International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Effect of Carbon Concentration in Batch Media on Up-Scaling Biomass Production

Nandini Banerjee

Abstract: ***Now a days recombinant bacteria (commonly used strain E. Coli BL21) is commonly used as a host and expression strain for protein expression and purification. When large quantities of fully deuterated proteins are required, E. coli is often grown in minimal media or customize chemical defined culture media with glucose or dextrose as the carbon source because these are less expensive. Despite the widespread use of BL21, we want to study the effect of different carbon sources concentration on BL21 growth. In this study, we assessed the growth behavior of E. coliBL21 in chemical defined media with different gluconeogenic carbon sources concentration. Though BL21 grew reasonably well on glycerol and pyruvate, it had a prolonged lag-phase on succinate (20 h), acetate (10 h), and fumarate (20 h), attributed to the physiological adaptation of E. coli cells grew well on all the substrates. We also examined the growth of E. coli BL21 in bioreactor to optimized bacterial batch growth using chemical defined culture media. The bioreactors is used to carefully control temperature, pH, and dissolved oxygen concentrations, in particular, makes them essential to efficient large-scale growth and expression of fermentation products. Scale-up from shake flasks to bioreactor has been hampered by the lack of knowledge, so before start commercial product, I have run bioreactor only for biomass production. Escherichia coli has a proven track record for successful production of anything from small molecules like organic acids to large therapeutic proteins, and has thus important applications in both R&D and commercial production. E. Coli is a very fast growing bacterial strain, for optimization study it is an ideal bacterial strain. We used chemical-defined media for the growth of E.coli rapidly in large quantities. In this experiment I used Carbon sources which is provide energy and major building blocks elements for the microorganism for growth and their choice is very critical for the production of high levels of antibiotics. For any lab-based experiment shake flask bacterial growth is good but when thinking about a large amount of production, means thinking about scale-up, then bioreactor is the only option. So that optimization study about up-scaling from shake flask to bench-top bioreactor is necessary.***

Keywords: Escherichia coli, wet cell mass, dry cell mass, bioprocessing, up-scale bench bioreactor, shake flask bioreactor, bacterial growth curve

Abbreviations/Acronyms

Agit- Agitation
WCM- Wet Cell Mass
DCM- Dry Cell Mass
DO- Dissolved Oxygen
E.coli- Escherichia coli
RTD- Resistance Temperature Detector
TMFC- Thermal Mass Flow Controller
OD- Optical Density
RPM- Rotation Per Minute
LB broth- Luria Bertani broth
PBS- Phosphate Buffer Saline
NaOH- Sodium hydroxide
NaCl- Sodium chloride
KCl- Potassium Chloride
Na_2HPO_4- Disodium phosphate/ Sodium hydrogen phosphate
KH_2PO_4- Potassium dihydrogen phosphate

1. Introduction

Bioprocessing operation system is an essential part of the food, chemical, and pharmaceutical industries. Bioprocess operations create use of microbic, animal, and plant cells and elements of cells like enzymes to manufacture new merchandise and destroy harmful wastes. The bioprocess industry is developed to produce low-cost products such as industrial alcohol and organic solvents, too costly specialty chemicals like antibiotics, therapeutic proteins, and vaccines. Industrially helpful proteins and living cells like baker's and brewer's yeast also are business merchandise of bioprocessing.

The use of microorganisms to rework biological materials for the production of soured foods has its origins in antiquity. *Escherichia coli* is a highly growing micro-organism in high density which is currently the method of choice for the production of therapeutic proteins and scientists are used *E.coli* for the store DNA sequence from other organisms, to produce protein, to test protein function. In most of the experiments, scientists used *E.coli* as a host for gene cloning due to the high efficiency of DNA molecules into cells. *E.coli* was originally founded by Theodore escherich in 1885, a German bacteriologist, and named bacterium coli commune. E.coli is a non-sporulation, gram-negative, facultatively anaerobic, and rod-shaped bacterium that is commonly found in a warm-blooded organism. The inner membrane surrounded by a murein wall and by an outer membrane. The space between both the membrane is called periplasm and significantly differs from the cytosol in terms of composition. E.coli is a mesophilic bacteria that can grow at a temperature ranging from 8 to 48oc with a maximum growth rate of 39oc at a maximum water activity of 0.95. E.coli is an acid-tolerant and can grow at pH values from 4.4 to 10. *E.coli* can be grown easily and inexpensively in a laboratory setting with useful equipments and useful raw materials.

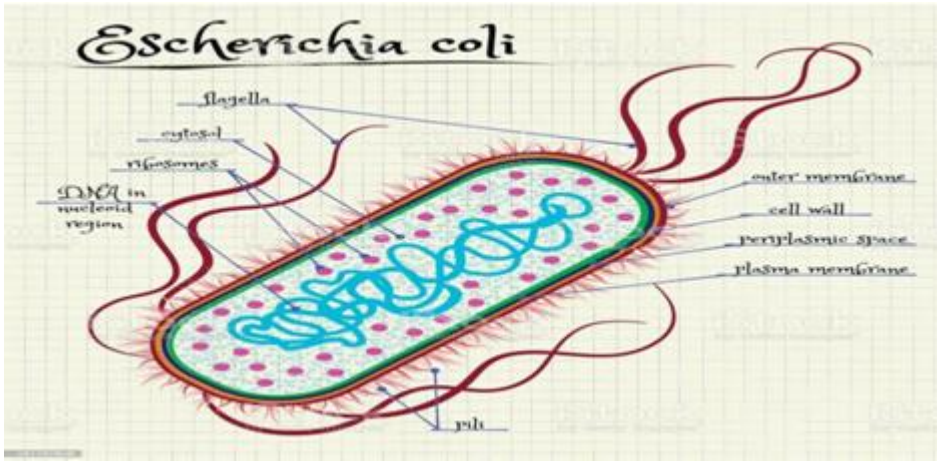

Figure 1: Schematic diagram of E.coli

E.coli can grow with different substrates like in the presence and absence of 02, in presence of glucose which is commonly used as a primary nutrient in media. In media, glucose is used as a primary nutrient because E.coli can be easily metabolized to provide carbon and energy for biosynthesis and also due to its low price. E.coli is a gram-negative bacillus that grows well on commonly used media like LB and Mac Conkey agar media. E.coli is a lactose fermenting and beta-hemolytic organism on blood agar. Most E.coli strains are non-pigmented; some of them are growing on mac Conkey agar. In nutrient agar plate it appeared as a circular, large, grayish, white, low convex, smooth, moist, and opaque. In liquid media, E.coli shows a homogeneous turbid growth within 12-18 hr. After some time of the experiment, we can see sedimentation forming on the bottom of the test tubes, which mainly contain E.coli cells. Under favorable conditions, E.coli cells take 20 minutes to reproduce.

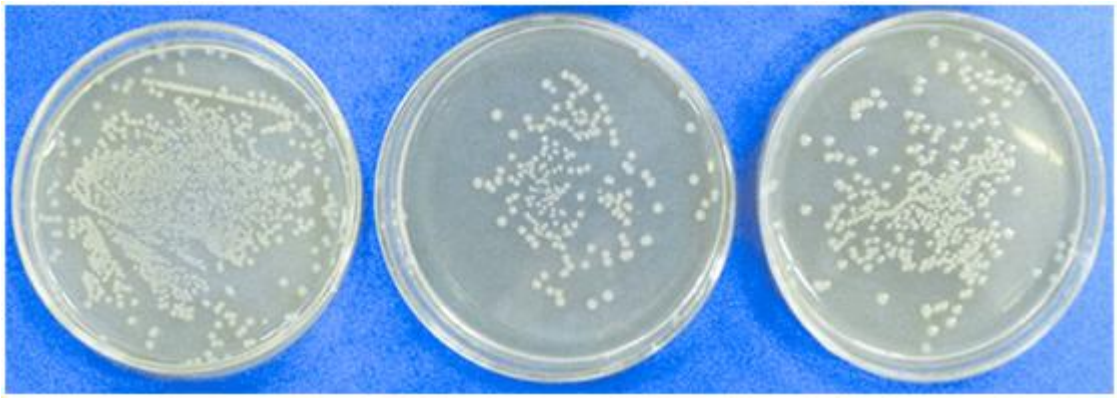

Figure 2: *E. coli* grow on luria bertani agar

In nowadays scientists are used chemical-defined media for the growth of E.coli rapidly in large quantities. E.coli is a chemoheterotrophic organism, so the chemical-defined medium which is used for the growth of these bacterias must include a source of carbon and energy. Chemical-defined media supplemented with ammonium salts, glucose, acetate, and glycerol. The production of 15N labeled protein is inexpensive, which is easily expressed in E.coli 13C label is costly, which is necessary for full analysis of larger protein. These label proteins are essential for exclude all-natural sources like nitrogen and carbon from growth media. They constitute chemical-defined media containing amino acid, vitamins, inorganic salts, buffer antioxidants, and energy sources, recombinant albumin, chemically defined lipids, zinc, iron. These media are designed for the cultivation of e.coli cells which additionally contain a suitable surfactant such as poloxamers [Paul N. Sanderson., 1999].

Bioreactor

Bioreactor is the core of biological processes. A bioreactor is a type of fermentation vessel that is used for the production of various chemicals and biological reactions. It is a closed container with adequate arrangement for aeration, agitation, temperature and pH control, and drain or overflow vent to remove the waste biomass of cultured microorganisms along with their products. To design an appropriate bioreactor for a particular bioprocess, intensive studies on the biological system, such as cell growth, metabolism, genetic manipulation, and protein or other product expression, are needed to understand the cells' requirement on their physical and chemical environment. It is also necessary to control and optimize the bioreactor environment via operating variables in order to favor the desired functions of the cells and achieve cost-effective large-scale manufacture. The specific demands are often conflicting and achieving optimal performance requires attaining the proper balance among the different requirements. Many different bioreactors and bioreactor applications are described, including cell growth, enzyme production, biocatalysis, biosensors, food production, milk processing, extrusion, tissue engineering, algae production, protein synthesis, and anaerobic digestion. Methods to classify bioreactors are presented, including

operational conditions and the nature of the process. References are included to enable the reader to find more information on each of the types of bioreactors and selected products. In all cases, the bioreactor must provide the environmental conditions necessary for the culture. Success of a bioprocess depends critically on good design and operation of the bioreactor. The variety of bioprocesses is tremendous and many different designs of bioreactors have been developed to meet the different needs.

Bioreactors are vessels that have been designed and produced to provide an effective environment for enzymes or whole cells to transform biochemicals into products. Fundamental design principles and various types of bioreactors including stirred-tank, pneumatically agitated, membrane, fixed- and fluidized-bed, and wave bioreactors are reviewed. The effects of process variables on biological performance, such as temperature, pH, mixing, oxygen transfers, and shear force, are discussed.Bioreactor is an indispensable part of any bioprocess irrespective of produces substances such as foods, feeds, chemicals and pharmaceuticals, and tissues and organs for use in biomedicine etc. The emphasis is on reactors that are produced commercially rather than on reactors that have evolved in the natural world. Bioreactors used for DNA and protein synthesis (biomolecular synthesizers) are included. Commodity products include all substances that are produced in bioreactors and marketed; however, the emphasis is on general principles and more widely used bioreactor types and products Bioreactor operation strategies include fed-batch, continuous, semicontinuous, and perfusion cultures. The basic fermentation system is designed to deal with all of those limitations. Most cell culture grow cell cultures in dishes, flasks or tubes. However if anyone needs to producelarge amount of cell, for secreting large amount of specific protein. At that time, it is sensible to scale up their culture conditions-growing lots additional cells to form lots additional protein. This can be best performed in a very larger vessel, referred to as a bioreactor, which might be used for a spread of applications, including: fermentation; bioprocess development; manufacturing antibodies, vaccines and recombinant proteins. A bioreactor is outlined as a system with a closed culture atmosphere that simulates totally different physiological, environmental, and mechanical factors.For the industrial application of bioreactors, bioreactor scale-up, multiscale study, and bioprocess monitoring, modeling and simulation are also very important. Finally, the trends in bioreactor engineering, including microbioreactor, cell as a super bioreactor, and plant and animal as powerful protein-producing bioreactors, are briefly summarized.

Basic fermentation technology is associate extension of the easy shake flask technique for growing cultures. Shake flask are wide employed in the study and improvement of biotechnology/ biology processes, permitting to the performance of experiments with minimal prices and material. However, shake flasks have many limitations, one in every of them, and possibly the foremost necessary, is that the complicated understanding of the individual environmental factors concerned. Shake flask grow out of the need to manage growth environments for live cultures during a lot of complete and quantitative manner. Batch culture shake flasks area unit typically restricted by inaccurate management of temperature uniformity in associate incubated shaker or heat area is very variable, generally lost 5°C or a lot of from the meant set-point. Shake flask is mostly agitated at a set speed, restricted O_2 uptake and gas exchange. Benchtop fermentors typically offer management of pH via liquid chemical agent addition through a pump. The pH worth is usually monitored in a shot to stay the atmosphere optimum for cell growth. Correct aeration is maintained by the infusion of air or element supplemented gas directly into the culture. With cultures, element supplemented gas is that the primary mechanism for maintenance of element level within the culture. mensuration of element in culture is typically achieved by a DO probe that isn't ordinarily on the market to be used in shake flasks. It's on the market in bioreactor system.

Most bacterium replica by associate degree apomictic method referred to as binary fission, which ends in doubling of the amount of viable microorganism cells. the quality microorganism growth curve describes many various stages of growth of pure culture of bacterium, starting with the addition of cells in sterile media to the death of the cells.

1.1. Background and context

During this period, in KIIT-TBI Bioprocessing Lab, I tried to study about scaling up of *Escherichia coli* biomass from shake flask to bioreactor and optimization bacterial culture media. If think about production scale, then we have to think about bioreactor and it's operation method so that we can get maximum yield using minimum consumable. For reducing the cost of culture media, we have to optimized the minimum amount of carbon source which is needed for maximum yield. Follow this path we do study about glucose concentration, in which concentration of glucose gives us the maximum yield. And also we operate bioreactor batch operation using same media composition.

*Escherichia coli*is commonly used as a host for protein expression as it constitutes a simple and well-studied system and provides a suitable choice of expression options. *E. coli*strain BL21 is well suited for protein over expression. This strain has been used extensively to express a variety of native and heterologous proteins. For many methods of protein analysis, large quantities of protein are often required and, therefore, the ability to achieve the maximum protein yield from a specific culture volume is important (Vanatalu et al. 1993; Sosa-Peinado et al. 2000; Zhong et al. 2005). To obtain proteins enriched in deuterium, they are often over expressed in E. coli cells such as BL21 grown in minimal medium supplemented with a completely deuterated carbon source (Gardner and Kay 1998; Sosa-Peinado et al. 2000). Among these deuterated carbon sources, deuterated succinate and acetate are less expensive than deuterated glucose or deuterated rich media such as algal hydrolysate (prices from Cambridge Isotope Laboratories, 2005), and thus d-succinate or d-acetate might be advantageous to use for large-scale protein production (LeMaster and Richards 1988; Vanatalu et al. 1993; Paliy et al. 2003). Although the glucose utilization pathways have been recently investigated for E. coli BL21 strain (Phue and Appl Microbiol Biotechnol (2007) genic carbon sources has

not been extensively studied. In this study, we characterized the growth of E. coli BL21 on different gluconeogenic carbon sources and in different minimal media. We also compared BL21 growth with that of K12 and JM109 strains.

Bacterial Growth Curve

The growth of bacteria and observing its growing pattern, thus forming a growth curve. For this experiment, bacteria are grown under controlled conditions in pure culture, that is, only one type of bacteria is grown. Bacteria grow with a rapid increase in the number of cells in a very short period of time. The measurement of the rate of the cell population is taken which increases over time forming a growth curve. The growth curve has four distinct phases as "lag phase," "log phase," "stationary phase," and "death phase." The importance of the technique lies in aiming to inoculate required numbers of the bacterial isolate, e.g., to enhance plant growth, increase biodegradation of toxic organics, and produce antibiotics or other natural products.

When a broth culture is inoculated with a small bacterial inoculum, the population size of the bacteria increases showing a classical pattern. When plotted on a graph, a distinct curve is obtained referred to as the bacterial growth curve.

- A population growth curve for any particular species of bacterium may be determined by growing a pure culture of the organism in a liquid medium at a constant temperature.
- Samples of the culture are collected at fixed intervals (e.g., every 30 minutes), and the number of viable organisms in each sample is determined.
- The data are then plotted on logarithmic graph paper.
- The logarithm of the number of bacteria per milliliter of medium is plotted against time.

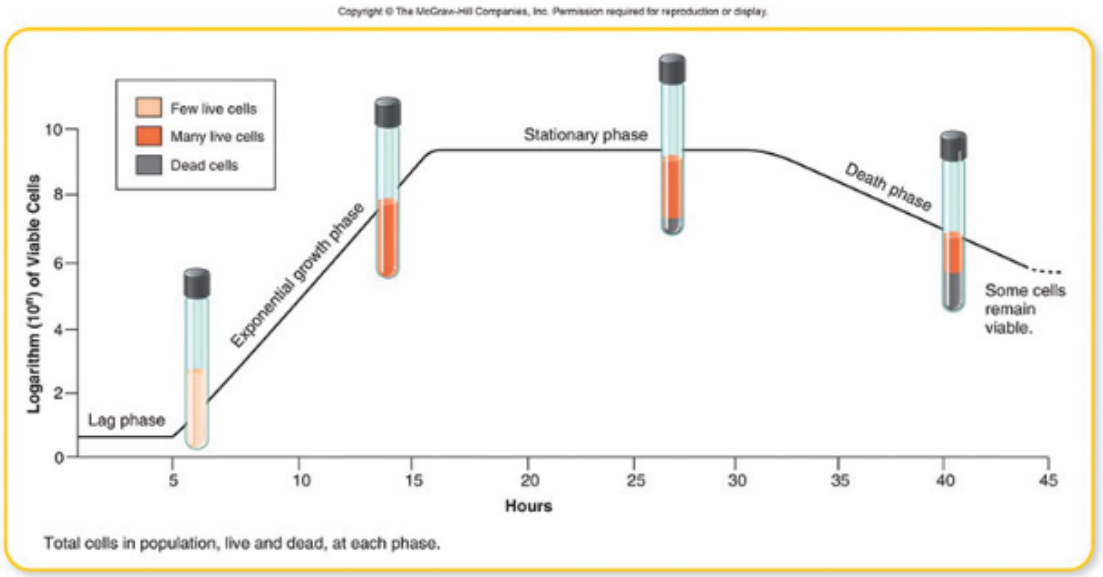

Figure 3: Bacterial growth curve

1) Lag phase:

- After a liquid culture broth is inoculated, the multiplication of bacteria does not start immediately. It takes some time to multiply.
- The time between inoculation and beginning of multiplication is known as lag phase.
- In this phase, the inoculated bacteria become acclimatized to the environment, switch on various enzymes, and adjust to the environmental temperature and atmospheric conditions.
- During this phase, there is an increase in size of bacteria but no appreciable increase in number of bacterial cells. The cells are active metabolically.
- The duration of the lag phase varies with the bacterial species, nature of culture medium, incubation temperature, etc.
- It may vary from 1 hour to several days.

2) Log phase:

- This phase is characterized by rapid exponential cell growth (i.e., 1 to 2 to 4 to 8 and so on).
- The bacterial population doubles during every generation. They multiply at their maximum rate.
- The bacterial cells are small and uniformly stained.
- The microbes are sensitive to adverse conditions, such as antibiotics and other antimicrobial agents.
- Growth rate is the greatest during the log phase.
- The log phase is always brief, unless the rapidly dividing culture is maintained by constant addition of nutrients and frequent removal of waste products.
- When plotted on logarithmic graph paper, the log phase appears as a steeply sloped straight line.

3) Stationary phase:

- After log phase, the bacterial growth almost stops completely due to lack of essential nutrients, lack of water oxygen, change in pH of the medium, etc. and accumulation of their own toxic metabolic wastes.
- It is during this phase that the culture is at its greatest population density.
- However, Death rate of bacteria exceeds the rate of replication of bacteria.

- Endospores start forming during this stage.
- Bacteria become Gram variable and show irregular staining.
- Many bacteria start producing exotoxins.

4) Death/Decline phase:

- During this phase, the bacterial population declines due to death of cells.
- The decline phase starts due to :-
  - Accumulation of toxic products and autolytic enzymes and
  - Exhaustion of nutrients.
- Involution forms are common in this stage. Some cells assume various shapes, becoming long, filamentous rods or branching or globular forms that are difficult to identify.
- Some develop without a cell wall and are referred to as protoplasts, spheroplasts, or L-phase variants (L-forms).
- When these involuted forms are inoculated into a fresh nutrient medium, they usually revert to the original shape of the healthy bacteria.

The study of bacterial growth curves is important when aiming to utilize or inoculate known numbers of the bacterial isolate, for example to enhance plant growth, increase biodegradation of toxic organics, or produce antibiotics or other natural products at an industrial scale. Knowledge of bacterial growth kinetics and bacterial numbers in a culture medium is important from both a research and commercial point of view. Growth kinetics is also useful for assessing whether particular strains of bacteria are adapted to metabolize certain substrates, such as industrial waste or oil pollution. Similarly, the slope and shape of growth curves produced from bacteria grown with mixtures of industrial waste products can inform scientists whether the bacteria can metabolize the particular substance, and how many potential energy sources for the bacteria can be found in the waste mixture.

Organism

Escherichia coli strain BL21 is commonly used as a host strain for protein expression and purification. For structural analysis, proteins are frequently labeled with deuterium 13C or 15N by growing *E. coli* cultures in a medium containing the appropriate isotope. When large quantities of fully deuterated proteins are required, *E. coli* is often grown in minimal media with deuterated succinate or acetate as the carbon source because these are less expensive. Despite the widespread use of BL21, we found no data on the effect of different minimal media and carbon sources on BL21 growth. In this study, we assessed the growth behavior of *E. coli* BL21 in minimal media with different gluconeogenic carbon sources. Though BL21 grew reasonably well on glycerol and pyruvate, it had a prolonged lag-phase on succinate (20 h), acetate (10 h), and fumarate (20 h), attributed to the physiological adaptation of *E. coli* cells. Wild-type strain NCM3722 (K12) grew well on all the substrates. We also examined the growth of *E. coli* BL21 in minimal media that differed in their salt composition but not in their source of carbon. The commonly used M9 medium did not support the optimum growth of *E. coli* BL21 in minimal medium. The addition of ferrous sulphate to M9 medium (otherwise lacking it) increased the growth rate of *E. coli* cultures and significantly increased their cell density in the stationary phase.

1) Reduced risk of contamination or cell mutation as the growth period is short.
2) Lower capital investment when compared to continuous processes for the same bioreactor volume.
3) More flexibility with varying product/biological systems.
4) Higher raw material conversion levels, resulting from a controlled growth period.

1.2 Scope and Objectives

The scope of this study was to optimize the bacterial culture media to get maximum number of cell from culture. We can make maximum number of cell, so that we can get maximum protein. That's why bioreactor is the heart of bioprocess facility. Here we want to observed that in which glucose concentration in culture media the bacteria will grow effectively.

Overview of Shake flask incubator:

Shake flask fermentation is one of the best examples of fermentation. Basic fermentation technology is an associate extension of the easy shake flask technique for growing cultures. Shake flask is widely employed in the study and improvement of biotechnology/ biology processes, permitting the performance of experiments with minimal prices and material. Shaking incubator used for cell culturing, increase aeration and solubility. The incubate is expose to oxygen continuously by which the nutrient can easily soluble and distributed efficiently to every place for proper growth of the microorganism. Batch culture shake flasks area unit typically restricted by inaccurate management of temperature uniformity in associate incubated shaker or heat area is very variable, usually 10% of volume per 24 hours of time at 37°C. The speed is 20-300 rpm in 1rpm increments, and the temperature operating range is 50c above room temperature to 70oc. Batch culture is a closed operate stage which contains an initial, limited amount of nutrient. The inoculation which is used in this process passed through a different number of phases. After inoculation, this period is called the log phase. In the lag phase, the cell grows at a constant, maximum rate and this period is also known as the exponential phase.

Over view of BioFlo/CelliGen 115 bioreactor:

Escherichia coli fermentation was conducted using the BioFlo 115 which is a bioprocess control station. By using this control system we can achieve high cell density in 12 hr as determined by a maximum optical density (OD 600) measurement of 215.6 *[Bin Li et al., 2015]*. This advanced bioprocess system was developed for both microbial fermentation and cell culture applications. It helps maintain the optimal performance of equipment over years and years.

1) System:

BioFlo/CelliGen115 could be a versatile bioreactor that provides a totally equipped system in one compact package. It will be applied for batch, fed batch or continuous culture with method management for pH, dissolved element (DO), agitation, temperature, pump feed, antifoam and foam/level. Systems will be organized as either management stations or

utility stations. every individual standalone system could be a management station. One management station will run up to 2 further utility stations, that ar obsessed on the control/management station.

2) Control panel:
In benchtop bioreactor controlled with a control panel system. control panel connected with bioreactor culture vessel which helps to control the inside environment of the culture vessel. In the control panel system, one screen is visible which shows different sections like summery screen, calibration screen, pumps screen, setup screen. In the summer screen different parameters are visible such as Agitation, Temperature, pH, DO, Air, O2. Pump screen is connected with 2 rota meter(one is responsible for controlling air and another one controlled the oxygen flowing), this rotameter help to control gases which are flowing inside the bioreactor. The pump provides a speed range from 50 to 200 rpm for fermentation with direct drive, from 25 to 400 rpm for cell culture. The whole control with the software. 3 pumps are places below the rotameter, these pumps are working as pumping the solution inside the bioreactor, which helps control pH concentration inside the vessel. In the calibration screen, we can calibrate the DO and pH.

3) Vessels:
There are 2 types of glass vessels used one is non-jacketed another one is water jacketed. It is a small bioreactor culture vessel with a maximum working volume is 7.5L and 40L designed for process characterization and optimization. This vessel is connected with the inoculation tube: for adding acid and base into the solution, resistance temperature detector, a foam probe, a sparger, harvest tube, a sampling tube, exhaust condenser, dissolved oxygen, and pH electrodes. An agitation motor is located on top of the head plate of a vessel which is connected with the agitator.

4) Agitation system-
On the head plate there's a removable agitation motor present on prime of the bearing housing, is connected to the agitation shaft with a right away drive coupling. The motor is simply disconnected before autoclaving the vessel and simply replaced once sterilization. The motor can give a speed vary from 50 to 1200 RPM for fermentation with direct drive, from 25 to 400 RPM for cell culture with direct drive, or from 25 to 200 RPM for cell culture with magnetic drive. the method management software package ensures agitation speed management throughout the speed vary. Agitation speed can vary between the user-specified minimum and most set points so as to keep up the set share of DO.

5) Temperature management-
The culture temperature setpoint is also elite among the vary from 20°C higher than fluid temperature to 70°C for 1.3- to 7.5-liter vessels, and from 20°C higher than fluid temperature to 65°C for 14.0-liter vessels. it's management by the method control package that then sends data to either a heater blanket and cooling coil or to a vessel. The media temperature is detected by a Resistance Temperature Detector (RTD) submerged within the thermowell.

6) Aeration-
Up to four gases, as well as air, nitrogen, CO2 and O2, are often introduced into the media through the ring sparger or elective microsparger. The rate of flow is controlled manually by one, two, three or four Rotameter (s) or mechanically by thermal mass flow controller (TMFC), in step with the definition of your system. The TMFC is regulated mechanically in step with values set via the control station touch screen. The gas combine will either be controlled manually by adjusting the flow of gases through their Rotameters or mechanically.

7) pH control
pH is controlled within the vary of 2.00-14.00. The pH scale is perceived by a gel-filled pH scale probe.Control is maintained by a controller that operates peristaltic pumps, appointed to perform acid or base addition.

8) DO control-
Dissolved chemical element (DO) is controlled within the vary of 0-200%. it's measure by the DO electrode and management is maintained by the controller by ever-changing the speed of agitation, the thermal mass flow controller-regulated flow and therefore the proportion of chemical element in aeration.

9) Foam/Level management-
Foam is monitored throughout batch fermentation by a foam/level probe set within the headplate. The controller operates the antifoam-assigned pump that adds chemical into the vessel as needed.

10) Tripod:
Tripod is attached with 3 pumps which are present in the control panel. Through this pump, the solution is pumped inside the vessel.

11) Harvesting tube:
It opens at the bottom of the culture vessel. Application of this tube is to harvest the culture media from the bioreactor culture vessel.

12) Temperature sensor holder:
It is put inside the holder. This holder has no open mouth. Temperature sensor direct connected with culture media. It is connected directly to culture media it creates contamination in media vessels.

13) Sample tube:
The sample tube is used to collect the samples and later its use to calculate CFU, nutrient content, glucose concentration, amino acid concentration, etc.

14) Baffles:
It will create a site for turbulence; it helps proper mixing of the solution.

- **Exhaust Condensor:**

Inside exhaust Condensor holes are there, which help in airflow into the vessel

- **pH control:**

pH is controlled within the vary of 2.00-14.00. The pH scale is perceived by a gel-filled pH scale probe. Control is

maintained by a controller that operates peristaltic pumps, appointed to perform acid or base addition.

Exhaust system-

The exhaust gases pass into the exhaust condenser wherever wetness is removed, then came to the vessel. The remaining gases then taste a zero.2 µm exhaust filter.

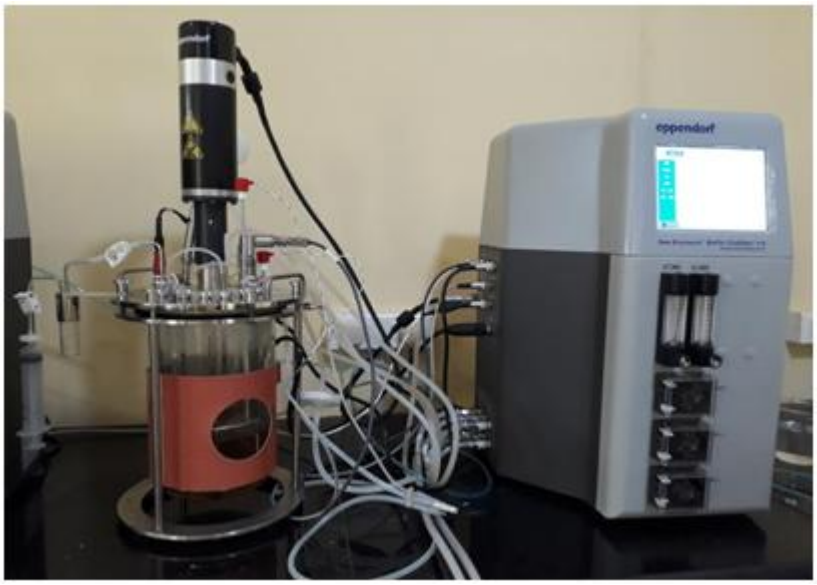

Figure 4: Bioreactor 7.5L

In long term, the stationary phase is successive in the growth of the viable cells in the population. Carbon sources provide energy and major building blocks elements for the microorganism for growth and their choice is very critical for the production of high levels of antibiotics. If bacteria can grow without a carbon source, that "autotrophic metabolism" and fix carbon in the form of co2 could thus support them. It is easily degraded by some bacteria. E.coli is a heterotrophic bacterium usually grows on glucose and produces co2. this strain uses co2 as the sole carbon source. For microbial growth different nutrients are needed like gases such as 02, co2, and H2 as well as alkaline and acidic titrants for pH control. In the bioprocess industry, the mode of operation of microbial processes can be categorized into 3 groups; fed-batch, batch, continuous. In fed-batch processes, the all nutrient which is needed during processes that add phase-wise in each process. In batch processes, the all required nutrient needed for the growth of microbes are initially added to the vessel, and the products are removed after the period of growth is completed. In continuous process nutrients are added to continuously manner, the rate is originally same and constant volume is maintained.

1.3 Overview of Dissertation

I have done scale up study from shake flask to bioreactor, and in each step we have to optimized the bacterial growth condition and media composition mainly carbon source. So, in this study I have done culture media carbon source optimization study in lab scale. And also I learn the operation of bench-top bioreactor (batch culture).

2. Aims and Objectives

To study the effect of carbon concentration in high cell density culture this project is design and to implement a scale up study of biomass production from shake flask to bench-top bioreactor.

- Culture media optimization
- Bacterial growth affect study using different carbon (glucose) concentration (1%, 1.5%, 2%, 2.5% & 3%) in culture media.
- Bacterial growth dependent carbon concentration study in shake flask (100ml culture in 500ml flask).
- Bacterial growth dependent carbon concentration study in bench-top bioreactor.
- Operate bench-top bioreactor and continuously monitoring using BioCommand software.

3. Materials and Methods

3.1. Bacterial strain used

The organism used in this study is Escherichia coli BL21. This strain is used as the host in all experiments of protein expression and plasmid stability, it is easy to genetically modify and inexpensive to culture. *E. coli* can also grow over a wide range of pH, from 4.4 up to 9.2, by adapting its metabolism to the environment. When adapting their metabolism the cells may take up acids or bases from the media, this can slow down growth and reduce the expression of recombinant proteins. *E. coli* can use many different carbon sources such as amino acids, glycerol, or glucose. Here I am using *E. coli* BL21(codon+) strain for my scale-up study.

3.2 Inoculum preparation

The bacterial culture was grown in LB broth (Lurial-Bertani broth) containing Trypton, yeast extract and NaCl. We need 1% inoculum for inoculation into fermentor. So for prepare inoculum autoclave LB media as needed with in a conical flask. A loop-full of 24 hour old culture was inoculated into above medium. Then flasks are incubated at 37°C in a incubator shaker at 150rpm for 12-14 h.

3.3 Preparation of LB& Chemically defined media

In a 1L flask of 250 ml of Lb and chemically defined media broth was prepared and 2.5 L media for bioreactor in which glucose(2.5%), KH_2PO_4, NH_4Cl, citric acid and trace element based CM media was added and it with the help of magnetic stirrer until a homologous mixture was formed .

3.4 Preparation of chemical define media

A chemical defined media for the growth of bacteria was prepared which contained primary nutrients required for bacterial growth and division. Such nutrients contain components such as Carbon, Nitrogen and Oxygen sources. Some trace metals were added to trigger the cell metabolism

3.5 Batch cultivation

In a batch cultivation is all components added at the beginning of the cultivation. The concentration of substrates is therefore high at the beginning. During the cultivation, when the components are consumed, the concentrations go down. *E. coli* grows faster and to higher cell densities if complex medias are used, that is because the nutrient content is high and the buffering better. The cells will in batch cultivation grow unlimited until some nutrition is depleted for example glucose. The oxygen can also become limited in shake flask, but *E. coli* can grow under anaerobic or oxygen limited circumstances but the production of recombinant proteins will then go down.

3.6 Optimization at shake flask level

Various time of incubation and volume of culture medium were employed to study their effect on growth of *E. Coli BL21* (codon+). But all other parameter are same like temperature 37°C, agitation speed 150rpm. Initially I have grow *E. coli* at 100ml medium in a 500ml conical flask.

3.7 Bench-scale bioreactor optimization

A stirred tank bioreactor (Bioflo 115, New Brunswick; Eppendorf) of 7.5L capacity was used for bench-scale studies. The reactor was equipped with direct drive dual impellers, temperature and agitation control, probes and controller for pH and DO. Temperature was control by heating jacket and cooling condenser, and temperature was monitor by RTD (Resistance Temperature Detector). After inoculate the inoculums, when the bacteria was start growing, DO was gradually decrease and then we have to increase agitation speed for stabilized/increase DO.

3.8. Sampling procedures

For induced cultivation samples for further analysis were taken at induction, every hours after induction. OD,WCM and DCM measurements were taken during the whole cultivation.

Shake flask: The samples were taken with a sterile pipette into a micro centrifuged tube.
Bioreactor: The samples were taken with a sterile syringe through a membrane. The membrane was sprayed with ethanol before the sample was taken.

3.9. DNS method

The DNS method is used to determine the concentration of reducing sugar in a sample. Reducing sugars have a free carbonil group and can decrease a wide range of agents. Reducing sugars include all mono saccharides (such as glucose) and some disaccharides (such as lactose). The alkaline solution of 3,5-dinitrosalicylic acid (yellow hue) is reduced by the free carbonyl group in reducing sugars to 3-amino,5-dinitrosalicylic acid, a red-brown colored molecule that absorbs light at 540. The amount of reducing sugars present in the reaction mixture determines the intensity of the color generated.

4. Result

Optimization of pH for *E. Coli* BL21in shake flask (100ml LB media in 500ml conical flask) was determined and then same optimization was done in 7.5L bioreactor (using 2.5 L of CDM media).

4.1 Table-01 Growth curve study using different carbon concentration (glucose) in shake flask, OD vs Time.

Time (min)	1% OD		1.5% OD		2% OD		2.5% OD		3% OD	
	Set-1	Set-2	Set-1	Set-2	Set-1	Set-2	Set-1	Set-2	Set-1	Set-2
60	0.101	0.101	0.099	0.105	0.097	0.109	0.1	0.1	0.098	0.11
120	0.129	0.142	0.115	0.139	0.109	0.122	0.108	0.132	0.101	0.125
180	0.178	0.183	0.157	0.175	0.142	0.138	0.121	0.17	0.117	0.147
240	0.21	0.221	0.196	0.212	0.175	0.161	0.189	0.216	0.156	0.196
300	0.315	0.304	0.298	0.238	0.218	0.24	0.325	0.292	0.183	0.251
360	0.692	0.462	0.621	0.394	0.568	0.329	0.575	0.483	0.371	0.398
420	0.875	0.816	0.85	0.544	0.791	0.548	0.769	0.718	0.658	0.674
480	1.06	1.83	1.11	0.812	1.02	0.892	1	0.946	0.931	0.919
540	1.89	2.59	2.01	1.68	1.91	1.75	1.85	1.31	1.27	1.06
600	3.53	3.48	3.68	3.82	3.51	3.22	3.47	2.98	3.35	2.82
660	4.45	3.79	4.57	4.05	4.45	4.61	4.49	4.21	4.52	4.11
720	4.56	3.94	4.98	4.16	5.08	4.87	5.89	5.31	5.78	5.45

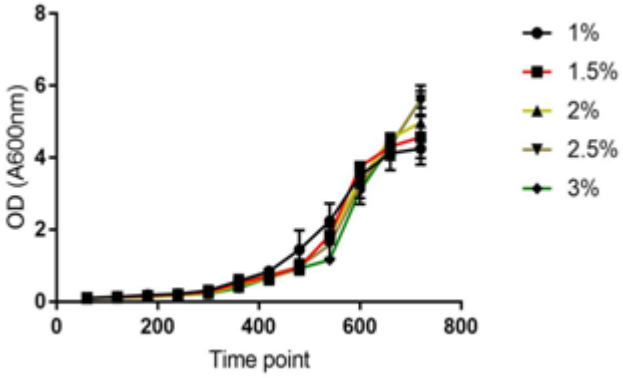

Figure 5: 100ml culture of E. coli BL21 in 500ml shake flask till 12 hour.
Using different glucose concentration (1%, 1.5%, 2%, 2.5% & 3%) OD & Time

4.2 Table-02 Growth curve study using different carbon concentration (glucose) in shake flask, WCM vs Time.

Time (min)	1% OD		1.5% OD		2% OD		2.5% OD		3% OD	
	Set-1	Set-2	Set-1	Set-2	Set-1	Set-2	Set-1	Set-2	Set-1	Set-2
60	0.39996	0.53328	0.53328	0.53328	0.46662	0.59994	0.59994	0.53328	0.53328	0.59994
120	3.59964	3.26634	4.06626	3.59964	3.26634	2.19978	3.46632	1.73316	2.86638	1.46652
240	6.06606	4.6662	6.39936	6.19938	4.86618	5.39946	4.73286	3.46632	3.6663	3.26634
360	8.9991	6.9993	8.26584	8.06586	7.73256	7.13262	6.3327	6.46602	5.26614	6.19938
480	14.86518	14.6652	13.79862	15.59844	14.73186	14.13192	9.86568	14.53188	6.73266	9.46572
600	16.73166	15.39846	17.46492	18.13152	19.79802	18.73146	20.13132	21.93114	17.19828	18.39816
720	17.6649	16.13172	19.13142	18.26484	22.13112	21.13122	28.86378	26.06406	28.06386	26.664

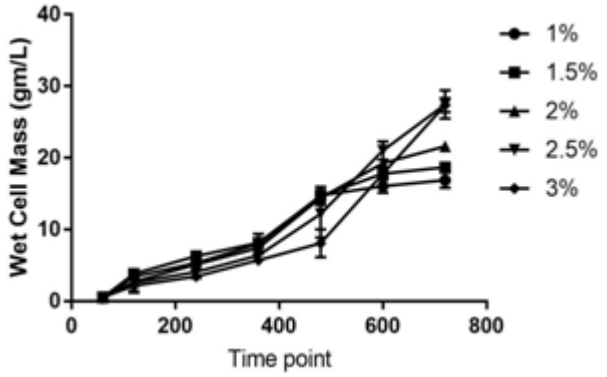

Figure 6: 100ml culture of E. coli BL21 in 500ml shake flask till 12 hour.
Using different glucose concentration (1%, 1.5%, 2%, 2.5% & 3%) WCM & Time

4.3 Table-03 Growth curve study using different carbon concentration (glucose) in shake flask, DCM vs. Time.

Time (min)	1% OD		1.5% OD		2% OD		2.5% OD		3% OD	
	Set-1	Set-2	Set-1	Set-2	Set-1	Set-2	Set-1	Set-2	Set-1	Set-2
120	0.06666	0.13332	0.06666	0.06666	0.06666	0.06666	0.13332	0.06666	0.06666	0.13332
240	0.26664	0.3333	0.19998	0.26664	0.26664	0.26664	0.3333	0.13332	0.19998	0.26664
360	0.73326	0.6666	0.86658	0.59994	0.46662	0.46662	0.59994	0.59994	0.19998	0.46662
480	1.3332	1.26654	1.19988	1.39986	0.6666	0.79992	0.9999	0.9999	0.39996	0.93324
600	1.3332	1.39986	1.39986	1.53318	1.53318	1.39986	2.06646	1.86648	1.39986	1.3332
720	1.46652	1.39986	1.46652	1.59984	1.73316	1.79982	2.6664	2.39976	2.39976	2.53308

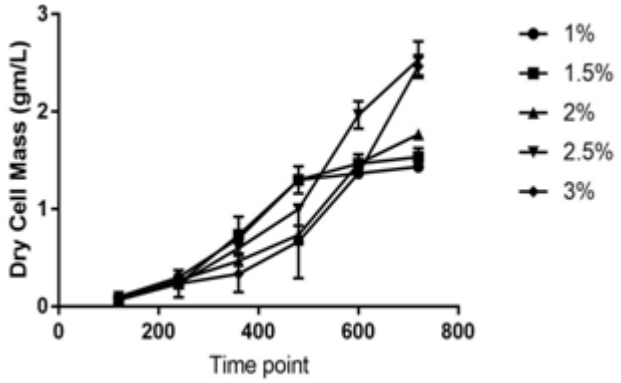

Figure 7: 100ml culture of E. coli BL21 in 500ml shake flask till 12 hour.
Using different glucose concentration (1%, 1.5%, 2%, 2.5% & 3%) DCM & Time

4.4. Table- 04: DNSA assay of shake flask study.

Time (min)	1%	1.5%	2%	2.5%	3%
0	1.0004	1.4728	2.0034	2.492	2.9176
60	0.9956	1.4704	1.997	2.4664	2.9064
120	0.9886	1.469	1.9856	2.4404	2.8632
180	0.9834	1.4618	1.9674	2.3428	2.7256
240	0.9776	1.4566	1.932	2.236	2.4432
300	0.9482	1.4396	1.8968	2.1732	2.2744
360	0.8616	1.4158	1.7966	2.0156	2.0808
420	0.821	1.308	1.4808	1.9064	1.9176
480	0.8252	1.3018	1.318	1.6916	1.7724
540	0.7075	1.0631	1.1872	1.3564	1.6
600	0.0166	0.0184375	0.0217	0.02955	0.0444125
660	0.0120125	0.01305	0.018375	0.02025	0.0413625
720	0.0067375	0.0107	0.0157	0.0141375	0.0373375

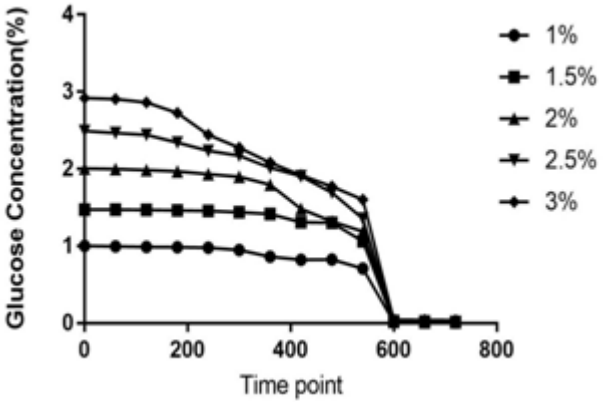

Figure 8: Glucose concentration vs. time study by DNSA assay from shake flask culture media.

4.5. Table- 05 Growth curve study (OD vs. Time) using different carbon concentration contain media in Bioreactor

Time (min)	1%	1.5%	2%	2.5%	3%
0	0.105	0.101	0.102	0.108	0.107
60	0.715	0.487	0.464	0.257	0.2
120	2.83	1.83	0.983	0.684	0.362
180	7.91	5.99	2.42	1.12	0.915
240	12.5	9.05	5.17	2.85	1.94
300	18.7	13.7	9.74	6.12	3.29
360	25.1	18.3	17.2	9.18	4.95
420	26.2	24.2	25.3	19.2	11.2
480	26.8	28.1	31.5	27.9	20.3
540	27.1	28.9	32.9	35.6	30.2
600	27.5	29.4	33.8	39.2	38.7
660	28.1	30.1	34.4	40.1	40.1
720	28.6	30.6	34.9	40.9	41.7

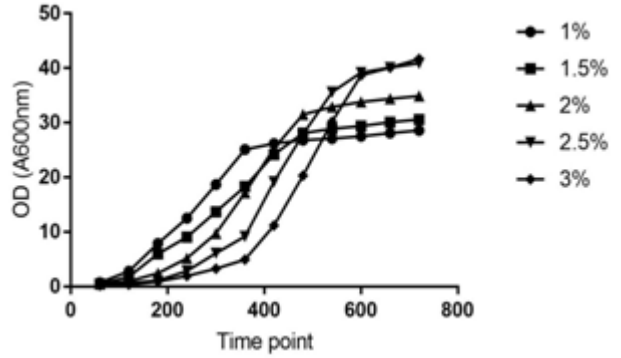

Figure 9: 2.5L culture of E. coli BL21 in 7.5Lbioreactor till 12 hour.
Using different glucose concentration (1%, 1.5%, 2%, 2.5% & 3%) OD & Time

4.6. Table- 06 Growth curve study (WCM vs. Time) using different carbon concentration contains media in Bioreactor.

Time (min)	1%	1.5%	2%	2.5%	3%
0	2.32	2.02	2.42	2.26	2.38
60	4.82	3.04	2.84	2.68	3.64
120	6.56	4.74	3.78	4.62	4.3
180	8.64	6.04	5.78	8.32	5.02
240	11.62	8.98	7.88	9.42	6.26
300	16.52	12.04	9.76	12.06	9.34
360	22.46	14.74	14.04	14.74	12.08
420	24.04	17.18	19.94	17.98	14.64
480	24.62	25.14	29.9	24.1	18.58
540	24.76	26.3	30.56	29.9	24.62
600	25.02	26.64	30.64	36.78	30.2
660	25.18	26.82	30.78	39.94	37.38
720	25.26	26.92	31.04	41.66	41.9

4.6. Table- 06 During bacterial **growth pH study in shake flask**

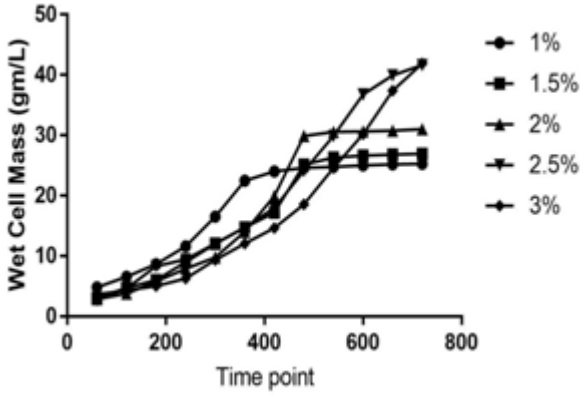

Figure 10: 2.5L culture of E. coli BL21 in 7.5Lbioreactor till 12 hour.
Using different glucose concentration (1%, 1.5%, 2%, 2.5% & 3%) WCM & Time

4.7. Table- 07 Growth curve study (DCM vs. Time) using different carbon concentration contains media in Bioreactor.

Time (min)	1%	1.5%	2%	2.5%	3%
0	0.48	0.42	0.66	0.66	0.52
60	0.98	0.98	1.22	0.9	0.9
120	1.72	1.6	1.86	1.18	1.42
180	2.24	1.88	2.3	2.06	2.04
240	2.78	2.74	2.68	2.62	2.52
300	3.62	3.9	3.46	3.58	3.06
360	5.16	4.72	3.98	4.24	3.86

420	5.24	6.04	5.14	5.06	4.16
480	5.34	6.72	7.22	6.54	5.18
540	5.4	6.82	7.56	7.36	5.9
600	5.48	6.94	7.66	9.18	7.02
660	5.54	7.06	7.78	9.64	9.1
720	5.58	7.16	7.86	9.78	9.86

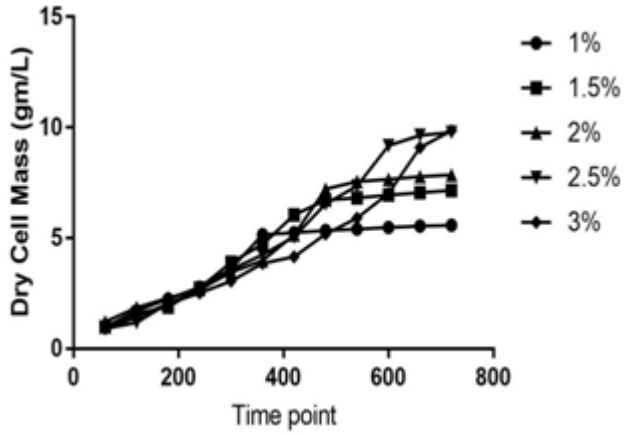

Figure 11: 2.5L culture of E. coli BL21 in 7.5Lbioreactor till 12 hour
Using different glucose concentration (1%, 1.5%, 2%, 2.5% & 3%) DCM & Time

4.8. Table- 08: DNSA assay of shake flask study.

Time (min)	1%	1.5%	2%	2.5%	3%
0	0.9556	1.5172	1.9722	2.5588	2.9656
60	0.8752	1.484	1.9052	2.4272	2.8352
120	0.7064	1.2706	1.849	2.3048	2.76
180	0.4682	0.9988	1.6302	2.1	2.6616
240	0.3296	0.5374	1.2366	1.8468	2.4232
300	0.1465	0.2174	0.9228	1.3326	1.8944
360	0.0773875	0.0709	0.405	1.126	1.5412
420	0.0543125	0.0615375	0.1068	0.7166	0.8694
480	0.0194625	0.0353	0.0839	0.4272	0.6159
540	0.015675	0.01635	0.0668875	0.1994	0.3442
600	0.0091625	0.010925	0.028325	0.01425	0.06885
660	0.005775	0.0064125	0.0095125	0.008975	0.05395
720	0.0045625	0.004525	0.0054375	0.0053375	0.0295375

Bioreactor monitoring by BioCommand software

BioCommand is a Windows® based, multi- bioprocess supervision program. It able to expanded programming enhanced graphing and reporting capabilities; and recipe-based process control.

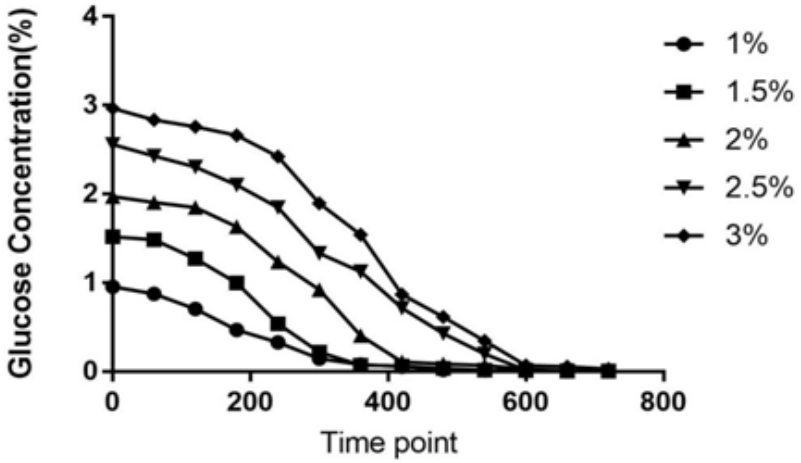

Figure 12: Glucose concentration vs time study by DNSA assay from Bioreactor culture media

Using BioCommand software we can monitor the present and past status and accordingly if some changes are needed then it control through control cabinet. Here we can monitor every parameter which has a great effect in bacterial growth, like agitation, dissolved oxygen (DO) level, pH level, temperature etc. And the bioreactor's control cabinet send the data to the BioCommand software, it continuously plot the graph of each parameter in respect to time.

Batch: 080518_BI21_2L - Batch Summary

Loop Name	SP	PV	Output	Mode	Totalizer	Deadband	Proportional	Integral	Units	Qua
Agit_0M	390.0	390.1321	31.9373	DO					RPM	Goo
DO_0M	0.0	100.7277	-100.0	Auto					%DO	Goo
pH_0M	7.0	7.9159	-100.0	Auto					pH	Goo
Temp_0M	37.0	36.9779	7.8919	Auto					DegC	Goo
Pump1_0M	0.0	0.0	0.0	Off					%	Goo
Pump2_0M	0.0	0.0	0.0	Off					%	Goo
Pump3_0M	0.0	0.0	0.0	Off					%	Goo

Figure 13: Batch summary monitored by BioCommand software

Initially when the batch was started the DO level decreasing very slowly, it means the bacteria was in lag phage and after 1-2 hour when it entered into the log phage bacteria will start dividing rapidly, so DO also decreasing rapidly. For maintain DO level into the vessel, control cabinet increase agitation speed when ever Do level was decreasing. And subsequently when bacteria grow the pH was decrease because when bacteria grow due to TCA cycle they relies pyruvate and acetyl-CoA in the media or environment. So that during bacterial growth culture media pH was dropped.

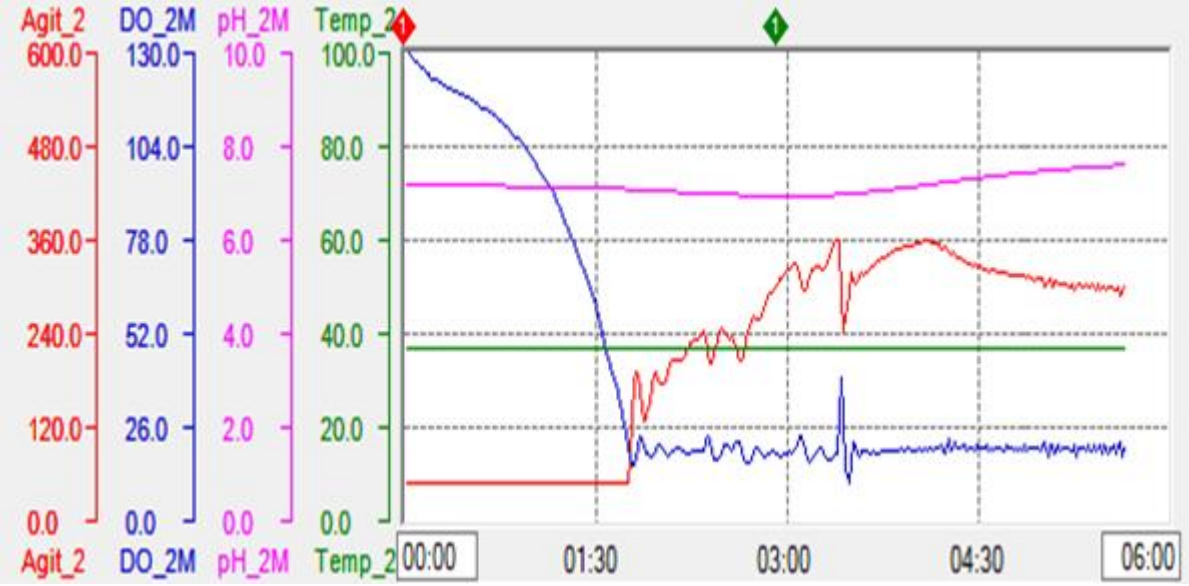

Figure 14: Batch summary of all loops assigned together into the graphical trend screen

5. Discussion

E. coli strain BL21 had a prolonged lag-phase during aerobic growth on glucose as the carbon source in chemical defined medium, though it grew well. It has been reported (Phue and Shiloach 2004; Phue et al. 2005) that there was a difference in gene regulation between the BL21 and JM109 strains for some of the genes of the TCA cycle and glyoxylate shunt (fumA, sdhABD, and acnA). In particular, fumA, coding for fumarase, was expressed at a significantly lower level in BL21 than in JM109 when cells were grown in low glucose medium. So, for better expression we have to induce it in a stage when the bacterial number was highest and the glucose concentration of the media was lowest. Here I had study the different carbon concentration in the culture media, from where got to know that 2.5% and 3% glucose concentration was shown the maximum biomass production. One thing we have to keep in mind that is cost of the consumable, means using minimum consumable get maximum yield. So, if we get same yield using 2.5% and 3% glucose concentration in culture media, we should carry our future study with 2.5% glucose concentration.

During bacterial growth we observed that there is a significant drop of pH. When bacteria grow due to TCA cycle they relies pyruvate and acetyl-CoA in the media or environment.

It is found that the growth of *E. coli* was more stable, log phage was very fast in bioreactor than shake flask.

6. Conclusion

The experiments are performed using shake flask but Scale up study is very necessary when larger amount of product is needed.Here I have done two sets of shake flask and one set of bioreactor study, where I observed using 2.5% of glucose in bacterial culture media, will provide maximum yield. The controlling machinery is automated in bioreactor so it makes the production process more hassle free than lab scale production as every parameter are needed to be maintained manually in shake flask. The bioreactor is a complete system, it has all the facility which is needed for fermentation and cell culture, like Aeration system for DO control, pH and Foam/level probes for monitoring Do, pH and Foam/level of the culture all time where pH ,foaming and temperature can be adjusted using the system itself to get the highest yield. So when we looking for commercial production bioreactor is one and only option. We can get ten tims more better yield from bioreactor then shake flsak.

7. Future Work

As I had mentioned before that I have complete only one set of bioreactor run now I have to complete two more sets of experiment and after optimized the culture media composition mainly carbon source in shake flask, now my future work is to optimize the culture media carbon concentration study in bioreactor.

Acknowledgement
I would also like to express my awe and sincere gratitude to my project guider **Dr. Rahul Modak, Assistant professor KIIT School of Biotechnology, KIIT Deemed to be University, Campus-11**for considering my candidature and providing Bioprocess laboratory facilities and guidance to successfully accomplish my dissertation project work.

I extend my gratitude to **Prof. Mrutyunjay Suar, CEO, KIIT-TBI, KIIT Deemed to be University, Campus-11,**

for giving me the opportunity to pursue my dissertation project in such an esteemed and prestigious institute of India.

I expand my hearty thanks to **Arindam Jana, Application Scientist, KIIT-TBI, KIIT University** for guiding me and analyzing my project data.

The internship opportunity I had with **KIIT-TBI Bioprocess facility** was a great chance for learning. I consider myself as a very lucky individual as I was provided with an opportunity to be a part of it. I am also grateful for a having a chance to meet so many wonderful people and my mentor and teacher, who led me through this dissretation period.

I would like to acknowledge **KIIT-TBI** for providing necessary equipments required for carrying out the experiments during the dissertation period.

I expand my hearty thanks to **Ritu Ghosh, JRF, Krea Food and Beverages Pvt. Ltd. Bhubaneswar, India** for her sincere supervision, guidance and flawless effort in helping me to complete my project in time.

Last but not the least I express my regards and respect to my parents for their support without which the work could not be possible.

References

[1] Parija S.C. (2012). Textbook of Microbiology & Immunology.(2 ed.). India: Elsevier India.
[2] Engelkirk, P. G., Duben-Engelkirk, J. L., & Burton, G. R. W. (2011). Burton's microbiology for the health sciences. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.
[3] Sastry A.S. & Bhat S.K. (2016). Essentials of Medical Microbiology. New Delhi : Jaypee Brothers Medical Publishers.
[4] Trivedi P.C., Pandey S, and Bhadauria S. (2010). Textbook of Microbiology. Pointer Publishers; First edition
[5] JoVE Science Education Database. Environmental Microbiology. Bacterial Growth Curve Analysis and its Environmental Applications. JoVE, Cambridge, MA, (2019).
[6] Gilsdorf J, Gul N, Smith LA (2006) Expression, purification, and characterization of Clostridium botulinum type B light chain. Protein Expr Purif 46(2):256–267
[7] Gyaneshwar P, Paliy O, McAuliffe J, Popham DL, Jordan MI, Kustu S (2005) Sulfur and nitrogen limitation in Escherichia coli K-12: specific homeostatic responses. J Bacteriol 187(3):1074–1090
[8] Lee SK, Keasling JD (2016) Propionate-regulated high-yield protein production in Escherichia coli. Biotechnol Bioeng 93(5):912–918.
[9] Paliy O, Bloor D, Brockwell D, Gilbert P, Barber J (2003) Improved methods of cultivation and production of deuteriated proteins from E. coli strains grown on fully deuteriated minimal medium. J Appl Microbiol 94(4):580–586.
[10] Phue JN, Noronha SB, Hattacharyya R, Wolfe AJ, Shiloach J (2020) Glucose metabolism at high density growth of E. coli B and E. coli K: differences in metabolic pathways are responsible for efficient glucose utilization in E. coli B as determined by microarrays and Northern blot analyses. Biotechnol Bioeng 90(7):805–820

Appendix

- **Media preparation (LB broth)**

Components	Shake flask(600ml)	Bioreactor(2500ml)
KH_2PO_4	7.98g	33.25g
$(NH_4)_2HPO_4$	2.4g	10g
Citric acid	1.02g	4.25g
EDTA	5.04g	21mg
Trace elements	0.6ml	2.5ml
Thiamine HCL	0.6ml	2.5ml
Distilled water	400ml	2000ml

- **NaOH solution**

Reagent/ chemical	0.1N (1L)	1N (1L)	2N (1L)	2N (100ml)	2N(50ml)
NaoH	4gm	40gm	80gm	8gm	4gm
Diistiled water	Volume make up upto 1L	Volume make up upto 1L	Volume make up upto 1L	Volume make up upto 100ml	Volume make up upto 50ml

- **PBS (Phosphate Buffer Saline)**

Reagent/chemical	Molarity(Conc)	(10X) 1L	(10X) 500ml	(1X) 500ml
NaCl	1370mM	80gm	40gm	4gm
KCl	27mM	2gm	1gm	0.1gm
Na_2HPO_4	100mM	14.4gm	7.2gm	0.72gm
KH_2PO_4	18mM	2.4gm	1.2gm	0.12gm