcDNA 3.1(+) and cut pG5 with *Bgl II* and inserted this CMV fragment in front of MCS2 in plasmid pG5.

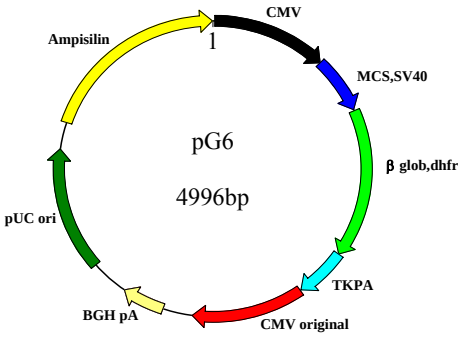


Fig . 4. pG3/TKpA/MCS.SV40pA/CMV (pG6) construct

7. pG5/S25.LC (pG7)

PCR amplification of LC from ps25 plasmid And cut pG5/S25 with *Apa1* and inserted LC fragment in MCS1 of pG5.

8. pG5/S25.LC/S25.HC (pG8)

PCR amplification of HC from ps25 plasmid
And cut pG5/S25/LC with *BamH1/HindiIII* and inserted HC fragment in MCS2 of pG7

9. pG6/S25/LC (pG10)

PCR amplification of LC from ps25 plasmid and cut pG6/S25 with Apa1and inserted LC fragment Into pG6

10. pG6/S25.LC/S25.HC (pG9-S25)
(See Fig. 5)

PCR amplification of HC from ps25 plasmid and cut pG10 with *BamH1/HindIII* and inserted HC Fragment into pG10 plasmid

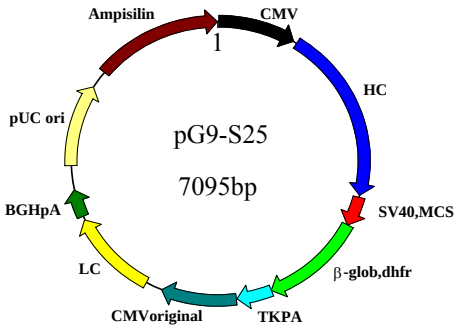


Fig . 5. pG6/S25.LC/S25.HC (pG9-S25) construct

11. pcDNA5/FRT/HC (see,Fig,6) PCR amplification of HC from ps25 plasmid

And cut pCDNA5/FRT with *Bam HI* and *Hind III* And inserted HC into this plasmid in the MCS of pc DNA 5/FRT.

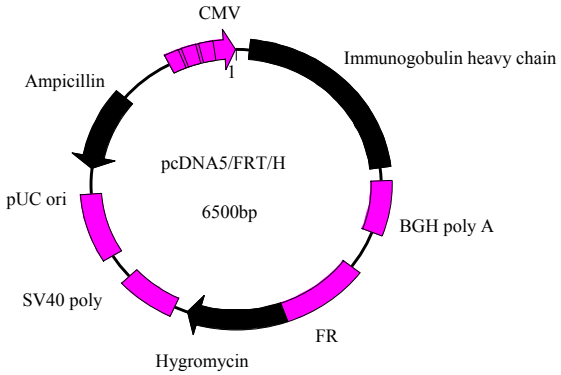


Figure 6. PcDNA5/FRT-HC construct.

12. pcDNA5/FRT/GFP PCR amplification of GFP from pTracerEF1α and Cut pc DNA5/FRT with Kpn *I* and *EcoRV* and Inserted GFP into this plasmid.

13. pF44/MMF Cut PcDNA5/FRT- F44 plasmid with *Mlu I/Mfe* and truncate and then *MluI / Mfe I* cut MMF-fragment ligate into this plasmid

14.	pF57/MMF	Cut PcDNA5/FRT- F57 plasmid with *Mlu I/Mfe* And truncate and then *MluI/Mfe I* cut MMF- fragment ligate into this plasmid
15.	pF44/MMF/GFP	PCR amplification of GFP from pTracerEF1α And cut pF44/MMF with *Kpn1/EcoRV* and inserted GFP into pF44/MMF
16.	pF57/MMF/HC	PCR amplification of HC from ps25 plasmid And cut pF57/MMF with *HindIII/BamH 1* and inserted HC into pF57/MMF
17.	pF57/MMF/GFP	PCR amplification of GFP from pTracerEF1α And cut pF57/MMF with *Kpn1/EcoRV* and Inserted GFP into pF57/MMF
18.	pF44/MMF/HC	PCR amplification of HC from ps25 plasmid and cut pF44/MMF with *HindIII/BamH1* and Inserted HC into pF44/MMF

19. Construction of PG6-GFP-Zeo Plasmids

To decrease the time and effort required to isolate high expressing gene amplified cell lines, we constructed the plasmid pG6-GFP and pG9-GFP (see Fig. 6 and 7), containing GFP-zeocin. It took 6 steps (see at the above) to construct of pG6. This plasmid contains two CMV promoters and two different multiple cloning sites for the insertion of the light and heavy chain. It also contains dhfr driven by a β- globin promoter and 3 different polyadenylation sites.

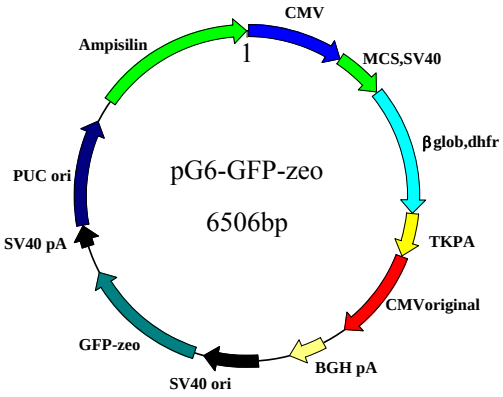


Fig. 7. Diagram of plasmid pG6-GFP-zeo.

20. Construction of pG9/GFP-Zeo.

pG9 is constructed after insertion of the light and heavy chain into plasmid pG6. After insertion of GFP to this plasmid, It is expected that this will result in high antibody production upon selection in both Zeocin and α-MEM (-). This plasmid will allow us to screen the amplified cells by flow cytometry, providing us the ability to analyze a significantly higher number of cell lines. This also gives us the ability to concentrate cells with high GFP production during the gene amplification process.

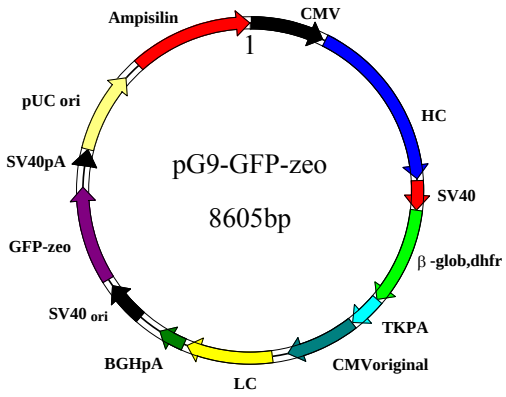


Fig.8. Diagram of P pG9-GFP-zeo

SELECTION OF CHO CELLS WITH HIGH MONOCLONAL ANTIBODY PRODUCTION USING TWO INDEPENDENT SELECTABLE MARKERS

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ABSTRACT

Isolation and selection of mammalian cell lines with high antibody production is one of the most critical and laborious steps in obtaining a high yielding cell culture process. Selection with a dual selectable marker and subsequent screening based on GFP fluorescence could decrease the number of clonal cell lines that need to be screened, and allow for the isolation of an optimized production cell line. CHO-DG44 cells were transfected with a plasmid (pS25-gfp) containing dual selectable markers, gfp-zeocin fusion and dhfr. Cells selected based on dhfr and zeocin showed a large increase in antibody production in comparison to cells selected based on dhfr or zeocin alone. Fifty-five percent of the cells selected showed antibody titers of greater than 1 μg/ml in comparison to less than 5% using either zeocin or dhfr alone. After three days in adherent culture, a cell line selected using both selectable markers produced 27 μg/ml S25 antibody and had a specific productivity of 15 pg/cell/day. The highest producing clone obtained using dhfr or zeocin alone resulted in a specific productivity of 3.1 pg/cell/day. To confirm that a high producing production cell line could be obtained the cells were transferred to suspension culture and were grown in both batch and fed-batch culture. An antibody concentration of 74 μg/ml was obtained after 6 days in batch culture, while 250 μg/ml was obtained in fed-batch culture. These correspond to specific antibody productivities of 5 and 15 pg/cell/day, respectively. These results show that a dual selectable marker can be used to obtain a high producing production cell line and further improvements could be obtained by selection based on GFP fluorescence.

INTRODUCTION

Production of therapeutic monoclonal antibodies and antibody derivatives constitutes 20% of the biopharmaceutical products in development (Roque, et al. 2004). These antibody products are typically produced in mammalian cells such as Chinese Hamster Ovary (CHO) and NS0, a mouse myeloma derived cell line. To obtain a stable antibody producing cell line, cells are transfected with a plasmid or plasmids containing the light and heavy

chain genes along with a selectable marker, such as an antibiotic resistance gene. Since a wide range of antibody productivities is achieved in the transfected population, a single cell clone is usually selected in order to obtain a culture with high monoclonal antibody productivity.

Various methods have been used to obtain cells with high productivities. These include the use of an amplification strategy using dihydrofolate reductase (dhfr) in CHO cells (Alt, et al., 1978; Urlaub and Chasin, 1980) and glutamine synthetase (GS) in NS0 (Cockett et al., 1990; Bebbington et al., 1992). Selection at increasing concentrations of methotrexate (MTX) in the case of dhfr, or methionine sulfoximine (MSX) for GS results in an increase in amount of integrated DNA and more importantly an increase in antibody or protein expression. The efficiency and stability of gene amplification is dependent on the location of insertion in the chromosomal DNA in the selected clone (Kim et al., 1998; Yoshikawa et al., 2000).

In addition to gene amplification, several methods of improved clonal selection have been employed. Meng et al. (2000) used green fluorescent protein (GFP), along with dhfr, as a second selectable marker for the isolation of CHO DXB11 cells with high protein expression. NT3, DNase or VEGF, along with dhfr, were expressed from a single SV40 promoter contruct separated by an internal ribosomal entry site (IRES), while GFP was produced from a second SV40 promoter. Protein productivity corresponded well with GFP fluorescence. In addition, a single clone with high VEGF production was isolated by selection only for GFP fluorescence by flow cytometry. FACS sorting produced a clone with a specific productivity of 4.4 pg/cell/day compared to 0.7 pg/cell/day selected by dilution cloning alone.

To obtain a clone with high antibody productivity, a plasmid was constructed for the production of the light and heavy chains of a chimeric antibody against botulinum neurotoxin serotype A. In addition to the light and heavy chain fragments, the plasmid contained dhfr and a gfp/zeocin fusion protein for selection of stable clones. Selection on dhfr and/or zeocin were used and GFP production was correlated with antibody production. It is suggested that two selectable markers on a single expression plasmid may allow for greater clonal selection and stability than a single selectable marker. GFP was included in order to quickly analyze numerous clones after the initial selection procedure.

MATERIALS AND METHODS

Contruction of plasmid vectors

CHO-DG44 cells, obtained from Dr. Larry Chasin (Columbia University), were maintained in α-MEM media containing 8% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). To construct the pS25-GFP plasmid (Figure 1), pTracer EF/V5 His A was cut with Csp I, blunted with the klenow fragment of DNA Pol I, and then cut to Bsm I. This fragment containing the full cDNA for the gfp-zeocin fusion was inserted into the Sma I / Bsm I site of the pS25 plasmid (Obtained from Jim Marks, University of San Francisco). The G418 fragment from the original pS25 plasmid was replaced by the gfp-zeocin fragment, allowing for selection using zeocin and detection of expression clones based on GFP fluorescence. The original pS25 plasmid contains the light and heavy chain fragments of the S25 antibody against botulinum serotype A driven by dual CMV promoters, in addition to dhfr driven by a β-globin promoter (Mowry et al., 2004).

CHO-DG44 cells were transfected with either pS25 or pS25-gfp plasmid using Lipofectamine 2000 reagent (Invitrogen). Briefly, CHO-DG44 cells were seeded in 1 ml α-MEM media containing ribonucleotides and 8% FBS at 2 x 10^5 cells/ml. One μg plasmid DNA, linearized with Nru I, and 0.5-2 μl Lipofectamine 2000 were combined in Opti-MEM media (Invitrogen) and equilibrated for 20 min. The DNA/Lipofectamine mixture was added to CHO-DG44 cells at 24 h after seeding and the cultures were grown overnight. The transfected cells were fed with fresh growth media 24 h after the transfection and selection media was added 3 days after the transfection. Cells were selected in α-MEM media lacking ribonucleotides, α-MEM media lacking ribonucleotides & 400 μg/ml zeocin, or α-MEM media lacking ribonucleotides with 400 μg/ml zeocin supplemented with 8% FBS. These conditions will select for stable clones based on dhfr alone, zeocin alone or the presence of both dhfr and zeocin resistance. After several passages, stable clones were obtained by dilution cloning at 0.5 cells/well in 96 well plates.

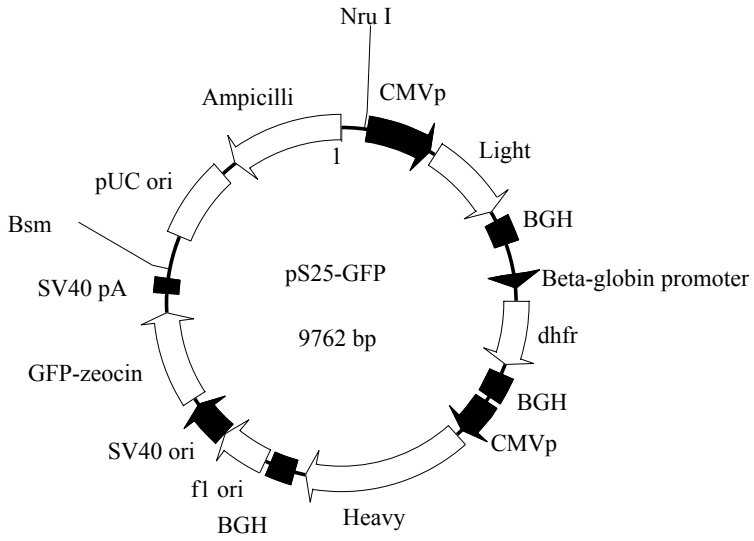

Figure 1. Diagram of pS25-gfp plasmid. Plasmid contains the light and heavy chains of the S25 antibody against botulinum neurotoxin serotype A, along with a GFP-zeocin fusion protein and dhfr as dual selectable markers.

ELISA

The concentration of the S25 antibody produced against botulinum neurotoxin serotype A was determined using an enzyme-linked immunosorbent assay. Botulinum neurotoxin serotype A H_C (BoNTA H_C) fragment was previously produced and purified from *Pichia pastoris* in our lab. BoNTA H_C was diluted to 5 μg/ml in coating buffer (100 mM $NaHCO_3$, 100 mM NaCL, pH 9.3). One hundred μl diluted BoNTA H_C was added to 96 well plates (Nunc, Rochester, NY) and incubated at 4°C overnight. The Plates were washed twice with dilution buffer (20 mM Tris-HCl, 50 mM NaCL, pH – 7.2) containing 0.1% Tween 20 and twice with dilution buffer alone. Blocking buffer (dilution buffer with 0.5% casein) was added to the plates and incubated at 37°C for 1 h. Diluted samples or S25

antibody standard were added to the wells and incubated at 37°C for 1 h. The plates were washed and 100 µl goat anti-human IgG-HRP conjugate (Sigma Diagnositics, St. Louis, MO) was added to each well of the plates. The plates were incubated for 1 h at 37°C, followed by the washing. The S25 antibody was detected by adding 100 µl of 1 mg/ml ABTS in ABTS buffer (Roche Applied Science, Indianapolis, IN). The absorbance was determined using an ELx800 plate reader (Bio-Tek, Winooski, VT) at 405 nm after incubating with shaking for 30 min at room temperature.

Flow Cytometry

CHO-DG44 cells were analyzed for GFP production using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Cells were trypsinized using 0.25% trypsin in HBSS and resuspended in α-MEM media containing 8% FBS. Cells were then centrifuged at 300 x g and resuspended in an equal volume of PBS. The relative fluorescence intensity of approximately 10,000 cells per sample was determined.

Growth of CHO-DG44 S25-gfp cells in suspension culture

The CHO-DG44 pS25-gfp cells were transferred to suspension culture in IS-CHO-V (Irvine Scientific) media by slowly decreased FBS content in spinner flasks. Cells were initially seed at 3 x 10^5 cells/ml in 50 ml IS-CHO-V with 1 % FBS. Cells were fed every 2-4 days with slowly decreasing levels of FBS. Cells were frozen and stored in liquid nitrogen after approximately 10-12 passages. For the batch runs, cells were taken for frozen stock and allowed to grow for 3-5 passages and were seeded in IS-CHO-V media at 2.2 x 10^5 cells/ml in IS-CHO-V media. Cell counts were determined using a hemacytometer and S25 antibody production was analyzed by ELISA.

Growth of CHO-DG44 S25-gfp cells in fed-batch culture

CHO-DG44 pS25-gfp cells were grown in 25 ml Wheaton spinner flasks to obtain cells for fed-batch culture. Cells were seeded at 2 x 10^5 or 1 x 10^6 cells/ml in 220 ml IS-CHO-V media supplements with glutamine and amino acids in a 1 L controlled spinner flask. The pH, dissolved oxygen (DO) and temperature were controlled using a Cellferm-Pro control system (DAS-GIP, Julich, Germany). The pH was controlled by the addition of CO2 and 0.5 M sodium bicarbonate. The glucose was controlled at 2 mg/L by the addition of a 30 mg/ml glucose solution. A second feed solution consisted of 3 mM glutamine in IS-CHO-V media supplemented with 10 x MEM essential and 10 x MEM non-essential amino acids (Invitrogen). The total amino acid concentration in the feed media was 46 mM including glutamine. The glutamine concentration was maintained at 0.5 mM. Glucose and glutamine concentrations were determined using a Bioprofile 400 analyzer (Nova Biomedical, Waltham, MA).

RESULTS AND DISCUSSION

Analysis of CHO DG44 cells transfected with pS25-gfp plasmid

CHO-DG44 cells were transfected with the pS25 plasmid and stable cells were isolated in selection media. Selection media consisted of α-MEM media containing ribonucleotides and deoxyribonucleotides (α-MEM(+)) supplemented with 400 µg/ml zeocin, α-MEM media lacking ribonucleotides and deoxyribonucleotides (α-MEM(-)), or α-MEM(-) media supplemented with 400 µg/ml zeocin. The three selection medias selected for stable transfectants based on zeocin alone, dhfr alone, or both zeocin and dhfr, respectively. Each of the selection media

were supplemented with 8% FBS. Single cell populations were isolated by dilution cloning at 0.5 cells/well in a 96 well plate. The transfected cells were allowed to grow for 14 days and the S25 antibody production was determined by ELISA. Resulted showed that very few cells selected based on zeocin alone or dhfr alone resulted in stable clones with high S25 antibody productivity (Figure 2). Three out of 40 cells isolated in zeocin had an antibody concentration greater than 0.5 μg/ml, with one having an antibody concentration of 2 μg/ml. Eighty clones isolated based on dhfr never showed an antibody concentration greater than 0.5 μg/ml, although a clone with a final antibody concentration of 2.3 μg/ml was previously obtained with a similar plasmid (pS25) (Mowry, et al., 2004). This plasmid was similar to the pS25-gfp plasmid, except the gfp-zeocin selectable marker was replaced with G418. This cell line (CHO-DG44 pS25 #56) provided a specific antibody productivity of 3.1 pg/cell/day. In comparison, 55% of 82 cells isolated using both α-MEM(-) and zeocin for selection resulted in a antibody concentration of greater than 1 μg/ml (Figure 2). This improvement in selection significantly reduces the number of clones that need to be analyzed to obtain the final production cell line. The S25 antibody concentration obtained 14 days after cloning was further analyzed in the cultures selected in the presence of α-MEM(-) and zeocin. Twenty-three percent of the clones had a final antibody concentration of 1-5 μg/ml, 15% resulted in a concentration of 5-10 μg/ml, 10% resulted in a concentration of 10-20 μg/ml and 5% resulted in an antibody concentration of greater than 20 μg/ml.

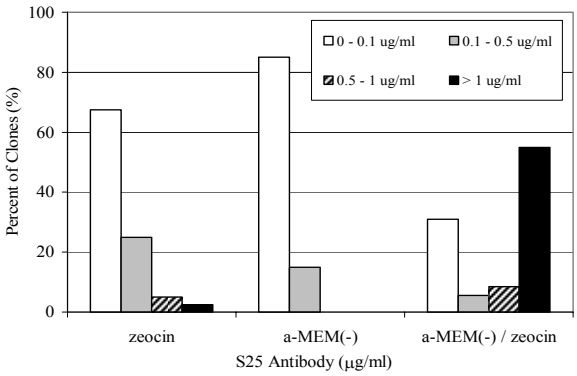

Figure 2. Isolation of S25 antibody producing clones based on zeocin resistance, dhfr, or both selectable markers. The use of both zeocin and dhfr resulted in a large increase in cells with high antibody production.

Analysis of S25 antibody production and GFP fluorescence

Fifteen CHO-DG44 pS25-gfp cell lines were analyzed for GFP fluorescence using a Becton-Dickenson FACScan flow cytometer in order to determine whether S25 antibody production correlated with GFP fluorescence. Cells with varying levels of S25 antibody production were compared and there was a definite correlation between S25 antibody production and relative GFP mean fluorescence (Figure 3). Cells were seeded at 2×10^5 cells/ml in a 75 cm^2 T-flask and allowed to grow for 3 days. The host CHO-DG44 cell line had a mean relative fluorescence of 12, while the highest S25 antibody producing cell line had a mean relative fluorescence of 121. The highest producing cell line had a S25 antibody titer of 27 μg/ml and a specific productivity of 15 pg/cell/day. Transfected CHO-DG44 cells with less than 1 μg/ml S25 antibody production after three days in adherent culture resulted in an average relative mean fluorescence of 30. These results suggest that cell sorting based on GFP fluorescence could allow for the isolation of cell lines with increased specific antibody production.

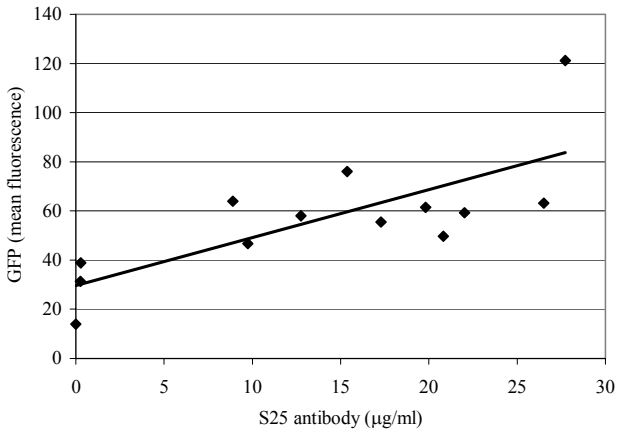

Figure 3. The correlation between the level of GFP and S25 produced.

CONCLUSION

These combined results show that cell lines with increased antibody production can be obtained by using dual selectable markers followed by flow cytometery to measure GFP to further isolate clones with high GFP production and antibody production. It should be noted that the gfp gene does not have to be on the same expression unit as the gene product of interest, in this case the S25 antibody.

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OPTIMIZATION OF SERUM-FREE MEDIA IN CHO-DG44 CELLS USING A CENTRAL COMPOSITE STATISTICAL DESIGN

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Abstract

Serum-free media was developed for the production of recombinant antibody against Botulinum Neurotoxin A (BoNT A) using dihydrofolate reductase deficient Chinese Hasmter Ovary cells (CHO-DG44) in suspension culture. A central composite statistical design, which is the most popular of the many classes of Response Surface Methodology designs (RSM), was used to optimize various media components. An initial control basal medium was prepared, which was similar in composition to HAM'S F12 : IMDM (1:1) supplemented with insulin, transferrin, selenium, and a lipid mixture. The vitamin concentration of the basal media was twice that of HAM'S F12 : IMDM (1:1). CHO-DG44 cells expressing S25 antibody grew from 2 x 10^5 cells/ml to a maximum cell density of 1.04 x 10^6 cells/ml after 5 days in this control media. A central composite design was used identify optimal levels and interaction between five groups of media components. These five groups were glutamine, essential amino acids (EAA), non-essential amino acids (NEAA), ITS (Insulin, Selenium, Transferrin), and Lipids. Fifty experiments were carried out in four batches with two controls in each batch. There was little effect of ITS and lipid concentration over the range studied, and glutamine concentration showed a strong interaction with EAA. The optimal concentrations of the variables studied were 2.5 mM glutamine, 7.4 mM (2X) EAA, 1.4 mM (0.5X) NEAA, 1X ITS supplement, and 0.7X Lipids supplement. The maximum viable cell density attained in the optimized media was 1.4*10^6 cells/ml, a 35% improvement over the control culture, while the final antibody titer attained was *22 μg/ml*, a *50%* improvement.

Introduction

Botulinum neurotoxin (BoNTA) produced by the anaerobic bacteria *Clostridium botulinum* is one of the most potent toxins known to humans (1, 2). The active neurotoxin contains two polypeptide chains, heavy chain (100KDa) and light chain (50KDa) connected via a disulfide linkage (3,4). Recombinant monoclonal antibodies (mAb) could provide unlimited supply of antitoxin free of infectious disease risk neutralizing deadly toxins with out requiring human donors for plasmapheresis (5). Recently, potent neutralizing monoclonal antibodies against botulinum neurotoxin serotype A (BoNTA) were identified, characterized, cloned and expressed in Chinese Hamster Ovary (CHO) cells to yield humanized mAb's against BoNTA (6).

The suspension culture based manufacturing process has become the method of large-scale, commercial production of therapeutically important proteins from rCHO cells (7). It is desirable to use serum-free media in suspension culture because serum can cause problems in the subsequent processes (8, 9). However there is no universal serum free media applicable to all cell lines, specific media suitable for each cell line need to be developed (10, 11).

The media used for an animal cell culture are very complex and the significance of particular compounds for cell growth or product synthesis is difficult to realize, therefore statistical methods are adopted to develop animal cell culture media. The traditional one factor at a time approach to optimization is time-consuming and incapable of reaching the true optimum especially because of interactions among the factors. Moreover, it assumes that the various growth parameters do not interact and the process response is a direct function of the single varied parameter (12, 13). In contrast, the observed behavior of growth results from the interactive influences of the various variables (14). Unlike conventional optimization, statistical optimization methods can take into account the interactions of variables in generating the process response. Response surface methodology (RSM), an experimental strategy for seeking the optimum conditions for a multivariable system, described first by Box and Wilson, is a much more efficient technique for optimization (15). RSM consists of a group of mathematical and statistical procedures that can be used to study relationships between one or more responses and a number of independent variables. In addition to analyzing the effects of independent variables, this experimental methodology generates a mathematical model that accurately describes the overall process (16, 17). Recently RSM has been employed to solve the multivariate problems and optimize several responses in many types of experimentation (18, 19, 20). In this approach, concentrations of medium components are the variables; each variable is referred to some base value and varies in a certain pattern. This pattern is designed by using statistical methods to yield the most information by a minimum number of experiments. In this study we adopt RSM approach to locate the optimum levels of Glutamine, EAA (Essential Amino Acids), NEAA (Non Essential Amino Acids), ITS (Insulin, Transferrin, Selenium,), Lipids, to gain an insight of interactions among these factors which were expected to have major influence on the response viable cell density. Glutamine acts as the primary source of nitrogen as well as additional carbon and energy source. It contributes precursors to the formation of the major intracellular binding blocks: amino acids, proteins. It has been estimated that between 30 to 65% of the cell energy requirement is derived from glutamine metabolism (21, 22). Amino acids are primary sources of nitrogen and they protect cells from nutrient deprivation (23) and elevated osmolarity (24) and elevated pCO_2 (25). Insulin serves as growth and maintenance factor (26) and is considered to be important for serum free cultures. The functions include stimulation of uridine and glucose uptake, and synthesis of RNA, protein and lipid, increases fatty acid and glycogen synthesis (27). Transferrin is considered one of the most essential growth promoting supplements in serum free media and its omission has led to severe inhibition of cell growth (28). It is iron binding glycoprotein, interacts with surface receptors and has an intimate role in the facilitation of the iron transport across the plasma membrane (29). It has additional in vitro functions such as chelation of deleterious trace materials, these functions are unlikely replaced by other components. Selenium is a trace element essential for mammalian cell cultures (30) mechanism is poorly understood. There was evidence that

selenium enhances growth rate in serum free cultures (31). Lipids are required for proliferation, differentiation, and antibody secretion. They play a major role in the cell membrane which is composed of phospo-lipid bi-layer, and helps in the transmission of nutrients into the cell and excretion of proteins out the cell (32). These major important functions exhibited by the variables made us to choose the response surface methodology to see the higher order interactions between these variables maximizing the response. Results were analyzed statistically by SAS program and optimum conditions were selected graphically. Interactions among these factors were also examined.

Materials and Methods

Cell Line and Media

The parental cell line was obtained by weaning CHO-DG44 off of serum according to standard cell culture techniques. The base medium used during the weaning process was commercial serum free medium CHO-S-SFMII, known to contain animal-derived proteins and hydrolysates. The process of weaning CHO-DG44 of its serum dependence lasted approximately 4-5 months. And the resulting cell line was used as the starting point for all subsequent development efforts including recombinant cell line generation, medium development studies. Recombinant cell lines were derived from our parent cell line using standard molecular biology techniques.

Media

The basal medium was prepared similar to the HAM'S: IMDM (1:1) media excluding Hypoxanthine and Thymidine, by adding components separately. The concentration of the inorganic salts, and other components like linoleic acid, lipoic acid, phenol red, putrescine 2HCl, sodium pyruvate, and HEPES is same as HAM'SF12:IMDM (1:1). The concentrations of the glucose and glutamine in the starting basal media were 4 g/L, and 4mM. For amino acids the media was supplemented with 1.75 X of Essential Amino Acids (EAA) and 1.75X of Non Essential Amino Acids, which come as 50X and 100X solutions from Gibco. The concentration of Individual Amino acids in EAA (50X) and NEAA (100X) solutions are given in Table 1. The additional components added to the basal media are vitamins, the concentrations of which were double to the concentrations in HAM'SF12:IMDM (1:1), 1X Insulin Transferrin Selenium (ITS), and 0.7 X Lipids Supplement, which come as 100X solutions from Gibco. The concentration of the Individual components in ITS and Lipids Supplements are given in the Table 1. The composition of the complete basal media is given in Table 1(a).

Cell Culture

The cell cultivation was performed in 37^0C humidified incubators supplemented with 5% carbon dioxide. The seeding density was $2*10^5$ cells/ml, and cell counts were performed every four days. The number of cells was determined using a hemocytometer. Spheroids would be enzymatically dissociated when spherical aggregates were observed. Two ml of sample was harvested from spinners and placed in 1.7 ml microtube and centrifuged at 1200 rpm for six minutes, and 1.5 ml supernatant was saved for antibody assays, 100 µl of trypsin solution (2.5% (w/v) Trypsin in PBS) was added to resuspend the cells of 400 µl. The cells were incubated at room temperature for fifteen minutes and the cell density and viability were then determined by then trypan blue exclusion method.

Antibody Assay

The concentration of the whole antibody, as well as the concentration of the light and heavy chain portions was determined by an enzyme-linked immunosorbent assay. Affinity purified rabbit anti-human IgG antibodies were diluted to 5 μg/ml in coating buffer consisting of 100 mM $NaHCO_3$ and 100 mM NaCl (pH = 9.3). 100 μl diluted antibody was added to 96 well plates (Nunc) and incubated overnight at 4°C. The plates were washed twice in Tris buffer (20 mM Tris-HCl, 50 mM NaCl, pH = 7.2) containing 0.1% Tween 20 and then twice in Tris buffer alone. Blocking buffer (Tris buffer with 0.5% BSA) was added to the 96 well plates and incubated at 37°C for 1 h. Supernatant samples were diluted in the blocking buffer and samples were loaded into the 96 well plates in triplicate. Plates were incubated for 1 h at 37°C and the washing procedure was repeated. 100 μl of a goat anti-human IgG-HRP conjugate antibody diluted to 0.5 to 2 mg/ml in the dilution buffer was added to the plates. The plates were incubated for 1 h at 37°C and the washing procedure was repeated. Lastly, 100 ml of 50 μg/ml ABTS in ABTS buffer (Roche) was added to the plates. The absorbance was determined at 405 nm using a ELx800 plate reader (Bio-Tek). This procedure was used for whole antibody, heavy chain (Fc specific), and light chain (κ specific). Whole, Fc and kappa rabbit anti-human IgG coating antibodies and whole, Fc and kappa goat anti-human IgG-HRP conjugated antibodies were used in the ELISAs (Sigma).

Experimental Design

Response surface methodology (RSM) was used to determine the influence of some medium components on the response of viable cell density and since we assume our product is growth-associated product, increase in viable cell density will ultimately increase the antibody production. Theoretical and fundamental aspects of RSM have been extensively discussed else where (16). The experimental design adopted Box's central composite design for five variables at five levels each. The five independent variables were X1 = Glutamine, X2 = EAA, X3 = NEAA, X4 = ITS, X5 = Lipids. The independent variable coded regions were -α (-2, Lowest Level), -1, 0 (middle level), I, and +α (2, highest level). The actual values, which were chosen from preliminary studies, and the corresponding coded and uncoded values of the five independent variables are given in Table 2. The complete design has 42 experimental points, which included 8 replications of the center point. The treatment combinations and observed responses are presented Table 3. The 50 experimental media runs were prepared in random order and the experiments were performed in four batches. The dependent variable (Y) was viable cell density and was assumed to be affected by the five independent variables. Based on data from this design we fit a second order or higher order polynomial regression model described as follows:

$$Y = b_l + \sum_{i=1}^{k} biXi + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_i \sum_j bijXiXj + \epsilon$$

where

Y = Viable Cell density

b_l = Intercept for block l

X = Factors (X1 = Glutamine, X2 = EAA, X3 = NEAA, X4 = ITS, X5 = Lipids)

b_z = regression coefficient ($z = i$, ii or ij, *where* $i<j$)

ϵ = Residual Error

k = 1,2,3…….

Statistical Analysis

Using ordinary least squares the regression model was fit to evaluate, the explanatory variables regarding linear, interaction, and quadratic effects of coded levels of Glutamine, EAA, NEAA, ITS, Lipids on cell density. The R^2 value was used to evaluate model sufficiency and the α-level was set as 5% at which every term in the selected model should be significant. The reduced model was evaluated using the R^2 and lack of fit and was used to attempt to find optimal conditions for all the variables maximizing the cell density. Canonical analysis was then used to evaluate the nature of the stationary point (maximum, minimum or saddle) and to find the ridge of steepest ascent. Further experiments were carried out in the direction of the maximum response along with alternate experiments where Glutamine was set to different coded levels from '0' to '-3', keeping EAA constant at coded level '2' and '4'. All statistical computations were done using SAS/STAT procedures, optimum conditions were found through SAS data-step programming. Response surface plots were generated by SAS/GRAPH

Results and Discussion

Regression analysis revealed that linear and quadratic effects were more significant than cross product interactions (Table 4). Among all the independent variables, Glutamine (negative effect) and NEAA (positive effect) had the greatest effects on the cell density, while EAA showed an effect when combined with Glutamine. Among the pair wise interactions EAA and Glutamine exhibited the greatest effect. NEAA squared and NEAA by itself were significant but when compared with the other variables did not showed great effect. The response surface plots are plotted to see the effect of EAA and NEAA (Fig. 1), EAA and Glutamine (Fig. 2), NEAA and Glutamine (Fig. 3) on the response which is viable cell density (Y). ITS and Lipids didn't have effects in anyway. The R^2 value for the total model is 0.6339. To simplify the model, the variables ITS and Lipids were removed from the model and the data were re-analyzed using the reduced model. The polynomial regression model used for three variables was

$$Y = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_{11}^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + \epsilon$$

When the variables ITS and Lipids kept constant, the lack of fit is insignificant suggesting that model is adequate to explain the effect of these three variables on the response. The R^2 value of the reduced model decreases to 0.502. From the Table 5, still the Glutamine had significant linear effects and interacted with EAA, EAA and NEAA had significant quadratic effects while NEAA also had a linear effect on the cell density.

Canonical Analysis:

Canonical analysis is a mathematical approach used to examine the overall shape of the response surface and to determine if the estimated response point is a maximum, minimum or a saddle point. If the stationary point is maximum or minimum, a corresponding increase or decrease will result in the response, and in the case of saddle point then we may get an increase or decrease in the response when we move away from the stationary point,

depending on which direction to be taken to get the maximum or minimum response. The maximization the viable cell density is of interest; however the stationary point was a saddle point, so we move on the ridge in the direction to get maximum response. Points on the ridge that increased the response were found using the RIDGEMAX option of the SAS/RSREG procedure and are as shown in the Table 6.

From the Table 5 and 6, glutamine showed a negative effect on cell density while EAA, NEAA showed a positive effect, so the Glutmine values for the ridge moved in the negative direction and in the positive direction for the variables EAA and NEAA. Following the ridge in Table 6 the highest cell density was 1.37E+06 cells/ml, but this prediction was not very reliable due to large standard error. Based on the ridge analysis, the Glutamine that had high cell densities were decreasing below 1 mM. We felt Glutamine values smaller than 1 mM were not reasonable and we were about conducting further experiments on the ridge below Gln values of 1 mM.

To further explore the surface, we used the reduced model from the Table 6, and obtained predicted cell densities with Glutamine constant at different coded levels from '0' to '-3', NEAA at '0.5' and various values of EAA. The results are shown in Table 7, where it suggests that with low glutamine values, the higher values of EAA would increase the cell density. Figure 4 shows the effect of EAA and NEAA on VCD when glutamine is controlled at coded level '–1'.

How ever EAA values up to 14.5 coded level (uncoded value =12.625 X) are biologically not reasonable because of osmotic effects or inhibition of metabolic pathways due to over feeding of the nutrients. But from the above results it is appeared that as Glutamine was lowered and at higher concentrations of EAA and NEAA at 0.5 coded level large cell densities could result

Alternate Experiments:

To further evaluate the surface we ran some alternate experiments at different levels of Glutamine (from coded level '0' to' -3'), keeping EAA constant at coded levels 2 and 4. We expected low cell growth at Glutamine value less than '-1.5 coded level' and no cell growth at Zero ('-2' coded level) glutamine concentration. We also expected the EAA coded level at '2' to result in higher cell densities compared to EAA coded level 4, because of osmotic effects and the inhibition of metabolic pathways due to over-feeding. Therefore 14 more experiments were carried out, four being on the ridge, four being at different level of Glutamine keeping EAA at coded 2, four being at EAA coded level 4, and two controls (Basal media) as shown in Table 8.

These experiments were with all other conditions the same as the initial experiments. The starting density of the cultures was $2*10^5$ cells/ml, and the cells were allowed to adapt to the media in four passages. The final viable cell densities are mentioned as average of the third and the fourth passage, as shown in Table 8.

The Media-6 had a higher cell density compared to the controls (13 and 14), but the last passage of the media's 3, 9, and 5 were nearly equal to the control media, shown in Fig 5. So, these media's, which were doing, better than (or equal to) Media-0, were carried out for one more passage (up to 8 days), to confirm their results. The results are shown in Figure 6. From the passage 5, the viable cell density attained in the media 6 after 5 days is about 1.6 times more than the control.

Replicate Experiments

The Media-6 resulted in higher cell densities than the control media, but to confirm the results, the cells in the control media were taken out of frozen, and the experiment was repeated three times with control media and Media-6. Cells were allowed to go for four passages and the final viable cell densities are taken as average of passage 3 and passage 4, the results are given in Figure 7.

So, for media-6 the viable cell density averaged over the replicates attained $1.45 * 10^6$ cells/ml, which is 1.4 times more than the control media and within two standard errors of 1.23E+06 from the original run of media 6 (se = 215928 cells/ml for media 6). After four passages if the cells were allowed to grow to passages of 5 to 7 the viable cell density increased to $1.6* 10^6$ cells/ml. The viable cell density on the control experiment in these passages (Passages 5 to 7) was between 9.2*106 cells/ml to $1.1 *10^6$ cells/ml, with in the standard deviation of 128103 cells/ml (data not shown).

Assuming the antibody production depends on the cell density, we should see an increase in the antibody production with the increase in cell density. The production is determined and standard deviation is calculated in these media's from triplicate experiments. The results are given in Figure 8. The production is determined for all the initial 50 media experiments, and the results were analyzed using SAS/STAT procedures. We saw the same trend for production also where the stationary point is a saddle point, and the ridge values for the glutamine are moving in the negative direction and in positive direction for EAA and NEAA, shown in Table 9. The R^2 value for the model was 0.75 which shows adequacy of the model in explaining the effect of the variables on the response which is antibody production.

So the antibody production in the Media – 6 was 1.6 times more than the control Media. So the Composition of the variables in the best Media – 6 is Table 10.

Conclusions

The increase in the viable cell density (Cells/ml), and the production of the antibody against BoNT-A was accomplished by using Box-Wilson's Central Composite Design. The viable cell density is increased by 1.4 times and the antibody production increased by 1.6 times. The lower values of the Glutamine and higher values of EAA are preferred. From the results it is expected that one of the amino acids is replacing the role of amino acid-Glutamine, and may be acting as limiting nutrient. So doing amino acid analysis, that limiting amino acid can be found, and if it is added separately, then we can decrease the concentration of the total amino acid, by that way we can make the media more economical and may also get higher cell densities and antibody production.

List of Tables

Table 1: Composition of the individual components in mg/L in the solutions EAA, NEAA, ITS, Lipids, concentrated solutions from Gibco.

Table 1(a): Concentrations of the components in the control media.

(50X) EAA [mg/L]	**(100X) NEAA [mg/L]**	**(100X) ITS [mg/L]**	**(100X) Lipids [mg/L]**
L-arginine [6320]	L-Alanine [890]	Insulin [1000]	Arachidonic acid [2]
L-Cystine[1200]	L-Asparagine[1320]	Transferrin [0.67]	cholestrol [220]
L-Histidine*HCl*H2O [2100]	L-Asparticacid [1330]	Selenium [0.55]	DL-alpha-tocopherol-acetate [70]
L-Isoleucine [2620]	L-Glutamicacid[1470]		Linoleic acid [10]
L-Leucine [2620]	Glycine [750]		linolenic acid [10]
L-Lysine HCl [3625]	L-Proline [1150]		myristic acid [10]
L-Methionine [755]	L-serine [1050]		oleic acid [10]
L-Phenyl alanine [1650]			palitoelic acid [10]
L-Threonine [2380]			palmitic acid [10]
Ltryptophan [510]			pluronic [0.1%]
L-Tyrosine [1800]			stearic acid [10]
L-Valine [2340]			Tween 80 [2200]

Components	**Composition (mg/L)**
CaCl2 (anhyd.)	99.1
CuSO4*5H2O	0.00125
FeSO4*7H2O	0.415
KCl	276.8
MgCl2 (anhyd.)	28.61
NaCl	5500
NaHCO3	2100
Na2HPO4 (anhyd.)	71
Na2HPO4*H2O	62.5
ZnSO4*7H2O	0.43
KNO3	0.038
MgSO4 (anhyd.)	50.8
Na2SeO3	0.0085
D-glucose	**4000**
Linoleic Acid	0.04
Lipoic Acid	0.105
Phenol Red	8.1
Putrescine 2HCl	0.0805
Sodium Pyruvate	110
HEPES	2979
L-Alanine	15.57
L- Arginine*HCl	221.2
L-Asparagine*H2O	23.1
L-Aspartic Acid	23.27
L-Cystine*2HCl	42
L-Glutamic Acid	25.72

L-Glutamine	**584**
Glycine	13.12
L-Histidine*HCl*H2O	73.5
L-Isoleucine	91.7
L-Leucine	91.7
L-Lysine*HCl	126.88
L-Methionine	26.4
L-Phenylalanine	57.7
L-Proline	20.12
L-Serine	18.37
L-Threonine	83.3
L-Tryptophan	17.8
L-Tyrosine*2Na*2H2O	63
L-Valine	81.9
Biotin	0.02
D-Ca Pantothenate	4
Choline Chloride	18
Folic Acid	4
i-Inositol	25.2
Niacinamide	4
Pyridoxine HCl	4
Riboflavin	0.4
Thiamine HCl	4
Vitamin B12	1.413
Insulin	10
Transferrin	5.5
Sodium selenite	0.0134
Arachidonic acid	0.014
cholestrol	1.54
DL-alpha-tocopherol-acetate	0.49
Linoleic acid	0.07
linolenic acid	0.07
myristic acid	0.07
oleic acid	0.07
palitoelic acid	0.07
palmitic acid	0.07
stearic acid	0.07
Tween 80	15.4
pluronic	0.10%

Table 2: Table 2: Actual factor levels corresponding to coded factor levels

		Actual factor level at coded factor level of:				
Level	**Symbol**	**-2**	**-1**	**0**	**1**	**2**
Glutamine	X1	1mM	2.5mM	4mM	5.5mM	7mM
EAA	X2	0X	0.5X	1X	1.5X	2X
NEAA	X3	0.25X	1X	1.75X	2.5X	3.25X
ITS	X4	0.25X	1X	1.75X	2.5X	3.25X

LIPIDS	X5	0.1X	0.4X	0.7X	1X	1.3X

Table 3: Table 3: Treatment combinations with variables in coded values and the values of Response.

Run	X1	X2	X3	X4	X5	Y
1	1	1	-1	-1	1	8.1E+05
2	1	1	-1	1	-1	7.2E+05
3	-1	-1	-1	-1	-1	6.5E+05
4	0	0	0	2	0	8.3E+05
5	-1	1	1	-1	-1	1.0E+06
6	1	-1	1	-1	1	8.0E+05
7	2	0	0	0	0	7.3E+05
8	0	0	0	0	2	7.1E+05
9	1	1	-1	1	1	5.4E+05
10	**0**	**0**	**0**	**0**	**0**	**9.5E+05**
11	1	-1	-1	1	1	6.6E+05
12	**0**	**0**	**0**	**0**	**0**	**9.3E+05**
13	-1	-1	1	1	1	8.40E+05
14	1	-1	1	1	-1	8.80E+05
15	1	-1	1	1	1	6.25E+05
16	-1	-1	1	-1	-1	7.00E+05
17	**0**	**0**	**0**	**0**	**0**	**1.18E+06**
18	-1	-1	-1	1	1	8.15E+05
19	0	0	0	0	-2	1.15E+06
20	1	-1	-1	1	-1	6.55E+05
21	**0**	**0**	**0**	**0**	**0**	**1.16E+06**
22	-1	-1	-1	-1	1	2.00E+05
23	-2	0	0	0	0	1.52E+06
24	0	0	0	-2	0	5.15E+05
25	0	-2	0	0	0	3.10E+05
26	-1	-1	1	1	-1	8.20E+05
27	1	-1	-1	-1	1	5.10E+05
28	1	1	1	-1	-1	8.10E+05
29	-1	1	-1	-1	-1	7.85E+05
30	**0**	**0**	**0**	**0**	**0**	**1.18E+06**
31	-1	-1	-1	1	-1	8.30E+05
32	0	0	2	0	0	9.55E+05
33	-1	1	1	-1	1	1.29E+06
34	0	0	-2	0	0	1.80E+05
35	1	1	-1	-1	-1	2.00E+05
36	1	1	1	-1	1	2.20E+05
37	**0**	**0**	**0**	**0**	**0**	**1.21E+06**
38	-1	1	1	1	1	1.03E+06
39	-1	1	-1	1	-1	9.00E+05
40	1	-1	1	-1	-1	9.85E+05
41	-1	1	1	1	-1	8.65E+05
42	1	-1	-1	-1	-1	8.75E+05
43	1	1	1	1	-1	7.70E+05

44	1	1	1	1	1	1.08E+06
45	**0**	**0**	**0**	**0**	**0**	**9.90E+05**
46	-1	1	-1	-1	1	9.35E+05
47	-1	1	-1	1	1	8.55E+05
48	-1	-1	1	-1	1	1.00E+06
49	0	2	0	0	0	8.90E+05
50	**0**	**0**	**0**	**0**	**0**	**9.85E+05**

Where α = '2'. Response = Viable cell density in cells/ml. '-1', '0', '+1' are coded factorial levels.

Table 4: The't' and 'p' values of full model with X1, X2, X3, X4, X5 as independent variables.

Parameter	t Value	p Value
Intercept	13.75	<0.001
X1	-2.79	0.0092
X2	1.53	0.1376
X3	3.07	0.0046
X4	1.2	0.2404
X5	-0.81	0.425
X1*X1	0.66	0.5167
X2*X1	-2.1	0.1446
X2*X2	-2.67	0.0124
X3*X1	-0.27	0.7855
X3*X2	-0.1	0.9206
X3*X3	-2.86	0.0078
X4*X1	0.31	0.758
X4*X2	0.22	0.8304
X4*X3	-0.74	0.4662
X4*X4	-2.21	0.0352
X5*X1	-0.8	0.4292
X5*X2	1.3	0.2026
X5*X3	0.25	0.8061
X5*X4	0.2	0.8427
X5*X5	-0.58	0.5675
Linear		0.0046
Quadratic		0.0055
Cross Product		0.6621

R^2 = 0.6339 for the total model

Table 5: The't' and 'p' values of the reduced model with X1, X2, X3 as independent variables.

Parameter	t value	p value
Intercept	15.56	<0.0001
X1	-2.81	0.0076
X2	1.54	0.132
X3	3.1	0.0036
X1*X1	0.66	0.5123
X2*X1	-2.11	0.0407
X2*X2	-2.69	0.0105
X3*X1	-0.28	0.7835
X3*X2	-0.1	0.9199
X3*X3	-2.88	0.0064

Linear	0.001
Quadratic	0.0035
Cross Product	0.2242

$R^2 = 0.5022$ for the reduced model

Table 6: Ridge of steepest ascent for X1, X2, X3 independent variables, and estimated response and standard error.

X1	X2	X3	Estimated Cell Density (10^5)	Std.Err
-0.15	0.07	0.11	10.1	62346
-0.33	0.13	0.18	10.4	61381
-0.52	0.20	0.23	10.7	60421
-0.71	0.26	0.26	11.0	60558
-0.91	0.32	0.28	11.4	63278
-1.10	0.38	0.30	11.8	70025
-1.29	0.43	0.31	12.2	81626
-1.49	0.49	0.33	12.7	98153
-1.68	0.55	0.34	13.2	119266
-1.87	0.60	0.35	13.7	144554

Table 7: Effect on EAA, NEAA and VCD when Glutamine is controlled at different coded levels

X1(level)	X2(Level)	X3(Level)	VCD
-0.5	4.5	0.5	1.27E+06
-1	6.5	0.5	1.56E+06
-1.5	8.5	0.5	1.95E+06
-2	10.5	0.5	2.43E+06
-2.5	12.5	0.5	3.01E+06
-3	14.5	0.5	3.69E+06

Table 8: Alternate experiments carried out on ridge and glutamine controlled at different coded levels, keeping EAA constant at coded level 2 and coded level 4.

Media	X1	X2	X3	VCD (105)	Standard Deviation
1	-0.52	0.196	0.225	8.13	203490
2	-1.1	0.375	0.299	8.81	179368
3	-1.487	0.491	0.329	8.61	60052
4	-1.874	0.604	0.352	8.01	152664
5	0	2	0.5	9.35	42230
6	-1	2	0.5	1.23	**215928**
7	-2	2	0.5	7.23	277564
8	-3	2	0.5	1.79	45162
9	0	4	0.5	7.93	102429
10	-1	4	0.5	4.49	152555
11	-2	4	0.5	5.7	144684
12	-3	4	0.5	2	52915
13	0	0	0	9.15	134722

14	0	0	0	9.48	121484

Table 10: Ridge of steepest ascent of the reduced model for getting maximum antibody production with independent variable X1, X2, X3. X4 and X5 kept constant at their zero-level

X1	X2	X3	Estimated Response (mg/L)
0	0	0	12.73
-0.172	-0.01	0.101	13.46
-0.34	-0.001	0.2	14.21
-0.522	0.03	0.293	15
-0.698	0.08	0.38	15.82
-0.874	0.149	0.461	16.69
-1.049	0.226	0.536	17.61
-1.22	0.31	0.607	18.58
-1.39	0.4	0.673	19.6
-1.56	0.496	0.736	20.6
-1.73	0.595	0.796	21.8

Table 11: Concentrations of the five variables of the optimal media, (total concentration of all amino acids in EAA and NEAA are given in g/L). Concentration of the remaining components same as given in Table 1(a).

Variables	Concentration
Glutamine	2.5 mM
EAA	3.25 X (1.67 g/L)
NEAA	2.125 X (0.168 g/L)
ITS	1 X (0.22 g/L)
Lipids	0.7 X (0.71 g/L)

List of figures:

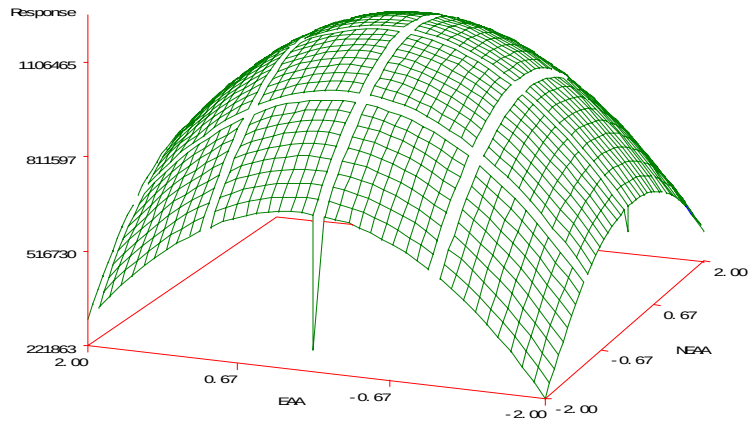

Fig 1: Response surface plot showing the effect of EAA, NEAA, and their mutual effect on the Response (Viable cell density). Other variables are held at zero level.

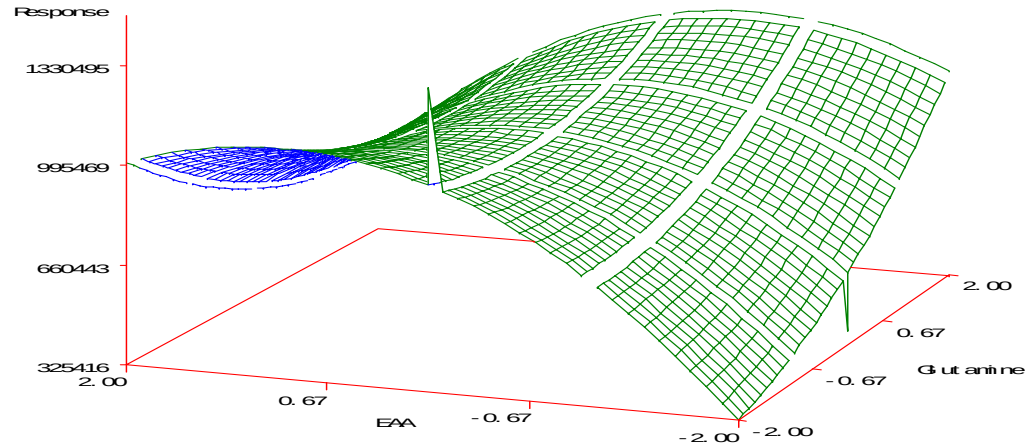

Fig 2: Response surface plot showing the effect of EAA, Glutamine and their mutual effect on the Response (Viable cell density). Other variables are held at zero level.

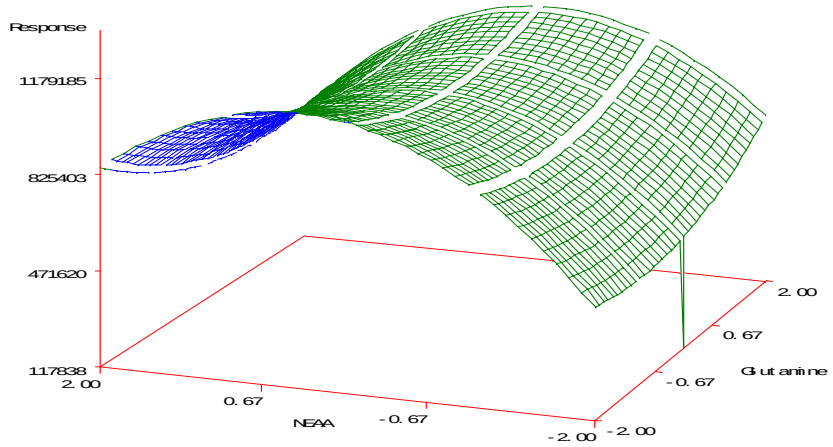

Fig 3: Response surface plot showing the effect of NEAA, Glutamine and their mutual effect on the Response (Viable cell density). Other variables are held at zero level.

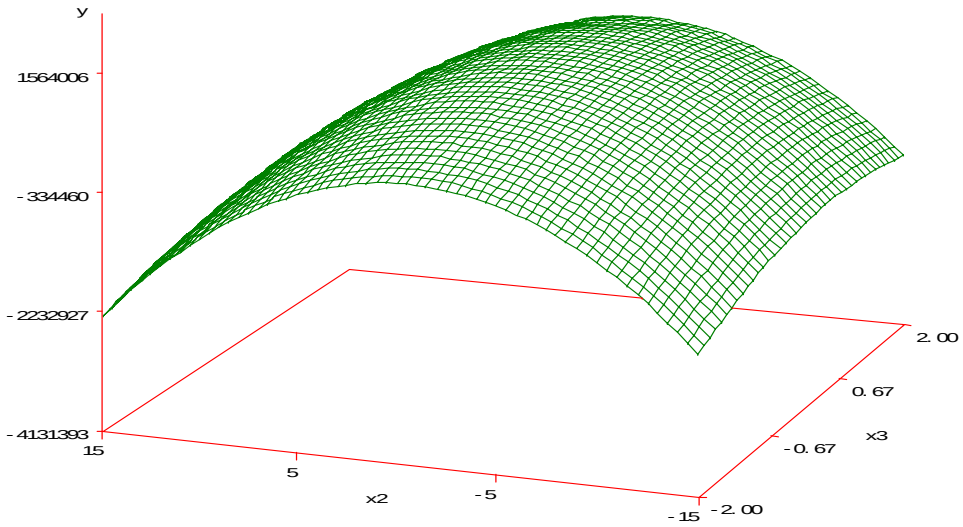

Fig 4: Response surface plot showing the effect of EAA (X2), NEAA (X3) and their mutual effect on the Y (Viable cell density), when Glutamine (X1) is controlled at '-1' coded level.

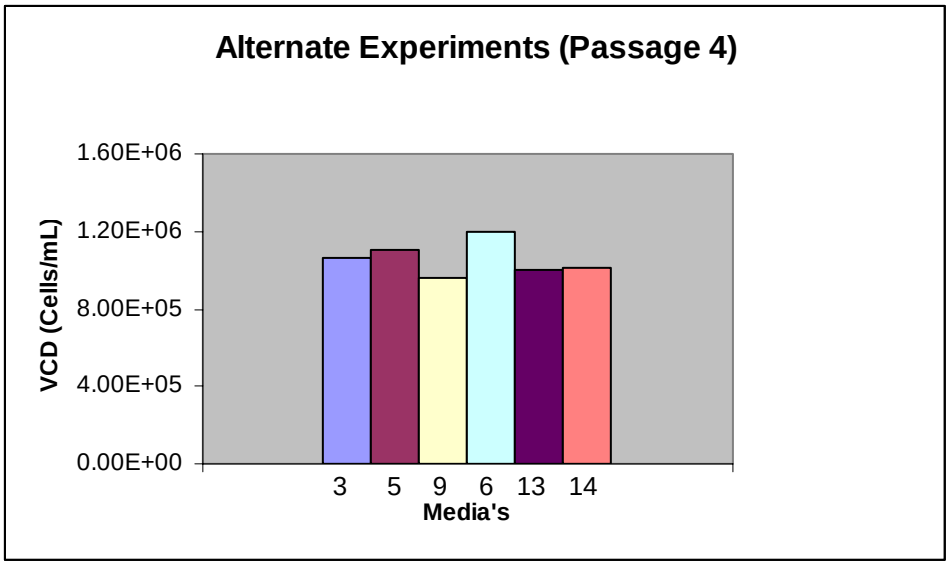

Fig 5: Results of last passage of Alternate experiments.

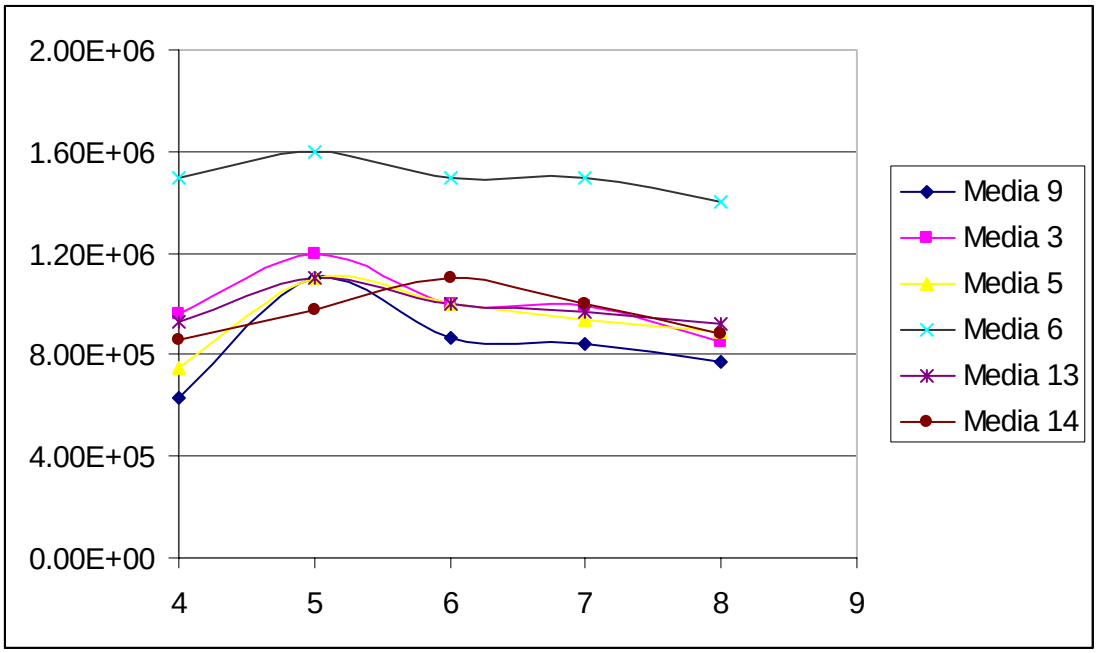

Fig 6: Results of the alternate experiments carried to further explore the surface.

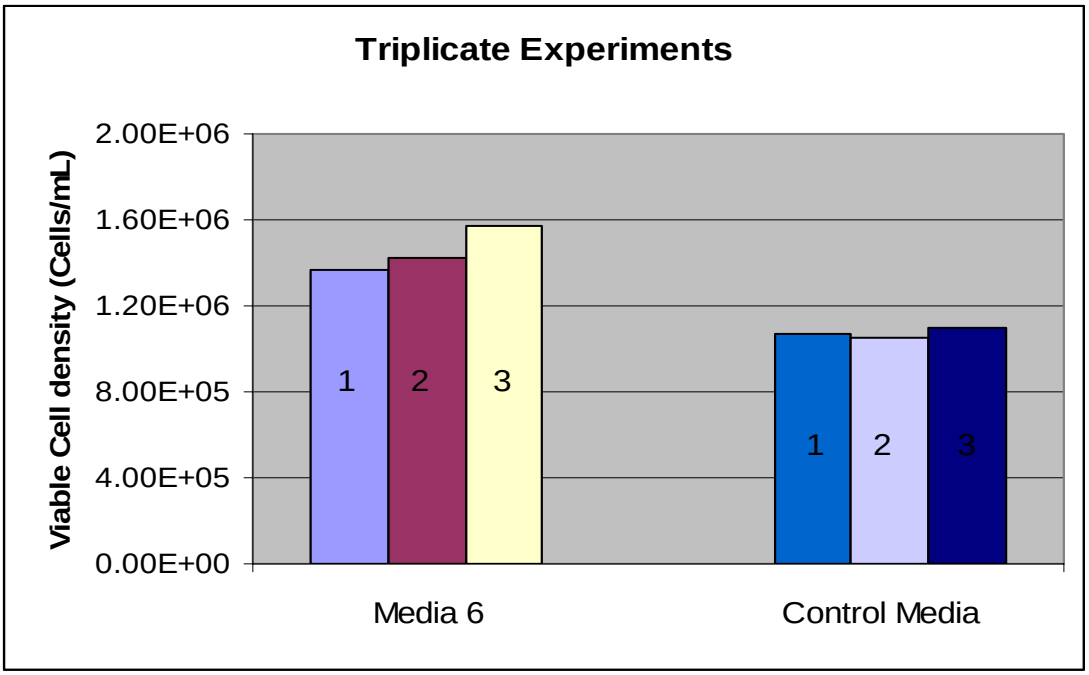

Fig 7: Results of the triplicate experiments of Media 6 and the Control Media.

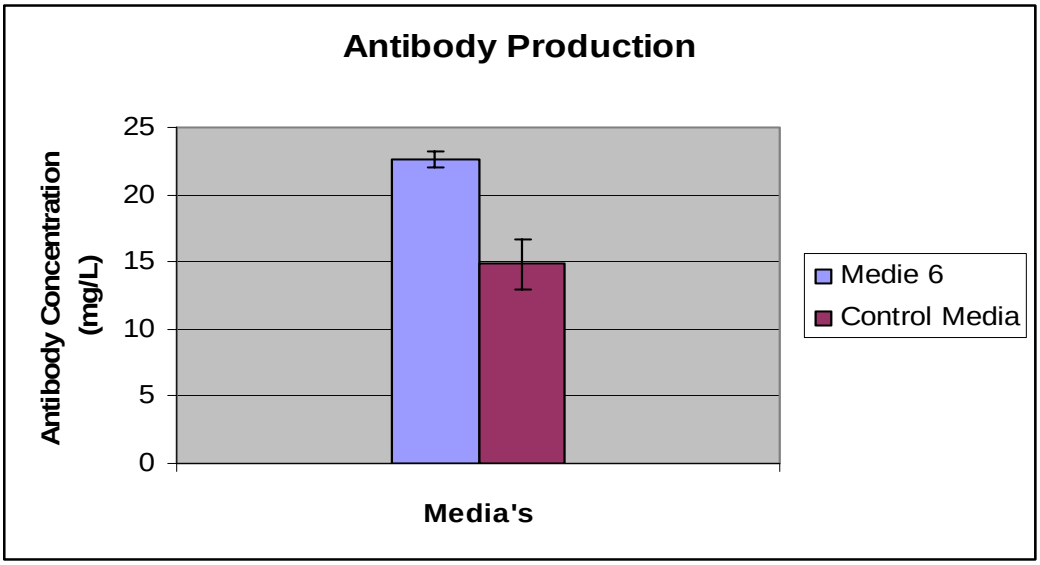

Fig 8: Antibody production in Media-6, and the Control Media.

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Key Research Accomplishments

- Design and preliminary budget estimate of CGMP facility is completed.
- Development of plasmids for easy insertion of light and heavy chain genes and transformation into CHO cells.
- Development of a rapid method to screen for higher producing CHO cell lines using both dfr and zeocin and selection using flow cytometery using GFP.
- Development of a defined media for production of mAb in a fed-batch bioreactor.