

CELL ENGINEERING AND CULTURE
MEDIA MODIFICATION STUDIES TO
IMPROVE THE CELL CULTURE
PERFORMANCE

by

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ABSTRACT

Mammalian cell cultures have become a universal path for producing therapeutic proteins, monoclonal antibodies in particular, in biopharmaceutical industries. There is a constant attempt to improve the productivity of the cell cultures through research in the industry and educational institutions.

The productivity of the cells can be improved through various techniques. The use of additives is the most economical option to pursue for improving the productivity of the cells. The additives, hydrolysates, were used in the cell culture at a final concentration of 0.4% and we have seen a two to threefold increase in the productivity of the cells. While soy hydrolysate doubled the productivity along with the cell growth, cotton hydrolysates have increased it three times when compared to the control flasks not supplemented with any hydrolysates.

Five different lots of cotton hydrolysates were tested simultaneously as media supplements for a lot variability study. The cotton lots 100NTPCR and 100PCHO performed the best among the five lots tested in terms of both cell growth and IgG productivity. Both these lots were selected to perform

proteomics analysis on the hydrolysate samples to understand the mode of action of these hydrolysates when added to the cell cultures.

Essential amino acids' biosynthetic pathways were to be engineered into mammalian cells. In particular, the research described in the thesis targeted branched chain amino acids- Leucine, Isoleucine and Valine. All the genes were cloned into the mammalian expression vector pBUD4.1 and later transfected into CHO-K1 and HEK 293T cell lines and their expression was tested using a western blot. Five out of the nine genes to be expressed, have worked. This opens up new avenues to explore the machinery in the mammalian cell and lead to the synthesis of a minimal cell.

Advisor: Dr. Michael Betenbaugh

Reader: Dr. Marc Ostermeier

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Chapter 1 INTRODUCTION

1.1 Objective of the Thesis

This thesis aims to develop processes which would improve the productivity of therapeutic proteins in mammalian cell cultures, especially Chinese Hamster Ovary (CHO) cell lines. Therapeutic proteins are produced using mammalian cells for the sole reason that they have to be compatible to the human body once injected into them. Chinese Hamster Ovary cells in particular produces proteins with a similar glycosylation pattern to the human cells. Having a compatible glycosylation pattern improves the efficacy of the therapeutic protein thus enabling its proper intended function in the human body.

Improving the productivity of monoclonal antibodies in CHO cells has been a burning problem since the time these cells were known to secrete these molecules. Even today, there is a gap between the raw material input and the product output from these cell cultures leading to the high cost of these therapeutics in the market. Keeping this broad aim in mind, this thesis has used various techniques in mammalian cell culture and molecular biology to achieve its two main objectives (to be described in the next paragraphs). All thesis techniques have been explained along with the protocols used in

Chapter 2. The two objectives whose end result would be to improve the productivity of the mammalian cell for the backbone of the thesis.

Objective 1: Addition of plant hydrolysates to improve the productivity of monoclonal antibody in CHO cells. In Chapter 3, mammalian cell culture techniques are employed to test the effect of plant hydrolysates on CHO cell cultures. The growth and productivity of the cultures are monitored to compare and contrast the impact of adding the hydrolysates from two different plant sources into a given cell culture media. The various lots of one plant source are also compared to provide a lot to lot variability when they are added to a culture media.

Objective 2: Engineering mammalian cells to express prokaryotic amino acid biosynthetic genes, thereby reducing the external supplementation of nutrients in the form of cell culture media rich in amino acids. In Chapter 4, molecular cloning is used to enable expression of the biosynthetic genes of essential amino acids from prokaryotic species (yeast), in mammalian cell lines like HEK and CHO-K1. The pathway genes of three branched chain amino acids have been cloned into mammalian expression vectors and tested for expression after transfection into CHO and HEK cell lines.

Chapter 5 summarizes the research work described in the previous chapters. Both the objectives include and involve extensive studies and thus

needs future work and improvements which have been described at the end of Chapter 3 and Chapter 4. These future objectives points to the fact that there is a wider scope to the abovementioned objectives which will be explored in the days to come.

1.2 Mammalian cell culture

We know that cells need a suitable environment to grow, which is provided by the living organism in which the cell resides. In the twentieth century, biologists began to provide this environment outside the living organism in the form of tissue culture. They started to believe that cells could also be cultivated and kept living even after the organism dies. This fact that we know today was just an idea hypothesized by Claude Bernard in 1878 [1].

Even though Bernard created this idea, it was put in practice by Ross Granville Harrison in 1907, working at Johns Hopkins Medical School, when he could grow nerve cells of frogs in sterile cultures in vitro. He named his breakthrough in biology research as “hanging drop” experiment and the cell culture of the nerve cells lasted for up to four weeks. This method created by Harrison was later used by his student Burrows, to establish the mammalian cell culture with chick embryos as the cell component of the culture and blood

plasma which has clotted as the cell culture media or nutrients supplement component of the culture. (Hydrolysate thesis) Since then, in vitro culturing of mammalian cells has been developed extensively [2].

After a great invention, several questions arise which lead to the betterment of the invention along with using the invention for a practical application. The same group of people thought of such questions associated with cell culture, which led to the concepts of sub-culturing of cells and changing media of cell cultures referred by them as media-exchange. Using complex media, they were able to subculture and maintain cultures for several months. They worked not only with normal adult mammalian tissues, but also with cancerous tissues. Various cell types and cell lines have been developed by them later using these in vitro culture techniques, epithelial cells, tumor cells to name a few [3]. These novel methods were a point of difference between Burrows' and Harrison's research and gave way to introduce the idea of continuous culture. These are the cultures created from the old ones, rather than starting primary cultures from new tissue explants. This continuous passage method to maintain cells could establish that cells could survive in vitro for days together provided depletion of nutrients is avoided through media replacement and suitable culture conditions like humidity and CO₂ supply.

After further advancement of research which was successful in generating new continuous cultures, it was observed that these cell cultures (after multiple passages) were morphologically different from the actual tissues from which the culture was developed. These morphologically different cells were termed to be 'transformed cell lines'. In 1951, the first human "transformed" cell line was developed from a patient who died with cervical carcinoma and the cell line was named the HeLa cell [4]. The immortality of HeLa cell line marked the beginning of modern cell culture.

The latter half of the twentieth century saw the development of other techniques that are commonly used to culture cells in the modern day like the use of trypsin to treat attached cells and passage them into on flask to the other, culturing cells in bioreactors, cryopreservation and subsequent revival of cells from cold preservation. Eagle started the development of cell culture media which needed supplementation of animal serum for adherent cultures [5]. Later, Eagle MEM and DMEM have become the standard media for mammalian cell culture.

Using cell cultures for production of biomolecules that are commercially important began with the production of vaccine for polio. Today, mammalian cell cultures are used for producing various biomolecules

important to mankind. As we have seen there are two major components to a mammalian cell culture: the cells the needs to be grown and the cell medium necessary for growth. There can be two major types of in vitro cell cultures based on their source. The first kind, primary cultures are obtained directly from the tissue of the organ. These cultures are difficult to maintain since their cell growth and division is slow. Such cultures are created aseptically from tissues of mammalian hosts which are embryonic, malignant or normal in nature.

After the first subculture, they are no longer considered as primary cultures. Such cultures have cells which are of different types since it is difficult to isolate one particular type from the tissue samples. Primary cell cultures are predominantly adherent in nature. These cultures cannot sustain without animal serum in the culture media. The present thesis has not utilized any of the primary cell cultures for research as these cultures are used for research involving genetics, morphological studies and other basic biology research.

The second type of cultures are, continuous cell lines which are created through following series of sub-culturing from the primary cultures. Their main purpose to create was to avoid extracting primary cell cultures

from tissue samples every time there is need for culture. These cell lines can thus be cryopreserved and used at later times. They can grow indefinitely if there is periodic change of media to ensure there is enough nutrient supply to the cells.

Continuous cultures are formed after the cells lose the character of death from their genetic makeup. This phenomenon is called immortalization and it can occur through a natural mutation that occurs in the cells due to serial passaging or through induced mutation. The cell lines which did not succumb to the mutations are finite cell lines which would eventually be terminated through cell death. Such cultures have practically no application other than characterizations of these finite cell lines. On the other hand, continuous cell lines can be used for a variety of application in biopharmaceuticals. Continuous cultures can be cultivated in both ways: adherent and suspension. In the modern times, suspension cultures are predominantly used to culture cells. [4]

The second important component of the mammalian cell culture is the cell growth media. Development of media has come a long way since the first time a growth medium was developed from a mixture of salts in the 1880s. Today, numerous formulations are available for different cell lines for

different levels of growth. In the early days, the serum from animal tissues or blood would be used as a medium of cell culturing.

When Eagles' invented a chemical formulation using amino acids, vitamins, minerals and growth factors, there was still a need to supplement this animal serum to sustain growth and proliferation in the cells. Early twenty first century obviated the use of serum in cell culture due to regulatory restrictions, with the creation of serum free media formulation [6]. Several chemically defined media are currently available for culturing mammalian cells for instance, CD CHO (Gibco), ExCell CD CHO (Sigma Aldrich), PowerCHO (Lonza), ActiPRO (GE Life Sciences) and so on.

1.3 Monoclonal antibodies as therapeutics

The biopharmaceutical industry has benefitted immensely over the last 20 years, from the recombinant DNA and hybridoma technologies. Before the invention of these technologies, the biomaterials necessary for medicine were extracted from plants and other natural resources. This leaves a very narrow arena to work around since we cannot create new materials or produce more of the existing material. Recombinant DNA technology created a potential for large scale production of a wide range of natural and modified

proteins and the hybridoma technology introduced a new class of protein therapeutics—the monoclonal antibodies (MAbs) [7].

Therapeutic proteins are an alternative solution to various types of cancer and autoimmune disorders [8]. The number of approved biologics in the market increased from 84 in the year 2000 to 148 in 2003. By 2010, the total number of biopharmaceuticals out in the global market increased to 200. [9]. Monoclonal antibodies are next to vaccines in the order preference for development in the biopharmaceutical industries [10]. Their growth as therapeutic proteins is the largest among all other biomolecules used in medicine, as the numbers suggest that 21 mAbs were approved by the FDA in 2008 in the United States alone [11]. This shows the global potential of mAbs as therapeutics of cancer and autoimmune disorders.

Mammalian cells are the host organisms for the production of therapeutic proteins. There has been constant effort in the industries to improve the titer even by the slightest amounts possible. [12]. Several ways have been employed for this purpose of which cell line improvement, media formulation modifications, bioprocess development through improvements in fed-batch culture, design of new equipment and modes of bioreactor operation are to name a few.

1.4 Mammalian cell engineering

Cell engineering of a mammalian cell is a growing field with new methods being developed from time to time with higher engineering capability and targeted gene modification potentials. One common method of engineering a mammalian cell is to clone the gene of interest into a mammalian expression vector and express the plasmid created in the cell line that is chosen. Cloning of a gene performs direct modification of the DNA in the cells which results in the expression of the gene of interest.

Many challenges occur during the gene expression stages which become even more predominant as the mammalian cell chosen for expression ranks higher in complexity. For instance, it is relatively simple to insert and express a gene into an E.coli than a CHO cell. New genetic engineering techniques like CRISPR-Cas9, TALENS, Zinc Finger Nucleases are bringing simplicity in mammalian cell engineering. The most recent addition to the field, the cell free systems are providing platforms to produce, mRNA through gene expressions using the contents of the cell outside the cell membrane in a small Eppendorf tube.

1.5 Summary

Mammalian cells in biopharmaceuticals are being used extensively for therapeutics production. The CHO cell line especially is utilized to its full potential to produce monoclonal antibodies. Monoclonal antibodies are the molecules which are used in cancer treatment and for the cure of various autoimmune disorders in human beings. Though CHO cells are being used to their full potential now, it is possible to further enhance the production and improve the product to raw materials cost index for mammalian cells. Use of media additives in cell culture and engineering mammalian cells are the ways to push the potential of CHO cells to produce monoclonal antibodies. The thesis explores these potential options and the extent of success obtained in these areas.

Chapter 2 MATERIALS AND METHODS

The work that will be described in this thesis, was performed using the materials and methods that have been detailed in this chapter. These protocols have been adopted from the companies like Thermo Fisher Scientific, New England Biolabs, Sigma Aldrich, Qiagen which provide support in the lab and thus reflect the protocol exactly given in their handbooks when the materials are purchased. The various cell lines used, the cell culture and molecular biology techniques utilized and the assays performed to obtain the final results of productivity or methods used for the detection are detailed in this Chapter.

2.1 Cell lines

Three different cell lines have been used for the studies performed in the duration of my research work. The Hydrolysate Project used a CHO cell line producing IgG (CL01) which has been purchased on a 18 month contract from Bioceros Ltd. The Cell engineering project used two mammalian cell lines, namely, HEK 293T wild type (Human embryonic kidney, CL02) and CHO-K1 wild type (Chinese Hamster Ovary, CL03) from ECACC (European Collection of Authentic Cell Cultures).

2.2 Cell Culture Media and Additives

The following cell culture media and additives have been utilized for the culturing of the cells that have been mentioned above:

- (A) DMEM (Gibco)
- (B) F12K Nutrient mix
- (C) ExCell CD CHO (Sigma Aldrich)
- (D) Glutamax (Gibco) [200mM L-Glutamine]
- (E) Poloxomer (Sigma Aldrich)
- (F) Zeocin
- (G) Blasticidin
- (H) FBS (Fetal Bovine Serum)

2.3 Cell culture Protocols

This section will describe the general cell culture protocols that were used during the course of research. These protocols are generally available in the literatures and can be adopted depending on the cell type being used for research. Many mammalian cell protocols are provided by thermo fisher scientific which also is the producer of cell culture media.

2.3.1 Cell Thaw

Description

Reviving cells from cryostorage is termed as cell thaw. Mammalian cells are fragile and the cell thaw procedure controls the way the culture behaves after thaw. The cryovials stored in liquid nitrogen are carefully removed and the contents of the vial are added to a new culture flask to grow the cells in a sterile environment. The general thaw protocol followed is given below.

Materials

- (A) Cryovial containing frozen cells,
- (B) Complete growth medium, pre-warmed to 37°C
- (C) Disposable, sterile centrifuge tubes,
- (D) Water bath at 37°C, 70% ethanol
- (E) Tissue-culture treated flasks, plates, or shake flasks for suspension cells

Protocol

1. Remove the cryovial containing the frozen cells from liquid nitrogen storage and immediately place it into a 37°C water bath.
2. Thaw the cells for nearly 2 minutes by gently swirling the vial in the 37°C water bath until completely thawed.

3. Transfer the vial it into a laminar flow hood after wiping the outside of the vial with 70% ethanol
4. Transfer 9mL of pre-warmed complete growth medium appropriate for your cell line into the centrifuge tube and add the thawed cells to the media.
6. Centrifuge the cell suspension at approximately 1200 rpm for 7 minutes. After the centrifugation, check the clarity of supernatant and visibility of a complete pellet.
7. Aseptically decant the supernatant without disturbing the cell pellet.
8. Gently resuspend the cells in complete growth medium, and transfer them into the appropriate culture vessel and into the recommended culture environment.

2.3.2 Cell Counting

Description

Once the cell thaw has revived, the cell number needs to be determined before sub culturing. Cell counting using hemocytometer with the principle of trypan blue exclusion method is a common cell counting procedure used which is quite economical when compared to the various automated cell counters available in the market.

Materials

- (A) Hemocytometer (Neubauer Chamber),
- (B) Trypan blue(0.4%),
- (C) Cell culture sample

Protocol

1. Clean the Neubauer chamber and cover slip with alcohol. Dry and fix the coverslip in position.
2. Dilute the cell culture sample with trypan blue such that the number of cells that you would count be not more than 150. Vortex the sample thoroughly.
3. Add 10 μ L of the trypan blue/cells mixture to the hemocytometer. Do not overfill.
4. Place the chamber in the inverted microscope under a 10X objective.
5. Use phase contrast to distinguish the cells.
6. Count the bright cells in the four gridded squares A, B, C, D in the four corners of each side of the chamber. The gridded squares are circled in the Figure below.

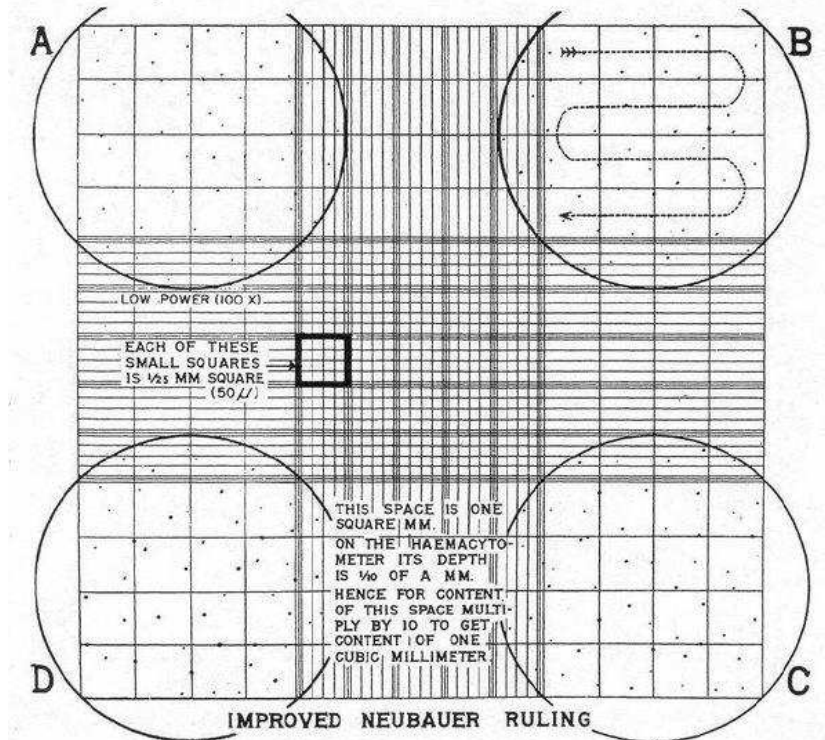


Figure 1 Hemocytometer chamber for counting cells

7. Multiply the average of the cell counts from two sides of the chamber by the dilution factor and divide by 400 to estimate the number of cells in millions per mL. This number estimated is called the Viable cell density and it gives the number of living cells in the culture
8. Using trypan blue dye exclusion, the dead cells are completely stained blue due to membrane permeation which I prevented in live cells. Count the blue dots in a similar way described in (7) which gives you the Dead cell density in the culture

9. Record the numbers on a sheet of paper and clean the chamber and cover slip with 70% ethanol. Dry the chamber and store it.

2.3.3 Cell Passaging

Adherent cell cultures description

The following protocol describes a general procedure for subculturing adherent mammalian cells in culture. Adherent cultures require cell detachment from the attachment surface before sub culturing. Care should be taken during the cell passaging stage because any deviation to usual culture behavior here, reflects in the end use of the culture.

Materials

- (A) Culture vessels containing your adherent cells,
- (B) Tissue-culture treated flasks, plates or dishes,
- (C) Complete growth medium, pre-warmed to 37°C,
- (D) Disposable, sterile 15-mL tubes,
- (E) 37°C incubator with humidified atmosphere of 5% CO₂,
- (F) Balanced salt solution such as Dulbecco's Phosphate Buffered Saline (DPBS), containing no, calcium, magnesium, or phenol red,

- (G) Dissociation reagent such as trypsin-EDTA (0.25%), without phenol red,
- (H) Trypan Blue and hemacytometer

Protocol

Note: All the materials in contact with the culture must be sterile. All the work with cells should be performed inside a Biosafety Cabinet of Biosafety Level 2.

1. Clean all the surfaces coming in contact with the cells with 70% ethanol.
2. Remove and discard the spent media from the culture vessel.
3. Wash cells using Material (F), approximately 2mL for T25 flask and 5mL for T75 flask, without disturbing the attached cells.

Gently add material (F) to the opposite side of the vessel where the cells are attached to avoid disturbing the cell layer, and rock the vessel back and forth twice or thrice.

Note: The wash step removes any traces of serum, calcium, and magnesium that would inhibit the action of the dissociation reagent.

4. Remove and discard the wash solution from the culture vessel

5. Add the pre-warmed Material (G) to the side of the flask; use enough reagent to cover the cell layer (approximately 0.5 mL to a T25 flask and 2ml to a T75 flask).
6. Gently rock the container to get complete coverage of the cell layer. Incubate the culture vessel at room temperature for approximately 5 minutes. For some cell lines like CL03, the incubation should be in the 37C incubator for 5 minutes
7. Observe the cells under the microscope for detachment. If cells are less than 90% detached, increase the incubation time a few more minutes, checking for dissociation every 30 seconds. You may also tap the vessel to expedite cell detachment.
8. When $\geq 90\%$ of the cells have detached, tilt the vessel for a minimal length of time to allow the cells to drain.
9. Add the one culture volume of pre-warmed complete growth medium.
10. Disperse the medium by pipetting over the cell layer surface several times.
11. Determine the total number of cells and percent viability using a hemocytometer.

12. Calculate the amount of inoculum necessary to start a new culture at a low cell density and add the calculated amount of culture to a culture flask containing fresh media.
13. Place the flasks in a 37°C CO₂ incubator and leave until the cells reach about 90% confluency

Suspension cultures description

The following protocols describe general procedures for subculturing mammalian cells in suspension culture. As these cells are in a suspension and are not attached, we do not need the step to detach the cells from the treated surface. These cell cultures are usually maintained in a shake flask which keeps agitating on a platform inside the CO₂ incubator.

Materials

- (A) Culture vessels containing your suspension cells,
- (B) New culture flasks,
- (C) Complete growth medium, pre-warmed to 37°C,
- (D) 37°C incubator with humidified atmosphere of 5% CO₂ with a shaker platform,
- (E) Trypan Blue and hemocytometer

Protocol

1. Take the flask out of the incubator and take a small sample from the culture flask using a sterile pipette.
2. From the sample, determine the VCD and percentage viability using a hemocytometer,
3. Calculate the volume of inoculum and aseptically add the appropriate volume of pre-warmed growth medium into the culture flask containing the inoculum.
4. Place the flask back into the incubator and measure the cell density after 72 to 96 hours of culturing.
5. Run the shaker at 130 rpm for a shake flask of 125mL capacity.

2.3.4 Mammalian transfection

Description

When we need to insert our gene of interest into a mammalian cells, we use transfection as the method of operation. The transfection reagent creates pores in the cell membrane without damaging it and the plasmid containing our gene of interest is then carried into the cell using the chemical molecules present in the transfection reagent.

Materials

- (A) Lipofectamine 3000
- (B) 6 well plates
- (C) DNA for transfecting (2.5ug per transfection)
- (D) OptiMEM
- (E) CO2 incubator with humidity
- (F) Biosafety Cabinet
- (G) 1.5mL Eppendorf tubes

Protocol

1. Seed the cells into a 6 well plate and grow until the cells reach 70-90% confluence.
2. Once the cells reach confluence, remove the culture media and wash the cells with PBS without disturbing the attached cells
3. Add 1.75mL of fresh prewarmed OptiMEM media and prepare the transfecting mixture in two different tubes
4. To the first tube labeled 'Tube 1', add 2.5ug of DNA, 5uL of Reagent P3000 (a part of the Lipofectamine 3000 kit) and 125uL of OptiMEM media in this order.
5. To the second tube labeled 'Tube 2', add 125uL of OptiMEM media and then add 5uL of reagent Lipofectamine 3000.

6. Add Tube 2 to Tube 1 and wait for 5 minutes. Do not disturb the tube or pipette it in this time.
7. After 5 minutes, add the contents present in Tube 1 dropwise into the culture well.
8. Transfer the well plate into the CO₂ incubator without disturbing it
9. After 24 hours, add 500uL of fresh culture media into the well to give enough nutrients for the cells to sustain in the culture.
10. The cells should be harvested after 72 hours of culturing either using trypsin or lysed using cell dissociation solution (either RIPA buffer or Cell lysis solution).
11. After harvesting the cells, the samples should be centrifuged at 13000rpm for 10 minutes and the supernatant can be stored in -80C as the cell lysate containing the protein expressed through transfection.

2.3.5 Sample preparation for proteomics

Once the cell culture experiment is performed, if we have to perform a proteomics analysis, the samples should be prepared before handing it to the Mass spectrometry experts for analysis. The following protocol should be followed to prepare the cells and later analyze them before sending the sample for proteomics.

Preparing cell pellets:

Materials

- (A) ice cold PBS,
- (B) Cell culture samples
- (C) pipette, conical tubes, eppendorf tubes,
- (D) bucket of ice,
- (E) bucket of dry ice

Protocol

1. Collect cell culture
2. Spin down pellet at 4C for 10min, 1200RPM
3. Remove media and resuspend in PBS
4. Divide 2-3e6cells into 1.5mL eppendorf tubes (1mL per tube)
5. Centrifuge eppendorf tubes at 4C for 5min, 5000RPM
6. Remove PBS and resuspend in PBS
7. Centrifuge eppendorf tubes at 4C for 5min, 5000RPM
8. Remove PBS and freeze on dry ice
9. Store frozen pellets at -80C

Protein digestion:

Materials

- (A) Frozen cell pellet,

(B) 2% SDS, TEABC, PMSF, EDTA, Iodoacetamide, TCEP,

(C) BCA assay kit, 9M urea, spin filters 10kDa, ammonium bicarbonate, speed-vac, hydroxylamine, centrifuge, pure water, bucket of ice, TMT 6plex kit, triethyl ammonium bicarbonate (90360 Sigma), glacial acetic acid (100%, A38-500 Fisher)

Protocol

1) Prepare 100mM PMSF aliquots dissolved in isopropanol, can store at -20C. PMSF (phenylmethanesulfonyl fluoride).

2) Use cell pellet frozen at -80C. Prepare 2 % SDS and then take out how much you need and measure pH (use ultrapure water).

3) Add 0.1 mM PMSF and 1 mM EDTA to final concentration (after you added PMSF in it, you should put it on ice but the SDS can get crystallized if it waits very long in ice. So be careful, either prepare it in small amounts or always check the tube and vortex it or bring it to room temperature.)

PMSF and EDTA was added to added the SDS before the cells were added in order to have a large volume (easier to pipette).

5) The lysates are denatured in lysis solution. Sonicate three times as 60 seconds sonicate and 90 seconds pause on ice (the sample should not get heated while sonication). Do the sonication on ice (Sonication for one minute then rest on ice while other sample was sonicated) Set sonicator to 40% so it

does spray as much. Have an ethanol napkin to dry off the sonicator between samples

6) SDS can get crystallized so be careful on the sample. If it is crystallized, vortex it well after sonication to get a good sample for BCA.

7) The protein concentration is measured with BCA assay

BCA Assay

This assay measures the total protein content in the sample. This is a colorimetric assay and needs to be performed at specific time points without delay. The BCA assay comes as a kit and the instruction to run the assay are given in the handout provided.

Protocol

1. Mix Reagent A and Reagent B in a 1:50 ratio and add 200uL of this mixture to each wells to be used in a 96 well non-treated plate
2. Add 25uL of the standards A through I (Prepared in advance from the BSA standard provided with the kit) in triplicates into the wells already containing 200uL of the reagent
3. In a similar fashion add, the protein sample you want to analyze at your desired dilution. Note: Vortex all the samples and standards before adding to the wells

4. Incubate the plate at 37C for 30 minutes to 1 hour and analyze the plate using a plate reader at 290nm

2.3.6 ELISA for IgG Quantification

Materials

- (A) DPBS (Dulbecco's Phosphate Buffered Saline) ready to use (Invitrogen Catno. 14190-094)
- (B) Tween-20
- (C) Pierce Protein-free block
- (D) Bovine serum albumin (BSA)
- (E) Capture antibody: Mouse anti Human IgG Fab specific, 1.5 mg/ml (Jackson 209 005 097, store at 4°C)
- (F) Detection antibody: Goat anti human IgG-HRP Fc specific, 0.8 mg/ml (Jackson 109 035 098, store at 4°C)
- (G) ABTS (=2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) single solution (ZYMED Cat no. 00-2024, store at 4°C)
- (H) IgG standard: Purified human IgG, 5 mg/ml (Zymed Catno. 02-7102) (store at -18°C)
- (I) Coating buffer: 5 µg Capture antibody/ ml in DPBS (prepare fresh daily) pipette 10µl Capture antibody in 30ml DPBS

- (J) Wash buffer: 0.05% Tween-20 in DPBS (prepare fresh daily)
pipette 0.5ml Tween-20 in a mixture of 500ml DPBS and 500ml demineralised water
- (K) Stock Protein solution: 6% (w/v) BSA in DPBS dissolve 3 grams BSA in 50ml DPBS,
- (L) Blocking buffer: 2% BSA in wash buffer (prepare daily) dilute 30ml stock EM-7 with 60ml wash buffer
- (M) Diluents buffer: 0.33% BSA in wash buffer (prepare daily) dilute 20ml blocking buffer with 100ml wash buffer
- (N) Detection buffer: Detection antibody 1:6500 in diluents buffer
Pipette 4 μ l detection antibody in 26ml diluents buffer Dilute directly before use.
- (O) Stop solution: 0.1 M citrate Dissolve 5.259 gram sodium citrate in 250ml MilliQ or sterile water, (store at 4°C.)

Protocol

1. Coating the Plates: Add 100 μ l of the coating buffer to every well of the 96 wells plate. Incubate the plate overnight (at least 12 hours) at 4°C, covered with aluminum sealing tape.

2. Preparation of calibration curve: Pipette 10 μ l IgG standard in a 50 ml flask and dilute with diluents buffer to 50 ml (standard solution A).

Concentration: 1000 ng IgG/ml.

Dilute standards in polypropylene tubes as follows:

Table 1: Standard preparation for ELISA

Sno.	Standard solution	diluents buffer in ml	end concentration IgG
1	1.0 ml of standard solution A	1.0	500ng/mL
2	1.0 ml of standard solution 1)	1.0	250ng/mL
3	1.0 ml of standard solution 2)	1.0	125ng/mL
4	1.0 ml of standard solution 3)	1.0	62.5ng/mL
5	1.0 ml of standard solution 4)	1.0	31.25ng/mL
6	1.0 ml of standard solution 5)	1.0	15.62ng/mL
7	1.0 ml of standard solution 6)	1.0	7.81ng/mL

8	1.0 ml of standard solution 7)	1.0	3.91ng/mL
9	1.0 ml of standard solution 8)	1.0	1.95ng/mL
10	1.0 ml of standard solution 9)	1.0	0.98ng/mL

Measurement procedure

1. Wash the coated plate 3 times with 200µl wash buffer in the plate washer
2. Dry the plate by tapping it gently on a tissue, lied flat on the bench
3. Pipette 200 µl blocking buffer into each well
4. Incubate plate, covered with aluminum sealing tape, for 60 minutes at room temperature.
5. Wash the plate 3 times with 200µl wash buffer in the plate washer
6. Dry the plate by tapping it gently on a tissue
7. Add the sample and the standard in the scheme that has been determined on the plate.
8. Incubate plate, covered with aluminum sealing tape, during 60 minutes at 37°C on the shaker at 350 rpm.
9. Wash the plate 3 times with 200µl wash buffer in the plate washer
10. Dry the plate by tapping it gently on a tissue

11. Add 100 μ l detection buffer/well
12. Incubate plate, covered with aluminum sealing tape, during 60 minutes at 37°C on the shaker at 350 rpm
13. Wash the plate 3 times with 200 μ l wash buffer in the plate washer
14. Dry the plate by tapping it gently on a tissue
15. Clean, if necessary, the underside of the plate
16. Pipette 100 μ l ABTS into each well
17. Incubate for 6 minutes
18. Pipette 100 μ l stop solution to each well
19. Measure the extinction at 410 nm (=E410), within 10 minutes after stopping the reaction

2.4 Molecular Biology Protocols

In Chapter 4, molecular cloning has been an integral part of the research. Genes have to be cloned into a mammalian expression vector for expressing in the mammalian cells. These protocols are discussed in the sections below.

2.4.1 PCR Cloning

Adding a gene to a plasmid is called cloning. When this cloning insert is obtained through a Polymerase chain reaction, it is termed as PCR cloning.

There are several steps involved in this process are:

(A) Designing primers for the gene of interest and performing a PCR to obtain the gene out of the genome or the plasmid is the first step in cloning. PCR reaction is performed in a thermos cycler by shifting the temperatures at the end of each cycle to perform the reaction. The reagents that were used for PCR are from New England Biolabs. The table below gives the protocol used to perform this step:

1. Add the components given in the table into an Eppendorf tube and mix them well
2. Place all the component of ice at all times along with the master mix prepared.
3. Add 25uL of the master mix to each PCR tube and start the thermocycler with the settings recommended and the annealing temperature set.
4. After the cycle ends, place the PCR tubes on ice for immediate use or store it at -20C for future use.

Table 2: Phusion Polymerase master mix recipe

Component	Final concentration	Volume for (50uL reaction)
Nuclease-free water		To 50uL
5x Phusion HF or GC buffer	1X	10uL
10mM dNTPs	200uM	1uL
10uM Forward Primer	0.5uM	2.5uL
10uM Reverse Primer	0.5uM	2.5uL
Template DNA	<250ng	Variable
Phusion DNA Polymerase	1.0 units/ 50uL PCR	0.5uL

(B) Gel Electrophoresis and PCR purification:

Once the PCR reaction is completed, the sample needs to be tested to check if the insert has been amplified or not. For this purpose, we perform gel electrophoresis with 0.1% agarose gel as follows:

1. Add 0.4g of ultrapure agarose to 40mL of 1X TAE buffer and boil the solution.
2. After the solution becomes lukewarm add 4uL of Ethidium bromide, pour the gel into the casting chamber and add the comb.
3. Once the gel sets, transfer it to the electrophoresis chamber and load the DNA ladder and the sample into the wells.

4. Run the gel at a constant voltage of 70 to 100 volts for 1 hour, ensuring that the DNA doesn't run off into the buffer
5. Once the run is completed, visualize the gel under UV light to see the DNA bands.

Once the DNA of interest is detected on the gel, it can either be extracted from the PCR product using gel extraction kit or PCR purification kit from Qiagen. The protocol is given along with the kit which can be followed to perform this step. In general PCR purification gives higher quantities of DNA when compared to gel extraction.

(C) Restriction digestion and Ligation

After the insert has been purified from the PCR mixture, the insert and the plasmid needs to be digested using restriction enzyme for creating a spot on the plasmid for the insert to attach. Digesting the DNA is immediately followed by ligation and the plasmid is ready for transformation.

2.4.2 Bacterial Transformation

The plasmid which contains the insert needs to be inserted into a bacterial organism for replication purpose. This step is termed as bacterial transformation. The organism chosen for transformation is an E.coli which is at its peak of production once thawed. Such cells are known as competent

cells. The protocol to transform bacteria to copy our plasmid is adopted from Addgene and is given below:

1. Take competent cells out of -80°C freezer and thaw them on ice for 30 minutes.
2. Prepare LB agar plates with antibiotics resistance and leave them at room temperature to set under sterile conditions (flame on)
3. Take 1-5 μL of DNA (containing 10pg to 100ng of DNA) and add to 25 μL of competent cells and place the mixture back on ice for 30 minutes
4. Place the cells containing the DNA in a water bath at 42C for 45 to 60 seconds and immediately transfer the tube to ice and incubate for 2 minutes
5. Add 500 μL of SOC media (without antibiotics) and incubate the tube at 37C for 45 minutes.
6. Pour the contents of the tube on to the agar plate and spread them using a cell spreader
7. Incubate the plates for 12 to 16 hours and check for colonies.
8. Once there are colonies formed, pick a few colonies to grow the E.coli in liquid LB medium.
9. After 12 hours, create glycerol stocks with the culture and extract the plasmid from the culture using minipreps

2.2.1 Western Blotting

Western blotting was performed on cell lysates in the research described in Chapter 4. Western blotting is a two step procedure. After performing BCA to know the total protein concentration in the cell lysate sample, they are used to run an SDS-PAGE gel which is the first step. Later the gel contents are transferred onto a PVDF membrane to detect if the gene inserted has been expressed or not.

(A) SDS-PAGE

Gel electrophoresis using a poly acrylamide gel is a common technique used to separate a mixture of proteins based on their molecular weights. The protocol used to perform this step is given below:

1. The samples to be loaded are prepared using lysate samples, PBS and 4X loading dye (prepared from lamellae buffer)
2. Samples are denatured using heat at 95C for 5 mins to linearize the proteins for the gel run
3. Load the samples and the ladder on to the gel and run the gel at 70 volts for 15 minutes until the samples get out of the well and later bump up the voltage to 100V for resolving the samples in the gel.
4. Run the gel until the blue line runs till the end of the gel.

(B) Blotting

After SDS-PAGE, the gel contents have to be transferred to PVDF membrane and later the membrane needs to be blocked. The blocking step is followed by treating with the primary and secondary antibody. After the secondary antibody incubation step is completed, the membrane can be visualized for the protein of interest. These activities can be performed through the following steps:

1. Assemble the transfer cassette by placing in the transfer buffer in this order: Cathode(Black) → Sponge pads → filter paper → gel → membrane → filter paper → sponge pads → Anode (white)
2. Run the transfer process at 100V for one hour using cold transfer buffer and an ice pack inside.
3. Once the transfer process is completed, block the membrane for one hour using 5% milk prepared using Blotting grade blocker from BioRad
4. Incubate the membrane with primary antibody overnight at 4C.
5. If the primary antibody is HRP conjugated, the membrane can be taken for detected at this stage. Otherwise, the membrane should be incubated with a secondary antibody conjugated with HRP for an hour to enable detection. Use chemiluminescence to detect the protein on the membrane with the help of Pierce kit.

Chapter 3 HYDROLYSATES IN MAMMALIAN CELL CULTURE

MEDIA

3.1 Introduction

Cell culture media provides the necessary nutrients for the cells to grow. It is a mixture of substrates like glucose, glutamine, and other amino acids together with other components important for the growth of mammalian cell cultures like vitamins, minerals and certain growth factors. The culturing of mammalian cells was done in early 1900s in naturally available serum from blood of different mammals. Such media are comprised of a balanced salt solution with natural growth promoting elements like serum, body fluids and exudates, and extracts of tissues and organs. Since the composition of these natural elements is not known, we introduce a lot of variability into the cell cultures. Such inconsistent processes are not suitable for producing large scale biomolecules since the bioprocess is not controlled by external factors. It is also difficult to track the metabolism of cells growing with serum due to unknown components though they are cultures in controlled conditions.

Eagle in 1950s made the most significant advancement in the nutrient requirement of cell cultures. He could create a chemically assembled medium combining amino acids, vitamins, cofactors and add serum to it to propagate

cell cultures. Even though a chemically defined media was invented by Eagle and group, it still used serum to grow the cultures [6]. Serum contains important components necessary for the growth of cell culture like proteins, growth factors, lipids and other trace elements. Use of serum to produce biologics has a major regulatory and economic concern. When serum is used to culture cells, there might be a possibility of bacterial, or viral contamination or a presence of mycoplasma which could not be eliminated to a 100% after multiple steps of purification. Also, Food & Drug Administration (FDA) will not approve any drug created in a process which uses animal components in its process [13]. This was a major drawback of the cell culture media until the development of serum free media which is fully chemically defined media without using any animal component in it. Serum-free media reduced the downstream processing cost for the industry and opened paths to design media with millions of different formulations. But the strength provided by the serum was still lacking in the cell cultures.

Strategies to replace this serum deficiency with another new component or nutrient enhancement has led to the introduction of hydrolysates into the cell culture media. Hydrolysates are derived from plant proteins like cotton, soy, wheat, rapeseed and so on. These hydrolysates not only were known to improve the growth by also were able to increase the productivity of the cell

cultures. With this unique feature, the era of serum-free media turned towards Hydrolysates for relief [14]. The hydrolysate supplementation to a serum free formulation enhanced the productivity of the cell lines similar to the case with serum containing media [15]. As soon as the utility of the Hydrolysates was discovered, there were various studies, proving their ability to improve the cell culture performance and different sources of hydrolysates were compared to find a winner among the troop, wheat hydrolysate giving 56% increase in productivity [16]. Even though this is a known fact today, the mode of action of the hydrolysates is yet to be explored. Several -omics tools exist to guide us through the metabolism that the hydrolysates undergo inside a mammalian cell. This chapter describes one such attempt to know the cause of the action.

3.2 Aim

Cell culture media composition can significantly influence the phenotype of a cell line. The influence of a component on the cell culture performance varies even with the chemical formula it is in. Media components can affect recombinant protein production yields, growth, and product quality. Optimal concentration of all the media components is a necessary condition for running a cell culture process. It is thus important to control media formulations in order to develop a robust process. Hydrolysates has been

known to improve the cell culture performance for a decade now. Plant protein hydrolysates are a replacement to animal serum in cell culture media. They have a concoction of nutrients that would potentially boost the cell culture performance. Commercial media providers have been using several kinds of hydrolysates to media. The effect of hydrolysates on the culture performance is also dependent on the cell line available. An IgG producing cell line was provided by Friesland Campina through Bioceros Ltd to test if there is an improvement in productivity in the CHO cell line due to hydrolysate supplementation. All the hydrolysate lots provided were produced by Friesland Campina from soy and cottonseed.

Every lot of the hydrolysate do not behave in the same way with the cell line available. The effect of hydrolysate might also vary with the type of cell line used. For this reason, various lots of hydrolysates have been manufactured by the Friesland Campina and these lots will be evaluated for the cell culture performance and final IgG productivity. While the cell culture performance will be evaluated using cell viability and viable cell density measurements, the antibody productivity will be measured using ELISA. Although the composition of the cottonseed hydrolysate and soy hydrolysate is known to Friesland Campina, the component(s) which enhance productivity, cell growth, and product quality are not defined precisely.

Therefore, the goal will be to verify the effects of hydrolysate supplementation primarily on recombinant protein productivity. Specifically, the effect of cottonseed hydrolysate during exponential and stationary phases will be distinguished. A secondary goal will be to establish any effects of the hydrolysate on cell growth. The intermediate aims will use transcriptomics and metabolomics to elucidate the mechanism of action of hydrolysates. Overall, the project will increase understanding of how hydrolysate supplementation affects bioprocess parameters. This will be accomplished using transcriptomics and metabolic flux analysis to delve deeper into cell culture metabolism the result of which will be worked out in the future studies.

The project aims to evaluate how hydrolysate supplementation affects the recombinant protein production yields and growth of a cell line, respectively. The cells will be supplemented with hydrolysates which have been manufactured from cottonseed and soybean at Friesland Campina. The different lots of hydrolysates behave in a different manner and influence the cell culture in a different way. The effect of different lots and types of hydrolysates will be evaluated for the given cell line producing IgG as the monoclonal antibody product. Cell growth will be monitored daily by hemocytometer using trypan blue dye exclusion method. The recombinant

protein production will be measured by an ELISA assay. We will look for differences in product yields as a result of cottonseed hydrolysate, soy hydrolysate, or no supplementation during both exponential and stationary phases. It is expected that cottonseed hydrolysate supplementation boosts the mammalian cell growth rate with increased recombinant protein production in comparison to soy hydrolysate supplemented culture media and media with no supplementation. The further section will describe the details of the experiment and the results and discussion

3.3 Culturing cells for the experiment

3.3.1 Cell thaw

Six vials were sent to JHU in April 2016. Cells were revived from liquid nitrogen (-196°C) using a cell thaw protocol used for mammalian cell cultures (Section 2.3.1). The cell media to during thawing cells is ExCell CD CHO media supplemented with 0.4% of Soy Hydrolysate. Take 48ml of ExCell CD CHO and add 1ml of 200 mM solution of L-Glutamine. Then add 1ml of Poloxomer 188 to the mixture. Then weigh 0.4% of soy hydrolysate which would be nearly 200mg and dissolve it in the culture media prepared above. This will darken the color of the media to golden.

Once the soy hydrolysate dissolves completely, filter the solution using a 0.22micron syringe filter to use it in a sterile manner. This thaw media should be freshly prepared for every cell thaw. The culture media after the addition of soy hydrolysate cannot be used after 96 hours. In a 15ml falcon tube, add 9mL of the culture media prepared. Thaw the cell vial in a 37°C water bath and add the contents of the vial to the falcon tube containing 9ml of culture media. Centrifuge the tube containing the contents of the vial at 1200 rpm for 5 to 7 minutes to remove the DMSO added to the cells while freezing. Discard the supernatant and resuspend the pelleted cells at the bottom of the falcon tube in 10ml of fresh culture media. Add this resuspended culture into a new ultra low attachment tissue culture flask and top it up with 5ml culture media to start the initial cell culture at 15mL.

Take the initial cell count and viability measurements. The cell density was observed to be around 0.2 million cells/mL and the cell viability was nearly a 100%. After 24 hours, it was observed that the cell culture viability has dropped to less than 40% and after 48 hours it further dropped to 0%. This was the first challenge encountered and had to be resolved in order to continue the project further. Several alternate procedures were tried to revive the cells.

Alternate procedure 1: Another vial was thawed and after 24 hours, the cell culture was centrifuged to replace the old culture media with new one.

This change in the thaw protocol was made through the hypothesis that, the cells are being exposed to some harmful contents in the culture media and after 24 hours all the necessary nutrients of the media were exhausted and replacement would provide extra nutrients to grow. On the contrary, the cell viability reduced further after 48 hours due to centrifugation and further resuspension of cells, some cells have been lost further reducing the cell density. This ruled out of hypothesis that the nutrients in the media were exhausted and we moved on to the next alternative.

Alternate protocol 2: After vial thaw, the culture in the tissue flask was decided to be moved to the shake flask before the culture viability hits 50%. This would give the culture enough cells to expand in a shake flask condition. We observed in the previous vial thaws that the culture viability dropped to almost 50% after 24 hours. Thus, we have decided to move the culture to a new shake flask and top it up with 10mL of cell culture media with no soy hydrolysate supplementation. This method gave less cells to begin with but the culture flask took 10 days to grow to a cell density greater than 1 million cells/mL. Once the culture was transferred to a shake flask after 24 hours, the cell density was less than 0.05million cells/mL. The culture media was replaced every three to four days to ensure that the nutrients like glutamine have not been depleted or degraded since the culture temperature is 37°C.

The cell counts and viability were monitored every three days to have enough time for the cells to grow. The doubling time was greater than 40 hours in the first few days and then by the end of 10 days the doubling time came up to 24 hours. The bank not being in good condition was the reason for our problems, not allowing us to revive the cells successfully. The cell thaw procedure was finally revised for these cells. The subsequent sections describe the maintenance of seed train and the details of the experiments performed to test the effect of hydrolysates on cell culture performance in CHO cells.

3.3.3 Subculture

After the cells fully recovered from the vial thaw, they were allowed to grow to a cell density of greater than 1 million cells/mL and then sub cultured at a density of 0.2 million cells/mL. Take 30mL of culture media containing 0.6mL of L-Glutamine and 0.6mL of Poloxomer 188 in a baffled shake flask. Based on the cell count of the old culture flask, calculate the volume of culture necessary to seed the new flask with 0.2million cells/mL. Remove the amount of media calculated from the new flask and add the same amount of old culture into the new flask.

Label the flask and place it on a shaker platform in an incubator shaking at 130 rpm and the conditions of the incubator are 5% CO₂ and 37C. Wait for

three days until the cells grow to a cell density greater than 1 million cells/mL. In case the viability of the culture is low, the culture cannot be used for future experimentation. One way to increase the viability in such cultures, if the value is below 50%, is to replace the old culture media and the dead cells along with the debris by centrifugation. The centrifugation process at 1200 rpm for 5 mins would settle only the cells and not the debris and lysates of the dead cells which are present in the supernatant. The proteases in the cell debris and lysate would stop damaging the healthy cells and fresh media would provide new nutrients to the cell culture for growth and cell division.

3.4 Cell culture experiments

3.4.1 Experiment 1

To determine the effect of hydrolysates on the CHO cells provided by Friesland Campina, a batch mode of operation for the cell culture should be performed and the growth characteristics of the cells should be observed. As a preliminary study to check if there is a difference between the growth of cell in normal media, soy and cotton hydrolysate supplemented media, a test experiment was performed with three flasks one for each condition. This experiment would give us more information on the growth profile of the CHO cells provided to us and confirm to us that all the materials provided are

behaving in a way in which they are intended to. For example, we have to check that the media is not killing the cells and is working well to let the CHO cells grow.

We also need to check whether the optimal conditions for growth of the cells is 5% CO₂, 130 rpm and 37C. There should also be a prior knowledge of the day to day cell growth before we perform the actual experiment with for our end goal. We should have the information of when the cells would crash and when the nutrients provided at the beginning of the culture in a batch process would be exhausted. For these purposes, the first experiment was planned and executed with three flasks one for each condition of media additive.

Table 3: Details of the plan of Experiment 1

Flask No.	Condition	Type of Additive	Lot No.
1	Control	No Additive	N/A
2	Hydrolysate 1	0.4% of Soy Hydrolysate	SE50MAU
3	Hydrolysate 2	0.4% of Cotton Hydrolysate	100NTPCR

To start the cultures, we have to follow the protocol described in the following. After determining the volume of inoculum needed to start the batch process at a seeding density of 0.2 million cells/mL, the old media should be replaced with the culture media containing the additive that the flask needs to start with by centrifugation and further resuspension. This would ensure that there is no media carry over from the passage flask and the concentration of hydrolysate in the media is indeed 0.4% and not less. This concentration of hydrolysate was prescribed by the collaborator company. Increasing the concentration would increase the osmolality in the culture leading to damage to the cells. As the hydrolysate is a bolus of nutrients comprising of sugars and proteins, the diversion from the optimal concentration would increase the variability in the expected result. (*See FR thesis)

Cell culture samples were collected daily for cell growth analysis using a hemocytometer and the samples of the later days nearing to the end of culture (day 5 to day 8) were centrifuged and the supernatant was stored to perform the titer analysis using ELISA and YSI analysis for Glucose and Lactate metabolism data.

Cell culture performance results

The cell culture was harvested on Day 11 when the viabilities of all the flasks was below 50%. The criteria for harvesting cells is that the culture should have viability less than 50%. Once this criterion is met, the culture is transferred into a 50mL falcon tube and centrifuged at 3000 rpm for 10 mins to pellet down all the cells and have just the media in the supernatant. The supernatant should be stored in -80°C for preventing it from degradation. All the samples collected during the cell culture should also be stored in -80°C so that there is no degradation of proteins in the samples. The number of freeze-thaw cycles should be reduced to minimize any damage to the samples due to temperature shock.

As to the results of the experiment, the cell culture performed well with the provided media ExCell CD CHO fusion. The culture was started at a cell density of 0.2 million cells/mL. There was exponential phase for the first five days and then the culture hit stationary phase for the next three days then the culture progressed to death phase. This was the trend with all the variants of the cell culture in this experiment. The distinction between the three conditions that was supposed to appear in the cell growth is shown in the cell growth plots.

When we see the VCD plot, we can see that Flask 1 has a slower growth when compared to the other two flasks. This is due to the absence of hydrolysate in the media. The presence of 0.4% of hydrolysate in the culture media is seen to have an effect on the cell count. We should look at the viability plot and observe that after the culture hits the stationary phase, the viability of flask 1 drops gradually whereas the other two flasks crash all at once. This observation is a clear distinction between the condition where hydrolysate is present or it is not present.

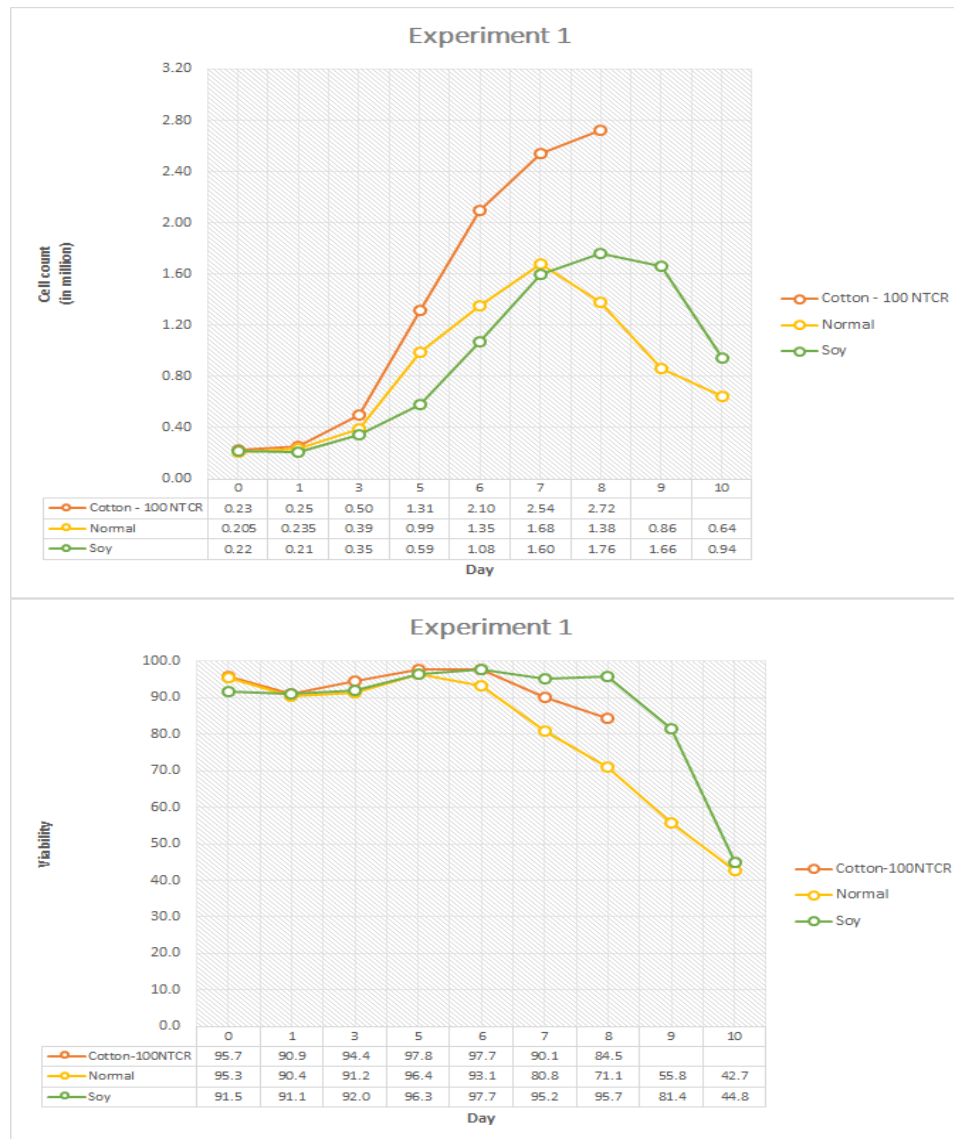


Figure 2 Viable cell density (Top) and Viability (Bottom) of the culture in Experiment 1

The peak cell density which is the maximum cell density attained in a cell culture process for the flasks containing hydrolysates is clearly higher than the one not containing the hydrolysate. Even between the two flasks

containing hydrolysates, the flask containing soy hydrolysate has a lower peak cell density compared to the one with cotton hydrolysate. The exponential phase is steeper in the cotton hydrolysate culture when compared to soy hydrolysate and the normal media flask is the least steep in the exponential phase region.

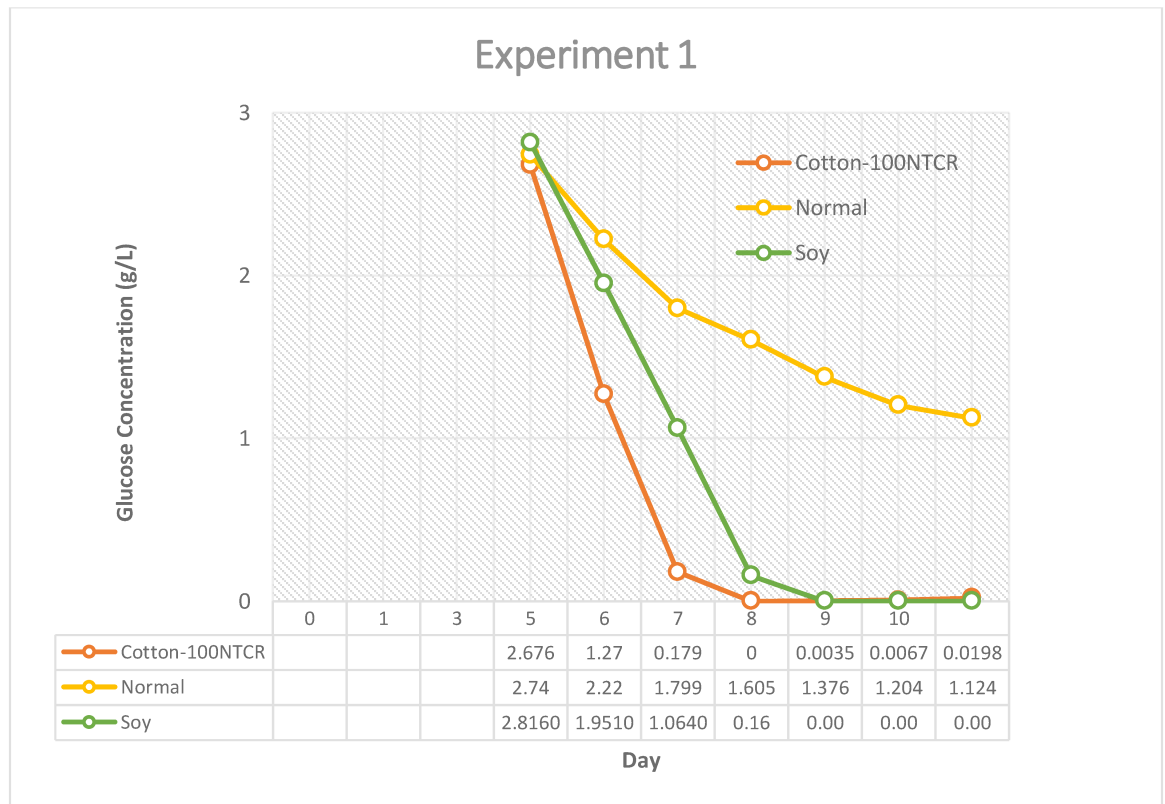


Figure 3 Glucose Concentration of the culture in Experiment 1 (g/L)

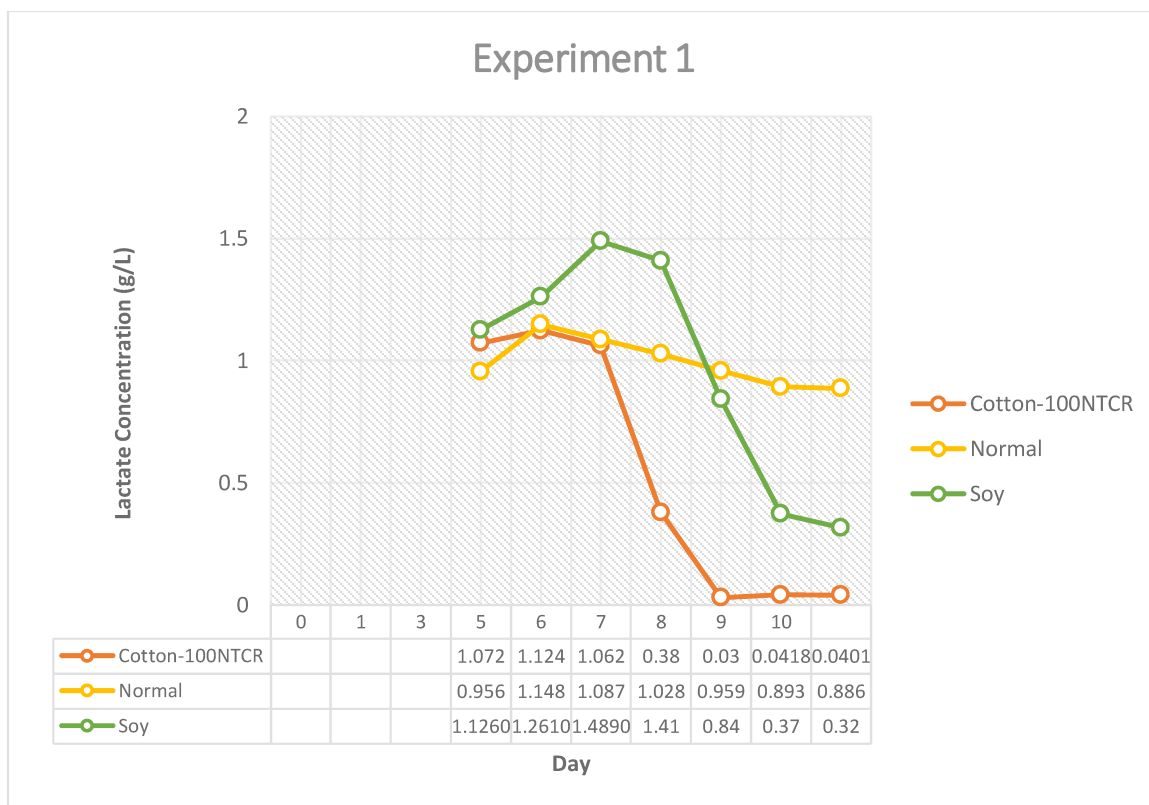


Figure 4 Lactate concentration in the culture for Experiment 1 (g/L)

When we analyzed the samples for glucose and lactate, we observed that in the soy and cotton hydrolysate, the time points of cell death coincided with the exhaustion of glucose in the culture medium. Since the cell density was high at those times, due to lack of primary energy source, the cell death was triggered. Since the samples for glucose and lactate analysis were only collected from day 5 as per the recommendation of the collaborator company, there is no data from this experiment for the time points before day 5. Thus, we do not have a full metabolite profile.

3.4.2 *Experiment 2*

Once there was a level of confidence with the cell culture batch process, the second experiment was planned with more conditions. The aim of the second experiment was to test three of the five lots of cotton hydrolysates manufactured by the collaborator company in 2016. These lots that were selected for this are 100NTCR, 100PCHO and 100P1HW for cotton hydrolysate. The three cotton lots were selected based on the fact that they were the most recent ones to be manufactured by the company.

This time of manufacture is an important factor because as the days of manufacture increases, there is a fairly higher chance for the hydrolysates to absorb moisture and become clumpy. When the hydrolysate powder absorbs moisture, it can no longer be used for the experiment. Thus, the hydrolysate powder should be kept away in a dry place and out of light. The experiment 2 was planned with 5 different conditions in a total of 9 cell culture flasks. The different hydrolysates were run in duplicates. The scheme of the experiment goes as follows:

Table 4 Details of the plan of Experiment 2

Flask No.	Condition	Type of Additive	Lot No.
1	Control	No Additive	N/A
2	Hydrolysate 1	0.4% of Soy Hydrolysate	SE50MAU
3	Hydrolysate 1	0.4% of Soy Hydrolysate	SE50MAU
4	Hydrolysate 2	0.4% of Cotton Hydrolysate	100NTPCR
5	Hydrolysate 2	0.4% of Cotton Hydrolysate	100NTPCR
6	Hydrolysate 3	0.4% of Cotton Hydrolysate	100PCHO
7	Hydrolysate 3	0.4% of Cotton Hydrolysate	100PCHO
8	Hydrolysate 4	0.4% of Cotton Hydrolysate	100P1HW
9	Hydrolysate 4	0.4% of Cotton Hydrolysate	100P1HW

*The culture conditions are 5% CO₂, 130 rpm and 37C for a duration of 8 days

Cell culture samples (of 200uL volume) were collected daily for cell growth analysis using a hemocytometer and the samples were centrifuged and the supernatant was stored to perform the titer analysis using ELISA and YSI analysis for Glucose and Lactate metabolism data.

Cell culture performance results

The second experiment was aimed at looking also at the lot variability between the cotton hydrolysate lots that were manufactured in 2016 and provided to us by the collaborator company. The cell culture plots of this experiment are given below. From the VCD plot we can see that the media without hydrolysate supplementation clearly has much lower cell densities compared to the other samples. We have seen a reduction in cell densities for all the flasks after day 0. When we inoculate the flasks at 0.2million cells/mL, the count on day 1 reduces to around 0.15million cells/mL and then the cell growth picks up. This slowing of growth is reflected as reduction in viability too on Day 1. Probing into this issue further revealed the reason for this observation.

The protocol provided by the company mentions that we need to centrifuge the inoculum to remove the old media so that exact amounts of hydrolysate is reflected in the culture. Centrifugation is a rough procedure

which is stressing the cells. Moving the cells into a new media which contains hydrolysate is not a reason for this viability crash is not a reason since the cells in the control flask have also shown a reduction in viability. Adding to this, the initial cell density is only 0.2million cells/mL. This is a low cell density to start with and when the viability drops at this cell density, the cells take some time to recover.

Owing to these reasons, the viability drop is justified. It is in line with this observation that, the cells culture containing hydrolysate showed faster recoveries when compared to the control flask. This shows us that the hydrolysates are in fact providing a boost of nutrients and extra factors necessary for growth for the cells in the culture.

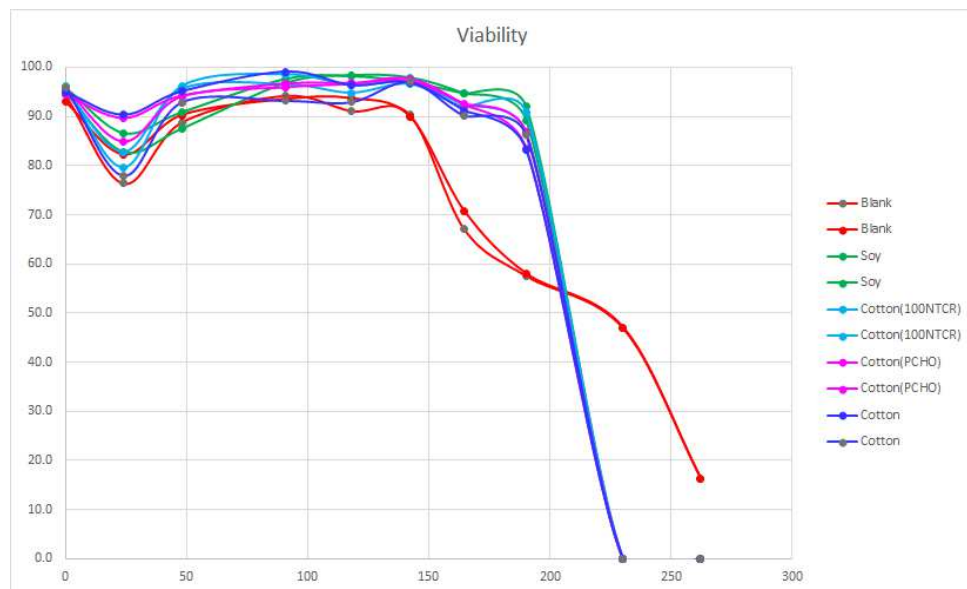


Figure 5: Cell culture viability for Experiment 2

We have tested three different lots of cotton hydrolysates in this experiment run. All the three cotton hydrolysate lots performed better than control flask in terms of cell density and cell viability. The peak cell density of the cotton lots were more than 100% higher than that of the control flasks. As this observation served half the purpose of our experiment, when the cotton lots were compared to the soy hydrolysate lot which also performed better than the control flask, there were some interesting observations made.

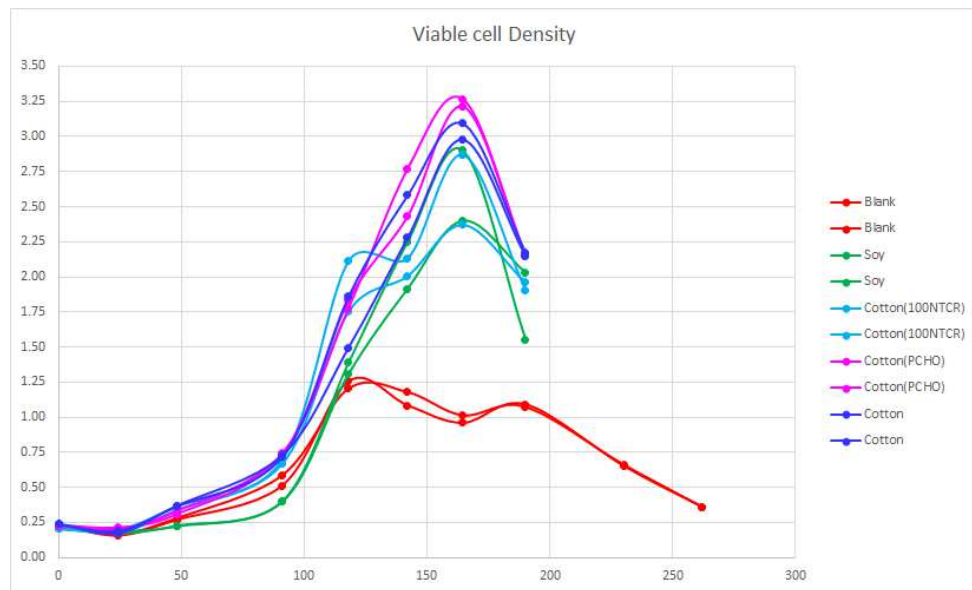


Figure 6: Viable cell density (million cells/ mL) for Experiment 2

Contrary to the result of the first experiment, the cotton lot 100NTCR performed similar to the soy hydrolysate lot. The other two cotton hydrolysate lots performed better than soy Hydrolysate flasks in terms of cell density. When the cell viabilities were compared, the observations were similar when compared to the previous experiment. The point of distinction in the viability profile is that this time the soy and cotton hydrolysates crashed at the same time. Backing up this observation is the glucose profile which was similar for all the hydrolysate lots when on day 6 all the glucose was exhausted. On the other hand, the glucose in the control flask was stagnated at a 1g/L value after the stationary phase where there is no further growth in the cell culture.

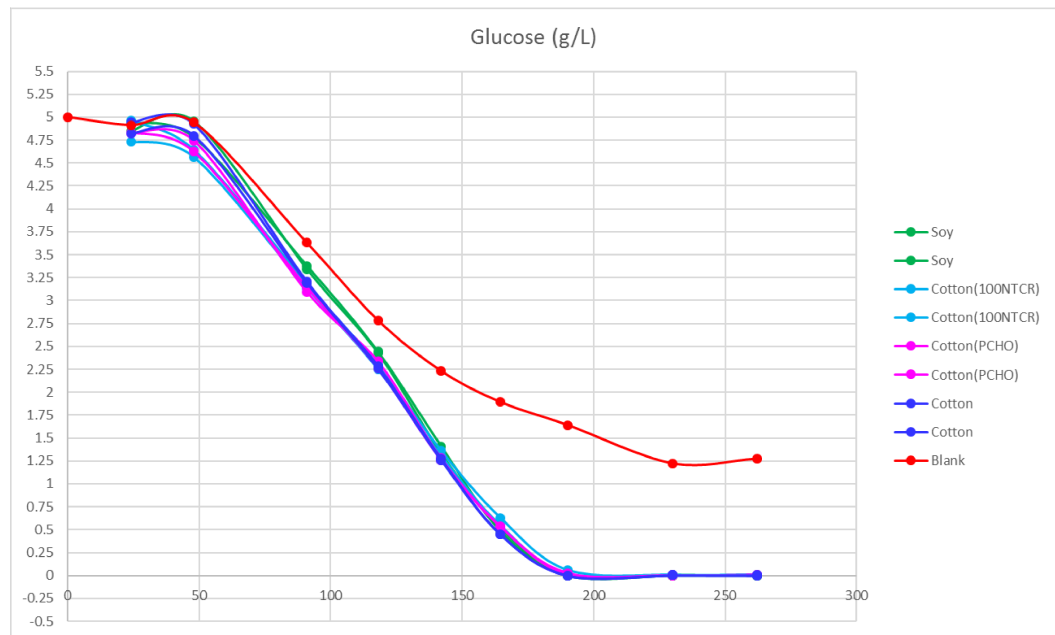


Figure 7: Glucose concentration profile for Experiment 2

But one thing that shows that there are no metabolism related changes due to the addition of hydrolysate is the fact the glucose consumption trend is the same among all the flasks. It is just that as there are more number of cells in the hydrolysate supplemented cultures that the amount of glucose consumed is more. Of we normalize glucose plot, it will have the same slope in all conditions.

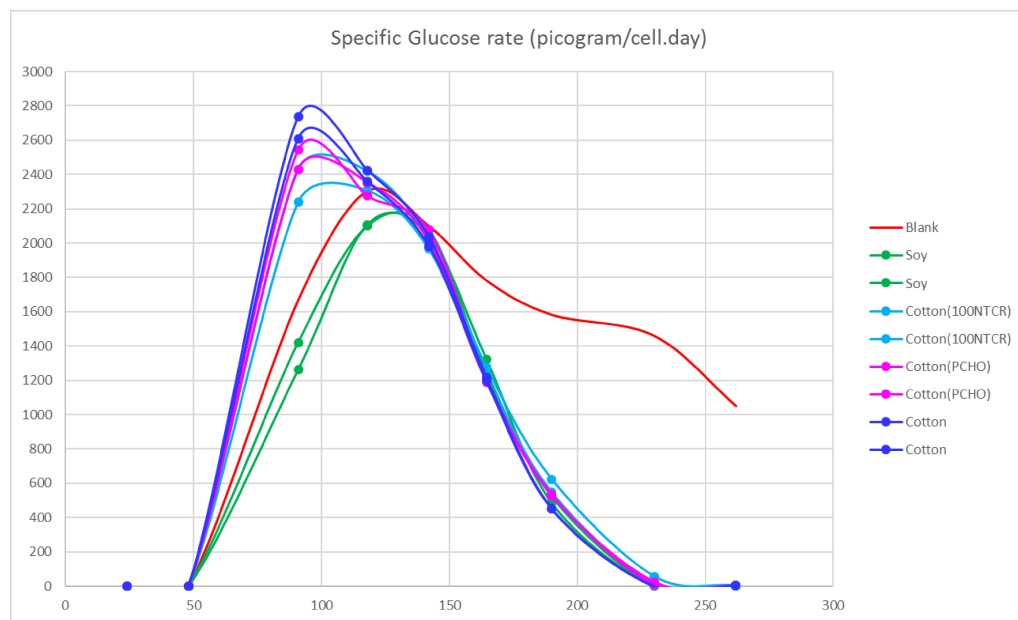


Figure 8: Specific glucose consumption rate (picogram/cell.day) for

Experiment 2

When we look at the lactate plot, an interesting observation is that the soy hydrolysate flask starts with almost double the amount of lactate when compared to the control and the cotton hydrolysate lots. The lactate consumption profile is still the same with an offset of the 2times the value that was found in the cotton hydrolysate and control samples. This increased amount of lactate had to be made sure that it wasn't interfering with the cell culture performance whatsoever.

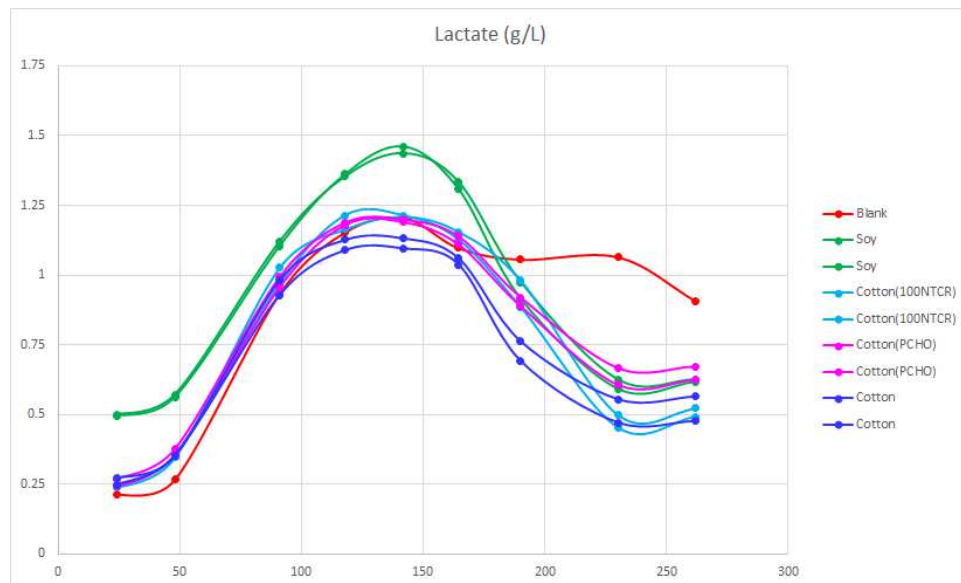


Figure 9: Lactate concentration profile Experiment 2

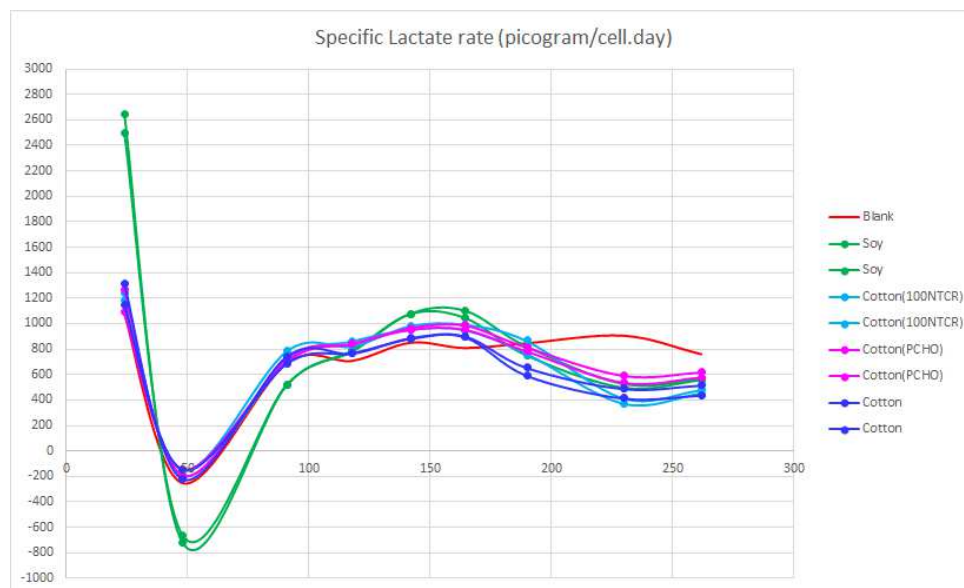


Figure 10: Lactate production rate (picogram/cell.day) in Experiment 2

For this reason, a pH experiment was planned if there is any variation in pH among all the different variants. Among the three lots of cotton hydrolysate, PCHO has performed the best in improving the cell growth in the common culture conditions. The next experiment would prepare samples for the proteomics analysis. The integral viable cell density (IVCD) plot of the current experiment are given below. This value helps in the evaluation of consumption and production rates of different substrates and metabolites along with the protein product.

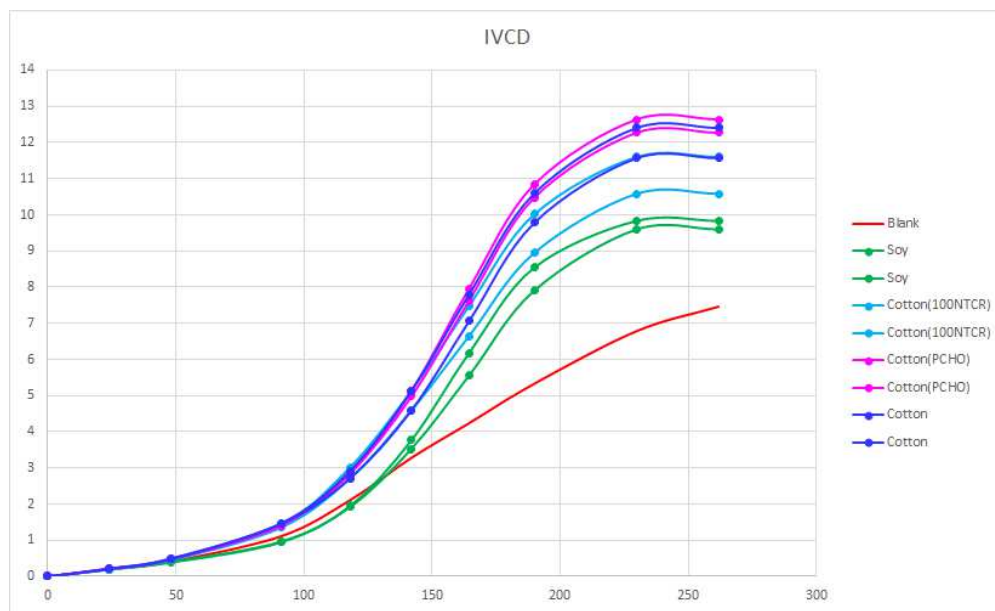


Figure 11: Integral Viable cell density (million cells.day/mL) for Experiment 2

3.4.3 Experiment 3

After checking the results of the previous two experiments, the next experiment had to give samples for proteomics analysis. The explanation will be described in the following sections. After two confident runs with the hydrolysates, the next experiment was also targeted to perform the pH experiments. For proteomics analysis, two samples were to be collected from each flask and two different time points - one sample at mid exponential and the other sample at early stationary phase. These two data points for proteomics would give us more information about the reason why hydrolysates influence the culture in the way it does. For this purpose, a cell

sample containing 1 million cells needed to be collected. So, we first take a sample for cell counting from the flask and then take another sample such that the total number of cells comes up to 1 million. Later these cells need to be prepared to run the proteomics analysis and handed over to the facility at Hopkins.

Keeping this aim in mind, a plan was made to run five flasks in each condition. Thus, in total there were 15 flasks, 5 flasks each for one condition. Two flasks out of the five were used to collect samples for proteomics, two flasks out of the remaining three were used to collect the samples for transcriptomics and the remaining one flask was used to study the variations in pH during the course of the experiment, particularly during the days when the proteomics samples were decided to be collected. Out of the five cotton hydrolysate lots tested, one cotton hydrolysate lot was selected for this experiment which was NTCR because, this cotton hydrolysate lot provided the same growth affect as soy hydrolysate but increased the productivity of the cells when compared to soy and control. Also, there was more data available for this lot from the first and the second experiment. The scheme of the experiment is given below.

Table 5: Experimental plan for Experiment 3

Condition No.	No. of Flasks	Condition	Lot No.
1	5	Control	N/A
2	5	Soy Hydrolysate	SE50MAU
3	5	Cotton Hydrolysate	100NTPCR

Cell culture performance results

This experiment's results were comparable to the previous experiments on terms of cell growth. NTPCR performed similar to Soy hydrolysate and both were better than the control flask. The samples were collected for proteomics analysis at day 4 and day 6 as recommended by the collaborators based on the previous experiment growth curves. The problem with this experiment was twofold because of which this was considered to be a faulty experiment and the samples were not taken forward for proteomics analysis. One reason was that, what was assumed to be the early stationary phase of the experiment, turned out to be the late exponential phase. Also, it was later decided that the cotton hydrolysate sample to be chosen for proteomics analysis should perform better than soy hydrolysate and not similar to it in terms of cell

density. For these two reasons, this experiment was not considered for proteomics analysis. Nevertheless, this experiment gave us valuable information on the pH of the culture at four different time points of the cell culture, i.e., Day 0, day 4, day 6, and day 8(harvest). The pH value was pretty much the same for all the flasks with minor .1 to .2 units difference which could be considered as the instrument error.

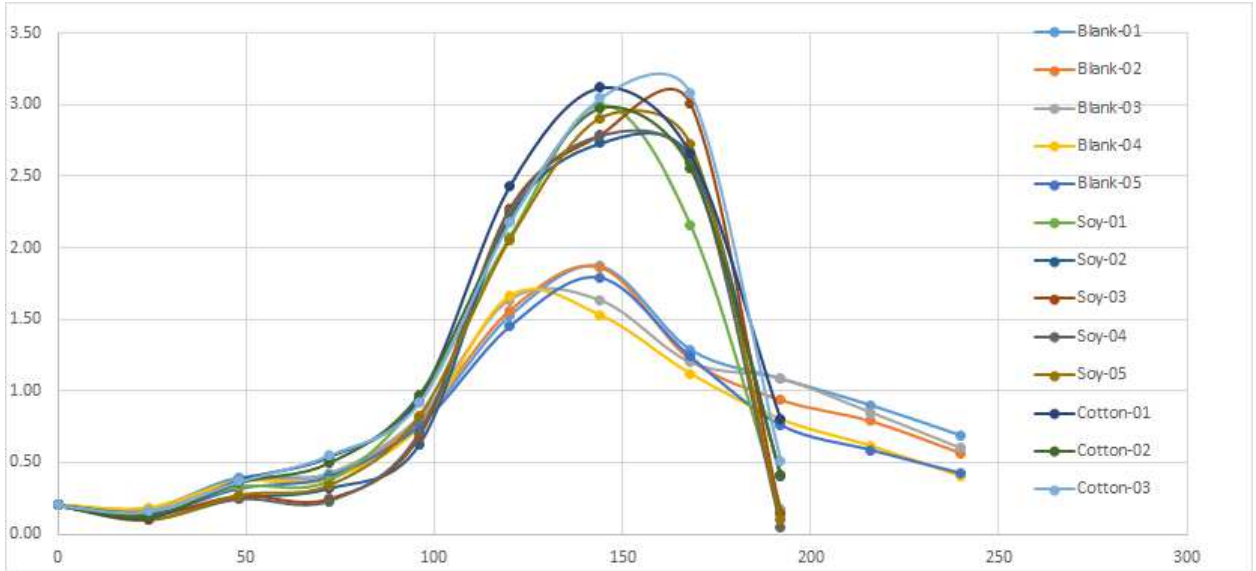


Figure 12: Viable cell density (million cells/mL) for Experiment 2

3.4.4 Experiment 4

The results in the previous experiment could not give good samples for proteomics due to sampling at insignificant time points. Also, the cotton hydrolysate lot used performed similar to the soy hydrolysate which was

assumed to not show any differences in the proteomics analysis. For these reasons, the scheme of experiment 3 was decided to be repeated but before that, we had to check for the lot variability of various cotton lots and select the one that performs the best to select for proteomics. As there are five cotton hydrolysate lots manufactured in 2016 by the company, these five lots has to be used in the cell culture to see how the performance of the cells was affected by these lots when compared to the soy hydrolysate and the normal media. For this purpose, each condition was run in duplicates, making it 14 flasks in total.

Table 6: Experiment plan for Experiment 4

Flask No.	Condition	Lot No.
1,2	Control	N/A
3,4	0.4% Soy Hydrolysate	SE50MAU
5,6	0.4% Cotton Hydrolysate	100NTPCR
7,8	0.4% Cotton Hydrolysate	100PCHO
9,10	0.4% Cotton Hydrolysate	100P1HW

11,12	0.4% Cotton Hydrolysate	100T1XJ
13,14	0.4% Cotton Hydrolysate	100SZHT

Cell culture performance results

Testing all the new cotton lots for choosing the best candidate for proteomics analysis was the aim of this project. Five different cotton lots behaved in different ways and there was a winner among all the candidates. The cell density plot clearly shows that both NTCR and PCHO were good lots among the cotton lots tested. SZHT underperformed that soy hydrolysate and T1XJ along with P1HW performed similar to soy hydrolysate. Thus these three cotton hydrolysates were not considered for proteomics. Since the aim of this experiment was to select candidates for the proteomics analysis there was no necessity for the metabolite analysis. The VCD and Viability are given here.

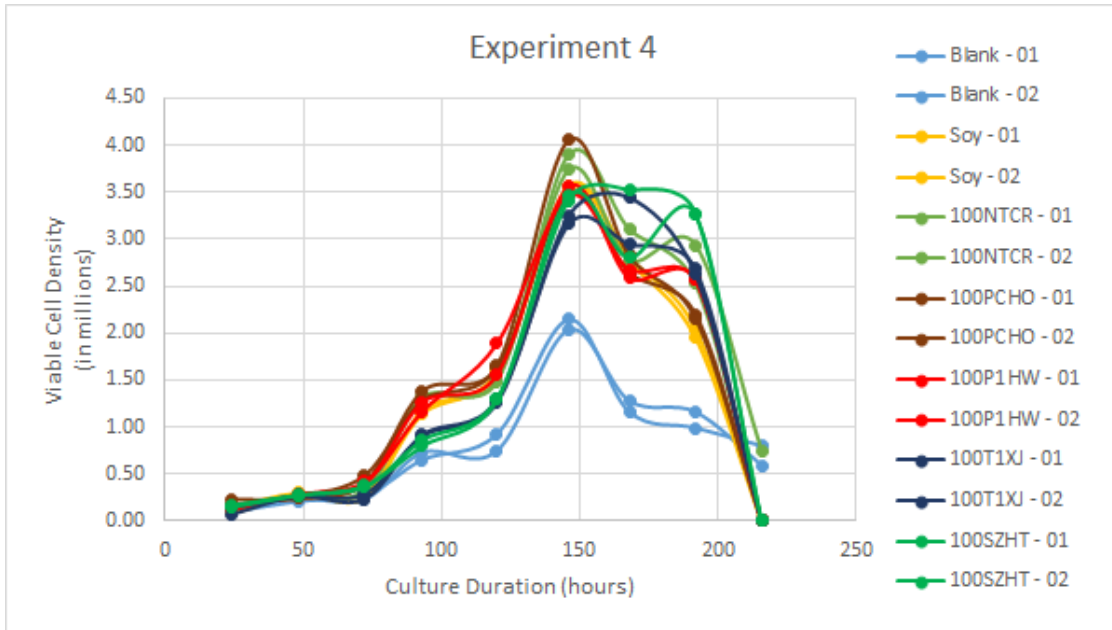


Figure 13: Viable cell density for Experiment 4

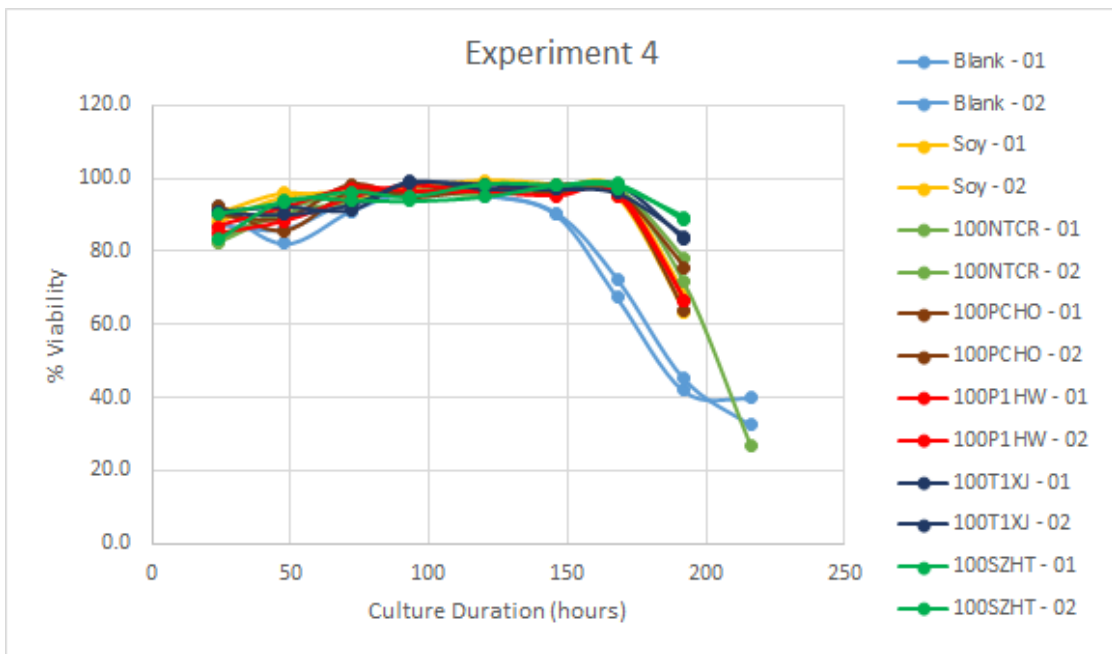


Figure 14: Viability plot for Experiment 2

3.4.5 Experiment 5

Four experiments have given enough evidence as to which lots of cotton hydrolysate should be selected for the proteomics and transcriptomics analysis. Based on the data we collected, it was decided to proceed with two cotton hydrolysate lots NTCR and PCHO for the -omics analysis. Thus, for the final experiment to get the samples for proteomics and transcriptomics analysis, each condition had four biological replicates, two flasks towards proteomics and two flasks towards transcriptomics. The scheme of experiment is given below.

Table 7: Experiment plan for Experiment 5

Flask Nos.	Condition	Lot No.
1,2,3,4	Control	N/A
5,6,7,8	0.4% Soy Hydrolysate	SE50MAU
9,10,11,12	0.4% Cotton Hydrolysate	100NTCR
13,14,15,16	0.4% Cotton Hydrolysate	100PCHO

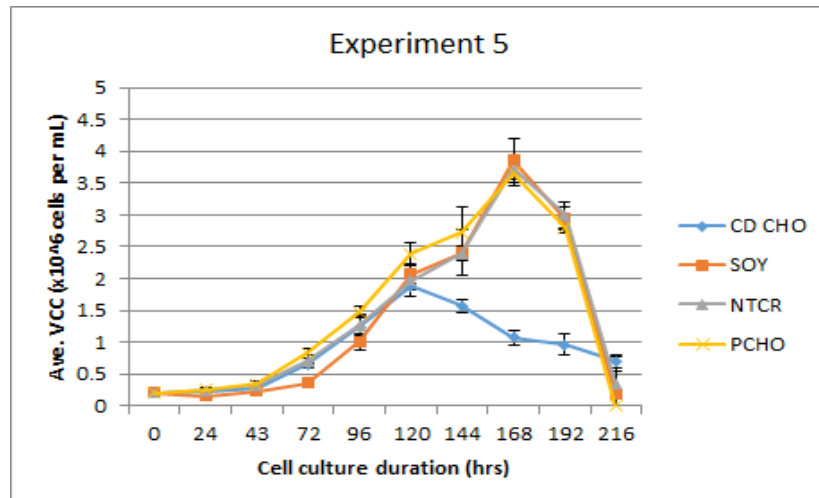
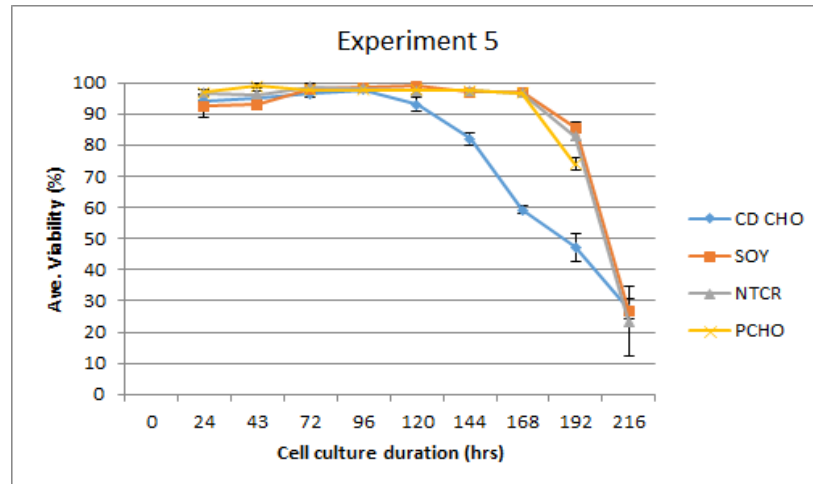
Samples were collected to analyze for cell growth and viability along with metabolites viz. Glucose, glutamine, glutamate and lactate. Based on the cell count, cell sample containing 1 million cells was collected at two time points of the culture, mid exponential and early stationary phase for proteomics. So, we first take a sample for cell counting from the flask and then take another sample such that the total number of cells comes up to 1 million. Later these cells need to be prepared to run the proteomics analysis and handed over to the facility at Hopkins.

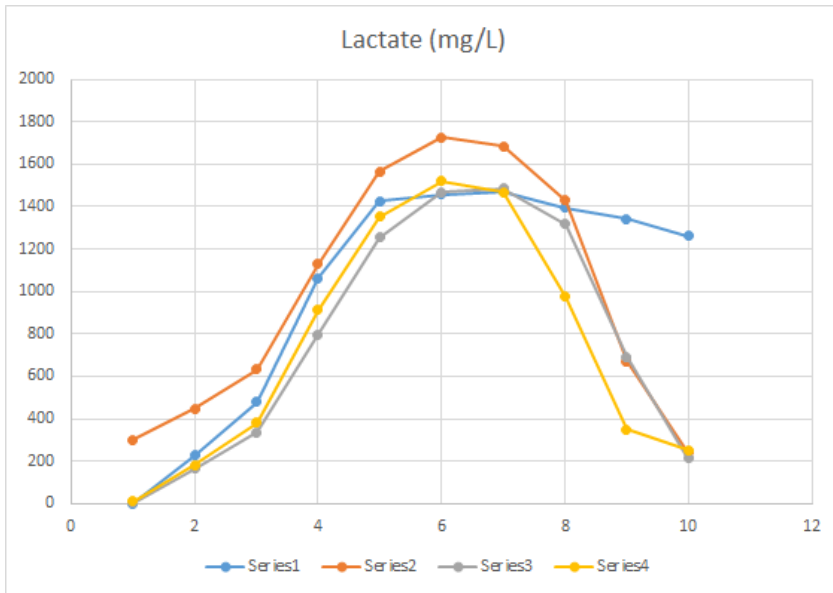
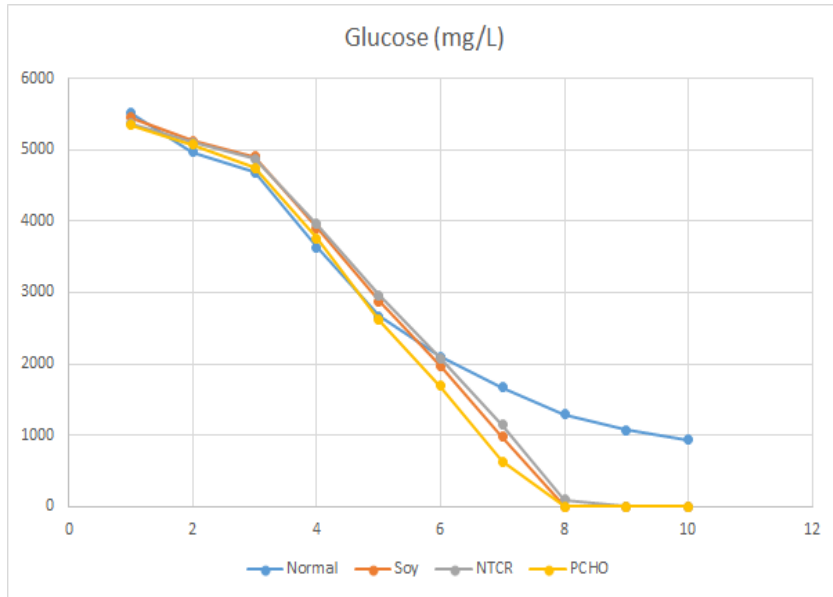
Cell culture performance results

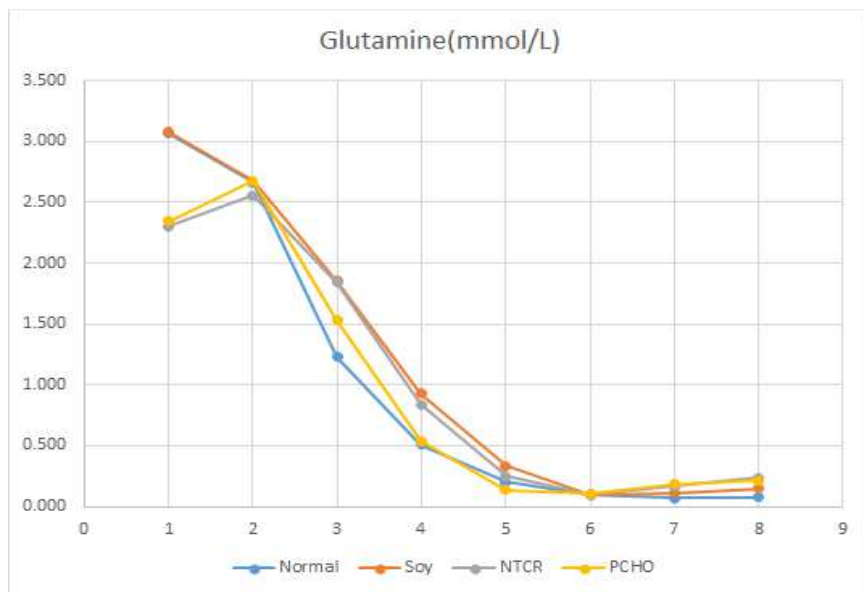
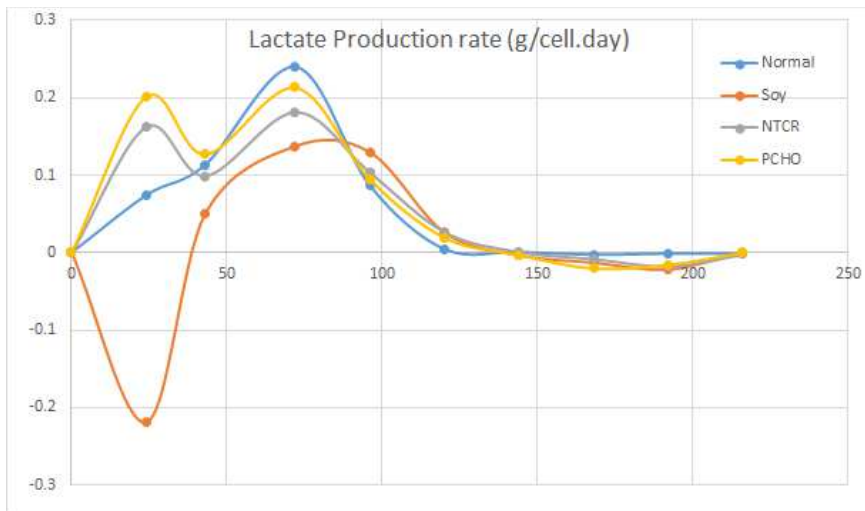
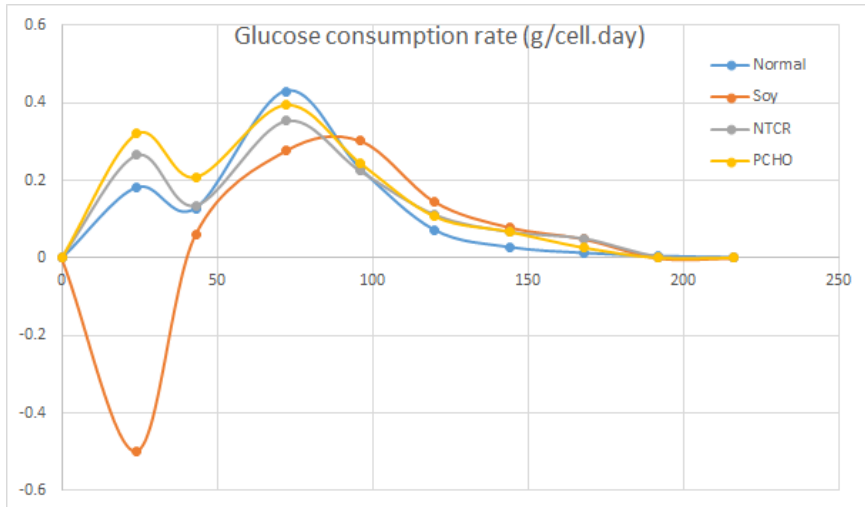
After running four experiments with these cells and hydrolysates, there was a level of confidence in the results and the plots given below show that the results have not been surprising in any manner. The viable cell density plot showed the control to grow lesser when compared to the hydrolysate supplemented flasks. The maximum peak cell density was attained by the cotton hydrolysate to 100PCHO.

The glutamine and glutamate measurements showed us that, glutamine exhaustion time coincides with glucose exhaustion and lactate metabolic shift from production to consumption. There is an inverse correlation between glutamine and glutamate levels in the culture. The six plot shown below

summarizes the experimental run. From this experiment, 100PCHO from the cotton hydrolysate lots was decided to be sent for proteomics analysis as this lot showed the maximum effect of cotton hydrolysate on cell culture growth and productivity.







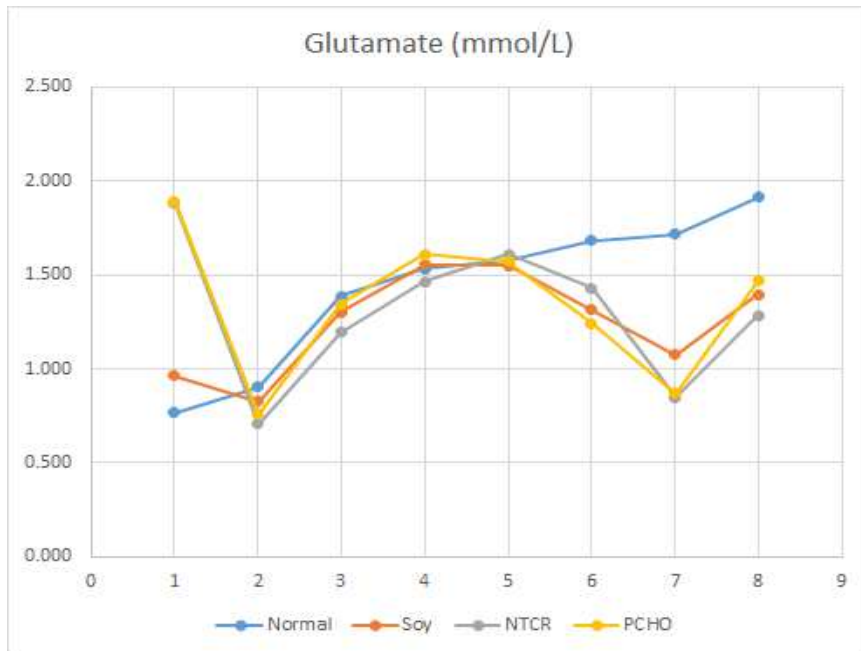


Figure 15: Cell culture characteristics of Experiment 5. (Top to Bottom plots in order) 1. Viability, 2. VCD, 3. Glucose concentration, 4. Lactate Concentration, 5. Glucose consumption rate, 6. Lactate consumption rate, 7. Glutamine concentration, 8. Glutamate concentration

3.5 Post cell culture analysis: ELISA, BCA and SDS-GEL

After the cell culture process and its growth performance has been completed, there needs to be an analysis of the IgG product that is generated by the batch culture. This would tell us if the hydrolysates are helping in increasing the productivity of CHO cells. This result of the protein productivity would put a final word on the decision to go for the proteomics

or not. Then following section will describe the Elisa protocols used for the experiment and further describe the proteomics related activities performed.

3.5.1 ELISA (Enzyme-linked immunosorbent assay)

The protein productivity of the cell culture was quantified using ELISA. The protocol detailed in Chapter 2 was used for the analysis. The samples of the last three days of cell culture in Experiments 4 and 5 were analyzed for protein productivity. The results of the assay are given below. There is a general trend seen in the analysis that, the culture no supplemented with any hydrolysate produced less quantity of IgG compared to the other flasks where there was hydrolysate supplementation. The IgG concentration reduced on the last day of culture since the viability of the culture crashed by that time and the IgG was broken down by the proteases released by the cell.

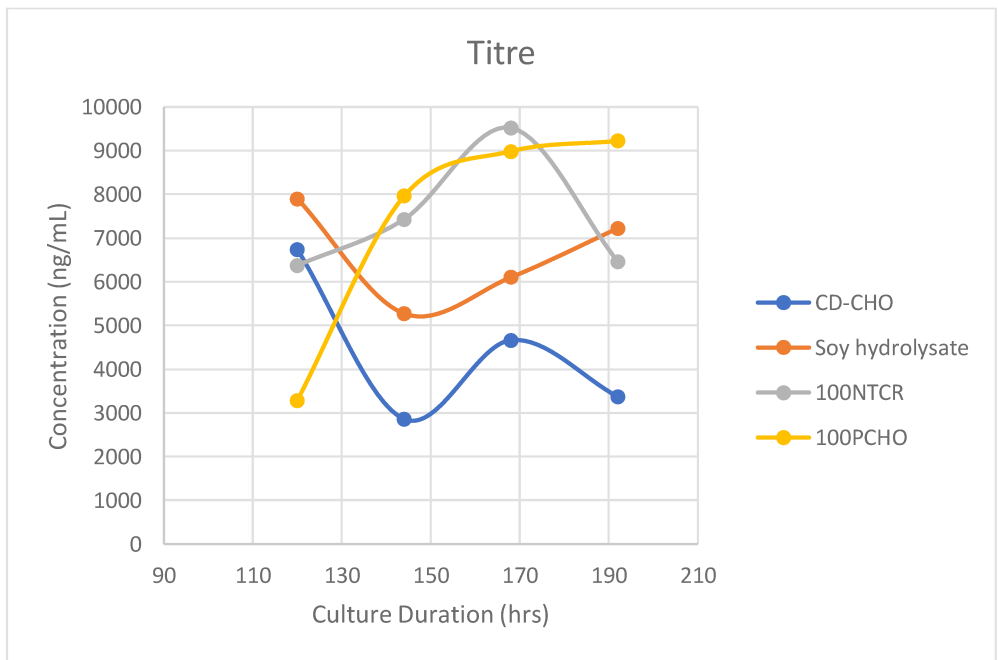
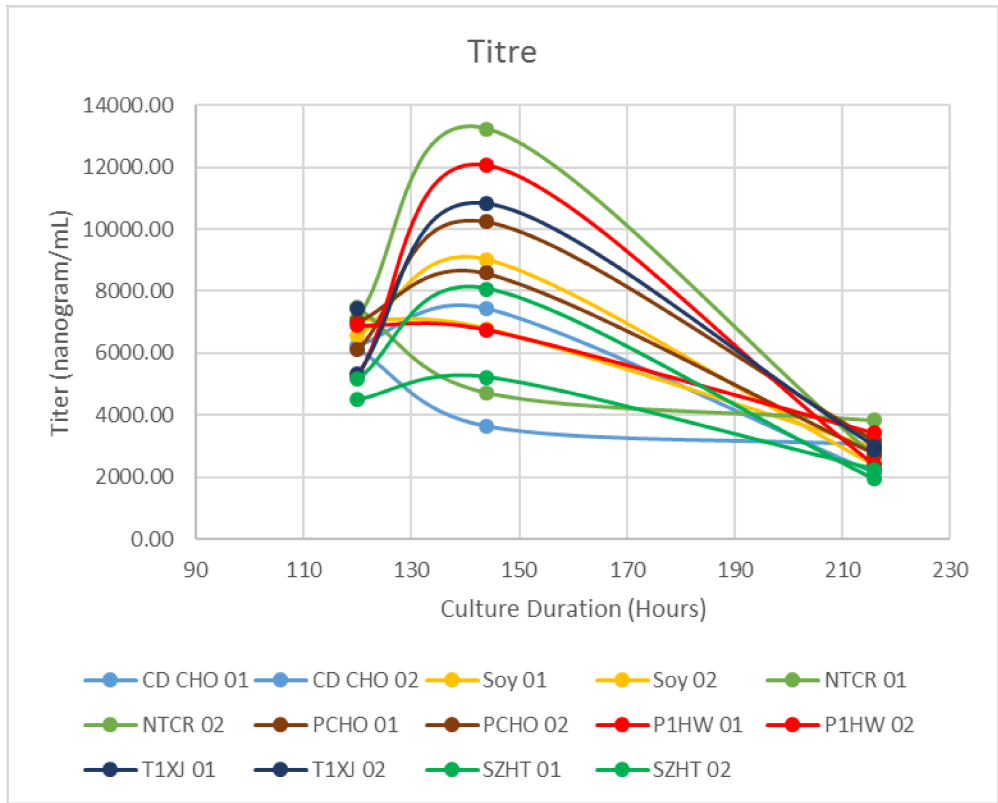


Figure 16: Titer results for Experiment 4 (top) and Experiment 5 (Bottom)

3.5.2 Proteomics

Until now, we have seen the effects of supplementing hydrolysates to the cell culture medium. The reason for this effect is still not known to us. This information could be obtained through proteomics analysis. Samples were collected during Experiment 5 on two days when the culture was in mid exponential phase (Day 5) and it was in early stationary phase (Day 6). The samples were processed according to the protocol detailed in Chapter 2. Later, BCA was performed on the samples to check for the total protein concentration in each sample. Depending on the total protein content, the loading volume would be determined for proteomics analysis. Later an SDS gel was run by the sample receiving Proteomics center and they have seen the spread of the sample to know what molecular weight range of proteins are present in the samples submitted. The samples have been submitted for proteomics analysis and the results are yet to be obtained for future analysis. The gel images for the SDS-PAGE analysis of the cell lysates utilized for proteomics run are shown below. The samples scheme is as follows:

SK1 (Flask 1 Day 5 Control), SK2 (Flask 4 Day 5 Control), SK3 (Flask 1 Day 6 Control), SK4 (Flask 4 Day 6 Control), SK5 (Flask 6 Day 5 Soy Hydrolysate), SK6 (Flask 7 Day 5 Soy Hydrolysate), SK7 (Flask 6 Day 6 Soy Hydrolysate), SK8 (Flask 7 Day 6 Soy Hydrolysate), SK9 (Flask 13 Day 5

100NTR), SK 10 (Flask 16 Day 5 100NTR), SK11 (Flask 13 Day 6 100NTR), SK 12 (Flask 16 Day 6 100NTR)

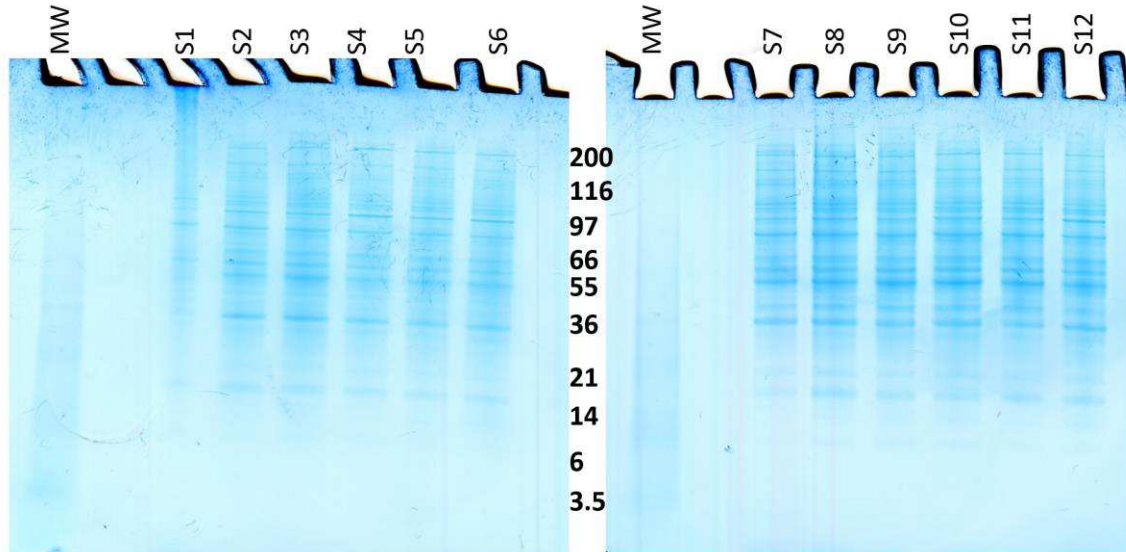


Figure 17: SDS-PAGE image for the samples submitted for proteomics. This gel was run by the proteomics center

3.6 Summary

Hydrolysates have proved to have improved the cell culture performance in terms of cell growth and productivity through the experiments conducted. Their supplementation is a good replacement to serum as the effect of serum is reproduced by using a hydrolysate. The present work has compared hydrolysates from two different sources in terms of effect on cell growth and productivity. The study also brought in the conclusion that there

is a lot to lot variability among the cotton hydrolysate sample. Each lot of cotton hydrolysate behaves in a different way. The productivity of hydrolysate supplemented culture is almost double and triple when compared to the productivity of the control batches. Once it was confirmed through experiments that the hydrolysates indeed are bringing in an improvement in culture performance and final productivity, the reason for this effect was decided to be explored. For this purpose, samples at two different time points were collected and sent for proteomics analysis. The results will give information on the proteome of the cell lysate which would enable us to map the pathways which are active in the cell. On comparing the proteomes of the soy and cotton hydrolysates with the control, there is hope to find some markers to cell growth and protein productivity to justify the effect produced by hydrolysate supplementation in the culture media.

3.7 Future work

Hydrolysates, since their inception, as media additives have been used in both batch and fed batch processes. Their supplementation at different phases of cell culture is the major area of focus in the future work. The project demands the design of a fed-batch process using soy and cotton hydrolysates both in combination and individually. These experiments would throw light

on the nature of action of these hydrolysates in a fed batch setup. Once the proteomics data is obtained, pathway analysis will be performed to look for the pathways which have been upregulated and downregulated with the addition of hydrolysates from different sources. Achieving these goals would be on priority which would then conclude once a fed-batch process has been locked down for the given CHO cell line producing IgG.

There is also a necessity of a good, precise IgG assay for quantification since, the ELISA method provided by Friesland Campina is found to show a great amount of variability. The source of this variability should be eliminated and an optimized assay should be developed. Finally, the findings of this research should be published to put forth our results in the scientific community.

Chapter 4 MAMMALIAN CELL ENGINEERING ESSENTIAL

AMINO ACIDS PATHWAYS

4.1 Introduction

Cells, in general, build higher level components like RNA, DNA from basic molecules like sugars and peptides. But a self-sufficient system should be built in a cell when a chemical system is built in it which is capable of replication and evolution with the help of small molecules. Such a cell system is called a minimal cell. Synthesizing a minimal cell would open many new paths in Bioprocess development. [17]. This ideology of self sufficiency exists in prokaryotic cells like bacteria. Incidentally, some of these pathways have evolved their way into higher eukaryotic cells. For instance, the division of amino acids into essential and non-essential amino acids is a result of evolution. The pathways of essential amino acids were lost when the eukaryotic organisms were formed. Compartmentalization in eukaryotic organisms was incorporated during this process of formation, which marks as one of the key difference between eukaryotes and prokaryotes [18]. Prokaryotic organisms have the ability to produce their own amino acids in a quantity that is necessary for the cell. This is another major difference between prokaryotes, like bacteria and yeast, and higher-level eukaryotes such as

mammalian cells. Surprisingly, the consumption of these essential amino acids is higher in mammalian cells. The amino acid demand for the prokaryotes is much less than that of eukaryotic cells [19, 20]. Figure below shows the amino acid pathways of biosynthesis for prokaryotic cells and Eukaryotic cells.

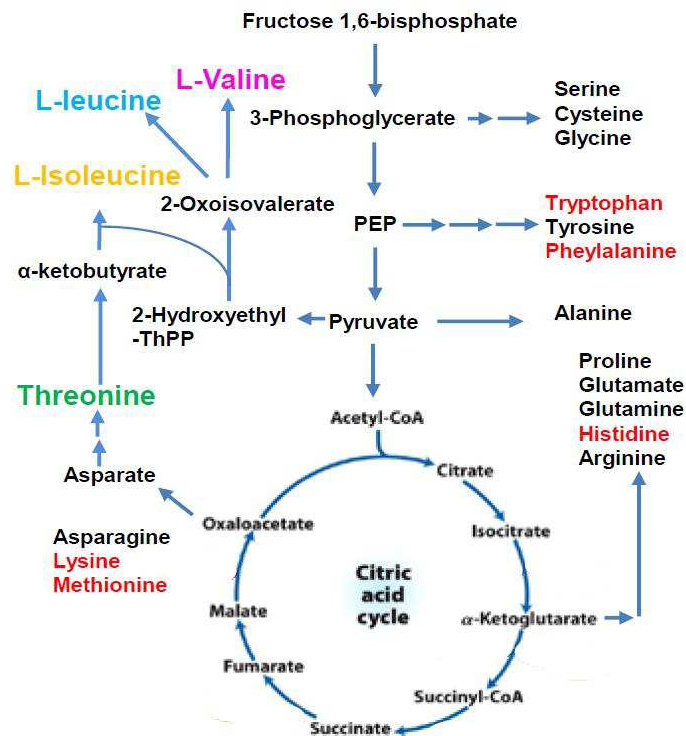


Figure 18: Biosynthesis of amino acids

Even though the essential amino acids are not produced by the mammalian cells, one-fourth (24.4%) of the amino acid content in the human body comprises of threonine and the three branched chain amino acids (valine,

isoleucine, leucine) [21]. Indeed, leucine, being an essential amino acid, has 6 codons encoding which occurs in one another amino acid serine. It is interesting to note that some of the non-essential amino acids require more energy for synthesis than essential amino acids like valine and threonine. If synthesizing the essential amino acids is less energy intensive, the reason for eliminating them from the mammalian repository of biosynthesis is still not evident [22]. Bringing back these pathways lost pathways would reconstruct a minimal cell which would make the mammalian cell self sufficient. It would be informative from a metabolic and evolutionary perspective and beneficial from a biomanufacturing perspective to evaluate the impact of reconstructing essential amino acid pathways on human and, more generally, mammalian cell physiology using a synthetic biology paradigm.

4.2 Aim

Mammalian cell cultures need all the nine essential amino acids for their growth and maintenance: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Apart from this high demand for nutrients to survive, these mammalian cultures require energy for protein production. The nutrients supplied to the cells as cell culture media fulfills these requirements even though the cell possesses the

machinery to create these amino acids. In order to address these challenges within mammalian cell culture, reconstructing the leucine, isoleucine, and valine biosynthetic pathways in mammalian cell lines could eliminate the dependence of the mammalian cell on these amino acids. With the existing cell machinery, if a path is created for them to synthesize and control the production of amino acids, there might be a higher productivity of the protein because there would be extra available energy for the cells to produce the protein.

The goal of the research described is to reconstruct the amino acid pathways for four essential amino acids: threonine, valine, isoleucine, and leucine in a Chinese hamster ovary (CHO) cell line, human embryonic kidney (HEK) cells. Since these biosynthetic pathways do not exist in the mammalian cells, they will be incorporated from cells which makes these amino acids by itself, the bacterial and yeast cells. The research work described here uses the biosynthetic genes of leucine, isoleucine and valine from yeast organism *Saccharomyces Cerevisiae* and clones them into a mammalian expression vector to eventually express them in two different mammalian cell lines. The cell lines chosen for research are highly significant for many reasons. Indeed, mammalian cell lines are responsible for the production of the vast majority of biotherapeutics including monoclonal antibodies, vaccines, and other

valuable biologics [23, 24]. In addition, cell lines such as HEK are used to supply complex mammalian membrane proteins needed in protein crystallography studies [25, 26].

The cell line HEK also gives a major advantage when it comes to mammalian transfections. The initial expression studies for a gene are performed in HEK cell line since, these cell lines work best for transient transfections. While it is far more complex to express a gene transiently in CHO cell lines. Keeping this in mind, initial expression studies are always performed in HEK cell lines. When the genes are known to have expressed in HEK cell line, transfections are performed in CHO-K1 cells to later create a stable cell line creating its own essential amino acids.

Three different amino acid biosynthetic pathways were dealt with in this study. Two sets of experiments were performed to achieve the following two aims:

A. Since the genes of isoleucine and valine were individually expressed in HEK cell line by my teammate Nathan, expression of these genes in CHO cells was to be explored. Since CHO cell are more complex when compared to HEK, the transfection efficiency is less. The first aim of this study was to express the biosynthetic genes of isoleucine and valine cloned from yeast organism *Saccharomyces Cerevisiae*, in CHO cells.

B. The biosynthetic pathway of leucine, branched out from the precursor of valine. This pathway genes were aimed to be cloned into a mammalian expression vector and expressed in HEK cells. Once the expression was achieved in HEK cell line, the study could be extended to CHO cell lines where the transfection efficiency is low.

The above two aims have been explored through molecular cloning techniques and various other protocols briefed in Chapter 2. The description of the amino acids worked with and the results of the study are detailed in the sections below.

4.3 Branched chain amino acids Biosynthesis

4.3.1 Leucine

Leucine is an essential amino acid which is one of the three branched chain amino acids, with an isobutyl side chain resulting in its hydrophobicity. Leucine is the amino acid which is encoded by six codons, UUA, UUG, UUC, CUC, CUA, and CUG. The biosynthetic pathway of leucine in yeast is given in the Figure.

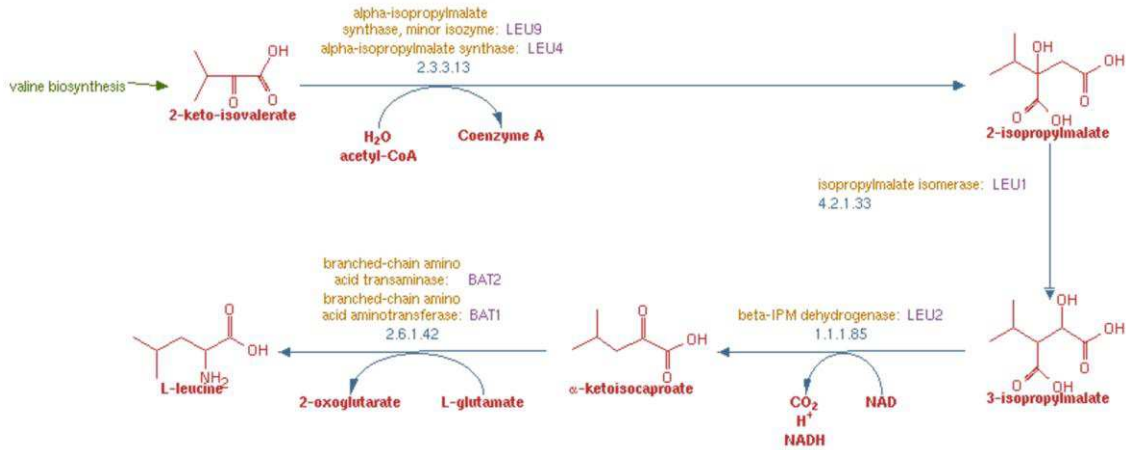


Figure 19: Biosynthesis pathway of Leucine in *Saccharomyces Cerevisiae*

[32]

In *Saccharomyces cerevisiae*, leucine is produced from the precursor of valine, 2-keto-isovalerate. The pathway is four steps long and there are six genes which help in the biosynthesis of leucine. Two out of the six genes are present in the mammalian genome. It is interesting to note that, leucine regulates the pathway by feedback inhibition of the first dedicated step catalyzed by LEU4 and LEU9. Mutants which have reduced this inhibition and increased leucine production have been created by researchers [31].

4.3.2 Isoleucine

Isoleucine is a hydrophobic essential amino acid which is one of the three branched chain amino acids. The codons, AUU, AUC, AUA encode

isoleucine. The biosynthetic pathway of isoleucine in yeast is given in the Figure.

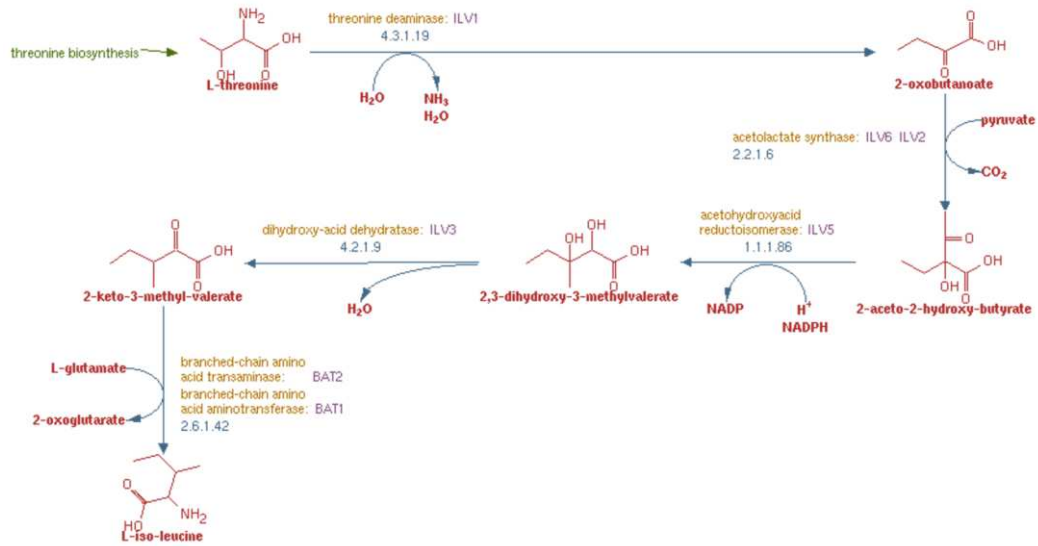


Figure 20: Biosynthesis pathway of Isoleucine in *Saccharomyces Cerevisiae*

[32]

In *Saccharomyces cerevisiae*, isoleucine is produced from another essential amino acid, threonine. The pathway is five steps long and there are seven genes which help in the biosynthesis of leucine. Two out of the seven genes are present in the mammalian genome. The initial enzyme in the pathway, threonine deaminase (ILV1), is subject to feedback inhibition by isoleucine and feedback activation by the valine [30].

4.3.3 *Valine*

Valine is an essential nonpolar branched chain amino acid (along with isoleucine and leucine) encoded by codons GUU, GUC, GUA and GUG. Valine is synthesized from pyruvic acid in the pathway shown below in Figure 3 [27]. Valine is a four step biosynthetic pathway with six genes necessary to produce it. Pyruvate produced from glycolysis is the substrate of the first step towards valine biosynthesis. Two out of the six genes involved are present in the mammalian genome. In *Saccharomyces Cerevisiae*, isoleucine and valine are synthesized from the same set of genes, except for *ILV1*, which converts threonine to 2-oxobutanoate.

The leucine biosynthesis pathway also utilizes the same pathway but diverges after 2-keto-isovalerate. Not surprisingly, the first dedicated step in the pathway catalyzed by acetolactate synthesis is feedback inhibited by both valine and leucine [28]. Degradation of valine and the other branched chain amino acids (BCAA) proceeds through the BC alpha-keto acid dehydrogenase complex. The complex degrades the BCAAs into Acyl-Co derivatives and eventually acetyl-Co or succinyl-CoA for entry in TCA cycle [30].

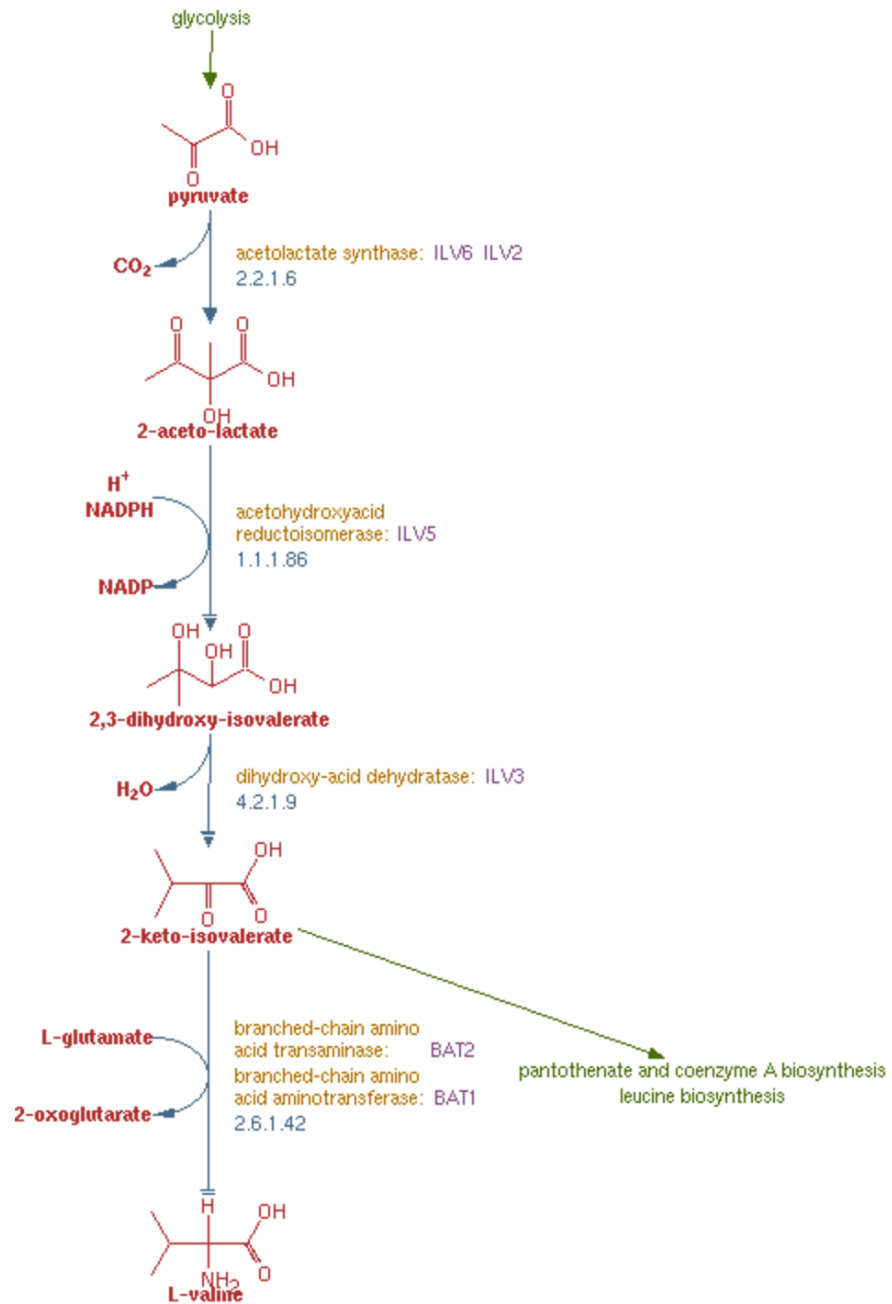


Figure 21: Biosynthesis pathway of Valine in *Saccharomyces Cerevisiae*

[32]

4.4 Experimental and Results

This section gives a detailed account of the different experiments that have been performed to achieve the aims (A) and (B). Experiment 1: The first set of experiments were the cloning of leucine genes into mammalian expression vector. The mammalian cloning procedure described in Chapter 2 was followed to clone four genes that are a part of leucine biosynthesis pathway in yeast, into mammalian expression vector.

Experiment 2: After the genes were successfully cloned six clones for each gene were created and sequenced for confirming the presence of the gene. Out of all the clones that were created, transfection experiments were performed to eliminate the clones which could not give the final protein product.

Experiment 3: For the isoleucine and valine pathway genes, after the expression was confirmed in HEK cells, transfection experiments were performed to express the individual genes in CHO-K1 cells. As isoleucine and valine have the same set of genes for biosynthesis, one single experiment was performed for expressing them.

The sections below will give a detailed account of both the experiments and the extent of success achieved through these experiments. The results of the experiments are also explained in detail.

4.4.1 Experiment 1

Four genes from yeast genome are necessary for the biosynthesis of leucine: LEU1, LEU2, LEU4, LEU9. The mammalian expression vector pBUD CE4.1 has two different multiple cloning sites which have a various restriction sites that were used for cloning the four genes. Design of primers was based on the vector map given in the figure below.

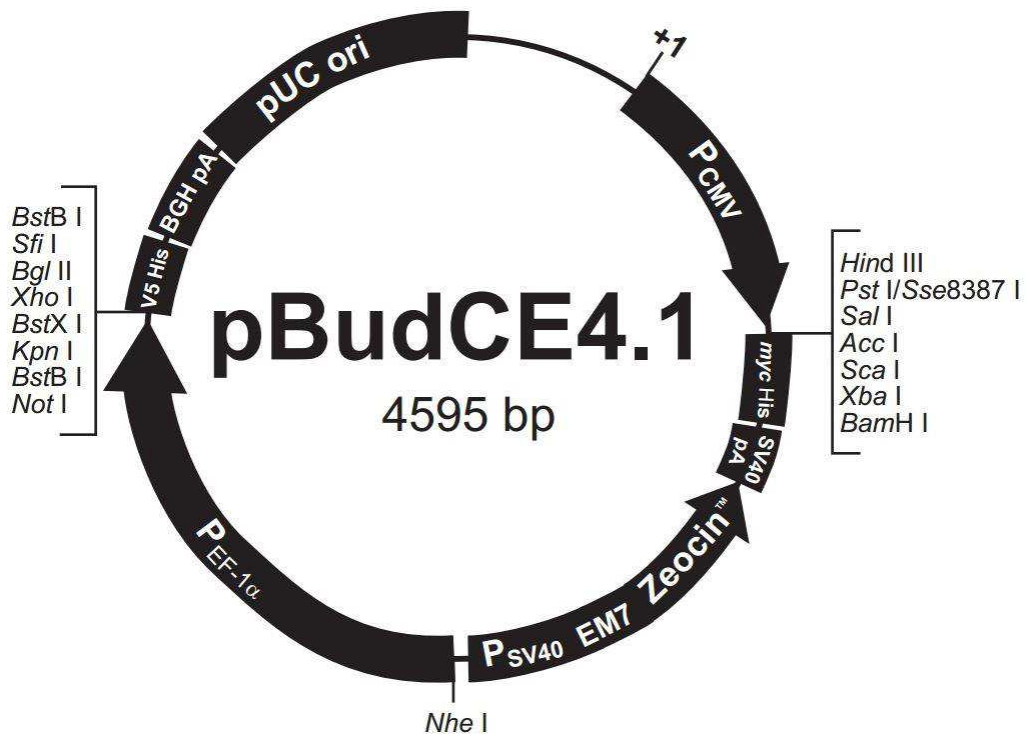


Figure 22: Vector map of pBudCE4.1, mammalian expression vector used in cloning the biosynthetic genes of all branched chian amino acid pathways

Though the vector is tagged with epitopes, it was recommended for designing the primers of the gene along with the epitope tag. Thus every gene was tagged with 6X Histidine which is the shortest epitope tag and it is known to not interfere with the functionality of the gene. The forward and reverse primers designed for the four genes and their respective annealing temperatures and GC content are given in the table.

PCR was performed with the primers designed with the protocol detailed in chapter 2. The output of the PCR was tested using gel electrophoresis. The ladder used for the gel electrophoresis is ‘GeneRuler 1kb plus DNA ladder’. The path of the ladder is shown in the figure below.

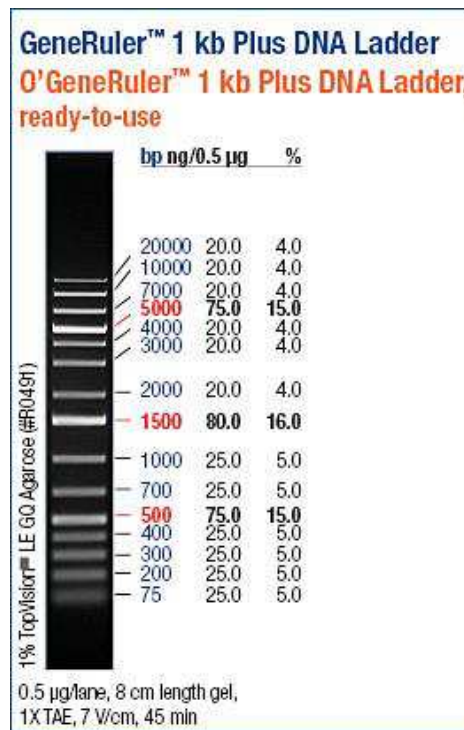


Figure 23: DNA ladder used for gel electrophoresis after PCR

Gene	Forward Primer	Reverse Primer	Properties
LEU2 1095bp	5'-ATTGATAAGCTTGCCACC ATGCTGCCCTAAGAAGATC-3'	5'- TGATAGGATCCCTA TTA ATGGTGGTGGTGATGATG AGCAAGGATTTTCTAACTTC -3'	Annealing Temperature 63 °C Hairpins Max T (F:R) 22 °C:54 °C Restriction Enzyme (F-R) HindIII-BamHI Tm (F:R) 61 °C:59 °C
LEU1 2340bp	5'-ATAGTATCTAGAGCCACC ATGGTTTACACTCCATCCAAG-3'	5'- CTAGCTGGATCCCTA CTA ATGGTGGTGGTGATGATG CCAATCCTGGTGGACTTTATC -3'	Annealing Temperature 63 °C Hairpins Max T (F:R) 35 °C:46 °C Restriction Enzyme (F:R) XbaI-BamHI Tm (F:R) 58 °C:63 °C
LEU4VH 1860bp	5'-TATATTGGTACCGCCACC ATGGTTAAAGAGAGATATTATGGTCC-3'	5'-TTAGTCTCGAGCTA TTAATGGTGGTGGTGATGATG TGCAGAGCCAGATGC-3'	Annealing Temperature 63 °C Hairpins Max T (F:R) 35 °C:35 °C Restriction Enzyme (F:R) KpnI-XhoI Tm (F:R) 58 °C:60 °C
LEU9VH 1815bp	5'-TCTATTGGTACCGCCACC ATGGTAAACATTCGTTTCATAGC-3'	5'- TTCATCTCGAGCTA TTAATGGTGGTGGTGATGATG CTCTGCCAGTAGAACATCC -3'	Annealing Temperature 63 °C Hairpins Max T (F:R) 35 °C:35 °C Restriction Enzyme (F:R) KpnI-XhoI Tm (F:R) 61 °C:60 °C

Legend:

Random Sequence

F Forward Primer

LEU2- <http://www.yeastgenome.org/locus/S000000523/sequence>

Restriction Enzyme Sequence

LEU1- <http://www.yeastgenome.org/locus/S0000002977/sequence>

Sequence for Promoter(F), Extra Stop(R)

LEU4- <http://www.yeastgenome.org/locus/S0000005048/sequence>

AT Gene of interest sequence

LEU9- <http://www.yeastgenome.org/locus/S0000005634/sequence>

Gene Sequences:

F Forward Primer

LEU2- <http://www.yeastgenome.org/locus/S000000523/sequence>

R Reverse Primer

LEU1- <http://www.yeastgenome.org/locus/S0000002977/sequence>

T Temperature

LEU4- <http://www.yeastgenome.org/locus/S0000005048/sequence>

bp Base Pairs

LEU9- <http://www.yeastgenome.org/locus/S0000005634/sequence>

Figure 24: Primers used for PCR in leucine biosynthesis pathway

The gel images under 60% UV light are given in the images below. The ladder was loaded in every gel for a better accuracy of the location of the gene.

LEU1 (Isopropyl malate isomerase) catalyzes the conversion of 2-isopropylmalate to 3-isopropylmalate and it is localized to the cytosol. The length of the gene is 2340 base pairs. The length of the insert including the restriction enzymes and the epitope tag along with the extra base pairs would be around 2360 base pairs. The gel image shows two sample tubes of PCR containing the insert of LEU1 gene.

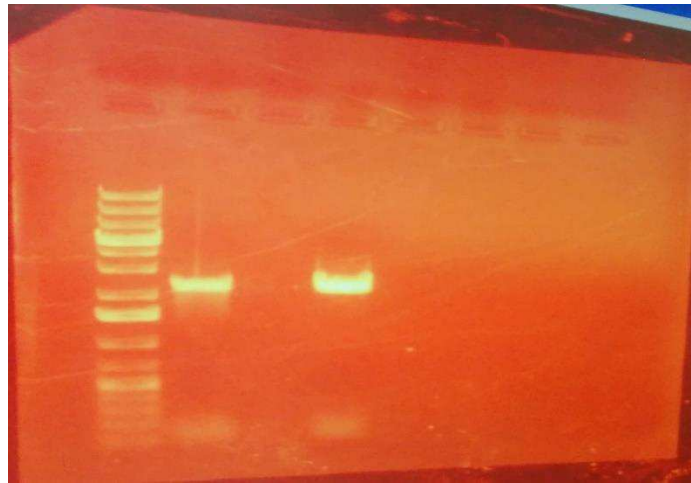


Figure 25: Agarose gel result Samples from left to right: DNA Ladder, LEU1 PCR Product samples

LEU2 (3-isopropylmalate dehydrogenase) catalyzes the third step in the leucine biosynthesis pathway; can additionally catalyze the conversion of

beta-ethyl malate into alpha-ketovalerate. The length of the gene is 1095 base pairs. The length of the insert including the restriction enzymes and the epitope tag along with the extra base pairs would be around 1120 base pairs. The gel image shows two sample tubes of PCR containing the insert of LEU2 gene.



Figure 26: Agarose gel result of LEU 2, samples from left to right: LEU2 PCR product samples, DNA ladder

LEU4 (2-isopropylmalate synthase) the main isozyme responsible for the first step in the leucine biosynthesis pathway; LEU4 has a paralog, LEU9, that arose from the whole genome duplication. The length of the gene is 1860

base pairs. The length of the insert including the restriction enzymes and the epitope tag along with the extra base pairs would be around 1900 base pairs. The gel image shows two sample tubes of PCR containing the insert of LEU4 gene.

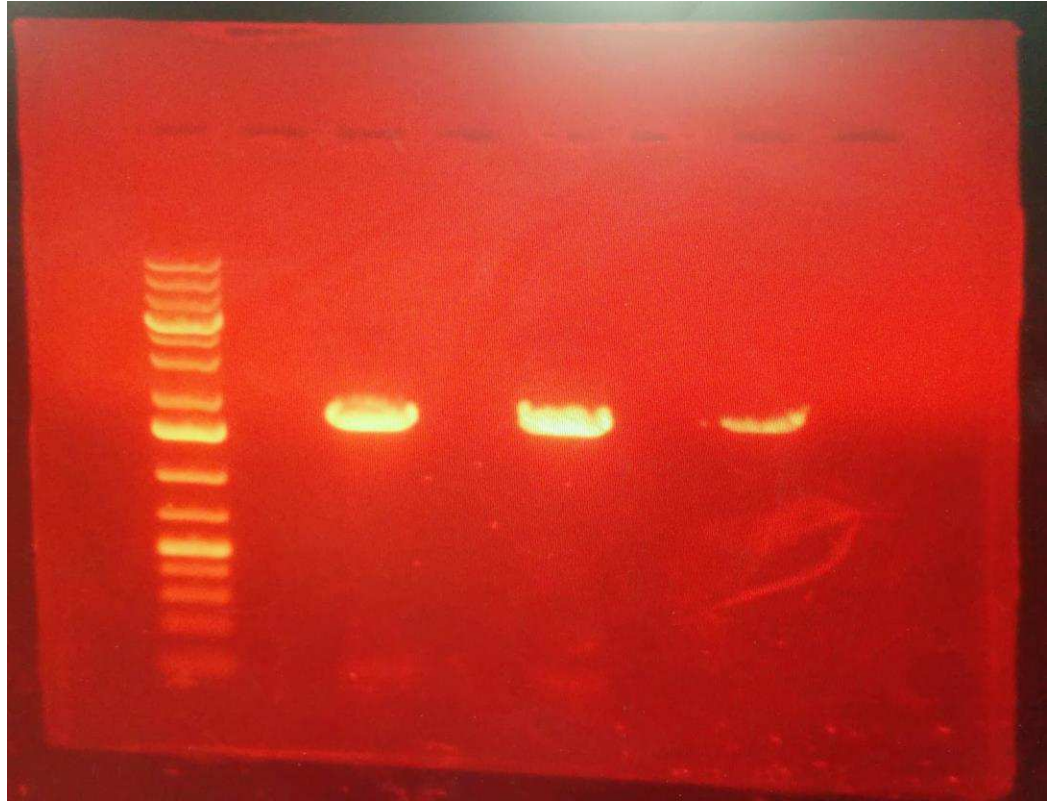


Figure 27: Agarose gel image of LEU4 and LEU 9, samples from left to right: DNA Ladder, LEU9 PCR Product sample (one), LEU4 PCR product samples (Two)

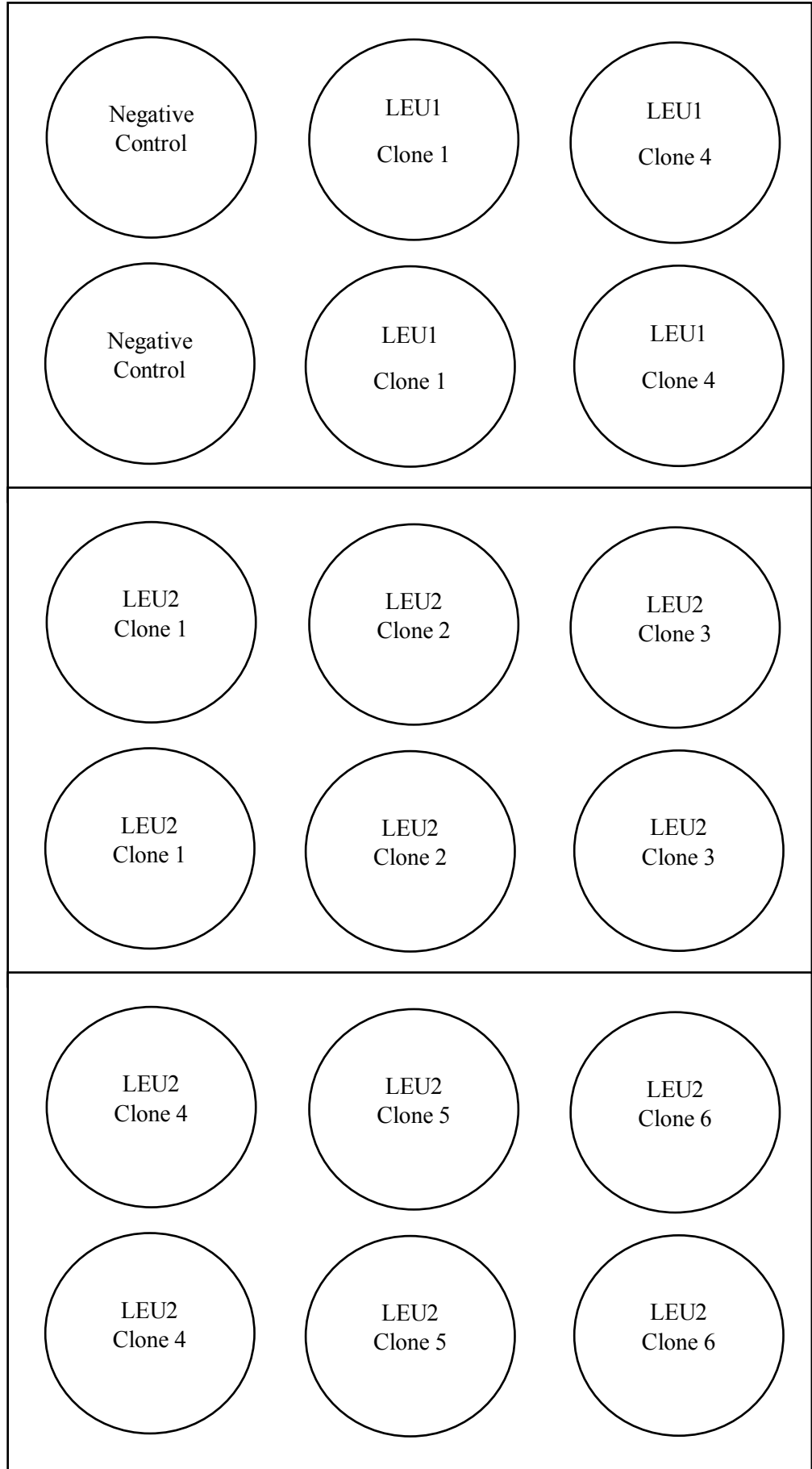
LEU9 (Alpha-isopropylmalate synthase II or 2-isopropylmalate synthase) catalyzes the first step in the leucine biosynthesis pathway. This

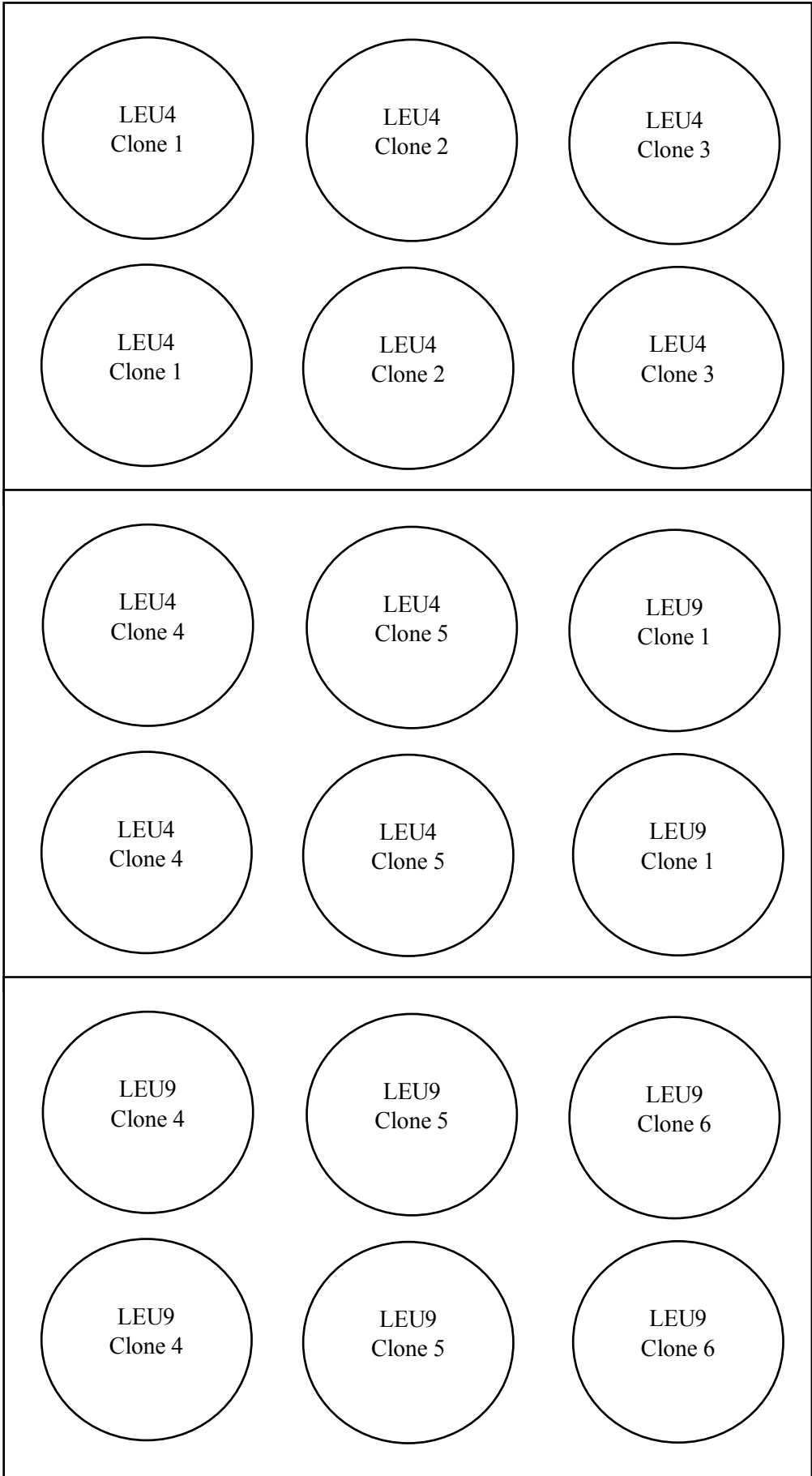
minor isozyme of LEU4 is responsible for the residual alpha-IPMS activity detected in a leu4 null mutant. LEU9 has a paralog, LEU4, that arose from the whole genome duplication. [Casalone E, et al. (2000) Identification by functional analysis of the gene encoding alpha-isopropylmalate synthase II (LEU9) in *Saccharomyces cerevisiae*. *Yeast* 16(6):539-45]. The length of the gene is 1815 base pairs. The length of the insert including the restriction enzymes and the epitope tag along with the extra base pairs would be around 1850 base pairs. The gel image shows one sample tube of PCR containing the insert of LEU9 gene.

These four inserts obtained from PCR were cloned into the mammalian expression vector pBudCE 4.1 and transformed to produce six clones for each gene. These clones were sequenced and out of all 24 clones, 17 clones had the respective genes in the right place on the plasmid. These clones were used to perform the expression studies.

4.4.2 Experiment 2

The 17 clones sequenced to find the right gene from experiment 1 were used to perform expression studies. HEK cells were plated and transfected in a 6 well plate. The scheme of the experiment in the 6 well plates is shown in the figures below.





The results were analyzed in two ways using an SDS-PAGE gel and subsequent staining of the gel using Coomassie Blue, and a western blot. Coomassie Blue staining was a preliminary study to check if the protein of interest was present or not. Unfortunately, the stained gels could not provide any point of differences when compared to the control. The ladder used for the gel image is ‘Spectra Multicolor Broad Range Protein Ladder’. The image of the ladder is given below.

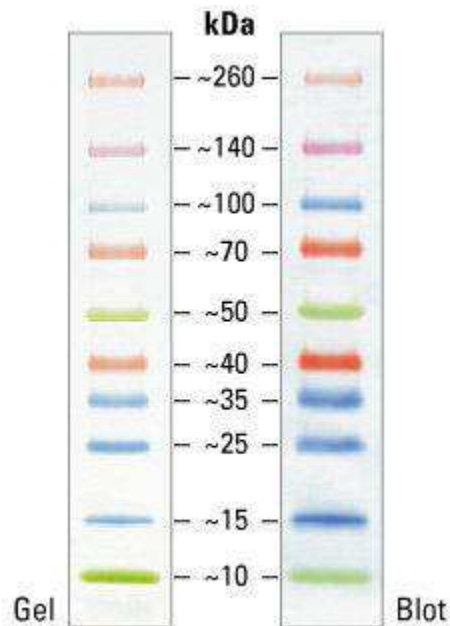


Figure 28: Protein ladder used for SDS-PAGE analysis

Figure 1 shows the gel with LEU1 and LEU9 clones. Sample order from left to right is as follows:

Ladder → Control → LEU1 Clone 1 → LEU1 Clone 4 → Blank → LEU9 Clone 1 → LEU9 Clone 4 → LEU9 Clone 5 → LEU9 Clone 6. The protein formed from LEU9 is 67kDa in size and that of LEU1 is 86kDa.

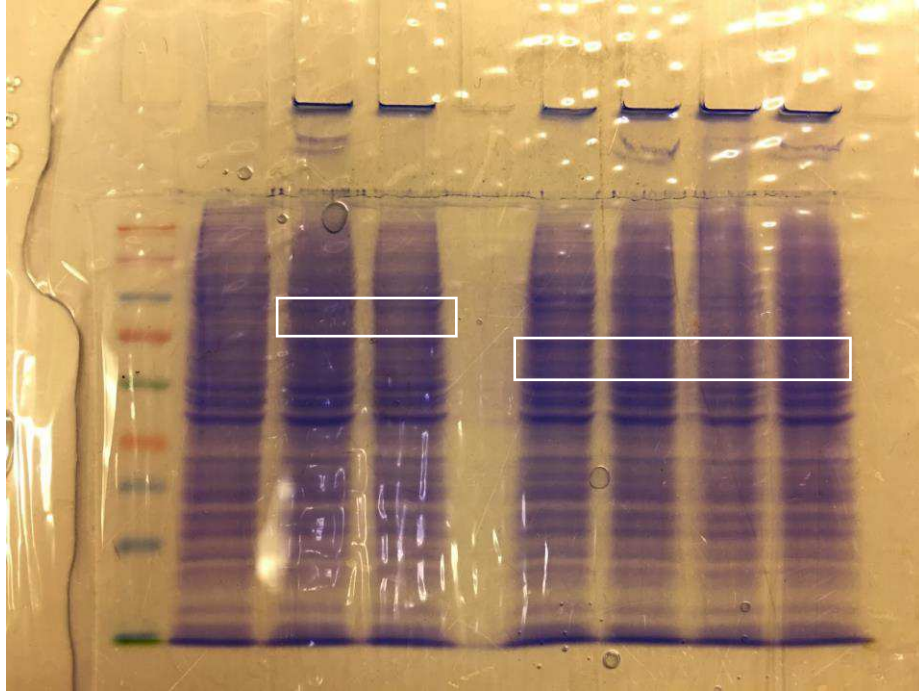


Figure 29: SDS-PAGE gel image stained with Commasie Blue of LEU1 and LEU9 gene clones

Figure 2 shows the gel with LEU2. Sample order from left to right is as follows: Control → Ladder → LEU2 Clone 1 → LEU2 Clone 2 → LEU2 Clone 3 → LEU2 Clone 4 → LEU2 Clone 5 → LEU2 Clone. The size of the protein translated by the gene LEU2 is 39kDa.

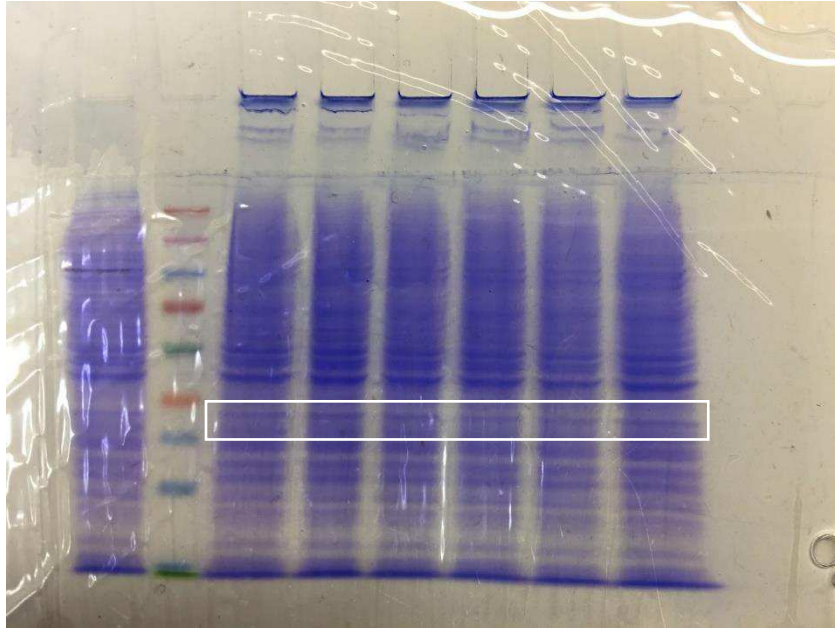


Figure 30: SDS-PAGE gel image stained with Commasie Blue of LEU2

Figure 2 shows the gel with LEU4. Sample order from left to right is as follows: Control → LEU4 Clone 1 → Ladder → LEU4 Clone 2 → LEU4 Clone 3 → LEU4 Clone 4 → LEU4 Clone 5. The size of the protein translated by LEU4 is 68kDa

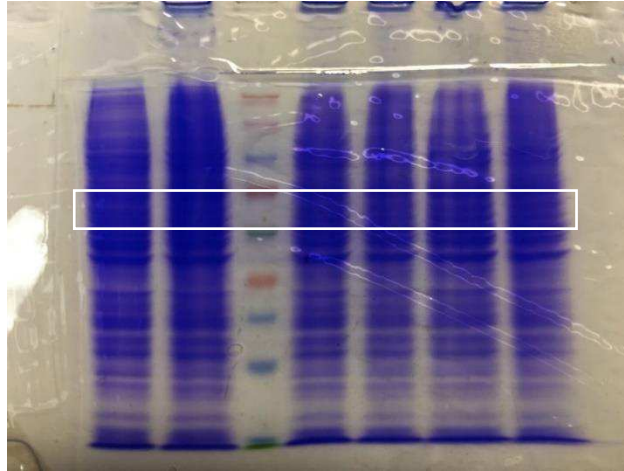


Figure 31: SDS-PAGE gel image stained with Commassie Blue of LEU4 gene clones

Since the gel staining could not provide valuable information regarding expression, western blotting was performed on the clones of LEU4 gene to check if the genes were expressed or not. The blotted membrane was incubated with ‘Rabbit IgG anti-HIS antibody’ as the primary antibody and the secondary antibody used was ‘Anti-rabbit IgG-HRP conjugated’. Then the image was taken using chemiluminescence techniques. Three clones out of the six clones transfected showed an expression of the gene translated by LEU4. The figure below shows the image:

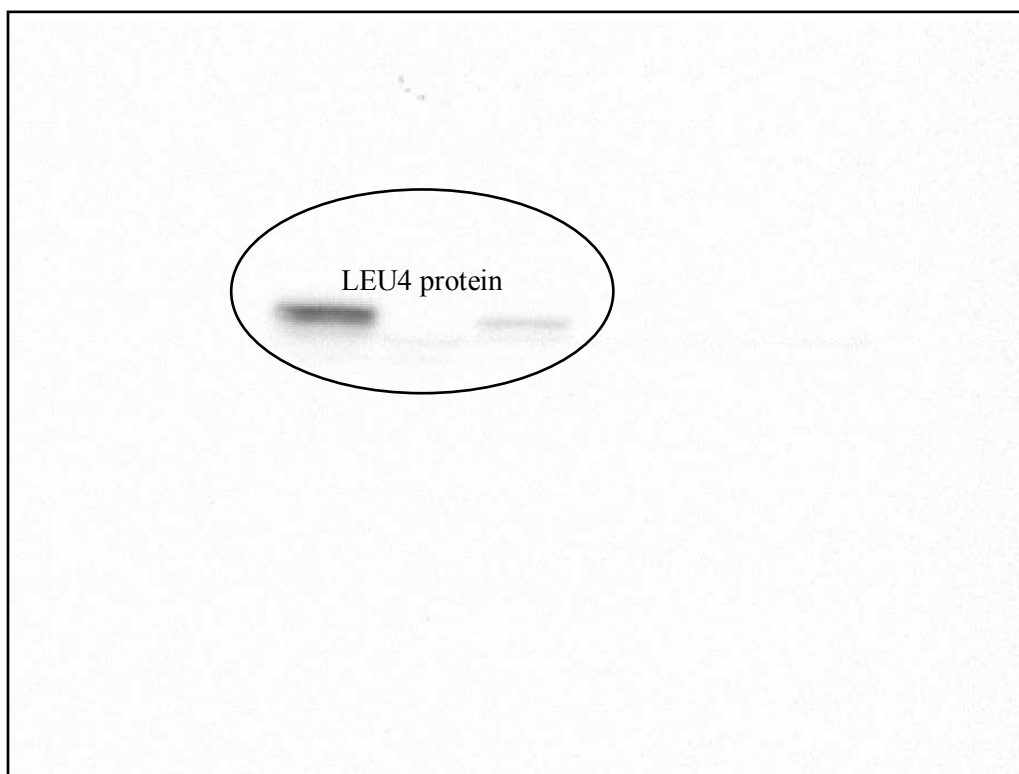
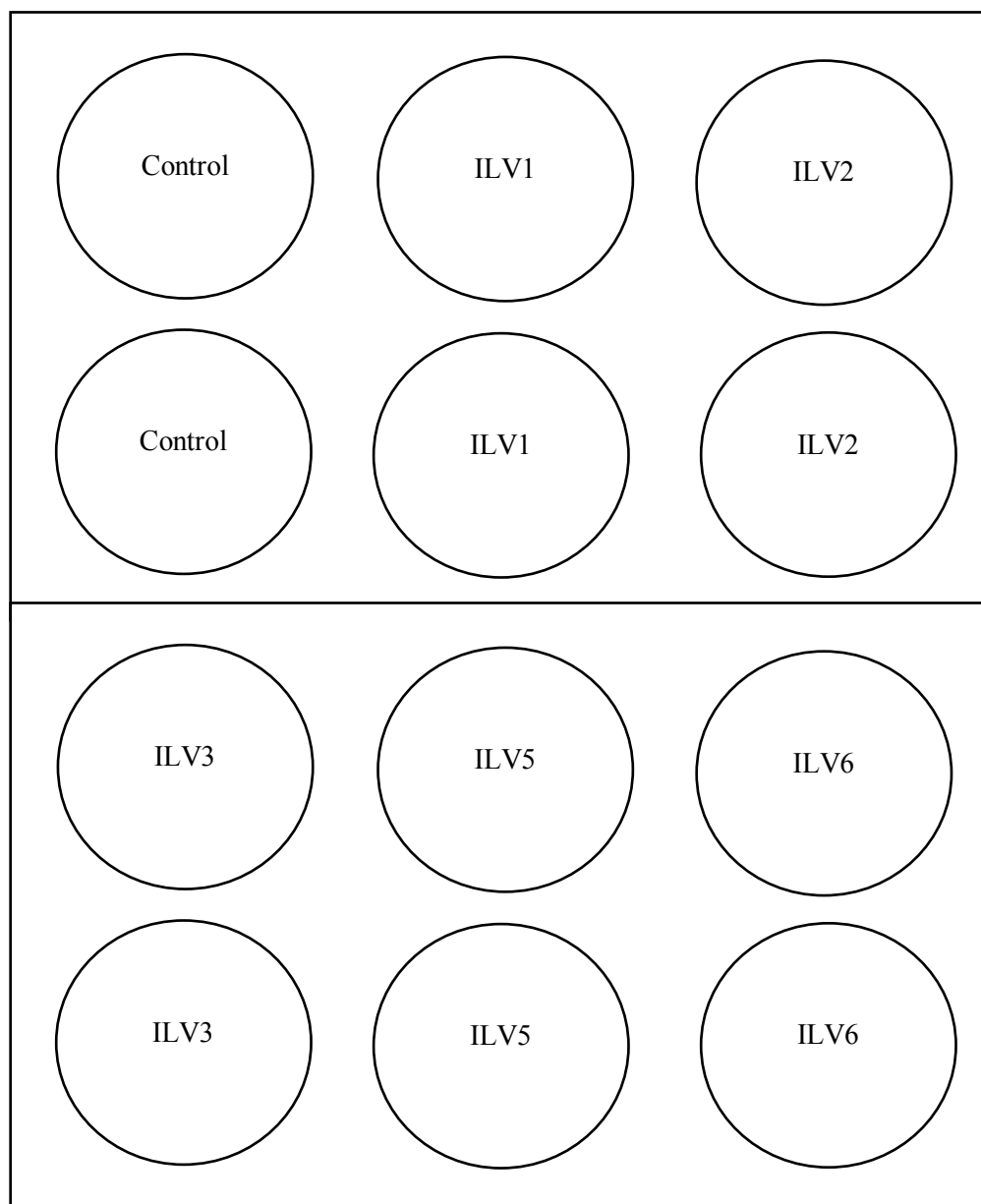


Figure 32: Western blot result showing the expression of LEU4 protein in three clones out of five clones transfected

4.4.3 Experiment 3

The 5 genes involved in the biosynthesis of isoleucine and valine were previously expressed in HEK 293T cell line. In this experiment, we have transfected these genes into CHO-K1 wild type cells. Cells were grown in a 6 well plate till they reached a 70% to 90% confluence. The plasmid containing ILV1, ILV2, ILV3, ILV5 and ILV6 were transfected using lipofectamine 3000. The scheme of the experiment is shown below.



The results were analyzed in two ways using an SDS-PAGE gel and subsequent staining of the gel using Coomassie Blue, and a western blot. Coomassie Blue staining was a preliminary study to check if the protein of interest was present or not. Unfortunately, the stained gels could not provide

any point of differences when compared to the control. The ladder used for the gel image is 'Spectra Multicolor Broad Range Protein Ladder'. The image of the stained gel is given below. The order of sample from left to right is: CHO-K1 Control → ILV1 → ILV2 → Ladder → ILV3 → ILV5 → ILV6

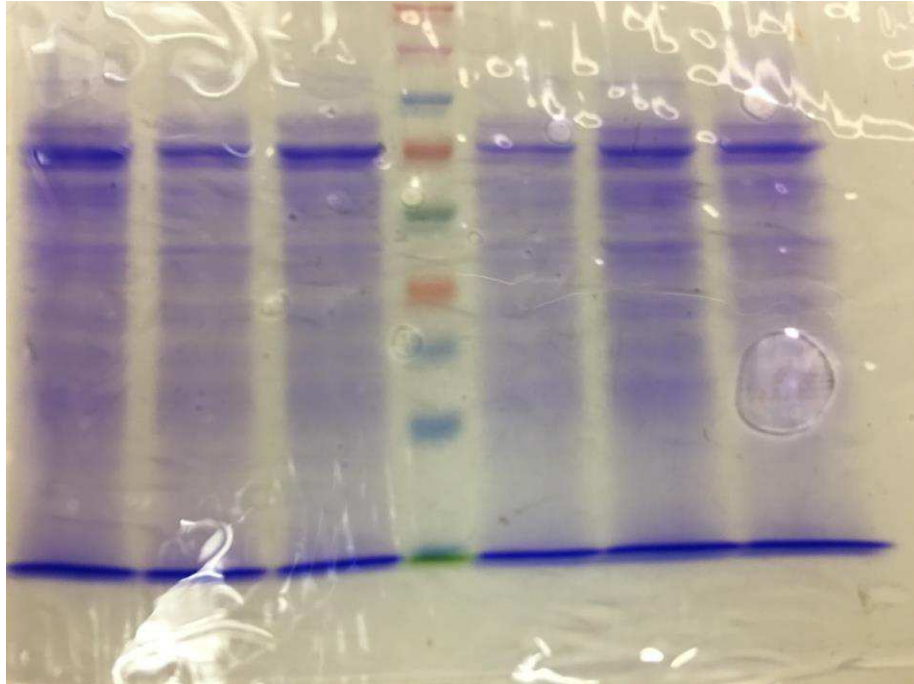


Figure 33: SDS-PAGE gel image stained with Coomassie Blue of ILV1, ILV2, ILV3, ILV5 and ILV6 genes transfected into CHO-K1

Since the gel staining could not provide valuable information regarding expression, western blotting was performed on all the genes to check if they were expressed or not. The blotted membrane was incubated with 'Goat-IgG anti-V5 antibody' and 'Rabbit-IgG anti-HIS antibody' as the primary

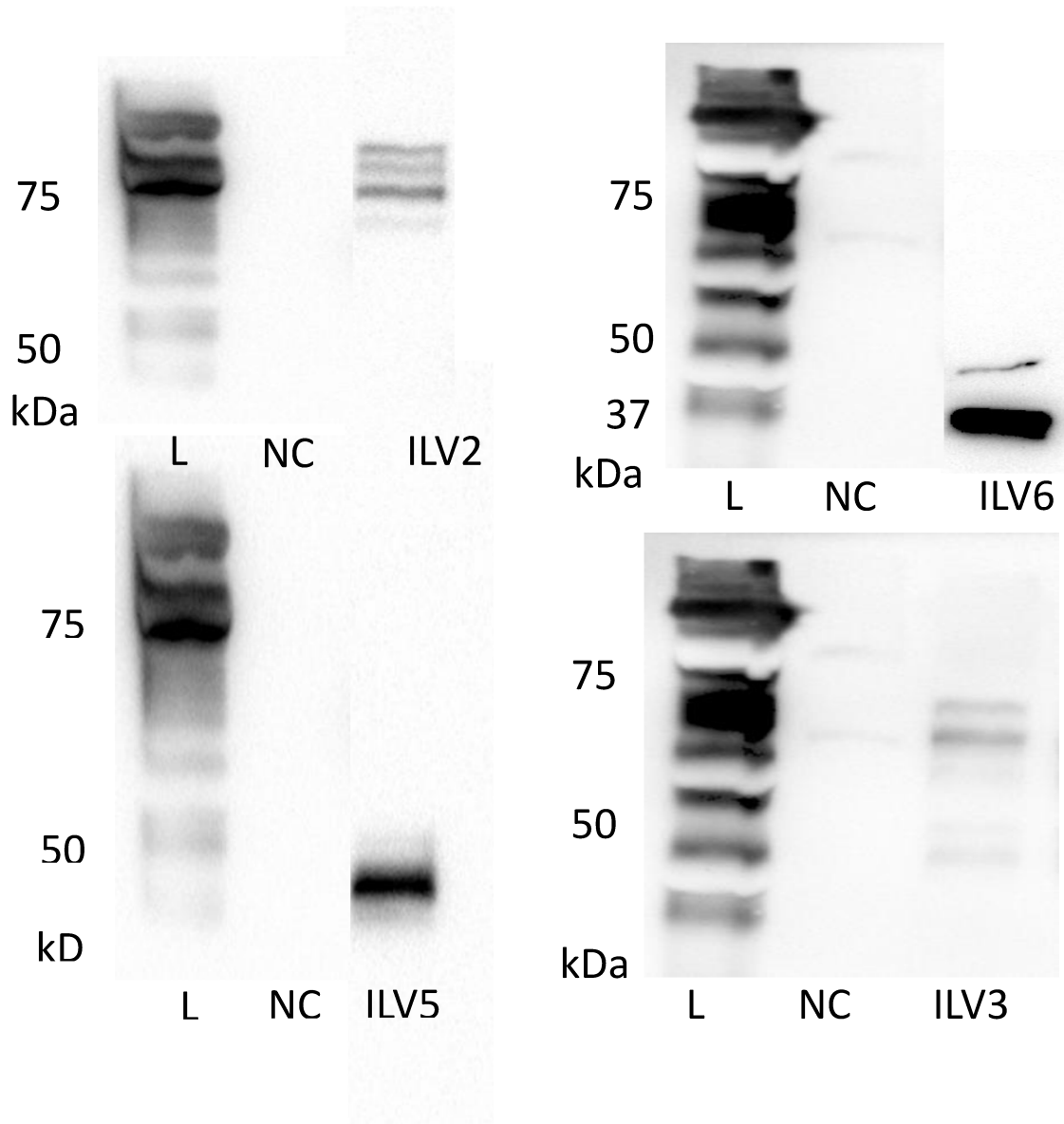
antibody and the secondary antibody used was ‘Anti-goat IgG-HRP conjugated’ and ‘Anti-rabbit IgG-HRP conjugated’. Then the image was taken using chemiluminescence techniques. The figure below shows the results. The bands that appear from left to right are ILV6, ILV5, ILV3 and ILV2 respectively. The expression of ILV1 is not seen thus it is concluded that ILV1 could not be expressed in CHO-K1 cells.



Figure 34: Western blot of isoleucine valine genes transfected into CHO-K1 wild type. Samples order from left to right: ILV6, ILV5, ILV3, ILV2 and ILV1 was not expressed

The discrete band that we see in the samples ILV2 and ILV3 has been a characteristic of the cloned gene since this pattern was also observed when these genes were expressed in HEK 293T cells. The results produced when

these genes were expressed in HEK 293T are given below (These experiments were performed by Nathan Calzadilla in the year 2016 and they have been included in his Master's essay).



From the above figure, we can clearly see the spread in the band of ILV3 and ILV2. These images stand as a reference for the result obtained in

Experiment 3 in terms of the positions of the band relative to the ladders in the above figure.

4.5 Summary

The issue of expensive media requirements continues to represent one of the major cost barriers to biopharmaceuticals for patients. If we could augment the synthetic capabilities of CHO cells to enable the production of key amino acids and other building blocks not naturally present, we would have the potential to transform the most important bioproduction platform currently available. Such a change would lead to a simpler media requirement for the CHO production platform, leading to lower costs to patients for life-saving monoclonal antibodies and other biopharmaceuticals. As a part of this change, the current research explored the essential amino acid producing capability of mammalian cells. Two aims were achieved in this respect. The branched chain amino acid (Leucine, Isoleucine and valine) biosynthesis pathway genes from yeast were cloned into mammalian expression vector and expressed in mammalian cells. The success of the experiments performed prove that prokaryotic amino acid genes could be expressed in mammalian cells and thus there is a possibility that these cells can produce their own amino acids when the pathway is engineering fully into the cell. These results

also provide us with a hope that, a minimal mammalian cell could one day be created such that it would not require any amino acid supplementation for growth and maintenance.

4.6 Future work

The ideology behind the research work described in the previous sections is an ocean whose exploration has just begun. There are 9 different essential amino acids which should be targeted. There are various challenges that researchers are facing when the biosynthetic genes are expressed together. This is impeding the development of cell lines which make their own amino acids. Cell-free systems have started being useful for synthetic biology research. Problems that could not be solved *in vivo* are being tackled *in vitro* because, the system converts into a chemical assembly rather than a biological assembly once the term cell-free comes into play. For instance, the isoleucine and valine pathway could produce the first intermediate but not the second intermediate when all the genes were transfected into a single well. If this reaction was performed *in vitro*, the information on the lacking component is easy to obtain. Thus cell-free systems provide a direction to find answers in this difficult problem statement.

Multicistronic plasmids using IRES could be created combining all the genes together into one single plasmid and checked for expression. This direction if works, would create stable cell lines producing amino acids. The future of this project could take two directions and both the directions will be explored to achieve the end goal of a minimal cell.

Chapter 5 CONCLUSION

Biopharmaceuticals around the world always work towards a higher throughput in a process they design, be it a upstream bioprocess, a downstream purification step or the final fill finish process. When it comes to upstream bioprocess improvement, target is set on increasing the productivity of the cells with minimal uptake of cell nutrients from outside. These two aspects have been explored in the research described in the current thesis.

Chapter 1 introduced the thesis aim and gave a brief background on various aspects of mammalian cell culture relevant to the current research work. All the protocols that have been used in the work are detailed in Chapter 2. These protocols were adopted from various cell culture websites and have been provided by the reagent suppliers like Sigma Aldrich, Thermo Fisher Scientific and New England Biolabs.

The productivity of the cells can be improved through various techniques. Changes can be made in the equipment used for culturing cells such that the mass transfer in the cultures is improved as in wave bag bioreactor, or the nutrients could be replenished at a regular basis through a perfusion system. These options involve heavy capital to begin with since the

equipment to be procured is expensive. The use of additives is the most economical option to pursue for improving the productivity of the cells. One such additive was manufactured and provided by Friesland Campina to explore its effect on the cell culture performance. The additives, hydrolysates, were used in the cell culture at a final concentration of 0.4% and we have seen a two to threefold increase in the productivity of the cells. While soy hydrolysate doubled the productivity along with the cell growth, cotton hydrolysates have increased it three times when compared to the control flasks not supplemented with any hydrolysates. Cell growth is also improved and the cells have a nutrient boost when hydrolysates are supplemented in the culture media. A trend observed in the hydrolysate cultures is that, they run out of glucose on day 6 and later the cells crash on day 8 to zero viability from a viability greater than 90%. Since the effect of hydrolysates was tested in a batch process, there was no possibility of adding extra nutrients like supplementing the cultures with glucose and glutamine as the primary energy source.

Five different lots of cotton hydrolysates were tested simultaneously as media supplements for a lot variability study. It was surprising to note that every lot of cotton hydrolysate behaved in a different manner but none of the lots underperformed when compared to the control and soy hydrolysate

supplemented flasks. The cotton lots 100NTPCR and 100PCHO performed the best among the five lots tested in terms of both cell growth and IgG productivity. Both these lots were selected to perform proteomics analysis on the hydrolysate samples to understand the mode of action of these hydrolysates when added to the cell cultures. The research performed on hydrolysates brought in confidence that, the deficiency created in the cell culture media due to the elimination of serum and compensating it with chemical formulations could be fulfilled by hydrolysates. Companies like Sigma Aldrich and Lonza, who are one of the leaders in the market of cell culture media, have already started exploring the potential of hydrolysates and a few media formulations are known to have hydrolysate supplemented in them. The future of hydrolysates looks promising in the cell culture industry.

The second major aspect of concern for the biopharmaceutical industries is to make the cells that produce their desired product self-sufficient. This would obviate media formulations with optimal amounts of various components and allow the cell to produce what it needs for its survival and maintenance. As the first step towards this end goal, essential amino acids' biosynthetic pathways were to be engineered into mammalian cells. In particular, the research described in the thesis targeted branched chain amino acids- Leucine, Isoleucine and Valine. The biosynthetic pathway genes were

cloned into mammalian expression vector and transfected into mammalian cells to check for expression. All the genes were cloned into the mammalian expression vector pBUD4.1 and later transfected into CHO-K1 and HEK 293T cell lines and their expression was tested using a western blot. Five out of the nine genes to be expressed, have worked. Out of these five genes, one was the gene for leucine biosynthesis, LEU4 and it was expressed in HEK 293T. The other four genes were from the isoleucine and valine pathway which were expressed in CHO-K1. These results of expression prove that prokaryotic genes could be expressed in eukaryotic organisms. New cell lines could be generated with non-dependence on the amino acids which are essential for growth, which would be our final target in this project.

Continued efforts to improve both the research statements will be put in through further experimentation as described in the Future works sections. Bioprocess development will benefit through the success of these research works leading to high throughput bioprocessing and thereby reducing the cost of innovation and production of novel therapeutics.

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32. KEGG Pathways for *Saccharomyces Cerevisiae*

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EDUCATION

Whiting School of Engineering, Johns Hopkins University (JHU), Baltimore, MD. Aug 2015-Present
Master of Science and Engineering in Chemical and Biomolecular Engineering

GPA: 3.91/4.00; Graduate Research Advisor: *Dr. Michael J. Betenbaugh*

Birla Institute of Technology and Science-PILANI, Hyderabad, India

Aug 2010-May 2014

Bachelor of Engineering(Honors) in Chemical Engineering

GPA: Overall- 8.58/10 (3.89/4.00), Major- 9.2/10 (3.92/4.00)

RESEARCH & PROFESSIONAL EXPERIENCE

Mammalian Cell Research, Dr. Michael J. Betenbaugh's Group, JHU

Oct 2015-Present

Graduate Researcher (Department of Chemical and Biomolecular Engineering)

- Metabolic pathway engineering (Project I)
 - Aimed to engineer biosynthetic pathways of prokaryotes into mammalian cells to produce biomolecules essential for the growth of mammalian cell.
 - Successfully constructed and expressed plasmids containing genes of three prokaryotic biosynthetic pathways into mammalian cells (HEK 293T) transiently.
 - Gained experience in PCR, bacterial transformation, mammalian transfection, ELISA and western blotting in the due course of the project
- Boosting Cell productivity through a novel cell culture media additive (Project III)
 - Aimed to evaluate the effect of different media additives (Proprietary) on CHO cell growth and productivity
 - Performed cell culture experiments towards transcriptomics and proteomics analysis on the cell culture samples to determine the cause of the observed effect on the cells.
 - Evaluated the lot variability between different batches of the media additive
 - Designed a fed batch process for maximizing productivity of the cells using different combinations and compositions of the media additive along with a commercially available feed

Biologics Development Centre, Dr.Reddy's Laboratories Ltd., Hyderabad, India. June 2014-July 2015

Technical Trainee (Cell Culture Process Development)

- Involved in the process development, characterization and scale down model establishment of Bevacizumab process (media and feed development, cell culture process conditions screening for optimal pH and temperature)
- Tested several cell culture media additives like Pluronic-F68, different compositions of amino acids and conditions like aeration rate, sparging rate, to improve the cell viability keeping the product quality and productivity constant
- Handled several cell culture experiments independently right from planning to bioreactor assembly stage until the signing of development report (Worked with Sartorius reactors integrated with MFCS)
- Gained expertise in mammalian cell culture methods necessary for upstream process development (15mL AMBR scale to 10L scale in GLP setting including perfusion systems and 200L in GMP manufacturing facility)
- Worked on cell culture experiments which necessitated the use of novel instruments like AMBR® 15 system, Guava® easyCyte Flow Cytometer, Octet® QKe System, ViCell XR, Nova Bioprofiler
- Used FACS (in Guava Flow Cytometer) to assist a study to improve culture viability in a bioprocess
- Established a validation methodology for automated cell counters like ViCell XR analyzer and drafted a protocol to execute the validation (in compliance with Good Manufacturing Practices)

- Drafted SOPs (Standard Operating Procedures) and established JHAs (Job Hazard Analysis) according to the guidelines in the organization
- Executed ProcessPad, a data analysis platform in Biologics, to ensure transparency and consistency in data across development and manufacturing facilities
- Learned Design of Experiments (DOE) analysis through Microsoft Excel and statistical tools JMP and MATLAB, to execute in media and feed screening for a pipeline product

Chemical Reaction Engineering Group, BITS PILANI, Hyderabad, India July 2013-April 2014
Undergraduate Researcher

- Aimed at Model identification of ‘Zeolite-catalysed Toluene Nitration’
- Obtained the real equations representing the observed kinetic and mass transfer rates ‘Incremental Identification’ method using MATLAB
- Enabled effective control, optimization, and scale-up of ‘Zeolite-catalysed Toluene Nitration’ in semi-batch mode (Results published in *Reaction Kinetics, Mechanisms and Catalysis 2015*)

Term Project sponsored by ATE India Ltd. BITS PILANI, Hyderabad, India Jan 2014-Jun 2014

Undergraduate Researcher

- Modeled Indirect Evaporative Cooling in various configurations
- Estimated the critical parameters in the evaporative cooling process in each configuration using models created in COMSOL
- Determined the key parameters for maximizing performance of the process

Chemical Reaction Engineering Group, BITS PILANI, Hyderabad, India May 2013-Nov 2013
Undergraduate Researcher

- Performed a stage-wise analysis of Chemical Looping Combustion i.e., oxygen carriers, reactor design, and overall efficiency for carbon capture incorporated power generation
- Compared CLC with Post-combustion, Pre-Combustion, and Oxy-fuel combustion methods for CO₂ capture to determine the most efficient method (Results published in *Renewable and Sustainable Energy Reviews 2015*)
- Created a ASPEN Plus design of carbon capture enabled power plant for facilitating the above comparison using thermodynamic, parametric, and efficiency analysis

SKILLS & HONORS

- **Skills:** Molecular cloning (PCR), DNA electrophoresis, ELISA, Western blotting, Cell culture techniques (CHO, HEK, Fibroblasts), Flow cytometry, JMP, MATLAB, ASPEN, COMSOL, GLYMMER
- **Awards:** ‘Graduate Student Excellence Award’, Department of Chemical and Biomolecular Engineering, JHU, 2017
- **Scholarships:** Awarded scholarships under ‘MHRD, Government of India, Scholarship Scheme’ and ‘Incentive Scheme-2010, State Bank of India’ for pursuing professional degree in elite institute in India
Tuition support for graduate student in the Department of Chemical and Biomolecular Engineering for two semesters (\$40,000), Johns Hopkins University (JHU)

JOURNAL PUBLICATIONS & CONFERENCE PROCEEDINGS

1. D V Gayatri and I Sreedhar, "Kinetics of solid acid catalyzed toluene nitration using incremental identification method, *Reaction Kinetics, Mechanisms and Catalysis*. 114 (1), 2015, 251-263. (A Springer publication)
2. B Sreenivasulu, D V Gayatri, I Sreedhar, K V Raghavan, "A Journey into the Process and Engineering Aspects of Carbon Capture", *Renewable and Sustainable Energy Reviews*, 41, 2015, 1324-1350. (An Elsevier publication)