Optimization of Bioreactor and Up-scaling Biomass production of E. coli BL21 from shake flask to benchtop bioreactor.

Dissertation submitted in partial fulfilment for the degree of

Master of Science in Biotechnology

Submitted By

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DECLARATION

I hereby declare that the dissertation entitled ***"Optimization of bioreactor and Up-scaling Biomass production of E. coli BL21 from shake flask to benchtop bioreactor."*** submitted by me, for the degree of Master of Science to KIIT University is a record of bonafide work carried by me under the guidance of, **Dr. Rahul Modak** *;* **,**Assistant Professor *KIIT School of Biotechnology, KIIT University, Campus-11 &* Consultant-Lab Technology *KIIT-TBI, KIIT University, Campus-11, Bhubaneswar-24.*

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Abstract

Fermentation systems are used to provide an optimal growth environment for many different types of cell cultures. The ability afforded by bioreactors to carefully control temperature, pH, and dissolved oxygen concentrations, in particular, makes them essential to efficient large-scale growth and expression of fermentation products. Major challenge in scale-up from shake flasks to reactor is primarily due lack of knowledge that leads to suboptimal growth conditions. It is essential to optimize conditions for biomass production before starting production of biomolecules. *E. coli* is most commonly used bacteria for biomolecule production. It very fast growing bacterium that makes it ideal candidate for initial optimization study. For many lab-based experiments shake flask bacterial growth is sufficient but it is not suitable for large scale production. For any scale-up processes, bioreactor is the preferred option, which requires optimization study for up scaling from shake flask to benchtop bioreactor. In the present study, I have shown that small volume of culture like 100ml media in 500ml flask gave good yield of biomass but when increasing the volume of culture like 500ml culture in 1L flask biomass yield had decreased. So when we think about 2.5L, 5L and above culture, bioreactor will be a better option for optimum biomass production. In bioreactor system, the product yield is good and we can quickly produce the maximum product. Bioreactor has a various advantages like rapid production, live monitoring and regulation of critical growth as well as production parameters. Live monitoring of pH, dissolved oxygen (DO) level, temperature, cell density etc. helps in yield optimization. Taken together this study helps in identification of critical parameters for scale up of bacterial biomass production from shake flask to bench scale bioreactors.

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Abbreviations/Acronyms

Agit- Agitation

CFU- Colony Forming Unit

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

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CHAPTER- 01

INTRODUCTION

1 Introduction

Bioprocessing is a necessary part of several food, chemical and pharmaceutical industries. Bioprocess operations produces microbial, animal and plant cells and elements of cells like enzymes to manufacture new products and destroy harmful wastes.

Use of microorganisms to rework biological materials for production of soured foods has it's origins in antiquity. Since then, bioprocesses are developed for a massive vary of business merchandise, from comparatively low cost materials like industrial alcohol and organic solvents, to costly specialty chemicals like antibiotics, theraputics proteins and vaccines. Industrially helpful proteins and living cells like baker's and brewer's yeast also are business merchandise of bioprocessing.

Basic fermentation technology is associate extension of the easy shake flask technique for growing cultures. Shake flasks 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 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. Once the accessible O_2 is depleted, most cultures fail to thrive. There's no pH management in shake flasks. In many cases, if the culture is not limited by feed stock, it becomes acidic to the point of detriment to the culture and respiration slows dramatically. Most shake flask cultures are run as a batch-culture, which suggests that they're fed one time at the start of the cultures inoculation. Shake flasks are typically subject to media evaporate loss in warmer culture environments, usually 10% of volume per 24 hour of time at 37°C.

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 produce large 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. Careful temperature management is achieved in fermentation vessels by RTD sensor and temperature can be controlled by cooling coil and heating jacket. A device referred to as Resistance Temperature Detector(RTD) inserted into the vessel and feedback management of heating and cooling of this jacket typically it's ends up in temperature management ±0.1 °C round the setpoint. Benchtop fermentors typically offer management of pH via liquid chemical agent addition through a pump. The pH of is usually monitored to maintain optimum atmosphere for cell growth. correct aeration is maintained by the infusion of filtered air or other gaseus element 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 in bioreactor system. DO probe is not commonly used in shake flask culture.

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. The phases of growth typically include: Lag phase; Exponential (log, logarithmic) section; Stationary phase; Death phase.

- **Bacterial growth** refers to a rise within the cells range. once microorganism are inoculated into a liquid medium and therefore the cell population is counted at intervals, it's attainable to plot a typical microorganism growth curve that shows the cells growth over time. It shows four distinct growth phases.
  - Lag phase: Throughout the lag phase; the microorganism cells adapt themselves to the new environment/media as they're inoculated into a new medium. The microorganism cells increase in size however there was no modification in range as they prepare themselves for biological process. They synthesize some new elastic enzymes to utilize new nutrients from the media. The length of the lag part is depends on the characteristics of

the microorganism species and conditions within the media, each the medium from that the organisms are taken . The microorganism cells then enters an energetic part of growth referred to as the power part.

- Log or exponential part: Throughout log phase, the microorganism cell divide quickly. Every cell mass will increase exponentially followed by multiplying replica. The amount of microorganism cells are increased, throughout every generation time microorganism cells are double. the expansion curve is linear throughout log part and therefore the cells are metabolically most active. Because the quantity of nutrients decrease, the atmosphere of the population changes and therefore the rate of replication decreases. The microorganism population then enters stationary part.
- Stationary part: Throughout this phase, because the nutrients get terribly less. The cells are secreting waste and secondary metabolic product, therefore the rate decreases to some extent wherever rate and death rate is equals to the death rate, therefore viable cells range remains same. If the culture continues to be continuing in apparatus the population then enters the death part.
- Decline or death part: throughout the death phase, population viable cell decreases as a result of the number of waste product will increase. The death of cells within the population abundant above the formation of latest cells.

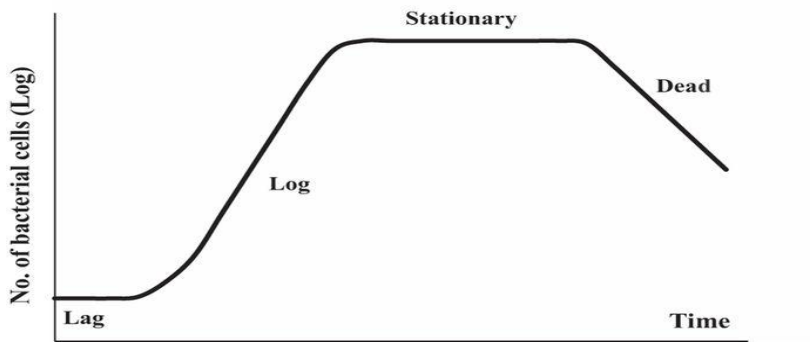

Fig 01- Bacterial growth curve

❖ **Overview of Bioreactor (BioFol 115)**

- 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 are obsessed on the control/management station.

- Vessels

There are 2 types of vessels, heat-blanketed and water-jacketed. every sort of vessel is accessible in four sizes: 1.3 liters, 3.0 liters, 7.5 liters and 14.0 liters, however we've got 7.5 & 14.0 liters vessels. Ports present within the headplate area unit provided for the subsequent purposes: inoculation; base and acid addition; a foam probe; a thermowell for a resistance temperature detector (RTD); a sparger; a harvest tube; a sampling tube; an exhaust condenser; and dissolved oxygen (DO) and pH electrodes. The drive bearing housing is additionally set on the headplate.

- Agitation system

On the headplate 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 setpoints so as to keep up the set share of DO.

- Temperature management

The culture temperature setpoint 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.

- Aeration

Up to four gases- 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 touchscreen. The gas combine will either be controlled manually by adjusting the flow of gases through their Rotameters or mechanically.

- 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.

- 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.

- 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.

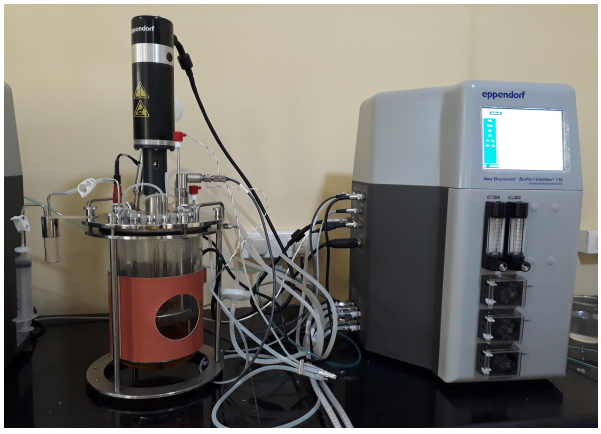

Fig 02- Bioreactor 7.5L

- 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.

1.1 Background and Context

A couple of the things to consider are the volume of cells planned to grow in bioreactor and how to scale up the culture in a bioreactor. "Most important when transferring cultures from dish of shake flask to bioreactor is a good understanding of the process," says Karl Rix, vice president of sales and support bioprocess at Eppendrof's Bioprocess Center.

So, the scale up is the most important part of any bioprocess facility and I am here KIIT-TBI Bioprocess lab trying to study about scale up *Escherichia coli* biomass from shake flask to bioreactor and optimization of bioreactor.

Escherichia coli is widely cultivated under aerobic conditions in laboratory and industrial processes. Scientists first chose to work with *E. coli* because it was easy and fast to grow in the laboratory.

There are several features of *E. coli* that make it easy to culture:

• **It likes warm** – however not too heat. Because *E. coli* is a gut bacteria, it grows best at blood heat (37ºC). this is often a simple temperature for scientists to figure with within the laboratory.

• **It isn't fussy regarding nutrition**- *E. coli* will acquire energy from a good form of sources. In its natural surroundings (the gut), it consumes digestible foodstuffs. In a laboratory context, *E. coli* are fed on various nutritional media that have smple compositions and available at cheep price.

• **It will grow with or without oxygen**- In the gut, *E. coli* grows anaerobically (in the absence of oxygen). However, in contrast to some anaerobic bacteria *E. coli* conjointly grows well in aerobic environments, like a culture flask during a laboratory.

• **It grows quick**- Under ideal conditions, individual *E. coli* cells will double at every twenty minutes. At this rate, it might be attainable to supply 1,000,000 *E. coli*

cells from one parent cell among regarding seven hours. Quick growth implies that experiments involving *E. coli* are often done quickly, handily and cheaply.

For a start up company it is best to start with *E. coli* strain for optimizing bioreactor properly. The fully automated bioreacter system we have, Eppendrof's Biofol 115 bioreactor 7.5&14L, it's have ability to keep track of what is going on inside the bioreactor via sensors, which then "talk" to computer software (Biocommand), makes for sophisticated monitoring system. This means that software can tell all the parameter inside the bioreactor are well or not, if any problem happened inside the reactor we can change the parameter by manually also.

1.2 Scope and Objectives

The scope of this study was to get maximum number of cell in culture medium. It means scale up the productivity from lab scale to commercial scale, if any one innovate some novel protein, for thinking about mankind, it should be produced in a large quantity, so that point of view we have to scale it up the culture from shake flask to bioreactor. We can make maximum number of cell, so that we can get maximum protein. That's why bioreactor is the heart of bioprocess facility.

1.3 Achievements

Now I know how to run a bioreactor, scale up from shake flask to reactor. The hole process of biomass production, vessel assembly, vessel sterilization, cascade control, monitoring by Biocommand software all this thing I have done properly.

1.4 Overview of Dissertation

I have done scale up study from dish culture to shake flask and then shake flask to bioreactor. To begin with vessel setup, a conventional fermenter has the following components that need to be installed.

pH probe - for measurement of pH in the live culture. Calibrate probe before installation and sterilize with the vessel. Install probe in the headplate.

DO probe - for measurement of dissolved oxygen content inside the vessel during fermentation. Install in the headplate.

Harvest pipe for sampling the culture. Install this adjustable height component in the headplate.

Gas sparger - resides at the bottom of the vessel and provides gas infusion to the culture. Hook sparger up to the gas source on the fermentor and mount in the headplate.

Impeller shaft and impeller - the impeller stirs the culture and is critical for maintaining culture uniformity in the vessel.

Exhaust gas condenser - for curbing evaporative loss in the vessel by cooling the exhaust gas path.

Clean the vessel and headplate with soap and water. A soft brush is recommended for scrubbing the vessel and headplate. The media is add to the clean vessel. Mount the vessel headplate, ensuring that the o-ring seal is properly seated. Install the antifoam probe into its 10 mm port. Install the harvest pipe into its 10 mm port. Put a piece of tubing on the top of it and clamp it off. Install tubing and a 0.20 μm filter on the sparge inlet.

Calibrate the pH probe:

- Press the CALIBRATION button to display the CALIBRATION screen.
- Rinse the pH electrode with distilled water, then immerse it into pH 7.00 buffer solution and allow a few minutes for the system to equilibrate. SET ZERO edit box enter 7.00 and press SET ZERO button.
- Rinse the pH electrode with distilled water.
- Immerse pH electrode into a second pH buffer solution which is several pH units above or below pH 7.00 (e.g., pH 4.00) and allow a few minutes for the system to equilibrate.
- Similar to step 7 above, touch the SET SPAN edit box. Use the touchpad that opens to enter the value of the second buffer solution (e.g., 4.00), then press the OK button.
- When the CURRENT VALUE reading stabilizes, press the SET SPAN button.

- To ensure accuracy, repeat Steps 4-11 a few times, using the same two buffer solutions.

Install the DO probe in the headplate and it will be calibrated after sterilization. Place tubing on all unused ports then clamp tubing off. Install a 0.2 μm filter on the exhaust gas condenser. This filter ensures that the vessel does not pressurize in the autoclave. Check that all tubes that go below the level of the media are clamped off to prevent media from coming out. Put the vessel in the autoclave for 25 - 30 min at 121°C, liquid cycle. Caution: When it comes out it will be very hot. Hook up the pH probe cable. Attach the DO probe cable. Hook the sparger up to the gas addition rotameter. Put the temperature sensor (RTD) in the thermowell on the headplate. Make sure that the fermentor is on. Attach all water tubing after that on the water cooling system. Scroll to the temperature parameter and set the temperature to 37 °C. Press the 'Enter' button to start the temperature control. The vessel will heat up in 15 min or less. Turn on the air supply to the fermentor. Ensure that the DO probe is polarized properly to display proper values, turn on sparger and vigorously sparge air into the vessel and on agitation at 50RPM, after 15-20 min enter 100 at SET SPAN box and press SET SPAN buttom. Start the control software package Biocommand on the PC. Draw the inoculum into a syringe and add it into the sterilized port. Close the port. Mark the time of inoculation in a log book. Open the clamp covering the harvest port and use a syringe to draw a sample. This first sample is usually discarded because it has been sitting in the pipe. Determine the cell density and pH and record the values. With microbial cultures, it is useful to log values every hour. Interval is culture dependent.

CHAPTER- 02

AIMS AND OBJECTIVE

2. Aims and Objectives

The aim with this project is to implement a scale up study of biomass production from shake flask to bench-top bioreactor, and optimization of bioreactor.

- *E. Coli* BL21 bacterial growth at 100ml culture in 500ml shake flask (lab scale)
- 500ml culture in 1L shake flask (lab scale)
- 2.5L culture in 7.5L benchtop bioreactor (large scale)
- 5L culture in 14L benchtop bioreactor (large scale)
- To find the bacterial growth kinetics at every stage of up-scaling.
- Optimize benchtop bioreactor.

CHAPTER- 03

MATERIALS AND METHODS

3. Materials and Methods

3.1. Microorganism

E. coli is a good host for recombinant protein expression though 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 its 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 loopful 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. 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.

3.4. 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 and after that 500ml culture in 1L conical flask.

3.5. Banch-scale bioreactor optimization

A stirred tank bioreactor (Bioflo 115, New Brunswick; Eppendorf) of 7.5L & 14L 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.

I have done three time of 2.5L culture in 7.5L bioreactor and 5L culture in 14L bioreactor.

3.6. Sampling procedures

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

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.

3.7. Calculation

CFU stands for Colony Forming Units, for measuring the cell number of every time-point sample's CFU calculation is ideal. Count the number of individual colonies.

cfu/ml = (no. of colonies x dilution factor) / volume of culture plate

CHAPTER-04

RESULTS AND DISCUSSION

4. Result

The first task was to try to find a suitable time-point of *E. coli* BL21(codon+) bacterial growth at which point the cells number will be highest in the culture medium. So, at first I am started with the 100ml medium in the 500ml flask, then 500ml medium in the 1L flask. After doing shake flask culture three-time repeated, then I go ahead with bioreactors. I have done 2.5L culture in the 7.5L bioreactor, and then 5L culture in the 14L bioreactor.

4.1.Table-01

100ml culture in the 500ml shake flask (OD at 600nm and CFU till 12 hour.)

Time (min)	OD(600) (19/02/18)	OD(600) (22/02/18)	OD(600) (23/02/18)	CFU (cells/ml) (19/02/18)	CFU (cells/ml) (22/02/18)	CFU (cells/ml) (23/02/18)
0	0.108	0.115	0.045	3.40×10^{7}	3.93×10^{7}	3.13×10^{7}
30	0.227	0.239	0.078	3.32×10^{7}	4.00×10^{7}	4.00×10^{7}
60	0.224	0.196	0.126	4.66×10^{7}	6.66×10^{7}	5.33×10^{7}
90	0.204	0.175	0.235	1.40×10^{8}	1.26×10^{8}	5.33×10^{7}
120	0.426	0.286	0.32	2.00×10^{8}	1.60×10^{8}	1.13×10^{8}
180	0.97	0.765	0.748	2.00×10^{8}	8.00×10^{8}	8.00×10^{8}
240	1.32	1.065	1.011	2.00×10^{9}	2.00×10^{9}	1.00×10^{9}
300	1.402	1.207	1.298	2.00×10^{9}	3.32×10^{9}	2.46×10^{9}
360	1.598	1.254	1.396	2.66×10^{9}	3.32×10^{9}	2.53×10^{9}
420	1.5	1.364	1.484	6.00×10^{9}	3.27×10^{9}	2.46×10^{9}
480	1.568	1.442	1.588	6.00×10^{9}	6.00×10^{9}	2.66×10^{9}
540	1.597	1.506	1.638	2.00×10^{10}	6.00×10^{9}	3.32×10^{9}
600	1.612	1.507	1.658	3.32×10^{10}	2.66×10^{10}	2.00×10^{10}
660	1.621	1.503	1.677	2.66×10^{9}	4.00×10^{9}	3.32×10^{9}
720	1.64	1.506	1.739	4.66×10^{9}	3.40×10^{9}	2.80×10^{9}

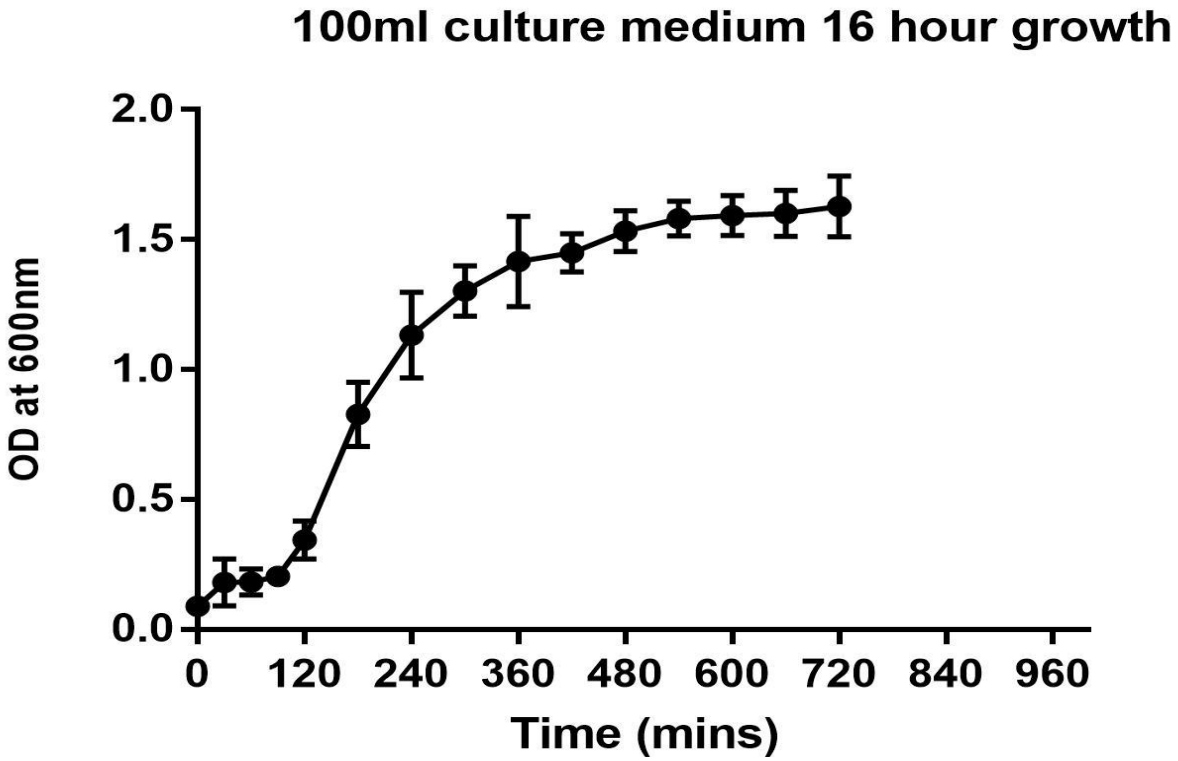

Fig 03- 100ml culture of E. coli BL21 in 500ml shake flask till 12 hour. OD & Time graph

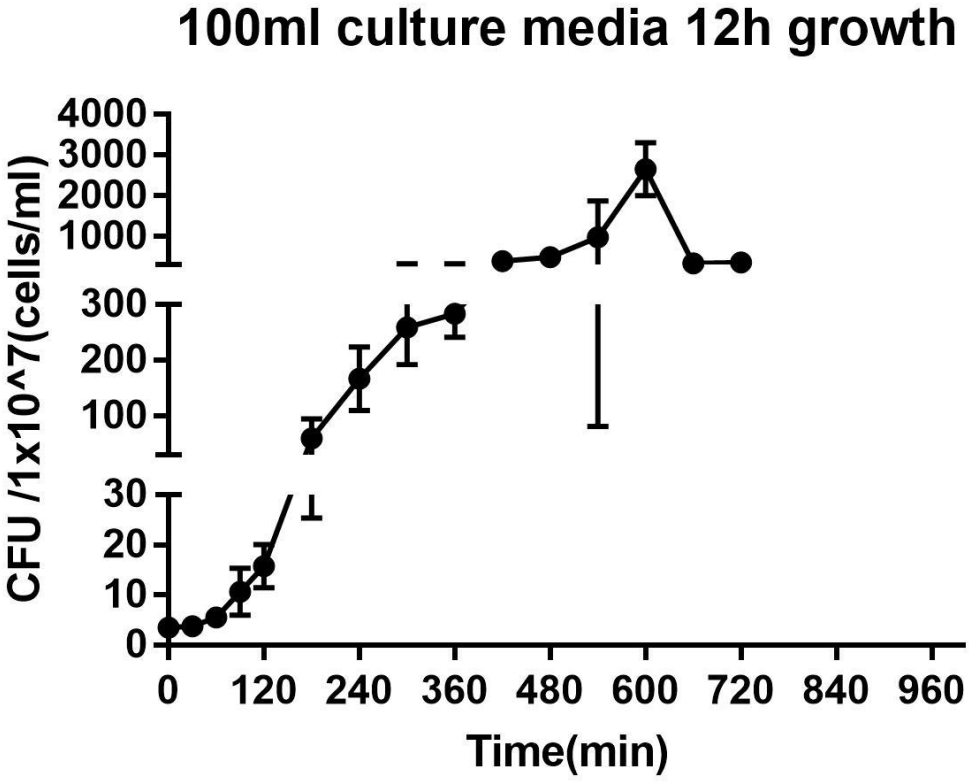

Fig 04- 100ml culture of *E. coli* BL21 in 500ml shake flask till 12 hour. CFU & Time graph

4.2.Table-02

500ml culture in the 1L shake flask (OD at 600nm and CFU till 16 hour.)

Time (min)	OD(600nm) (01/03/18)	OD(600nm) (08/03/18)	OD(600nm) (15/03/18)	CFU (cells/ml) (01/03/18)	CFU (cells/ml) (08/03/18)	CFU (cells/ml) (15/03/18)
0	0.15	0.05	0.068	1.80×10^{7}	2.20×10^{7}	2.06×10^{7}
15	0.128	0.048	0.057	1.40×10^{7}	2.53×10^{7}	2.13×10^{7}
30	0.161	0.11	0.065	1.40×10^{7}	2.60×10^{7}	2.27×10^{7}
60	0.168	0.115	0.076	2.66×10^{7}	2.66×10^{7}	2.40×10^{7}
90	0.209	0.129	0.145	3.80×10^{7}	2.66×10^{7}	3.06×10^{7}
120	0.297	0.21	0.25	6.00×10^{7}	7.32×10^{7}	5.32×10^{7}
150	0.356	0.239	0.424	1.80×10^{8}	1.07×10^{8}	1.26×10^{8}
180	0.585	0.561	0.515	2.00×10^{8}	2.07×10^{8}	3.06×10^{8}
240	0.66	0.582	0.682	3.32×10^{8}	4.00×10^{8}	3.32×10^{8}
300	0.808	0.824	0.75	9.32×10^{8}	6.00×10^{8}	6.00×10^{8}
360	0.84	0.914	0.826	1.00×10^{9}	1.60×10^{9}	1.13×10^{9}
420	1.08	0.918	0.858	1.06×10^{9}	1.60×10^{9}	1.13×10^{9}
480	1.12	0.923	0.987	1.33×10^{9}	2.00×10^{9}	1.27×10^{9}
540	1.142	0.985	1.13	1.40×10^{9}	2.66×10^{9}	2.00×10^{9}
600	1.194	0.998	1.15	2.00×10^{9}	1.60×10^{9}	2.66×10^{9}
660	1.303	1.03	1.22	1.86×10^{9}	1.40×10^{9}	1.46×10^{9}
720	1.355	1.059	1.226	1.00×10^{9}	1.27×10^{9}	1.27×10^{9}
780	1.398	1.083	1.22	9.32×10^{8}	1.20×10^{9}	1.20×10^{9}
840	1.446	1.1	1.23	8.00×10^{8}	1.20×10^{9}	1.20×10^{9}
900	1.458	1.12	1.24	7.66×10^{8}	1.13×10^{9}	1.00×10^{9}
960	1.476	1.1	1.26	1.00×10^{8}	9.32×10^{8}	1.00×10^{9}

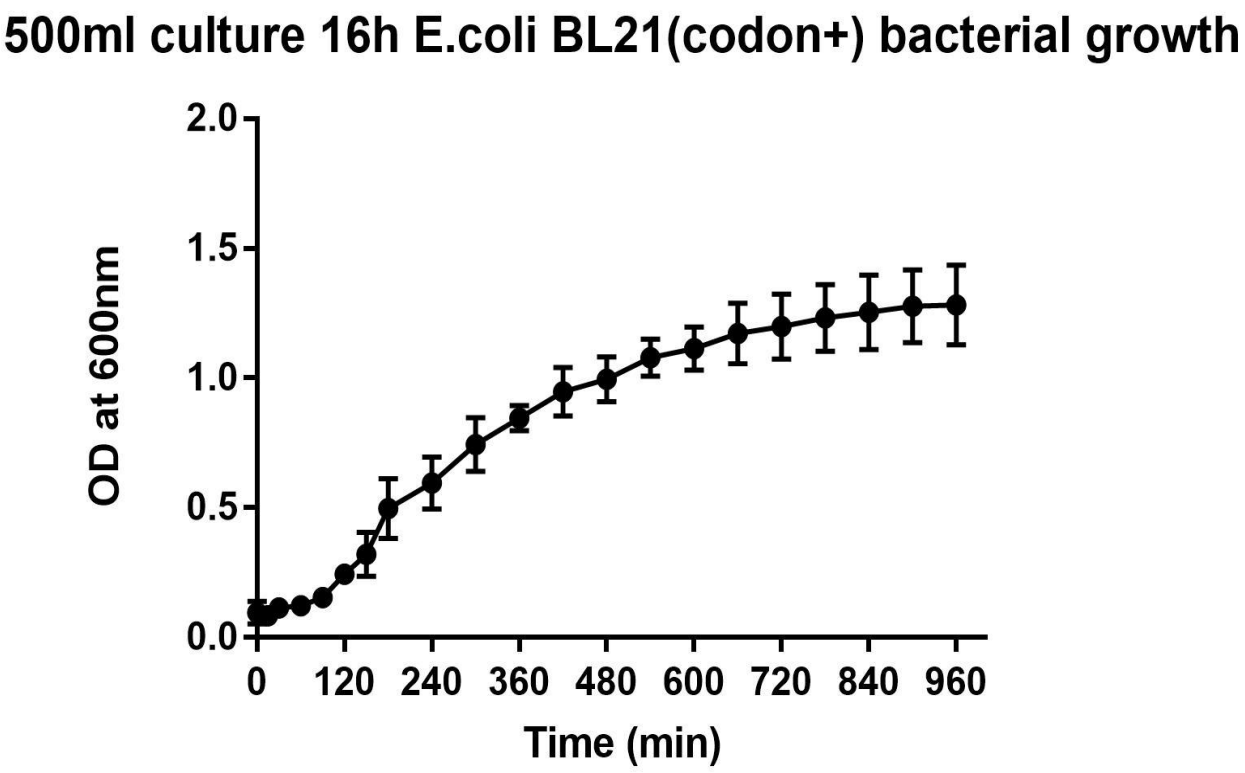

Fig 05- 500ml culture of E. coli BL21 in 500ml shake flask till 16 hour. OD & Time graph

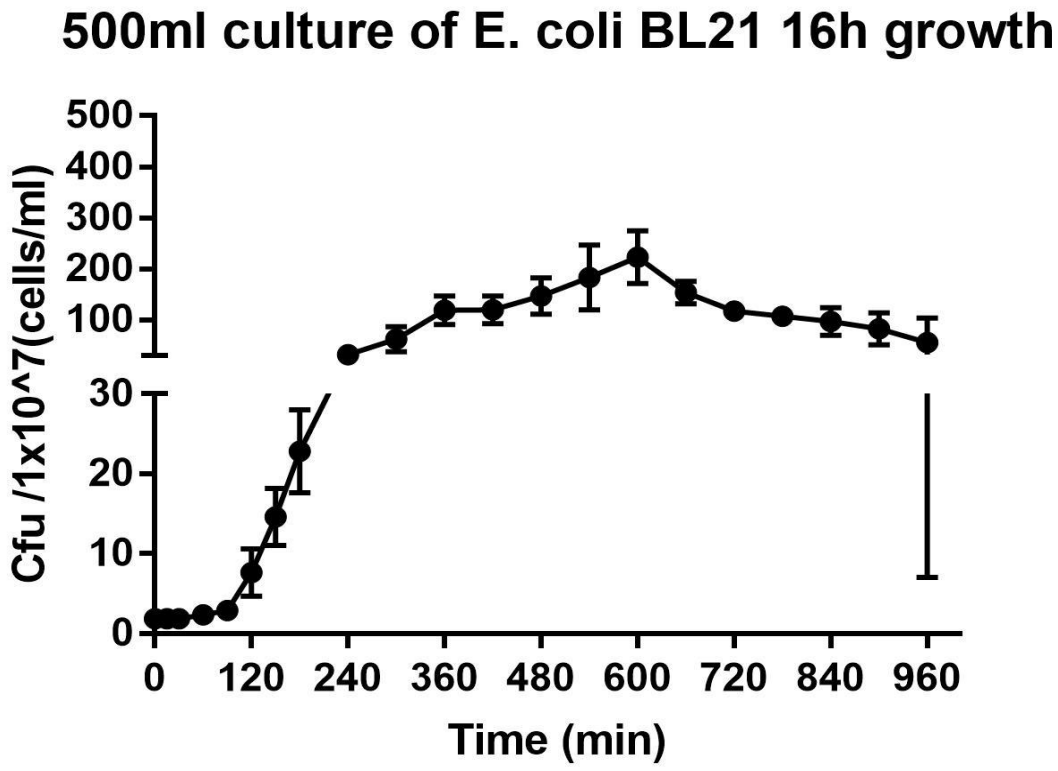

Fig 06- 500ml culture of E. coli BL21 in 500ml shake flask till 16 hour. CFU & Time graph

So after doing shake flask study we can conclude that, in case of 100ml culture cell density was more higher than 500ml culture, because the ratio of culture volume and flask volume was not same in both cases. In case of 100ml culture in 500ml flask the ratio of culture volume and flask volume was 1:5, means there have good amount of spacing for aeration and air mixing into the culture. But in case of 500ml culture in 1L flask the ratio of culture volume and flask volume and flask volume was 1:2, means there are no such spacing for air mixing, so the bacterial cells are not produced at very high amount like 100ml culture.

Comparison between 100ml and 500ml culture

Factors	Time(min)	100ml	500ml
Maximum CFU(cells/ml)	600	2.66×10^{10}	2×10^{9}
OD at 600nm	600	1.612	1.15
Culture and flask volume ratio		1:5	1:2

4.3.Table-03

2.5L culture in the 7.5L benchtop bioreactor (OD at 600nm and CFU till 16 hour.)

Time (min)	OD(600nm) (06/04/18)	OD(600nm) (11/04/18)	OD(600nm) (08/05/18)	CFU (cells/ml) (06/04/18)	CFU (cells/ml) (11/04/18)	CFU (cells/ml) (08/05/18)
0	0.02	0.012	0.015	1.00×10^{7}	2.66×10^{6}	6.00×10^{6}
15	0.022	0.01	0.014	1.07×10^{7}	2.00×10^{6}	6.00×10^{6}
30	0.062	0.015	0.016	1.00×10^{7}	4.66×10^{6}	8.00×10^{6}
60	0.1	0.028	0.045	2.13×10^{7}	6.66×10^{6}	1.13×10^{7}
90	0.152	0.051	0.092	3.00×10^{7}	2.00×10^{7}	2.13×10^{7}
120	0.222	0.096	0.125	1.40×10^{8}	4.66×10^{7}	5.34×10^{7}
150	0.375	0.203	0.289	2.40×10^{8}	1.07×10^{8}	1.13×10^{8}
180	0.572	0.351	0.496	8.00×10^{8}	2.13×10^{8}	2.00×10^{8}
240	1.06	0.76	0.758	1.60×10^{9}	4.66×10^{8}	4.00×10^{8}
300	1.315	1.193	1.133	1.60×10^{9}	1.47×10^{9}	1.00×10^{9}
360	1.399	1.361	1.4	2.07×10^{9}	2.00×10^{9}	2.66×10^{9}
420	1.405	1.452	1.428	4.00×10^{9}	4.66×10^{9}	4.00×10^{9}
480	1.424	1.454	1.431	2.66×10^{9}	4.00×10^{9}	3.34×10^{9}
540	1.439	1.455	1.435	2.66×10^{9}	3.32×10^{9}	3.34×10^{9}
600	1.441	1.453	1.437	2.66×10^{9}	2.66×10^{9}	2.66×10^{9}
660	1.444	1.454	1.442	2.00×10^{9}	2.00×10^{9}	2.66×10^{9}
720	1.443	1.454	1.445	1.93×10^{9}	2.00×10^{9}	2.00×10^{9}
780	1.44	1.453	1.444	1.60×10^{9}	2.00×10^{9}	2.00×10^{9}
840	1.444	1.455	1.448	1.07×10^{9}	1.53×10^{9}	1.80×10^{9}
900	1.445	1.456	1.446	8.66×10^{8}	1.40×10^{9}	1.60×10^{9}
960	1.446	1.455	1.449	8.66×10^{8}	1.13×10^{9}	1.60×10^{9}

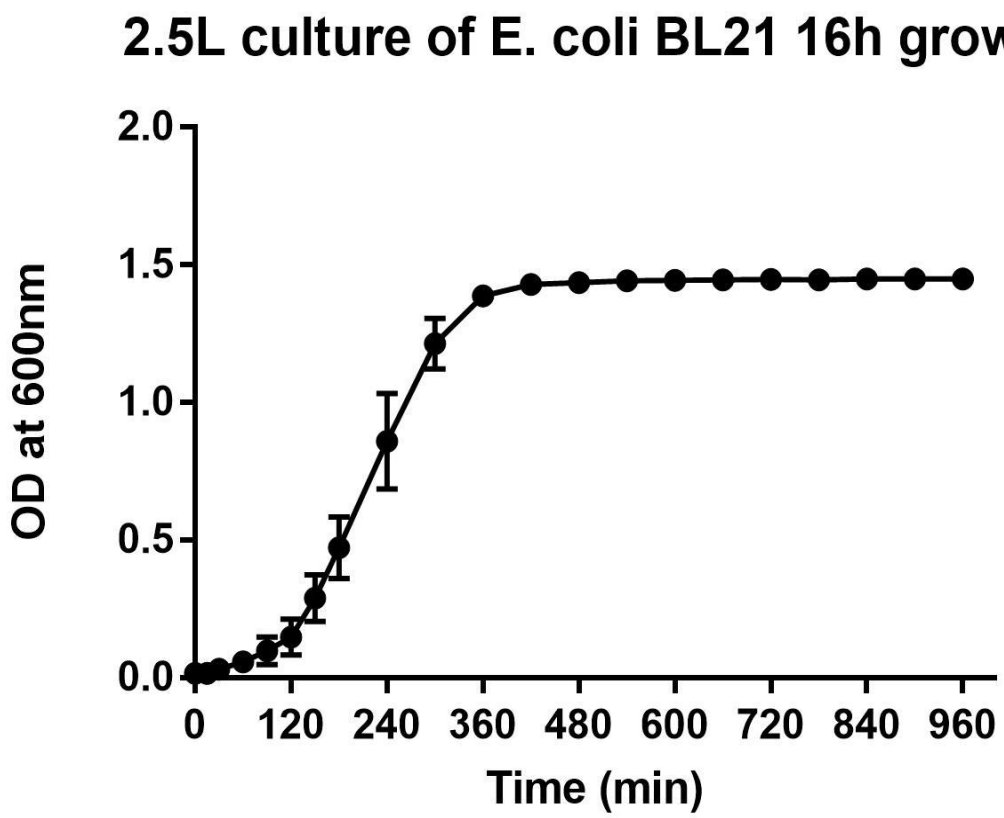

Fig 07- 2.5L culture of E. coli BL21 in 7.5L benchtop bioreactor till 16 hour. OD & Time graph

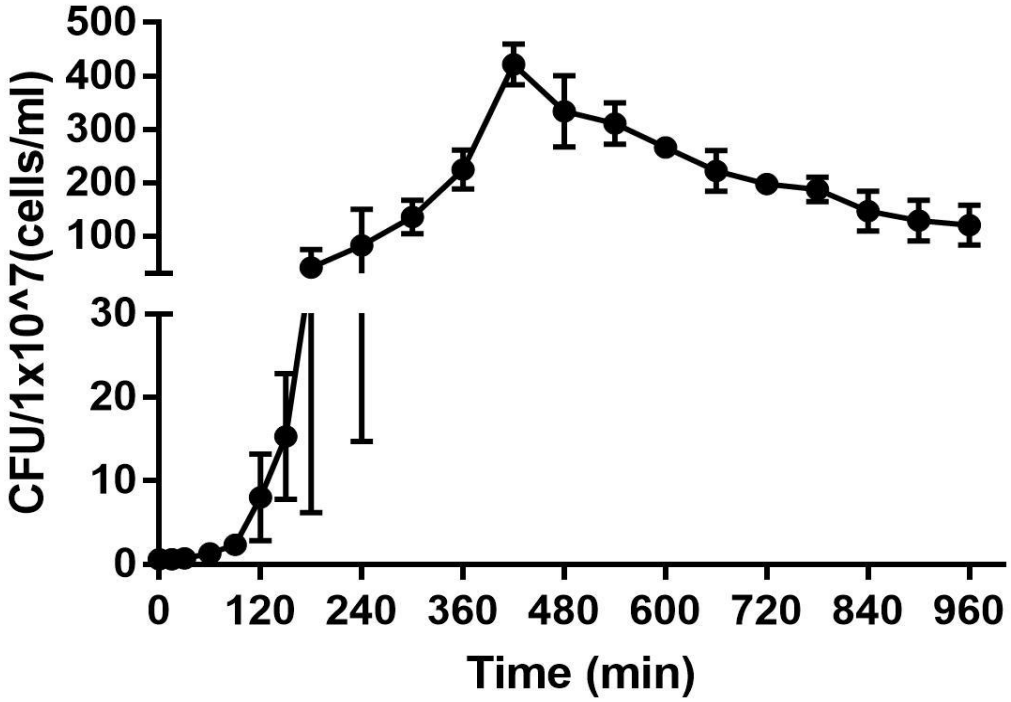

Fig 08- 2.5L culture of E. coli BL21 in 7.5L benchtop bioreactor till 16 hour. CFU & Time graph

4.4.Table-04

5L culture in the 14L benchtop bioreactor (OD at 600nm and CFU till 16 hour.)

Time (min)	OD(600nm) (18/04/18)	OD(600nm) (25/04/18)	OD(600nm) (01/05/18)	CFU (cells/ml) (18/04/18)	CFU (cells/ml) (25/04/18)	CFU (cells/ml) (01/05/18)
0	0.018	0.01	0.016	7.34×10^{6}	1.00×10^{6}	5.34×10^{6}
15	0.021	0.011	0.018	6.66×10^{6}	1.07×10^{6}	8.00×10^{6}
30	0.023	0.008	0.03	1.00×10^{7}	2.66×10^{6}	1.27×10^{7}
60	0.061	0.015	0.053	1.80×10^{7}	4.00×10^{6}	1.73×10^{7}
90	0.073	0.023	0.084	2.00×10^{7}	6.66×10^{6}	4.00×10^{7}
120	0.135	0.071	0.162	5.34×10^{7}	1.00×10^{7}	1.13×10^{8}
150	0.266	0.171	0.292	1.07×10^{8}	2.00×10^{7}	1.73×10^{8}
180	0.429	0.347	0.589	2.66×10^{8}	4.66×10^{7}	3.34×10^{8}
240	0.811	0.715	0.845	6.66×10^{8}	2.66×10^{8}	6.66×10^{8}
300	1.237	1.115	1.244	1.34×10^{9}	2.00×10^{9}	1.60×10^{9}
360	1.39	1.412	1.389	2.00×10^{9}	3.34×10^{9}	2.66×10^{9}
420	1.451	1.467	1.427	4.00×10^{9}	2.66×10^{9}	3.34×10^{9}
480	1.455	1.476	1.437	2.66×10^{9}	2.00×10^{9}	2.00×10^{9}
540	1.46	1.479	1.449	2.66×10^{9}	2.00×10^{9}	1.53×10^{9}
600	1.47	1.488	1.465	2.66×10^{9}	1.93×10^{9}	1.33×10^{9}
660	1.469	1.494	1.482	2.00×10^{9}	1.80×10^{9}	1.27×10^{9}
720	1.468	1.501	1.497	1.87×10^{9}	1.80×10^{9}	1.07×10^{9}
780	1.471	1.508	1.499	1.66×10^{9}	1.60×10^{9}	8.66×10^{8}
840	1.47	1.509	1.499	1.20×10^{9}	1.27×10^{9}	9.43×10^{8}
900	1.472	1.511	1.501	1.07×10^{9}	1.47×10^{9}	7.34×10^{8}
960	1.471	1.512	1.5	1.07×10^{9}	1.27×10^{9}	8.00×10^{8}

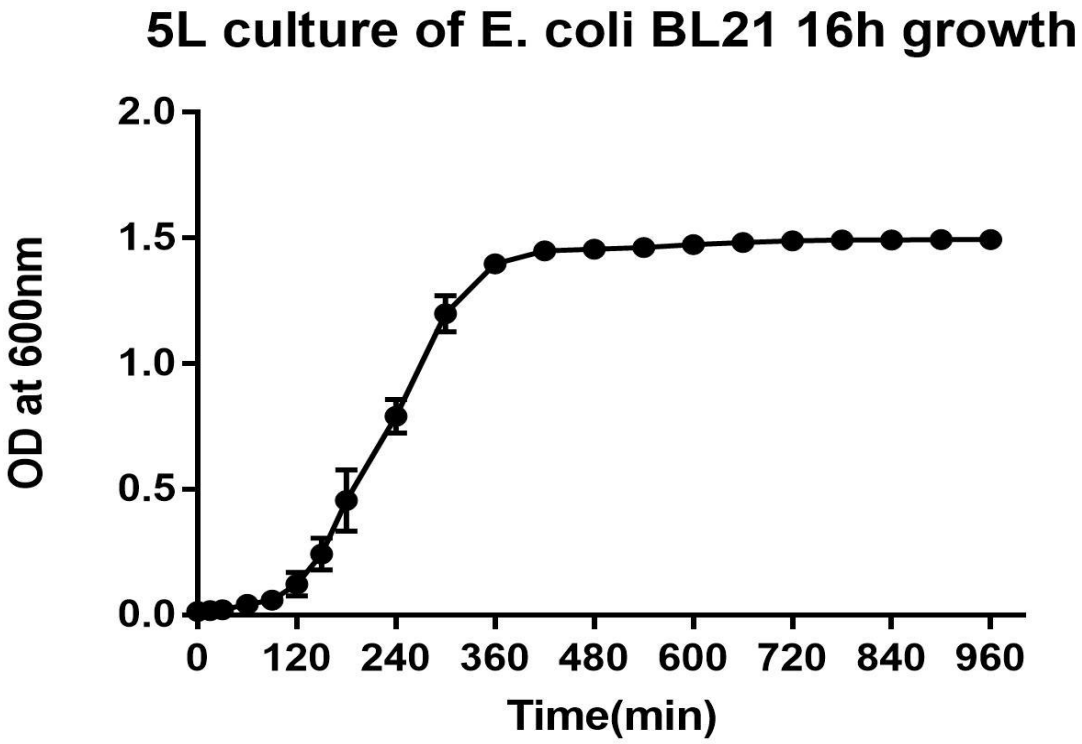

Fig 09- 5L culture of E. coli BL21 in 14L benchtop bioreactor till 16 hour. OD & Time graph

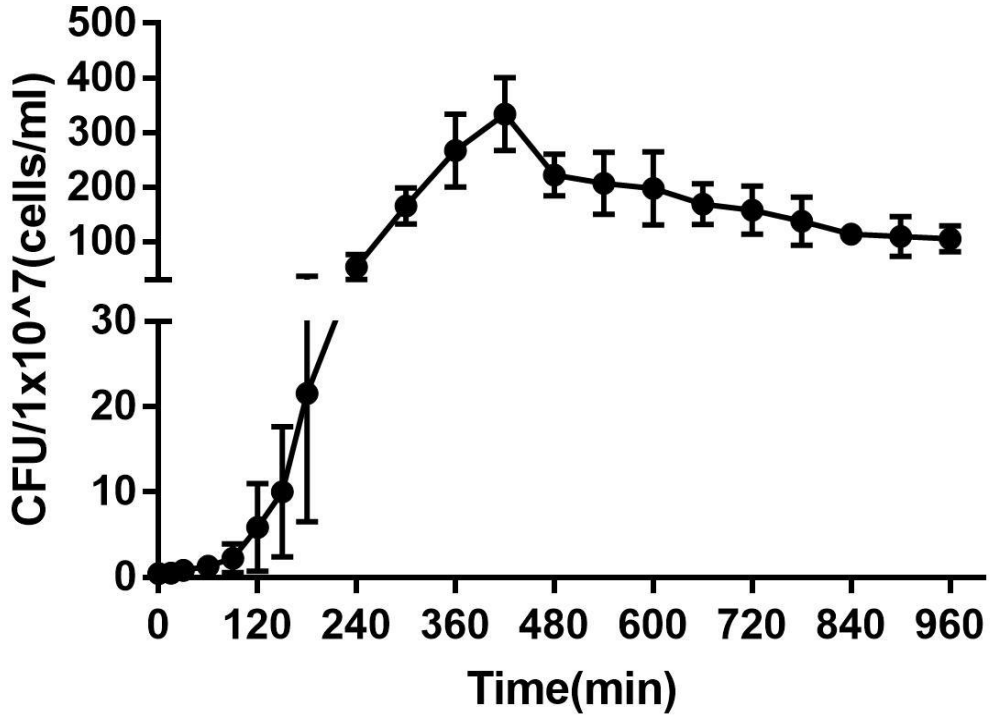

Fig 10- 5L culture of E. coli BL21 in 14L benchtop bioreactor till 16 hour. CFU & Time graph

After running bioreactor we get very stable and reproducible data of biomass production. Lag phage time duration was 1-2 hour after inoculation in bioreactor, shake flask and bioreactor in both cases the bacterial lag phage was same. But we can modify the log/exponential phage where bacteria grow and divide rapidly. In bioractor all facility like DO control, Temperature control, pH control systems were present, so bacteria divide rapidly in bioreactor and that why the exponential phage was significantly shorter, means it take very short time to produced highest yield.

Comparison between shake flask and benchtop bioreactor culture-

Factors	Shake flask	Benchtop bioreactor
Consuming time for giving highest product yield.	10 - 11 hours	6 - 7 hours
Highest CFU (cells/ml).	2.66×10^{10} for 100ml 2×10^{9} for 500ml	4.66×10^{9} for 2.5L 4×10^{9} for 5L
OD at 600nm in highest productive point.	1.612 for 100ml 1.15 for 500ml	1.453 for 2.5L 1.488 for 5L

4.5. 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.

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

Fig 12- Batch summary monitored by BioCommand software

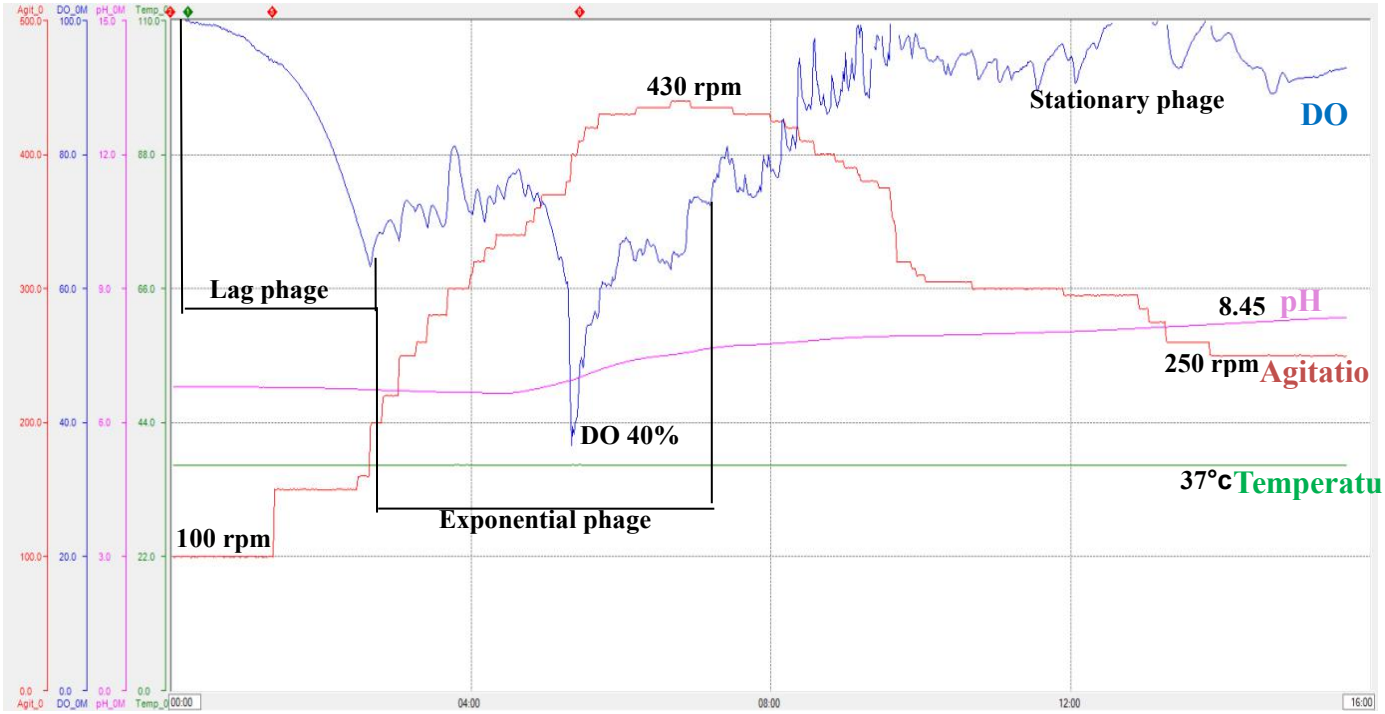

Fig 13- Batch summary of all loops assigned together into the graphical trend screen.

Using this software we can monitor the present and past status and accordingly if some changes are needed then it control through control cabinet. 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. But we have to maintain DO level into the vessel, so that we have increase Agitation speed when ever Do level was decreasing. When the culture reached stationary

plase O2 consumption decreased and DO started to increase. To maintain optimum DO level we reduced agitation speed till DO level stabilized. Interestingly we observed gradual increase in pH from 6.95 to 8.45 during log phase, which indicated accumulation of secondary metabolites or excretory products secretion from the cells.

4.6. Sigmoid model

A sigmoid model is a mathematical function having a characteristic “s”- shaped curve or sigmoid curve. By using sigmoid modeling we can differentiate the real data, which is not fits into the sigmoid model curve. So, average of all data set (100ml, 500ml, 2.5L, 5L) put into sigmoid model to verify that it fits into the model or not.

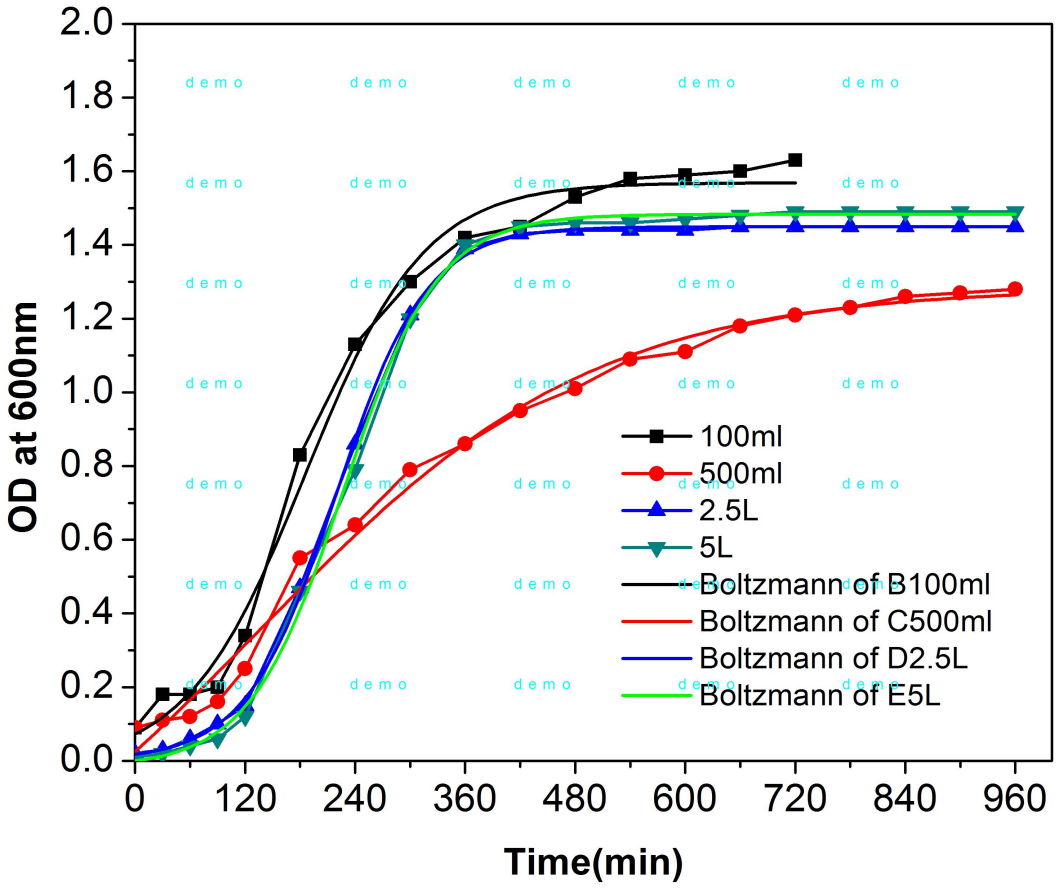

Fig 11 - Sigmoid modeling (Boltzmann)

We have fitted our experimental data with Boltzmann growh curve model and compared deviation from theoretical values. Experimental values from 100ml and 500ml shake flask experiments showed ~90% fit whereas 5L and 2.5L growth curve data fit most appropriately (~98%) into the model. This indicates that run conditions of bioreactors are highly optimized for large E. coli biomass production.

Discussion

To apply a scale-up strategy based on the volumetric biomass production from shake flask to benchtop bioreactor, it is necessary to study about growth kinetics of a bacterial strain in each up-scaling stage, like I have done at first 100ml culture in the 500ml flask, then 500ml culture in the 1L flask. After that 2.5L culture in the 7.5L bioreactor and 5L culture in the 14L bioreactor.

Stirred tank bioreactors are the standard in the Biotechnology industry and have been used for over 40 years. The small stirred tank has been important for scale-up, scale-down, strain optimization, characterization, and process development. It may also have an important role in the development of individualized medicine. Most often, experiments are performed using shake flasks but the conditions in the small-scale bioreactor differ significantly from the shake flask. In bioreactor experiment we found that the growth of E. coli was more stable, log phage was very fast than shake flask. The maximum cell number observed at a very early stage, like in between 6-7 hour mostly. But in the cause of shake flask maximum cell number observed at 10-11 hour and growth rate also slow. In shake flask, there was no aeration, only a fixed agitation system by shaking the flask, so the growth rate of the bacteria was slow. The bioreactor is a complete system, it has all the facility which is needed for fermentation and cell culture, like Agitation and Aeration system for DO control, pH, DO and Foam/level probes for monitoring pH, DO and Foam/level of the culture all time. Accordingly, if anything needs to add to the vessel, like acid, base or antifoam agent there have three pumps. Also, have temperature control and exhaust system. All the facility are present in the bioreactor, so that it is easy to grow bacteria into the bioreactor, very quickly we can get the highest yield. So when we looking for commercial production bioreactor is one and only option.

When we operate the reactor, all time we have to monitor each parameter. If DO is started decreasing then we have to increase Agitation speed accordingly and also increasing Aeration by using rotameter. Due to the growth rate of bacteria is very high so that DO also decrease very rapidly. There is lots of foaming and pH also higher than normal at stationary phage, for controlling that we have to add acid or base for pH and antifoaming agent for foaming. But as we are doing optimization study so we do not add anything to the vessel.

CHAPTER- 05

CONCLUSION

5. Conclusion

Scale up study is very necessary when anyone wants a larger amount of product. Operating bioreactor is quite hard, every day we have to face a new challenge, one of the big challenges is autoclaving the full vessel with media. However, once it starts running the system is automatically control everything except DO control. So the benchtop bioreactor is a very good platform to scale up from lab-scale to commercial-scale.

5.1 Summary

I have optimized bioreactor, now I know how to run bioreactor and what is the problem that has to face during the run reactor.

5.2 Evaluation

Basically, I have done bioreactor optimization by using E. Coli BL21 growth kinetics. My first experiment shows the lab scale bacterial growth, and the same experiment was done in bioreactor only the volume of culture increased. And find a positive result, that bacterial cells are growing rapidly so maximum cells number observed at a very early stage.

5.3 Future Work

Lots of future work have to, I have studied only up-stream bioprocessing, and optimization of different biomolecule production is my future work.

CHAPTER- 06

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Appendix

◆ Media preparation (LB broth)

Reagent/chemical	For 500ml	1L	2L	5L
Tryptone	5gm	10gm	20gm	50gm
Yeast extract	2.5gm	5gm	10gm	25gm
Nacl	2.5gm	5gm	10gm	25gm

pH of media should be in the range of 7.0-7.4, the media is slightly acidic so we have to add 2N NaOH solution until media pH rich 7.0-7.4

For preparation of LB agar add 7.5gm agar-agar per 500ml LB broth.

◆ NaOH solution

Reagent/chemical	0.1N (1L)	1N (1L)	2N (1L)	2N (100ml)	2N(50ml)
NaoH	4gm	40gm	80gm	8gm	4gm
Distilled 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