UNIVERSIDADE DE LISBOA

INSTITUTO SUPERIOR TÉCNICO

Miniaturized platform to capture bio product

Malik Abdul Wahab

Supervisor : **Doctor** Maria Raquel Murias dos Santos Aires Barros

Co-Supervisor : **Doctor** Ana Margarida Nunes da Mata Pires de Azevedo

Thesis approved in public session to obtain the PhD Degree in

Biosciences and Biotechnology

Jury final classification: Pass with Distinction

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Abstract:

With increasing focus on the production of biopharmaceuticals there is need for developing novel cost-effective biomanufacturing processes and the focus have been on developing continuous process as they lead to high yield and reduce cost. Moreover, there is need to consider the holistic approach rather than considering one step at a time. Microfluidics with its advantages like low reagent consumption and low residence time have been at the forefront of process development as it accommodates both continuous and integrated approach.

The goal of this work has been on the design, fabrication and operation of an integrated microfluidic platform, using GFP produced by *E. coli* as model system, for production and subsequent purification of biomolecules, allowing integration of upstream with downstream process. Three modules are considered; a microbioreactor to produce protein continuously; a lysis module for intracellular proteins release and recovery and a purification module to concentrate and purify the protein of interest using liquid-liquid extraction.

The microbioreactor consisted of two layered devices with a fluid layer as bioreactor channel and a control layer with valves and integrated peristaltic pump. Microbioreactor has the ability to work both in semi continuous and continuous mode. In case of continuous mode, which is of greater interest here, it was found that optimum flowrate of 0.5μL/min provided the highest cell concentration at steady state.

An integrated chip was designed for continuous chemical lysis and extraction using aqueous two-phase extraction to process cell samples for lysis and subsequent purification of GFP. The goal of this part of work was to optimize conditions that can be subsequently used for fully integrated system.

The three-unit operations were than integrated on a single chip and optimum conditions were used to produce GFP using E. coli as host in a microbioreactor. After the production of cells were first lysed and then PEG-phosphate ATPS was used to concentrate and purify GFP in the PEG rich phase, demonstrating an integrated approach that can be used for evaluation of the whole process at once and understand the cascading effect of multiple modules.

Keywords: Continuous biomanufacturing, integrated processing, microfluidics, microbioreactor, cell lysis, aqueous two-phase extraction.

Resumo

Com o foco crescente na produção de biofármacos, existe a necessidade de desenvolver novos processos de biofabricação com boa relação custo-benefício e o foco tem sido o desenvolvimento de processos contínuos, pois levam a um alto rendimento e reduzem os custos. Além disso, é necessário considerar a abordagem holística em vez de considerar uma etapa de cada vez. Os dispositivos microfluídicos com vantagens em termos demenor consumo de reagentes e baixo tempo de residência, têm estado na vanguarda do desenvolvimento de processos, pois permitem implementar tanto a abordagem contínua quanto a integrada.

O objetivo deste trabalho foi o projeto, fabricação e utilização de uma plataforma microfluídica integrada para produção e posterior purificação de biomoléculas, que permite a integração do processo a montante de produção com os processos a jusante de purificação Foram considerados três módulos: um micro-quimiostato para produzir proteína continuamente; um módulo de lise para liberação e recuperação de proteínas intracelulares e um módulo de purificação para concentrar e purificar a proteína de interesse usando extração líquido-líquido.

O micro-quimiostato é constituído por duas camadas: a camada fluídica com os canais que constituem o bioreator e a camada de controlo com as válvulas e a bomba peristáltica. O microbiorreator funciona tanto no modo *descontínuo* como no modo contínuo. *E. coli* foi cultivada em modo descontínuo e contínuo. No modo contínuo, o foco de maior interesse neste trabalho, verificou-se que um caudal de 0,5 μL/min produziu a maior concentração de células em estado estacionário.

Além disso, foi desenhado um dispositivo para a lise química contínua e extração de proteína GFP Neste dispositivo as células começam por ser lisadas e de seguida entram no módulo de extração aquosa de duas fases usado para purificação da proteína extraída. O objetivo desta parte do trabalho foi otimizar as condições que podem ser utilizadas posteriormente no sistema totalmente integrado.

Os três módulos desenvolvidos foram depois integrados num único dispositivo e as condições otimizadas foram usadas para produzir a proteína GFP usando *E. coli* como hospedeiro no micro-quimiostato. Após a cultura de E. coli durante 24h, as células foram lisadas e por fim o sistema de extração aquosa de duas fases PEG-fosfato foi usado para concentrar a GFP na fase rica em PEG demonstrando uma abordagem integrada que pode ser usada para a avaliação de todo o processo de uma só vez e entender o efeito em cascata dos vários módulos.

Palavras-chave: Biofabricação contínua, processamento integrado, microfluídica, microquimiostato, lise celular, extração aquosa bifásica.

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Acronyms

ATPE aqueous two-phase extraction.

ATPS aqueous two-phase system.

B-PER® Bacterial Protein Extraction Reagent.

CAD Computer Assisted Design.

CNC Computer Numerical Control

CVD chemical vapor deposition

DC direct current

DI de-ionized.

E. coli Escherichia coli.

GFP green fluorescent protein.

MEMS microeletromechanical systems.

OD optical density

PDMS polydimethylsiloxane

Pe Péclet number

PEG polyethileneglycol

PMMA polymethylmethacrylate.

Re Reynolds number.

RIPP Recovery, Isolation, Purification and Polishing.

TTL tie line lenght

1

Introduction

1.1 Project Motivation:

Biological processes have been used for producing various food products for centuries, but it was not until first biopharmaceutical recombinant human insulin (trade name "Humulin") was approved in 1982 that the interest in using biological process and organisms for production of therapeutics increased [1]. Since then, the focus on production of biopharmaceutical have increased significantly and it is anticipated that in next 5-10 years 50% of all drugs in developments will be biopharmaceuticals [2]. Therefore, there is a need to for novel and economical bioprocessing techniques. There has been paradigm shift for continuous processing in biopharmaceuticals production and purification as it can increase yield, reduce cost and reduce environment footprint [3]. Moreover, the focus has also been shifted to development of holistic approach by considering the whole manufacturing process at its entirety rather than optimizing each unit operation separately [4,5]. However, process development at larger scale is quite expensive because of costly infrastructure and huge material consumption, thereby needing cost effective screening and process development methodologies.

Microfluidics refers to science and technology used to manipulate small volume of fluids in channels that ranges from tens to hundreds of microns [6],[7]. Microfluidics have advantages like low residence time, low reagents consumption, precise control over the conditions, portability, and integration of various components on same device which makes is a cost-effective method for screening various conditions and process development. [8-10].

The main goal of this work is the establishment of miniaturized platform for continuous bioprocessing to aid process design and development and intensification of the bioprocessing of biological products through integration of upstream and downstream processing.

The specific objectives of the work are:

1) Development of microfluidic devices for continuous bioprocessing both and upstream and downstream
2) Integration of upstream and downstream to understand the cascading effect.
3) Design and fabrication of single microfluidic chip capable of handling more than one unit operation.

In literature, various studies can be found where miniaturization of unit operation of bioprocessing is done for process development and optimization, however the novelty of this work is to develop a microfluidic platform with three steps; Microbioreactor, cell lysis and extraction of protein; on a single chip and all the steps working in continuous manner.

1.2 Bioprocessing:

Bioprocessing can be broadly divided into two parts, upstream processing and downstream processing. Upstream processing deals with production of product of interest while downstream processing is involved in recovery and purification of the product. Figure 1.1 shows the general schematic of the bioprocess as a whole. In order for biopharmaceuticals to be approved for use the purity requirements are very strict, hence making downstream processing the bottle neck in decreasing cost of biopharmaceuticals as it accounts for 70% of the total cost [11].

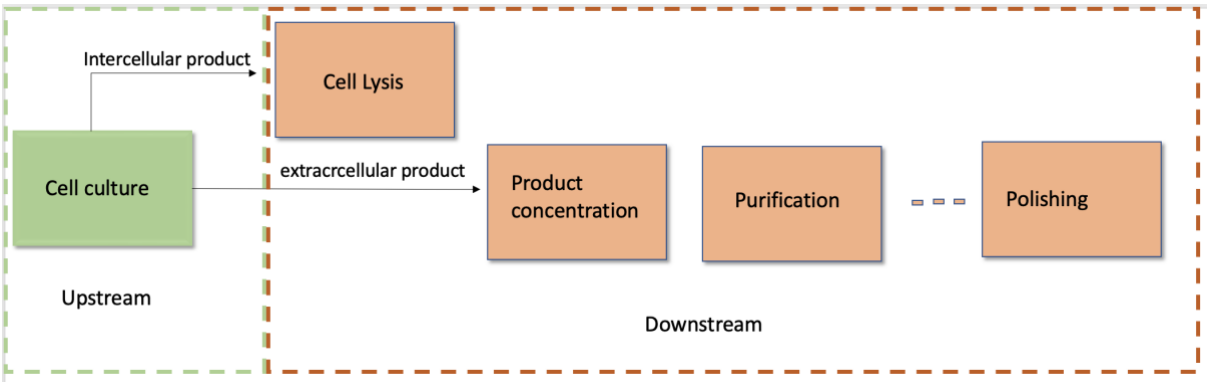

Figure 0.1:General schematic of bioprocessing

There have been significant improvements in upstream processing due to process control, bioreactor design and cell engineering which resulted in higher titers of biological products [12] but the downstream processing still needs to catch up. Moreover, optimization process

should take into account the whole process rather than optimizing upstream or downstream. But there is a need to bridge the gap between both processes where this work comes in to address the cascading issues.

1.3 Upstream processing:

Upstream processing deals with the production of biomolecule and most important factor to consider here is the selection of organism. Though any protein can be produced using any organism, reverse is not true, hence choice of organism depends on the product of interest [13]. Initially the microorganisms like E. coli were the most common choice but there has been shift in using mammalian cells due to the complexities of the products being produced [14]. As the product produced in this step needs to purified hence the choices made in this step have huge impact on downstream processing, which must be taken into account [15].

1.3.1 E. coli as model organism:

Bacteria has been used by for centuries for process like production of dairy products and lately for the development of biopharmaceuticals. *E. coli* in particular has been used to produce majority of biological products especially in case of therapeutic proteins where one third of the all approved therapeutic proteins are produced using *E. coli* [15]. *E. coli* not only has high growth rates but it is also very robust and have proved to provide high yield with simple and cost-effective growth medium. Moreover, being a pioneer host in production of recombinant protein [15], this microorganism has been the subject of biological advances and highly efficient *E. coli* strains have been created for specific purposes. Like any other organism it also comes with its challenges. Table 1.1 summarize it advantages and challenges.

Table 1.1: Advantages and challenges of E. coli

Advantages	Disadvantages
Safe	Inclusion body formation
Easy handling	Improper protein folding
High growth rates	Complex downstream processing
Well characterized genetics	Endotoxin contamination
Short processing time	No post translational modifications
Cheap medium requirement	Plasmid instability
High product yield	Limitations in single use technology

1.3.2 GFP as model protein:

The green fluorescent protein (GFP) is found in a jellyfish that lives in the cold waters of the north Pacific. GFP has revolutionized cell biology and have been used as biological marker in molecular biology, cell biology and medicine [16]. GFP is composed of 238 amino acids and a have molecular weight of 26.9 kDa, and emits green fluorescence under blue to ultraviolet light [17,18]. It can be rapidly produced in many organism and have multiple variants [19]. Fusion tags can also be easily added without changing its characteristics thus making it very useful in separation processes [16].

GFP is dimeric proteins and have a single α-helix containing a covalently bonded chromophore, which is surrounded by 11 β-sheets. The top and bottom are closed by α-helical sections which contributes to stability by protecting the chromophore from surrounding medium [16]. GFP has a major excitation peak at 395 nm and emission peak 509 nm [21].

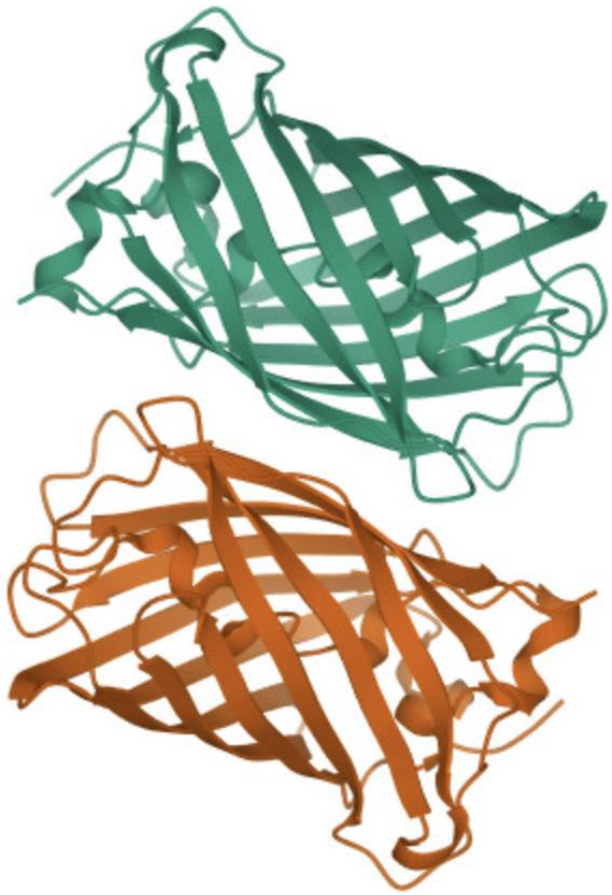

Figure 0.2:3D Structure of GFP [20]

1.3.3 Bioreactors:

A bioreactor is typically a vessel with the ability to maintain a unform environment for growth of microorganisms and maintain a balance in biochemical reactions going on in the microorganism to ensure production of desired biomolecule [22]. The applications of bioreactors involve either bio mass production like proteins and therapeutics or metabolite formations like acids, ethanol, and even to production of enzymes [23]. The most important factor that distinguish bioreactor from any other vessel is the ability to provide meticulous environment for optimal growth of the cells and production of product of interest [24].

Bioreactors are designed to maintain certain parameters like temperature, pH, aeration rate and agitation rate and in case of any deviation from set point an output is usually provided to specified control element [25]. Hence there are various sensors and control elements that monitor and control these parameters [26]. The pH conditions are controlled by flowing acid or base or by including pH control elements in medium [27], aeration or oxygen levels are controlled by agitation [28] and temperature is kept uniform by employing a thermal jacket with water flow [29].

All these parameters are dependent on the microorganism being used, however there are certain general requirements that must be taken into account while designing the bioreactor. There must be enough biomass concentration so that product yield is high but the upper limit of biomass is affected by nutrients availability. Another important condition is sterile environment. Contaminant organism not only utilize nutrient and diminish the growth of desired organism but also produce byproducts that will affect recovery and purification of desired product. Agitation is important for homogeneity and aeration but excessive agitation can cause shear stresses in cells. Another factor to consider is the removal of the product, as certain products can accumulate and effect cell growth so the product should be removed periodically.

Bioreactors can be broadly classified into aerobic and anaerobic. Anaerobic bioreactor is operated with mechanism of breaking complex molecules into simpler molecules [30], while aerobic bioreactors grow cells and produce product of interest [31].

1.3.1.1 Types of bioreactors:

Stirred tank bioreactors: Stirred tank reactors are the most commonly used bioreactor. As the name suggests an impeller is usually used for mixing. Due to this they can reach a well-mixed state quickly and have homogeneous environment throughout [32]. However, power requirement in this type of bioreactor is high. Figure 1.3 shows schematic of typical stirred tank bioreactor [33].

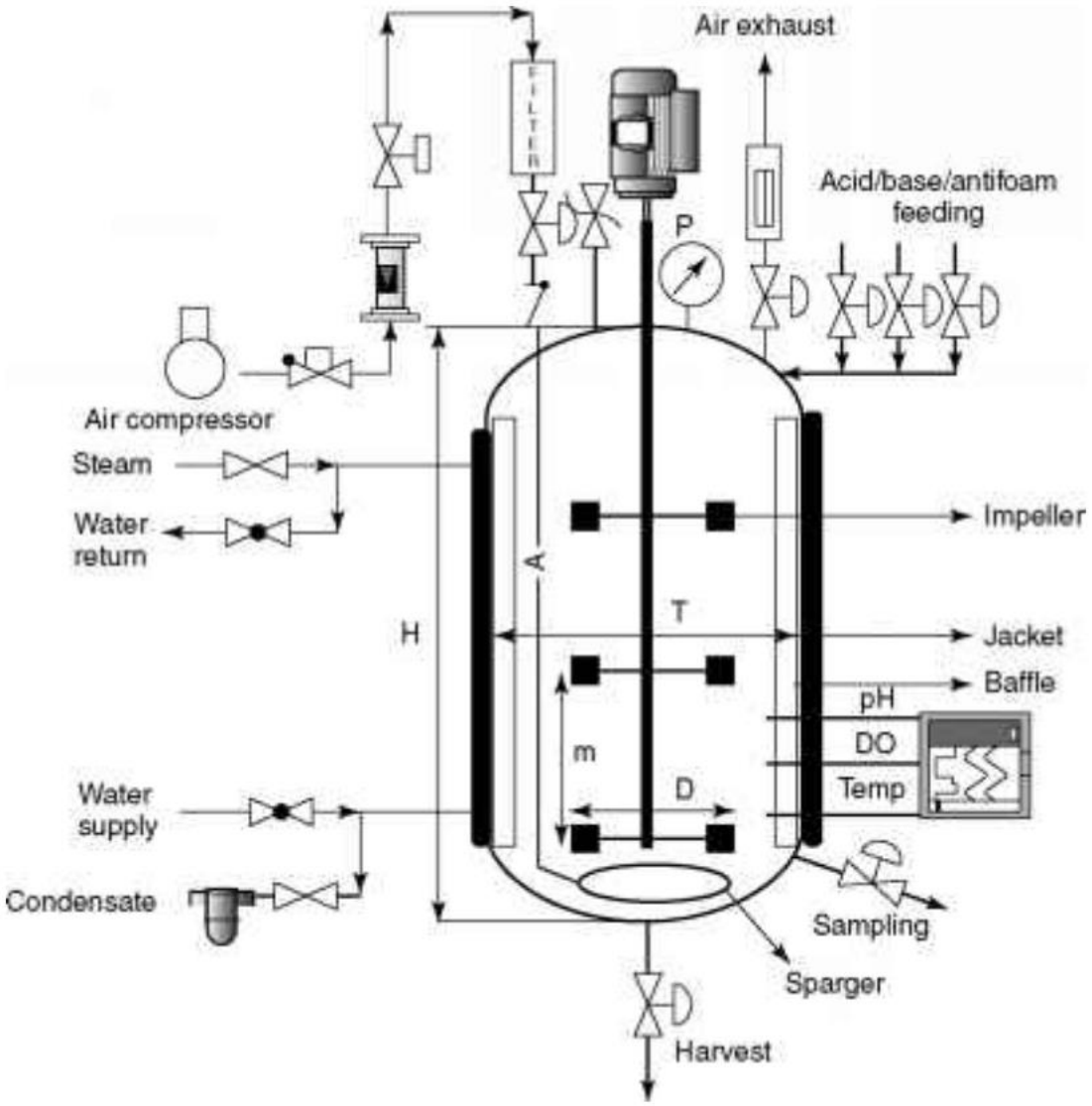

Figure 0.3: Schematic of typical stirred tank reactor, Adopted from [33]

Bubble column reactors: Alternative to stirred tank reactors are bubble column reactors. In this type of reactor, the agitation is provided by raising bubbles rather than a mechanical impeller [34]. In construction they contain an air distributor at the bottom. As the air is injected from bottom the fluid density changes resulting is convection motion which results in mixing at high gas flowrates. The lack of mechanical parts like stirrers decrease cost of maintenance however the reactor design is complex due to bubble formation and some limitations can also arise in the availability of enough oxygen.

Air lift reactors: Airlift reactors are similar to bubble column reactors with one distinction that air is sparged into only a part of vessel called the riser [34]. Fluid density decreases causing

liquid to move upward and the bubble detach from the liquid at the top of vessel and density increases causing the liquid to move through downcomer. This difference in density in riser and downcomer results in liquid circulation [35].

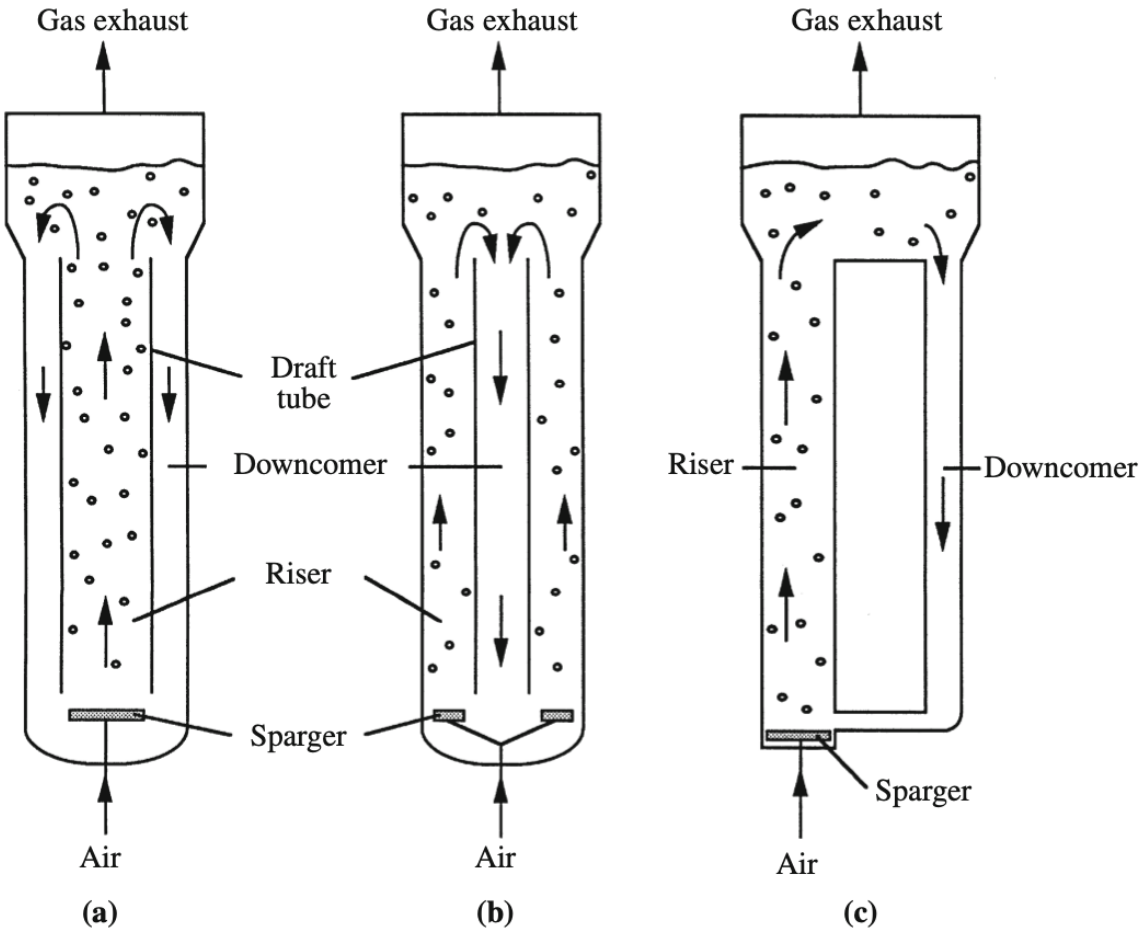

Figure 0.4: Airlift reactor configurations: (a) and (b) internal loop vessels; (c) external loop airlift. Adapted from [35]

Solid state fermenter: In solid state fermenter, growth of microorganism takes place on a solid substrate with very low moisture content which result is high product concentration and very low energy requirement [36-39]. But these types of reactors are more suited for fungus and algae rather than bacteria.

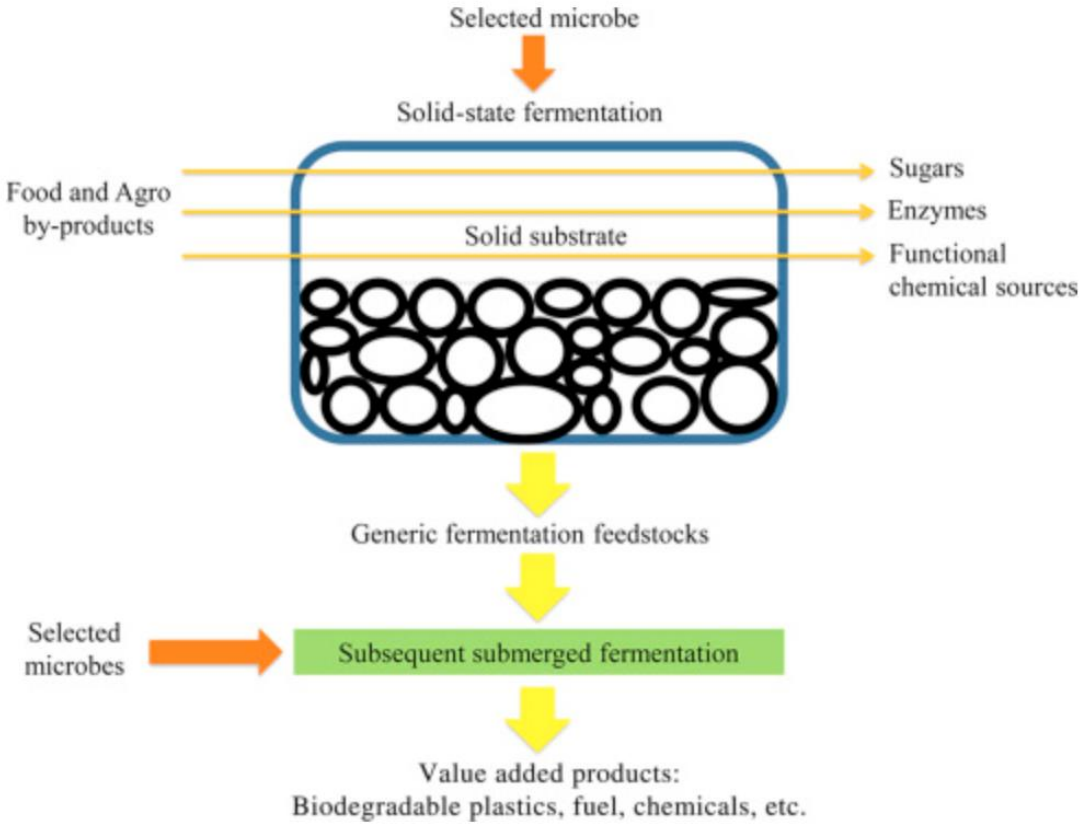

Figure 0.5: Schematic of solid-state fermenter. Adopted from [40]

1.3.4 Modes of operation:

Bioreactors can be operated in batch, fed batch and continuous mode.

1.3.4.1 Batch:

Batch mode is the most common mode of operation, where substrate is added in the beginning and the final product is extracted at the end [28,35,41]. It acts as partially closed system where nothing is added in between except for oxygen supply and solutions to control pH hence the volume of the reactor remains constant throughout [28,35]. Being a closed system, it is less prone to contamination and operation is quite simple, however conditions might change during the operation and may not be optimal for the growth of cells [35,41]. Also, there is significant down time between batches making it quite expensive [28].

1.3.4.2 Fed Batch:

In fed-batch operation, a portion of medium or nutrients are added periodically and some of the product or cells are removed [28,35]. This addition of nutrients provides control of substrate condition which in turn help control the growth conditions of cells. Injection of medium also help maintain the oxygen requirements of the process [35]. Fed batch bioreactors can operate at optimum growth rate all the time providing a high cell density but at the same time needs skilled operator and increase cost of the process indirectly [28].

1.3.4.3 Continuous mode:

In continuous mode nutrients are added and cell or products are removed continuously keeping the volume of the reactor constant throughout the operation [28,35]. Continuous mode reactors are classified inti chemostat, where the chemical composition of medium is kept constant or turbidostat, where biomass concentration is kept constant [28,35,41].

1.4 Downstream Processing:

Downstream processing refers to recovery, isolation and purification of bio product and usually costs about 70% of the total cost of the process [11]. There can be many unit operation in downstream processing depending on the purity requirement for the product. General purity requirements of enzymes are 80-90%, for diagnostic proteins is 95% and therapeutic proteins its 99% [42].

Downstream processing is usually divided into recovery, intermediate purification and polishing also known as RIPP strategy. Initial recovery focus on separating solids and liquids by using techniques like filtration, sedimentation and centrifugation and then cell disruption if the product is intercellular. Cell disruption method is chosen based on the organism and location and characteristics of the product. Intermediate purification focuses on reducing volumes and removing some impurities and polishing is done to remove remaining impurities and reach the desired purity goal [42].

1.4.1 Cell Lysis:

As significant part of products is intercellular, hence cell disruption becomes the first step in downstream processing. In order to release the product cells are to be disintegrated. There are many methods available and the selection of the method depends on various factors. First

and foremost, the chosen cell disruption method should be able to provide high recovery and at the same time should be able to retain the integrity of product. [42].

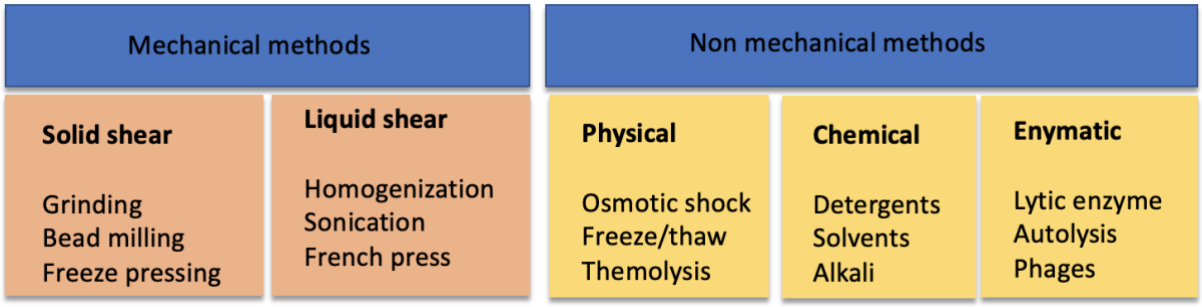

Figure .0.6: Lysis mechanisms. Adapted from [42]

While selecting the most appropriate method for cell disruption following factors must be considered [42].

1) Resistance of cell wall which will vary depending on cell type age and structure of membrane and walls.
2) Whether the product in organelle, periplasmic space or cytoplasm.
3) Product application and its stability to temperature and shear stress
4) Product recovery facility
5) Cost of the operation
6) Amount of cells

Chemical methods are currently being used in laboratory, however at industrial scale mechanical methods are more suited due to large scale operation and low cost [43, 44].

1.4.1.1 Mechanical methods:

Mechanical methods are the most common used methods for cell disruption and are used in industrial scale. These methods rely on shear stress either solid or liquid shear to disrupt the cell. The need specific equipment and also energy requirements for these methods make them expensive and shear stress and high temperatures can result in protein degradation [45]. Also, these methods tend to release cell components like host protein and nucleic acid which bring challenges for further downstream processing [46,47].

Bead mill:

Bead mill is the most effective method for cell wall disintegration and it is very commonly used at industrial scale due to the availability of the equipment and continuous operation [48]. It consists of milling chamber will with beads and an agitator and cell disruption happens due to shear stress caused by milling between the beads and a cooling system is required as temperature increase can result in protein degradation [42]. Bead size is an important parameter [49], with smaller beads being more effective but larger beads tend to release periplasmic products only and thereby making further downstream process less challenging [50].

Homogenisation:

It involves passing cell suspension through a small restriction under high pressure and then impinging it against a stationary phase. Mechanism behind cell disruption is shear stress when the cells are passed through restriction and sudden change in pressure when they pass [48]. This process has quite high efficiency but require expensive equipment [45].

Freeze Pressing:

As the name suggests, in this method cell are frozen and then passed through a small orifice under high pressure. This method is quite efficient and high rupture occurs even in single pass. Moreover, it creates larger fragments which are easy to remove in further downstream processing.

Sonication:

Another liquid shear method very commonly used at laboratory scale utilizes 20 MHz of ultrasonic waves to create liquid shear [51]. The working phenomenon of this method is cavitation. Ultrasonic waves result is local vapor cavities which lead to local pressure changes that causes cell disruption. However, it causes extreme local temperatures resulting in protein and enzymatic degradation and therefore it has not been used in industry. Debris created as the result of this method is quite small thereby hindering further downstream processing [52].

1.4.1.2 Non mechanical methods:

Freeze and thaw:

Freeze and thaw is the most common method used in laboratory. Cell disruption is due to the formation and melting of ice crystals. But it is very slow process and there have been reports of loss in enzymatic activity [49].

Thermolysis:

Thermolysis is similar to freeze and thaw however it is faster as it is based on instant high temperature shocks. But this method is suited for heat resistant molecules only. Its not only a very effective method of DNA isolation but also can be the extraction and purification process for heat resistant proteins as other protein will desaturate and cell structure remain intact [53].

Osmotic shock:

Another laboratory technique which is usually used in combination with another cell disruption method. It works on rapid dilution of cell sample in high osmatic pressure environment so that there is inflow of water which increases pressure until the cell is disrupted. But this method can only release product from periplasmic region and contamination in the sample increases by using agent for high osmotic pressure.

Alkali solution:

This method was developed for DNA extraction in laboratory. Use of alkaline solutions acts by saponification of lipids present in cell walls [54]. High pH solution like NaOH is used in combination with detergents and high temperature, which leads to total disruption of the cells and increase viscosity. As this method is detrimental to most of the products it has only be used for extraction of plasmid DNA at limited scale [54].

Solvents:

Various solvents like urea and guanidine hydrochloride have been used in product release of bacteria [46,47, 50, 55]. These methods act by solubilizing the hydrophobic membrane proteins and eventually destabilizing the cell membrane. Guanidine hydrochloride has been used in combination with Triton as it solubilizes membrane proteins and high cell disruption can be achieved with quite lower concentration [50]. But different solvents work in different

ways like toluene effects the cytoplasmic membrane while chloroform is used for periplasmic proteins. Nevertheless, the toxicity of these solvents and effect on product of interest have made these limited to lab scale applications [56].

Detergents:

Detergents contains surfactants which act both the water and lipid and solubilize cell membrane and proteins present in membranes [56]. Surfactants have been used extensively for cell disruption. Some surfactants completely solubilize membranes and proteins and therefore results in complete disruption of cells while others weaken protein- protein interaction and make cell disruption easier with other methods [43].

Lytic enzymes:

Lytic enzymes are predominantly used for extraction of nucleic acid but they have been also used for extraction of proteins from bacteria [57]. They don't require high temperatures or shear stress but they become another contaminant and must be removed in downstream processing. The most common enzyme being used is egg white lysozyme [57]. It acts on peptidoglycan and hence effective for disintegration of cell walls.

Autolysis:

Autolysis refers to self-digestion of cell wall by peptidoglycan hydrolases named autolysins. [58]. There are various methods to trigger this like osmotic shock, nutrient limitations and may be antibiotics but this only work on growing cells and hence have very limited applications [58].

Phages:

Bacteriophages have been also used for lysis of cells where an enzyme endolysins present in bacteriophages is responsible for lysis [58]. The lysis starts at the end of the reproductive cycle of phage. The enzyme accumulates in cytoplasm until membrane is disturbed by a different enzyme and then endolysins act on peptidoglycan and results in cell disruption [59]. Phage lysis has very high specificity and activity but again phages themselves are also the part of impurities that must be removed in downstream processing.

1.4.2 Purification:

As cell disruption results in release of bioproduct the next steps are employed to remove contaminants and reach the desired purity level. Most of the biomolecules needs a series of separation and purification techniques. There exist many differences between the product and impurities and these differences work in the favor of separation and purification. Factors like size and shape, density, polarity, net charge, solubility, volatility and partition coefficient are very important when it comes to separation and purification process. Key issues that are to be considered while designing purification process are as follows [11,15].

1) Product concentration is very low hence initial efforts must be tailored towards decreasing volume an concentrating the product in order to save cost in using expensive purification process towards the end.
2) There may be various impurities with similar physiochemical properties as the product hence requiring highly selective process.
3) Quality requirements for the desired product must be met at the end.
4) Due to the fact that it's a multistep process, the interactions between various steps must be considered.
5) The solvents and other reagents should nit interact with the desired product in any way.
6) Cost and environmental footprint of process must be taken into consideration.

1.4.2.1 Types of separation techniques:

Separation process can be broadly classified into following categories.

1) Solid-Liquid separation: Methods like filtration, sedimentation, centrifugation comes under this umbrella where the goal is to separate the suspended solid particles from liquid medium and these methods are one of the first methods applied right after cell culture or cell lysis.

2) Solute-solvent separation: This method focuses on removing solvents along with dissolved impurities or changing solvents that facilities further purification.

Evaporation and distillation are the most common methods however adsorption and precipitation are used when temperature is an issue.

3) Solute-solute separation: The methods that fall under this category are complex as usually different solutes have similar physical properties like solubility so the separation relies on more complex chemical properties. Liquid-liquid extraction and membrane filtration methods comes under this category.

4) Liquid-liquid separation: These methods are used to separate different liquids from each other and methods like decantation and distillation lies under this category.

1.4.2.2 Common separation techniques:

Although the development of complete separation strategy depends on the biomolecule being produced but there are some common separation and purification unit operations which serves e as building blocks for the complete purification process.

Centrifugation:

Centrifugation is use to separate solid particles based on the size and density by applying artificial gravitational fields. Depending on the particles to be separated, different magnitudes of gravitational field can be created by centrifugation. At laboratory scale centrifuge can reach up to 1000-20000 RCF with working volume of up to 5L, however industrial scale centrifuges can work with thousands of liters in continuous or semi continuous way.

Precipitation:

This method allows the soluble particles to become insoluble so that they can be removed by filtration or any other solid liquid separation methods. The precipitate can either be the product of interest or the impurities. Precipitation is induced by adding a precipitating agent like salt or changing the solubility by changing physical parameters like temperature, pH, ionic strength or dielectric constant. Precipitation has the potential to be scaled up and use as continuous process.

Filtration:

Filtration is the most common methods used for solid liquid separation. In this method suspension is flown through a porous medium called filter, which allows liquid to pass through and solid particles are retained. It can be easily adjusted to remove desired particles by changing the size of pores. Only the particles that are bigger than filter pores will be retained while other will pass through. Also, several filters with different pore size can be used in succession to separate various solid particles of different size. This process needs pressure differential between sides of filter which can either by provided by positive or negative pressure upstream or downstream of filter respectively.

Membrane bio separation:

If the filter in filtration is replaced by semipermeable membrane, then the process can be used for various separations like particle-liquid, particle-solute, solute-solvent and solute-solute separations. In this case separation is not only based on solute size but also electrostatic charge and diffusivity can also be used as separation factor.

Adsorption:

Adsorption relies on binding of molecules onto a solid surface and uses to separate the molecule from mixture of molecules or sometimes solute from solvent. The adsorbent material usually contains specific ligands for separating specific molecule. But the adsorption process must be reversable in order to retrieve the molecules. The adsorption can utilize several factors like size, molecular weight, polarity, net charge and hydrophobicity etc.

Chromatography:

Chromatography is highly selective separation technique for solute fractionation. It consists of stationary phase and mobile phase. The stationary phase can be made of small particles or fibers or polymerized structure called monolith. Mobile phase is the sample that need to be purified and it exploits the different interaction of the molecules in mobile phase with the stationary phase. Depending in interaction mechanism there exist various types of

chromatography like size exclusion which works on size, ion exchange which works on charge on the molecules, and affinity chromatography [60].

Extraction:

Extraction process is liquid-liquid separation method that relies on distribution of the products between two immiscible phases [11]. The process consists of three steps: a contact stage where the extraction solvent is mixed with the feed phase (containing the biomolecule of interest), followed by separation of the two phases and equilibrium establishment and finally the removal of solvent containing the desired product. The product distribution at the final stage is given by following equation:

$$K_p = \frac{C_E}{C_O}$$

Where K_p is partition coefficient and C_E is the concentration of product in the extraction solvent and C_O is the concentration of product left in the original solvent at the end of process.

Most of the separation techniques described above present excellent performance in laboratory but when it comes to scale up there are several problems like cost of equipment, efficiency loss, problems in integration, mass transport issues and continuous operation. At the same time advances in upstream processes has made it possible to have high titers which all these methods are unable to handle. Liquid-liquid extraction on the other hand is quite promising for such transition [61].

Aqueous two-phase extraction:

Aqueous two-phase extraction is special case of liquid-liquid extraction in which both the phases used are aqueous making them very useful for biomolecule extraction. Aqueous two-phase system has been widely used for extraction of biomolecules. ATPS are created by mixing two solution containing incompatible solutes are mixed above a critical concentration [62,63]. Various compounds can be used to create two phase system like [61].

1) Polymer-polymer system like PEG and dextran
2) Polymer-salt system like PEG and salts like phosphates, sulfates.

3) Alcohol salt system
4) Polymer-ionic liquid system
5) Surfactant water system

Out of all of these most common are polymer-polymer system typically PEG-dextran and polymer salt typically PEG-phosphate and PEG-citrate [61,62]. ATPS are easily emulsified by agitation where droplets are formed and then those droplets merge into larger phase. Denser phase in this case will sediment while lighter phase is on top. The mechanism behind partitioning of molecules to one phase or the other is quite complex and depends on many factors like molecular weight and type of polymer, concentration of polymer and salt, temperature, pH, ionic strength, and the characteristics of the molecule itself like size, charge, hydrophobicity, conformation and the presence of specific ligands [61-63].

The composition of an aqueous two-phase system is represented in terms of (X, Y) where X and Y refers to system concentration in %w/w. Phase diagram is used to describe the phase separation as shown in figure 7. Phase diagrams consist of binodal curve “B”, the concentration below binodal curve will exist in single phase and two phases start to form when the composition is increased above the binodal curve [62,64]. A line joining the points on binodal curve like line XY in figure 7 is called tie line and its length is called “TTL” and any point on a single tie line will have the same overall composition but phases will be formed at different volumes and phase ratios. Zero TTL refers to critical point marked by ‘C’ in figure where composition of both phases is equal and greater the length of TTL the different will be properties of the two phases [62, 64].

Though phase diagrams are very useful tools in ATPS, however determining binodal curve is quite challenging [62,64]. It is usually done by adding one of the solutions into the other one until turbidity starts appearing which gives a signal that system is about to enter two phase region and the composition at the point is noted [62]. Though it is less precise method but it is less laborious than determining the composition of each coexisting phase in multiple systems. This method has been adapted to use in microfluidic which requires less time and less material consumption [61].

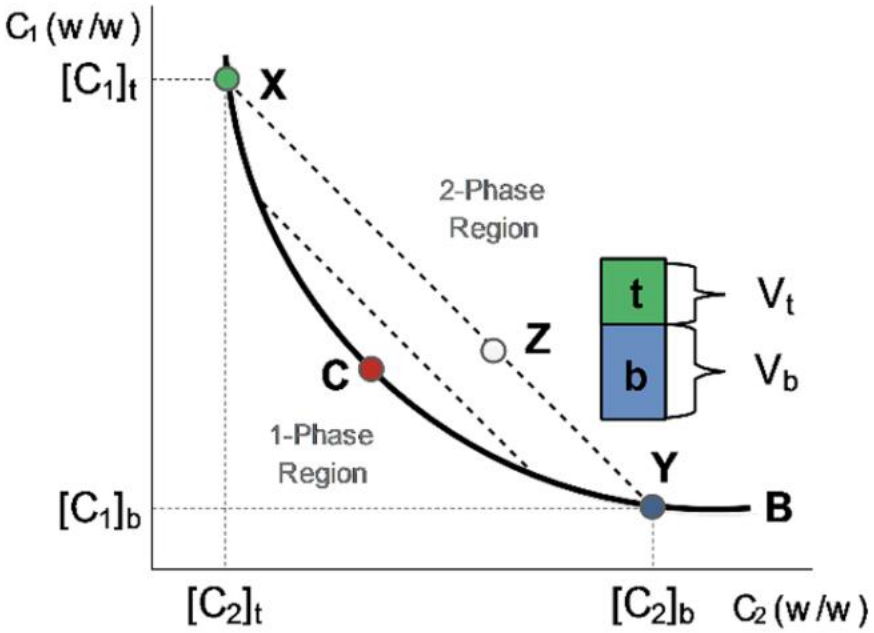

Figure 0.7: A typical phase diagram for an ATPS, showing the binodal curve "B" which separates system compositions which form one single phase or two immiscible phases and tie-lines (XY). Pont C refers to critical point. Adapted from [64]

The use of ATPS for selective partitioning of biomolecules have been the research interest for quite some time and different molecules shows affinity to different phases depending on various factor which can be quantified by partition coefficient (K_p):

$$K_p = \frac{C_T}{C_B}$$

Where C_T refers to the concentration of molecule in top phase while C_B refers to the concentration in bottom phase. ATPs are not only used for purification based on high selectivity but have been used for pre concentration of the biomolecule before progressing to further downstream processing as the concentration factors of two to three orders of magnitude are reported [64].

Table 1.2 shoes a comparison of different techniques and it can be seen that ATPS has the ability to offer solution for various challenges in downstream processing. Advantages of ATPS like use of inexpensive material and equipment, high selectivity and biocompatibility, ease of scale up and integration and potential to be used as continuous process has made it method of interest not only in research but also in industry.

Table 1.2: Comparison of various separation techniques Adapted from [61]

Property	**Chromatography**	**Crystallization**	**Precipitation**	**Polymer-polymer ATPS**	**Polymer-salt ATPS**
Technical simplicity	No	Possibly	Yes/No	Yes	Yes
Ease of integration	Yes	Possibly	Yes/No	Yes	Yes
High throughput	No	No	Yes	Yes	Yes
Affordability	No	Yes	Yes	Possibly	Possibly
Nontoxic reagents	No	Possibly	Yes/No	Yes/No	Yes/No
Removal of separation agent without Extra downstream steps	Yes	Yes	Possibly	No	No
Unconditioned sample loading	Yes/No	No	Yes	Yes	Yes

1.5 Microfluidics:

Microfluidics refers to science and technology used to manipulate small volume of fluids in channels that ranges from tens to hundreds of microns [6,7].

By reducing space, labor and time for experiments, microfluidics has the potential to revolutionize biology, bioprocessing particularly process development. Microfluidics have open door for automation and parallel processing and have proven to be critical steps towards sustainability with low reagents consumption. The impact of microfluidics can already be seen in biosciences to allow for easier and fast whole genome sequencing and analysis [65] and Lab on chip (LOC) devices have been developed for biochemical analysis faster than standard method and using low volumes of reagents and samples [66]. A more specific application of LOC devices called organ on a chip are being developed for streamlining drug development and moving towards personalized medicines.

With new technology comes new challenges and hence its essential to understand the basic working principles of microfluidics so that it can be adopted to new process. Though microfluidics has found applications in various areas such as physical sciences and display technology [66], here the focus will be on the applications that are related to bioprocessing and biotechnology. As the scale decreases some of the physical phenomenon which were dominant at larger scale becomes negligible and vice versa. In order to use the technology for useful purposes these phenomena and their impact on scale change needs to be understood.

1.5.1 Flow at microscale:

Fluids are continuum materials and as a consequence of continuum assumptions fluid properties like density ρ, velocity v , are continuous function. In case of Newtonian fluids, velocity field is described by Navier stokes equation:

$$\rho \frac{D\,v}{Dt} = \rho(\frac{\partial v}{\partial t} + (v.\nabla)v = -\nabla P + \rho g + \mu\nabla^2 v + F$$

Where v is velocity, P is the pressure, ρ is density and μ is dynamic viscosity of the fluid. Term $\rho(\frac{\partial v}{\partial t} + (v.\nabla)v$ represents inertial force, ∇P is pressure field, $\mu\nabla^2 v$ is viscous force, and F represnts any external force acting on the fluid. Another important equation if the equation of continuity.

$$\frac{\partial \rho}{\partial t} + \nabla(\rho v) = 0$$

With its special case where density is constant for example in case of water:

$$\nabla v = 0$$

Though Navier stokes equation is on the millennium prize problem but at microscale it can be simplified to

$$0 = -\nabla P + \mu\nabla^2 v$$

Where only pressure field and viscous forces are applicable.

1.5.2 Dimensionless numbers:

In fluid flow a series of dimensionless numbers are used to describe various flow phenomena and the dependence at various scale. Some of these numbers are important in case of flow at microscale Reynolds number being the most significant one. Reynolds number describe the relation between inertial and viscous forces and is used to classify the flow regime into laminar, turbulent or transition region.

$$Re = \frac{\rho v \, d_h}{\mu}$$

Where ρ is density, v is velocity, μ is dynamic viscosity of the fluid and d_h is hydrodynamic diameter. At Re<2300, flow regime is laminar, but in case of microfluidics Re are even lower than that making viscous forces the dominant force while neglecting inertial forces. Hence almost all the microfluidic devices reported works in laminar regime. In case of laminar flow multiple streams flowing in contact only mix by diffusion. A typical flow profile in microfluidic channel is parabolic where the fluid away from the channel wall has higher velocity than the one close to channels walls due to no slip condition [67]. This can be explained by the stringer adhesion forces between the solid walls and the fluid particles than the interaction between particles. It is important to note when modeling diffusion within microfluidic devices.

1.5.3 Diffusion and mixing at microscale:

Diffusion is defined as movement of particles from a region of high concentration to. region of lower concentration. At large scale diffusion is not a dominant mode of mixing because advection and turbulence are the driving factors of mixing but at microscale diffusion is the only significant mode of mixing. In order to understand and control mixing at microscale it's very important to understand diffusion. In some cases, for example where a chemical reaction is supposed to take place, it is important to mix the reagents efficiently and quickly but in cases like separation, its desirable to avoid and delay mixing. Peclet number defines the relation between convection and diffusion and is given by [68]:

$$Pe = \frac{U_o w}{D}$$

Where U_o is flow velocity, w is the width of microchannel and D is the diffusion coefficient of the molecule to be transported. As Peclet number is depending on the flow velocity hence it can be seen that the low flow velocity near the walls of channel provide a very different diffusion and mixing profile than the rest of channel. In case where fast mixing is desirable the narrow channel width will be helpful but decreasing the channel width will increase the effect of no slip boundary conditions and will lead to high pressure drop within channel.

1.5.4 Fluidic resistance:

From Ohm's law the relation between voltage applied across a conductor, electrical resistance of the conductor and current is given by:

$$V = IR$$

In case if fluid flow voltage is analogous to pressure difference (driving factor) and flowrate is analogous to current then above equation can also be written as

$$\Delta P = QR$$

Where ΔP is pressure difference, Q is the flowrate and R represents hydrodynamic resistance of the channel. The hydrodynamic resistance depends on the dimensions of the channel and the fluid properties. For a circular pipe it is given as:

$$R = \frac{8\mu L}{\pi r^4}$$

Where μ is dynamic viscosity of the fluid, L and r are channel's length and radius respectively [68]. For a rectangular channel where width greater than the height, the resistance can be given by:

$$R = \frac{12\mu L}{wh^3}$$

Where w is channel width and h is channel height.

1.5.5. Surface area to volume ratio:

An important factor in microfluidics is high surface area to volume ratio, which is one of the biggest advantages of microfluidics. Surface area does not vary linearly with volume hence there can be several orders of magnitude increase. In case of the applications where surface adsorption is used to facilitate the process, this increase in surface area provides a very high efficiency [69-71]. But in cases where low concentration is an issue this high adsorption can lead to depletion of analyte beyond detection limits [67]. Another aspect of higher surface to volume ratio is the effect on the surface tension force. Surface tension force is the consequence of between the molecules of fluid at the surface which is negligible at macroscale. But with increase in surface to volume ratio, surface tension force becomes dominant. It has its applications in capillary microfluidics which can be used for liquid insertion and circulation and assay development [72].

1.6 Microfabrication techniques:

Novel technology like microfluidics require novel fabrication techniques that can fabricate structures in the range of ten to hundreds of micrometers. Some of these techniques are adapted from semiconductor industry but they have been modified to be used for microfluidics [73].

1.6.1 Micromachining:

Basic fabrication techniques are the same as the one used in microchip industry. Micromachining techniques were used for silicon and glass [83] and include various processes as described below:

Thin film deposition:

It involves depositing a layer of another material onto a substrate for patterning, isolation or structural purposes. Techniques like sputtering, physical vapor deposition (PVD) and chemical vapor deposition (CVD) are very common.

Lithography:

Lithography is used for pattern transfer. Generally, computer generated pattern called photomask is transfer on to a substrate. Usually, a photoresist material is spin coated on to the substrate and then a focused beam is used to transfer pattern. If laser is used as the focused beam, it is called Direct write lithography (DWL) and electron beam lithography in case of electron beam [74].

Etching:

Etching refers to removal of material by using a chemical called etchant. Etching can be wet or dry depending on whether liquid or gas etchant is used. Two most important characteristic of etchant to be considered are selectivity and directionality. Selectivity refers to how quickly it etches the desired material as compared to masking layer and directionality is concerned with whether it etches uniformly in all directions (isotropic) or have varying etch rate in different directions (anisotropic).

1.6.1.1 Photolithography:

Though micromachining technology using silicon is quite versatile but it has high manufacturing cost and the properties of silicon like opacity and less than ideal surface for biological applications limited its use. The focus then shifted on using polymeric material for fabricating microfluidic devices. Polymers like PDMS, PMMA and COC have been used because of their low cost [75]. The most common method for fabricating polymer based microfluidic structures is soft lithography [73]. Most of the microfluidic devices have been fabricated from PDMS due to its properties like permeability to gas and light, impermeable to liquids, chemical and thermal stable and biocompatibility [74]. For fabricating a PDMS based structure a mold is fabricated first and then that mold is used for fabricating PDMS replicas.

The photolithography used to fabricate molds is similar to micromachining. Silicon wafer is used as substrate and the process start with coating a photoresist layer of desired thickness. SU-8, an epoxy resin based negative photoresist, is used most commonly. It can be used to spin coat thick layers. After coating SU-8, it is patterned with UV lithography [74]. Later on, propylene glycol methyl ether acetate (PGMEA), etchant for SU-8, is used to reveal final microfluidic structure. More complex structures are also possible by using different

photoresist and different heights and repeating the process many times [76]. These molds can be used to fabricate multiple microfluidic structures by pouring PDMS mixed with curing agent and then baking at specified temperature for specified time. After curing it can be peeled off and sealed against glass or PDMS membrane.

1.6.2 CNC Micro milling:

Micromachining is based on a combination of additive and subtractive manufacturing, however recently a well-known method of milling has been adapted for microfabrication. Computerized numerical control (CNC) micro milling is a subtractive fabrication method where miniaturized milling tools are used to fabricate molds by removing material from substrate [77-79]. Instead of using expensive silicon or glass substrate, polymer-based substrate made of PMMA, polycarbonate and polystyrene are used which are very cheap as compared to silicon substrate. CNC micro milling also does not require clean room as compared to its counterpart soft lithography and have advantages like low cost, high flexibility and is even faster than soft lithography [80,81]. CNC micro-milling can be used to directly fabricate final device using thermoplastic [82], or fabricate molds with positive features for PDMS patterning [83,84] or fabricate molds with negative features [85,86]. Recently a study was performed to fabricate 3-D microcolumns for chromatography and the process was optimized to reduce surface roughness up to 0.223 microns [87].

The process starts with making 2-d CAD drawing with all the device features and then converting that to G-code which is machine language and then using end mill tools of various dimensions mold is fabricated by removing material from a substrate. As the desired depth material removal is defined while milling so one can fabricate molds with different channel heights in a single process.

1.7 Microfluidic Components:

With the advancement in technology various flow components like valves [88-91], pumps [91-93] have been adapted for use in microfluidic devices. Valves and pumps are not the only components used in microfluidic devices but here the focus will be only on these two as they have been used in this work.

1.7.1 Valves:

Valves are flow control devices and are widely used in any fluidic system. It is not a surprise that flow control will also be required in microfluidic device and hence the requirements of valves. Valves can be classified into two broad categories: passive valve where actuation is provided by the fluid or pressure rather than an external stimulus, and active valves which are actuated by providing external source.

Passive valves:

Passive valves are operated by the fluid flowing in the channels itself rather than any external actuation mechanism. Feng et al. [96] used hydrophobicity to fabricate passive valve. Microfluidic structure was fabricated out of silicon dioxide and surface of the channel were modified to be hydrophobic while the rest of channel was hydrophilic and the hydrophobic part acted as passive valve only allowing the flow of water at certain pressure threshold. Jeon et al. [95] used diaphragm and flap made of PDMS to develop a passive check valve which allowed the fluid flow in one direction only and if back flow closed the valve stopping the flow.

Active valves:

Though passive valves are easy to fabricate and no external energy source is required to open and close the valve, the application of passive valves is limited to check valves. In order to use valves with more control active valves are fabricated which usually consist of a flexible membrane with magnetic [97-101], electric [102-108], thermal [109-116] and other actuation method, but the simplest are the one that are actuated by applying air pressure. Grover et al. [117] developed an air actuated valve by micromachining on glass substrate and used Teflon as a membrane to close and open the valve. The flow in the channel was controlled by applying positive and negative pressure. Unger et al. [118] used the same mechanism but using PDMS chip. In this fluid layer and control layer both fabricated from PDMS were separated by a PDMS membrane and applying pressure in the control layer PDMS membrane will be pushed against the fluid channel and flow will stop and releasing the pressure will move PDMS membrane back to original position and allowing the flow.

1.7.2 Pumps:

Pumps are the flow driving devices that are essential for creating a pressure difference to maintain flow. Syringe pumps are the most common external pumping devices being used in microfluidic but recently there have been increased interest in developing integrated pump on chip which will be very helpful for portability of devices. Like valves pumps can also be actuated by various mechanisms.

Like valves the first attempts pump fabrication were also done using micromachining and Lintel et al. [119] used piezoelectric diaphragm on micromachined silicon to fabricate a pumping chamber capable of providing flow up to 8μL/min. Grover et al [117] combined one-way valves to act as pump and were able to reach a flow rate of 1.1μL/sec. Jeon et al [95] applied the concept of check valves in combination with a reservoir and developed a pump with the pressure of reservoir as actuating mechanism. Unger et al. [118] used three pneumatic valves in series and by actuating them in sequence were able to create peristaltic motion with flowrate of 2.35nL/min. Another peristaltic pump was reported by Liu et al. [120] by using magnetic material embedded in layer above the microchannel. When magnet is rotated under the microchannel the magnetic material will push the membrane creating peristaltic motion. A flowrate of 50μL/h was reported in this study. Juncker et al. [121] utilized capillary action to develop an autonomous microfluidic pumping system. In this case a solution deposited at inlet will flow by capillary cation until it reaches capillary retention valve and this process can be repeated for several fluids in succession and has application in multi-step immunoassays.

1.8 Microfluidic applications:

With all the advances in microfluidic technology from use of novel materials to novel fabrication method to development of microfluidic components, it has been used extensively in different fields including biotechnology. In case if biomanufacturing, microfluidics has already been applied in both upstream, for designing microbioreactors, and downstream, for screening various conditions and process optimization.

1.8.1 Microbioreactors:

First step in bioprocessing and biomanufacturing is the selection of best microbial system and screening of various conditions to decide on optimal growth conditions for production of desired product. This is a tedious and laborious work involving several rounds of experiments and utilizing several liters of reagents. Microfluidics have provided a solution to this problem by reducing volume and time and use of parallel processing to screen various conditions simultaneously [122,123]. Various microfluidic chips have been described in literature using bacterial cultures and as well as other types of cell cultures to monitor growth, oxygen requirements and biomass production.

Kostov et al [124] used a 4 ml polystyrene cuvette for fermentation of E. coli which was mounted with sensors for optical density (OD) and pH measurement. Data for microbioreactor was compared with 1 L shake flask and it was observed that there was agreement in cell density, oxygen utilization and pH hence concluding that microbioreactor can be successfully used for process development at very low cost. Lamping et al [125] designed a microbioreactor inspired by a micro titer plate. Microbioreactor was machined from plexi glass with working volume of 6 ml and 6 bladed open flat turbine impeller was used for mixing. Microprobes were installed to measure fermentation parameter like DO, pH, temperature and OD. The device was not only used to study mass transfer coefficient in air-water and E. coli fermentation but also growth curve of E. coli was compared with 15-L bioreactor and both the data sets were in agreement.

M. Maharbiz et al [126] realized an array of 8 250μL microbioreactors from PCB strips. E. coli was cultured and OD was measured by in house sensor after every 30 seconds. In this work growth curve was generated for various oxygen concentration to study the growth of E. coli. Szita et al [127] designed a multiplexed microbioreactor system for parallel operation of multiple microbial fermentation. Microbioreactor had working volume of 150 μL and was fabricated from PMMA and PDMS. PMMA was used to house the chamber for cultivation with a magnetic spinner and PDMS membrane was used ensure the supply of oxygen. In situ pH and DO measurement were done using fluorescence lifetime sensors. E. coli was used as model microorganism and OD was measured for a period of 20 h.

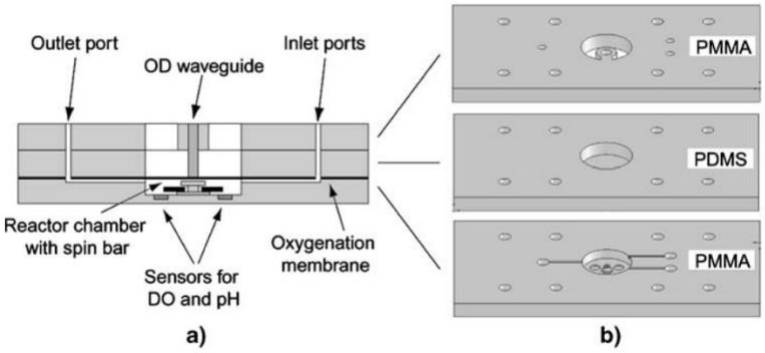

Figure 0.8: Schematic of microbioreactor (b) Exploded view of multiple layers. Adapted from [127]

Zanzotto et al [128] fabricated microbioreactor from PDMS and sealed the channels against glass. The volume was varied from 5-50μL. Multiple sensors were used to measure OD, DO and pH. In this study modeling of DO and OD was done and the model was compared with real time data obtained from microbioreactor. Also, a comparison of E. coli fermentation with a 500 ml bench top fermenter and microbioreactor was done and it was observed that pH, OD and OD in both the cases are quite similar hence scaling down does not affect the growth conditions and are a valuable tool for modeling. Lee et al [129] demonstrated microbioreactor to be used for 500h. Microbioreactor was fabricated in Polycarbonate (PC) by CNC milling and then a PDMS membrane was used to close the channels. An integrated peristaltic pump was used which not only controlled the flowrate but also was responsible for mixing within the channels. The device was operated in sequential steps like input, mixing and loss through evaporation was compensated. HPLC analysis of concentrations were compared with 1 mL bioreactor. Steady state production of various products was analyzed in chemostat mode while maximum cell growth was measured from turbidostatic experiments. Lou et al [130] realized a microturbidostat for E. coli. PDMS was used to house the channels and the devices had integrated input output valves and in situ agarose filter was used to ensure the transport of nutrients into channel and avoid the loss of bacteria. Instead of measuring OD, florescence intensity of GFP (produced by E. coli) was used to quantify cell growth. It was observed that cell numbers reach a steady state quite earlier than a bench top reactor but there was no explanation provided for that behavior.

Balagadde et al [131] realized a nano-liter scale bioreactor for continuous monitoring for E. coli. It consists of a peristaltic pump for circulation of the medium and valves to control

the inlet and outlet. A control on input and output prompted the study of cell growth for various dilution rate. The focus of the study was cell count but such low volume reactors can be used gene expression dynamics and distributions. Kim and Lee [132] fabricated a silicon based microreactor using microfabrication process like photolithography, material deposition and lift off etching process. Various fermenters were fabricated on single chip with each having a volume of 20 μL. Microsensors for continuous measurement of pH, dissolved oxygen (DO) and glucose were employed, however no data on the cell growth were presented. Figalo et al [133] realized an array of 12 wells within a device that is the size of a microscope slide (26×76 mm). Each well has a volume of 30 μL and are fabricated using soft lithography. The devices were used to culture primary rat cardiac myocytes C2C12 and human embryonic stem cells (hESCs). Thew device was also used to study vascular differentiation of hESCs hence showing that such microbioreactors are fast and economical tool to study cellular interactions.

Zhang et al [134] designed 150 μL microbioreactor from PMMA and PDMS. Both PMMA and PDMS surfaces were modified with poly (ethylene glycol) (PEG)-grafted poly (acrylic acid) (PAA) copolymer so that there is no cell adhesion. In situ measurement of OD, DO and pH were done by integrated sensors. Data from microbioreactor was compared with bench top stirred tank reactor and were found to be consistent. Schapper et al [135] designed a single use microbioreactor with working volume of the order of microwell plate. A free-floating stirrer was used for mixing and air was provided through a semipermeable membrane. Online sensors were used to control temperature and pH of medium and DO and OD were also measured. Saccharomyces cerevisiae was cultivated for validation and resulting growth curve were similar to those seen in bench scale reactors. Edlich et al [136] designed 8μL microbioreactor for microscale cultivation and to understand its potential for screening biological process. A PDMS membrane were used for passive supply of oxygen. A florescent dye complex DO sensor was used to monitor oxygen supply. An online OD sensor was also integrated for continuous measurement. Saccharomyces cerevisiae was used and parameters like OD, glucose and biomass was studied. However, mixing was not adequate resulting is limited growth rate. Seo et al [137] designed a bioreactor for fermentation with immobilized cells. A hydrophilic quartz channel was used to immobilize cells on 0.4% polyethyleneimine. Saccharomyces cerevisiae was used to study ethanol fermentation and carbon dioxide was

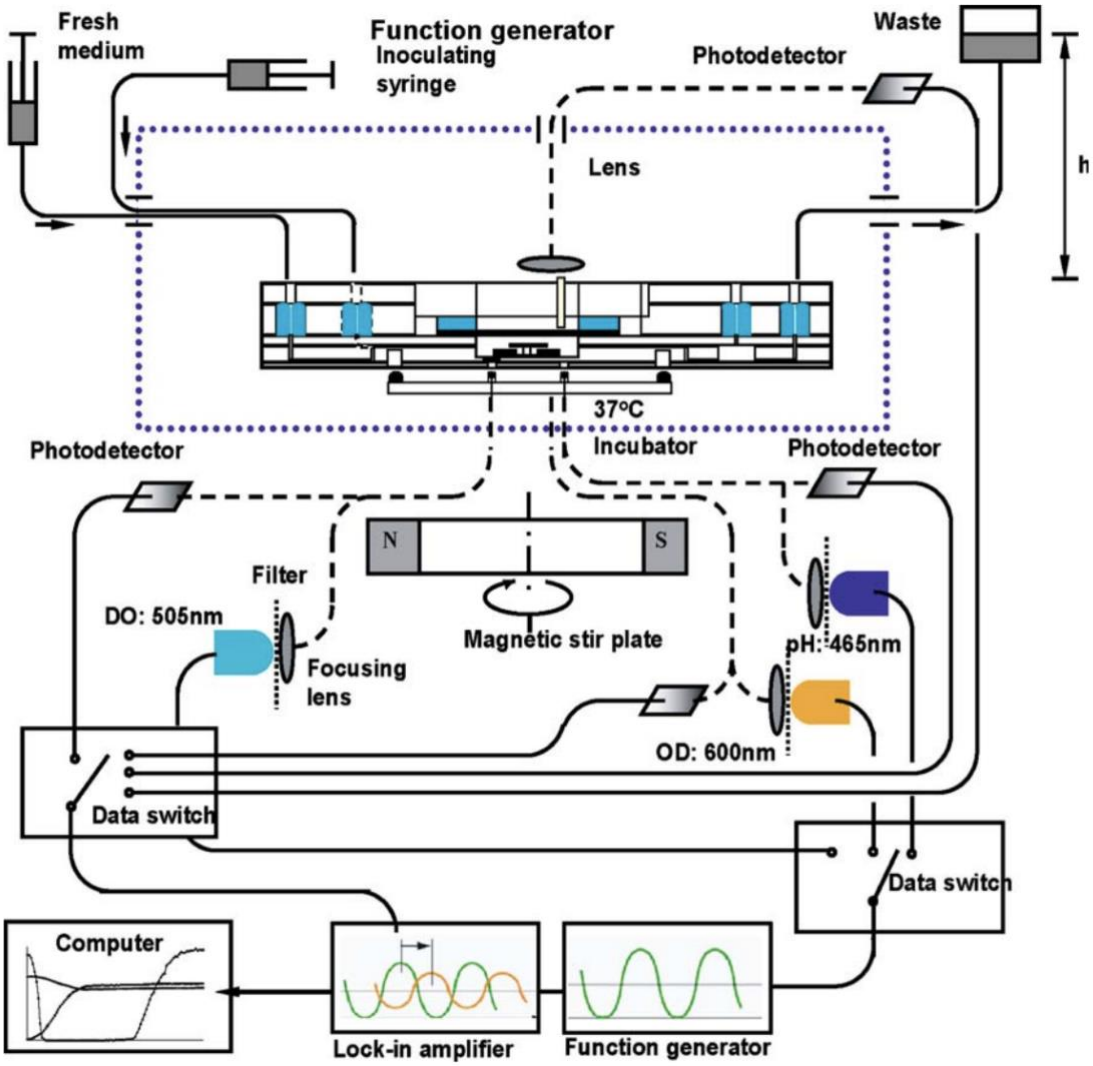

Figure 0.9: Schematic of microbioreactor experimental set up with tubing for fluid delivery and sensors for DO, pH and OD measurement. Adapted from [134]

removed using hydrophobic polypropylene. Microreactor was operated for a period of over a 1 month at steady state.

Leeuwen et al [138] operated two microbioreactors each with working volume of 100 μL. Candida utilis was used for validation and the dimensions were kept similar to 96-well microtiter plate so that comparison can be established. Integrated electrochemical sensors were used for continuous monitoring of temperature, pH, DO and viable biomass. A similar set of experiment were performed in 4L bench scale bioreactor and the data was compared; all the parameters showed reproducible results in microbioreactor though there was some deviation in bio mass which need further investigation. Suberbie et al [139] investigated an array of 24 15mL microbioreactors for cultivating various strains of E. coli. In this study various carbon sources were used to and the results were compared with 1L bench top bioreactor

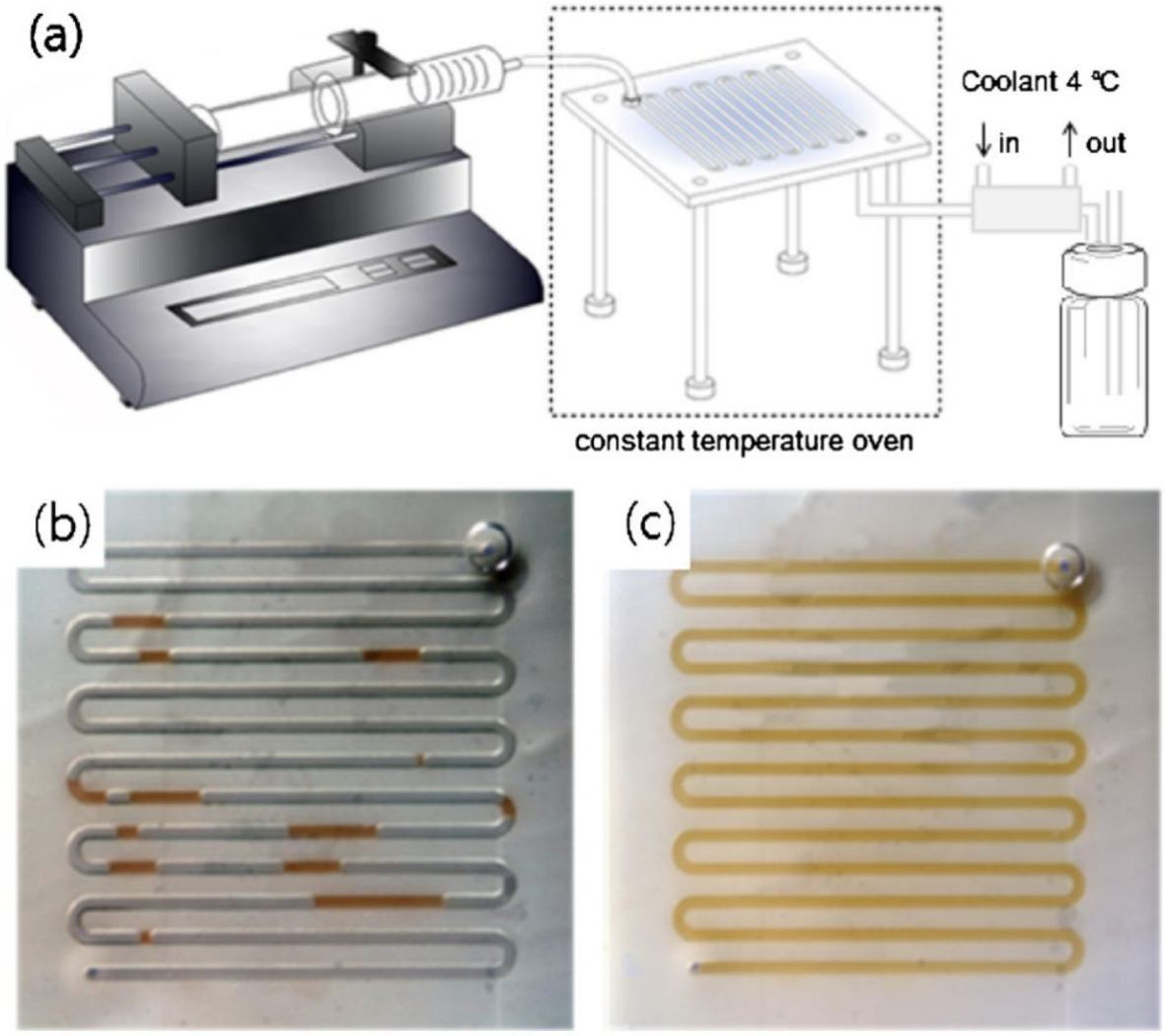

Figure 0.10: (a) Schematic of experimental setup, (b) air interrupting flow when both layers are made from quartz (c) no air flow when top layer is made from polypropylene. Adapted from [137].

and were found to be similar. Amanullah et al [140] used SimCell™ platform for cultivation of CHO cells and compared with 3-L bench scale and 100-L pilot scale bioreactor. 114 parallel microbioreactors were employed and under similar culture condition the results were similar to bench scale and pilot scale reactor.

Reis et al [141] used a 4.5 mL microbioreactor with oscillatory flow mixing (OFM). Saccharomyces cerevisiae were used as model organism and fermentation was done in aerobic and anaerobic environment. The results were compared with stirred tank reactor and it was observed that higher efficiencies of biomass production were obtained in novel micro-bioreactor, however further studies are required to understand if OFM technology can be used for parallel processing. Lee et al [142] developed an integrated array of microbioreactors, with 100 μL working volume, comprising a peristaltic oxygenating mixer and microfluidic injectors. Bubble free oxygenation were done via PDMS membrane and real

time cell density was measured using integrated sensor. The results were compared with bench top reactor and it was concluded that the cell density in this microreactor are four times higher than previously reported microfabricated arrays. Ostrovidov et al [143] used a membrane based microreactor to study the rat hepatocyte cultures. In this study in vivo liver environment was mimicked by approximating the perfusion conditions and architectural properties found in in vivo hepatic tissue. Microbioreactors were validated by call growth and major liver function and it was concluded that the platform can be used for liver studies ranging from drug interactions and liver tissue engineering. Au et al [144] designed a unique microbioreactor for culturing various microorganisms. Microbioreactor is digital microfluidic based reactor where droplets are generated and they serve as bioreactor. As it is capable of growing bacteria, algae and yeast therefore it is called BAY microbioreactor. There is no need of valves mixers or pumps and microorganisms were grown for 5 days and cell densities were determined by measuring absorbance and also with florescent assay. Cell densities were compared with macroscale and were found to be similar.

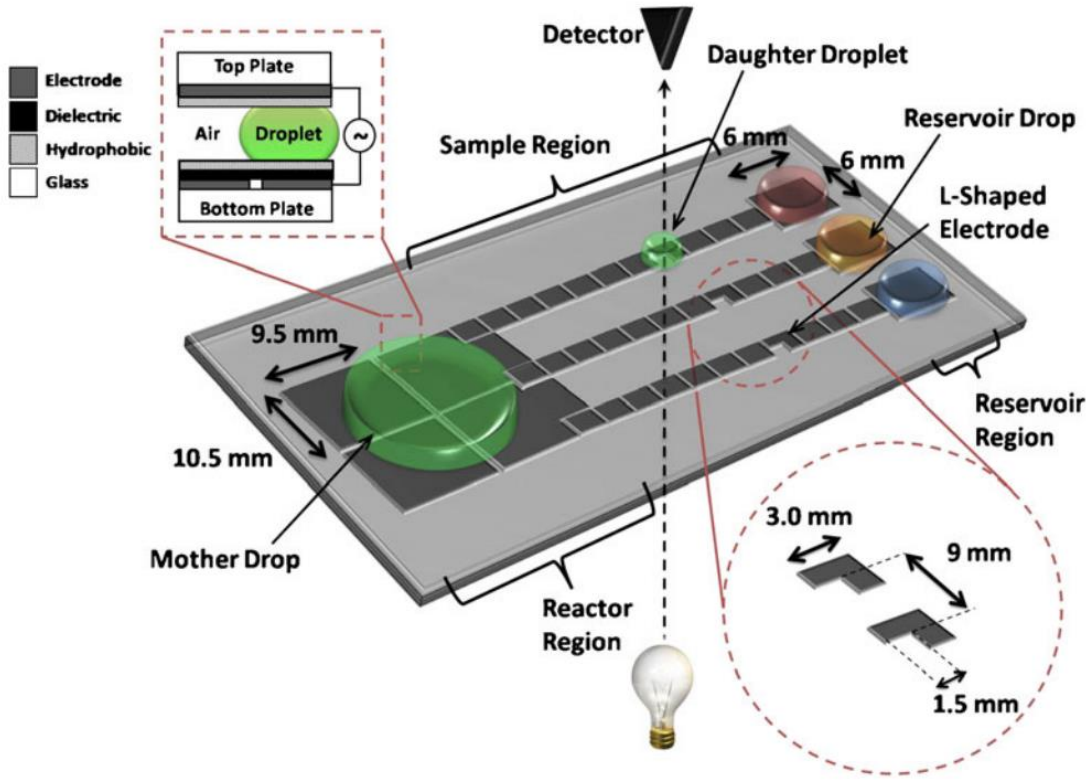

Figure 0.11: Schematic of BAY reactor. Adapted from [144]

Miniaturized bioreactor described above have shown how microfluidics is revolutionized process development and parameter validation. With help of microfluidics optimization of parameters like DO, pH, temperature, dilution rate for cell growth can be performed at low cost and with small reagent volume. However there have been limited applications to current problems and there is not much work done in relating these microscale findings with large scale bioreactors. Most of these devices are solely focused on the reactor and there has been little to no work on understanding and developing methods to integrate it to further processing where whole process can be optimized.

1.8.2 Lysis:

In order to release intercellular product, the next step in bioprocessing is cell lysis. In order to integrate this process on chip, it must be scaled down as well. There are various lysis methods that have been used on a microscale. The most common methods adapted on microscale are chemical, mechanical, thermal, electrical and laser lysis. In this section a brief review of literature is presented highlighting advantages and disadvantages of each method.

Chemical lysis:

Chemical lysis primarily uses detergents or alkaline solutions of lysis and is the most common method to be used in microfluidics due to its simplicity, Chen et al. [145,146] used a microfluidic device to compare lysis efficiency of guanidine and Triton X-100 on rat red blood cells. Lysis time for guanidine and Triton X-100 was found to be 174 s and 161 s and a mixture of both reduced the lysis time to 108s and it was concluded that lower flowrate leads to higher lysis due to increased time of contact. Hein et al. [147] used a different approach to compare lysis efficiency of different chemicals for lysis of Arcella Vulgaris cells where various chambers were used to capture and lyse cells. In this case high flowrate leads to faster lysis as there is more detergent available as compared to lower flowrates. A complete list of detergents is available in [148]. Irimia et al. [149] also used a similar process to capture and perform single cell lysis where a bifurcation channel was used to capture the cell and then mix the lysis solution. Gregor Ocvirk et al. [150] developed a Y shape microfluidic device for lysis of HL-60 cells. Triton X-100 was used as lysis buffer and cells were lysed in 30 seconds.

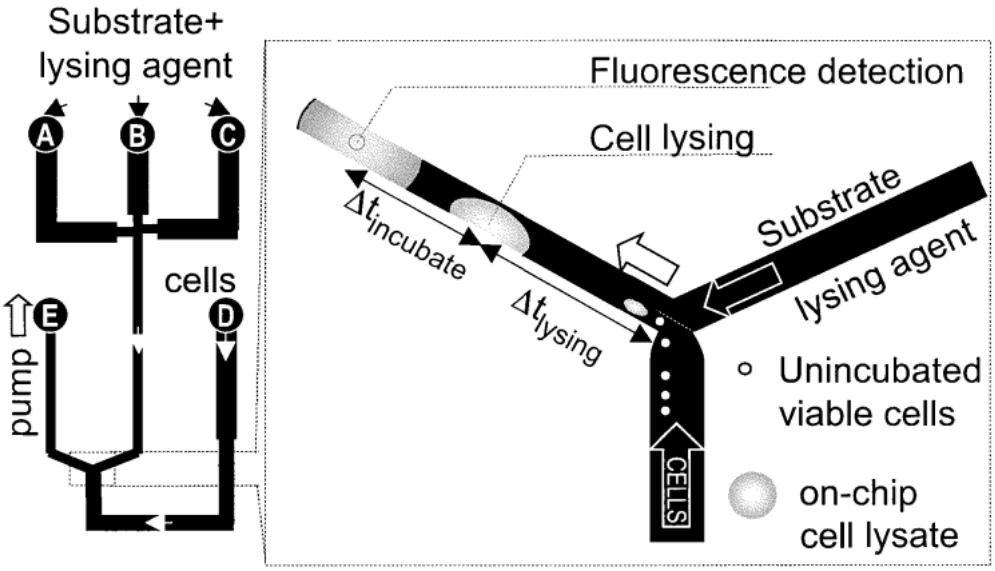

Figure 0.12: Scheme of device for lysis of HL-60 cells with Triton X-100 Adapted from [150].

Another chemical lysis technique was reported in [151] which mixes 25 pL of two fluids using cell crossover (CCO) technology. When cell pass through CCO region they move to lysis solution and are immediately lysed and based on comparison of extracted DNA quantity the device had 86% capacity of conventional chemical lysis methods. Yasuhiro Sasuga et al. [152] developed a microfluidic chip where cells and lysis solution were injected successively and the cells were trapped in microwells. Using inverted fluorescence microscopy, it was observed that cell membrane was gradually lysed in a period as short as 12 s. SooHoo et al. [153] developed a microfluidic platform for detailed kinetic analysis of chemical lysis. 20 observation points on 23 mm channel were used to observe the effect of lysis on cells and lysis concentration and rates were measured. It was concluded that lysis was initiated even at concentration less than 1% but will require longer lysis time and lysis rate almost plateaued above 10% concentrations. Ramji et al. [154] devised a droplet based microfluidic device consisting of cell focusing channel, droplet generator and lysis solution inlet. In this single cell were encapsulated in droplets and lysed by high pH buffer. Klein et al. [155] developed a similar platform where cells were encapsulated in droplet filled with lysis buffer.

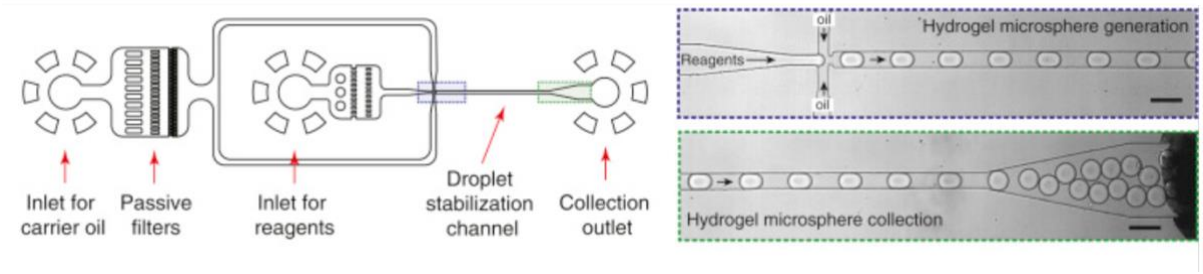

Figure 0.13: Microfluidic platform to encapsulates cell into droplets with lysis buffer. Adapted from [155]

Mechanical Lysis:

In mechanical lysis shear force is used to disrupt the cell and release intercellular product. In case of microfluidic devices, the shear force is provided by patterning nano scale obstacles in the channel to make sure there is enough shear force to break down the cell wall.

Dino Di Carlo et al. [156] reported a microfluidic device with sharp nanostructures in the channel which constrict the flow and squeeze the cell causing cell membrane to disrupt. The fabrication of the device is simple and it caused no harm to extracted proteins.

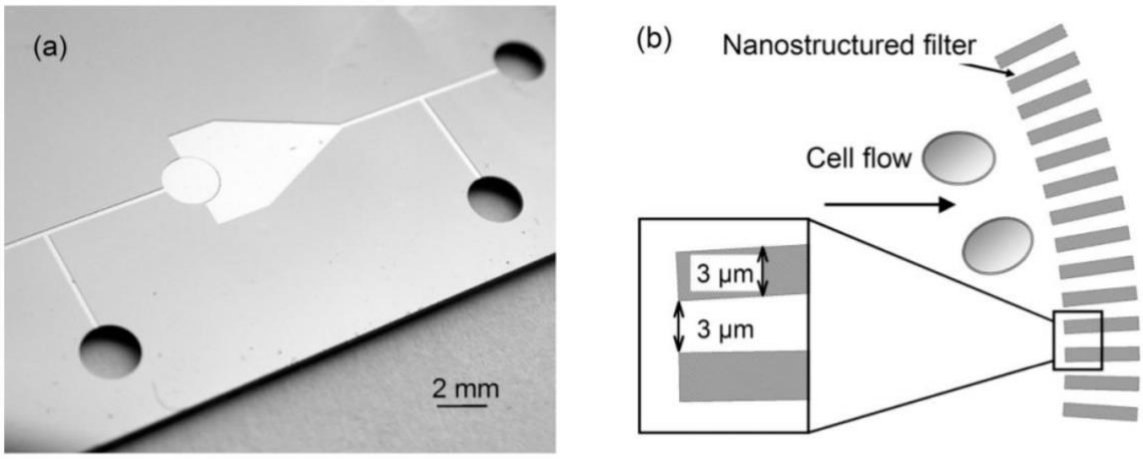

Figure 0.14: (a) Schematic of microfluidic device (b) nanostructured pillars Adapted from [156]

Han et al. [157] utilized a porous silica monolith for lysis of blood cells from whole blood sample and at the same time isolating bacterial cells. Bacterial cells being smaller than blood cells passed through the monolith intact while larger size of blood cells could not pass and were disrupted which resulted in lysis of 99.9% blood cells and 100% bacterial cells were recovered. Huang et al. [158] reported a silicon glass device with point constrictions for cell

lysis and isolating intact nuclei. In this work the results from chemical lysis on an identical chip without constrictions and mechanical lysis with constrictions were compared with mechanical lysis device having four to eight constrictions performing better than average chemical lysis. It was concluded that the size and number of constrictions must be tailored according to the cell type. Yun et al. [159] designed a disposable lysis device with ultra-sharp nano blade array. The performance of device was compared with conventional chemical lysis and it was observed that this device was not only faster but were able to achieve 18% higher protein concentration than conventional chemical lysis method. Other mechanical lysis devices with similar obstacle-based design are also available in literature [160-162]

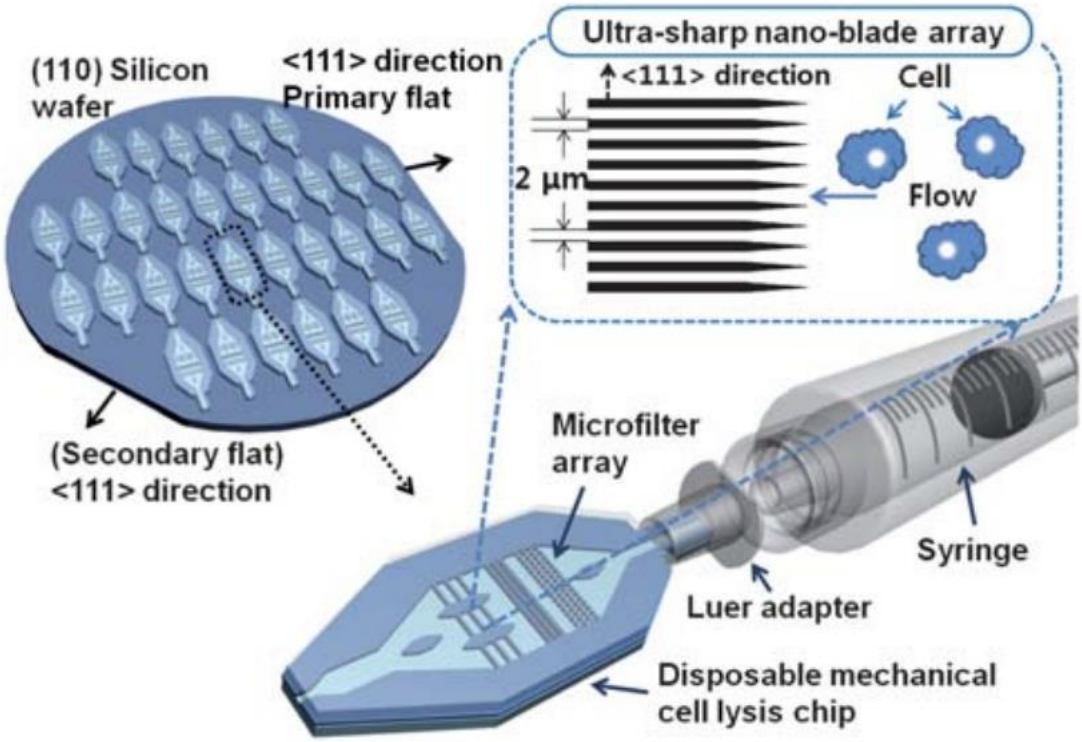

Figure 0.15: Schematic of chip with ultra-sharp narrow blade array. adapted from [159]

Kim et al. [163] developed a microfluidic where compressive force by deflecting a polymer membrane. A pressure range of 0-50 KPa was used to observe the deformation and rupture of cell membrane. It was observed that cells were mechanically lysed by compressive forces at 35KPa. Other mechanical method that has been used at microscale is bead beating and sonication. Chen et al. [164] used bead beating in microfluidic device by employing an on chip micropump. The rotary motor contained three electromagnets and three steal balls were

evenly mounted on motor head and were in contact with channel fluid to disrupt the cells. Cell lysis was evaluated by varying pressing depth and rotation speed with maximum of 56.5% at pressing depth of 250pm and 56.2% at 112 rpm. Flaender et al. [165] developed a microfluidic device for grinding lysis on chip where the cells were grinded against frosted glass using spatula. The whole operation took 1 minute with 30 s for loading the sample and 30 s for grinding. Wang et al [166] demonstrated the use of acoustic to create liquid shear as done in sonication at larger scale. A sharp-edged structure was acoustically oscillated which resulted in shear force from fluid motion and resulting forces were controlled by controlling applied voltage. Lysis efficiency of higher than 90% were reported and even up to 98% in some cases.

Electrical Lysis:

Electric methods also known as electroporation is another method for cell lysis where a transmembrane potential (TMP) is created by applying external field and when TMP exceeds threshold pores are formed on membrane and intercellular product is released. Electrodes are usually made of gold [167] or platinum [168] and the electrode potential for lysis depends on cell type [169]. Use of direct current (DC) will lead to maximum possible TMP but causes bubble formation around electrode [170], however this can be avoided by using alternating current (AC) with a frequency more than 0.15Hz [171].

Kim et al. [172] used DC field but resolve the bubble formation by using polyelectrolyte salt bridge and a pair of plugs on both sides of channel separate cell suspension and hypertonic solution and ionic solution completes cell. This results in low impedance and a high electric field is achieved by small DC bias avoiding bubble formation. Wei et al [173] utilized ITO electrodes build into a transparent microfluidic device for individual cell lysis. A low voltage of 16V was used to provide safe and reliable cell lysis and it was concluded that optimized frequency could increase the speed and efficiency of lysis. Lo et al. [174] developed a microfluidic device with planar electrode in bottom wall and used AC voltage of 20V peak to peak to lyse human blood cells in 7 seconds. Church et al. [175] reported a microfluidic device which utilized DC voltage to concentrate red blood cells in single constricted channel and then used AC voltage for lysis. Both constriction and lysing were used to isolate leukemia cells from sample of red blood cells.

Ramadan et al [176] developed a microfluidic chip for lysis of human white blood cells and murine clonal cells. Electric field was applied by sinusoidal voltage and it was observed that lysis rate decreases with increase at flow rate at constant electric field value and maximum lysis occurred at 10V above which bubbles start forming due to electrolysis. Boa et al. [177] investigated bacterial cell lysis using electric field. E. coli was lysed by applying electrical pulses and release of GFP was observed at intensity of 10 V/m and such release was finished within first few pulses if the intensity was 12.5 V/m, however these intensity values were just approximation and real intensity value could be higher. Hung et al [178] investigated the effect of different osmotic environment and electrical conditions on cell lysis and DNA release and concluded that hypotonic solution of 75mM glucose solution and AC voltage of 100 V at frequency of 10KHz.

Droplets based devices are also used in electrical lysis methods to increase efficiency. Delange et al. [179] designed a microfluidic device to lyse the cell using electric field right e before encapsulation in the droplet then lysozyme solution was added to digest bacterial cell wall. E. coli was also used in this device and it was observed that 99% cells remain intact without electric field and to 104 V/m, 70% cells were lysed.

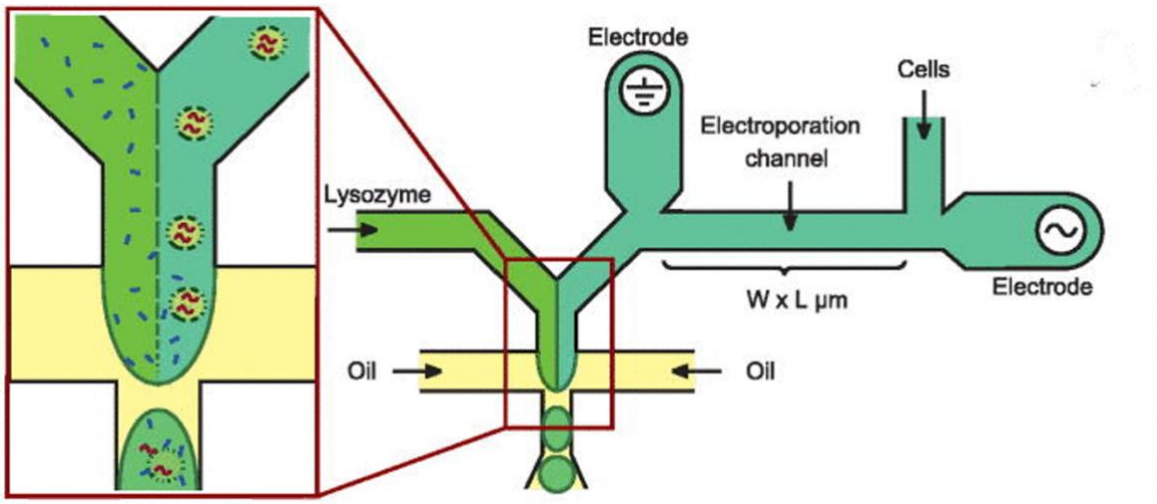

Figure 0.16: Schematic of electrical lysis device. Adapted from [179]

Laser lysis:

Laser microsurgery has proved that cell membrane can be ruptured while keeping the interior of cells intact [180]. Laser lysis works by producing localized cavitation bubble then the bubble expands and finally it collapses causing cell membrane to rupture. Rau et al. [181] used time resolved imaging and mechanical analysis to study the physics and kinetics behind cell lysis and bubble deformation. It was found that site of bubble formation defines the starting point of lysis. And it is the fluid flow during bubble expansion that causes lysis when the formation point is close to cell but in case the laser is away the cell lysis is due to shock wave.

Wan et al. [182] used a UV light array and titanium oxide particles. By exciting electron in valance band by UV, electron hole pair was created which reacts with aqueous solution to create free radical which reacts with proteins in cell membrane and lyse the cell. However, this process is very slow. Huang et al. [183] reported microfluidic device for optically induced cell lysis and nucleus extraction. Lysis efficiency was around 80.9% and overall nucleus extraction efficiency was 60%. In this study multiple light spots were projected from digital projector with intensity of 3.2W cm^{-2}, which caused cell lysis and kept the nucleus intact.

Thermal lysis:

Thermal lysis utilized high temperatures to denature the membrane proteins and disrupting the cells to release intercellular products but the temperature should be controlled such that it does not denature the cell proteins or the products.

Lee et al. [184] developed a microfluidic device for cell lysis and DNA amplification. Microheaters and sensors were used to lyse the cells in 2 minutes by at 95°C. Extracted DNA is then amplified in micro-PCR chamber and the device showed that sample pretreatment for DNA amplification can be automated. Packard et al. [185] reported a thermal lysis of E. coli on microfluidic chip. A serpentine channel was used for cell lysis and membrane compromise, protein and DNA release were used to quantify the extent of lysis. Significant cell lysis was observed for temperature of 95°C for residence time as short as 3.75 seconds and extent of lysis comparable to standard techniques were noticed for temperature greater than 65°C with residence time between 1 and 60 seconds.

Natalaya Privorotskaya et al. [186] demonstrated the use of microcantilever device for cell lysis. Cells were immobilized on microcantilever and were lysed in 30 s by utilizing localized heating. It was very effective for mammalian and bacterial cells but failed to rapture cell wall some microorganisms. Burkland et al. [187] developed microfluidic chip for highly controlled lysis of bacterial cells. In this work iron oxide nanoparticles were used and heating was accomplished by applying alternating magnetic field. Exposure to alternating magnetic field caused bacteria to thermally lyse and extracted DNA was later amplified downstream. This device could reach temperature up to 110°C and lysis efficiency of 90% was reported. Baek et al [188] also used alternating magnetic field for heating but used nickel as heating element. The amount of protein extracted were proportional to applied magnetic field and lysis percentage was almost 80%.

Comparison of lysis methods on chip:

Various methods described above have their advantages and disadvantages. Chemical lysis is cheapest methods with high efficiency and is also easier to handle but require more time and needs extra steps in downstream processing to remove reagents used for lysis. Laser lysis methos is very expensive and therefore only limited to lab scale experiments. Thermal lysis, though being the oldest technique, has limited application because of heat diffusion which can denature the protein of interest. Mechanical lysis uses the same phenomenon that is usually used in large scale but the fabrication of devices is quite complex and expensive. Electrical lysis, though being quite selective, has challenged like bubble formation and heat generation and also the electric potential required to lyse cell wall is quite high.

Table 1.3: Summary of Lysis methods. Adapted from [189]

Method	Efficiency	Time	Technical difficulty	Cost
Chemical	High	Slow to moderate	Low	Low
Mechanical	Medium	Moderate	Medium	Medium
Electrical	High	Fast	High	High
Laser	High	Very fast	Very high	Very high
Thermal	Medium	Moderate	Medium	Medium

1.8.3 Separation:

After the release of intercellular product, the next step is concentration and purification of the product. Microfluidics have been used for separation of biomolecules due to its fast-processing time low volume consumption and also the ability to translate these results obtained at microscale to larger scale operation. Hence microfluidics has been playing vital role in process development and almost all the unit operations in case of bioprocessing has been scaled down.

Pinto et al. [190] demonstrated the use of nanoliter scale device for multimodal chromatography. The device was used to for analysis and optimization of process with very low amount of resin and were able to test various conditions of pH and conductivity using a very fast essay time. Pons Royo et al. [191] designed a milliscale device for continuous precipitation of proteins and screened various conditions for precipitation and concluded that multiple addition of the precipitating agent leads to better results. This device was not only able to screen various conditions with low residence time but also less reagents consumption as compared to full scale process development. Silva et al. [192] demonstrated extraction of monoclonal antibody using aqueous two-phase system (ATPs) on a microfluidic chip and concluded the process is faster at microscale while the yield remains the same as in macroscale. M.J et al. [193] used a microfluidic device to screen out various ATPs system for purification of Human Immunodeficiency Virus (HIV)-like particles and concluded that PEG-ammonium sulfate was the most promising system for this purification. The results obtained at microscale were consistent with batch scale methods which highlights the use of microscale platform for process development.

Huft et al. [194] reported a multilayer, fully integrated PDMS-based chromatography device for purification of fluorescent labelled DNA fragments and this device was capable of automated sample loading, programmable gradient generation, separation, fluorescent detection, and sample recovery. Chan et al. [195] demonstrated pressure driven liquid chromatography on a PDMS chip, by separating dextran and BSA using silica beads. Yang et al. [196] reported integrated PMMA microfluidic chip for isolation and quantification of proteins from human sera using affinity chromatography and anticipated that the device can be used to detect up to 10 biomarkers simultaneously provided that number of immobilized antibodies are increased, which shows the potential of microfluidic devices in parallel

processing of various conditions simultaneously. Nge et al. [197] designed an ion exchange monolith in a microfluidic device for preconcentration of protein before electrophoretic separation and concluded that enrichment factor increased by over 10-fold in comparison to without pre-concentration. A Javidanbardan et al. [198] deigned a round section chromatography column with integrated impedance sensor and compared the performance of microcolumn with 1 mm prepacked column and concluded that pre-packed column showed better results than microcolumn but designed microcolumn showed significant improvement in efficiency and reproducibility over previously reported columns and microcolumns could replace single unit optimization methods with holistic methods.

1.9 Thesis outline:

This thesis is divided into five chapters: the first chapter (Introduction) gives a brief overview of bioprocessing and the most common approaches being used for manufacturing and purification of bioproducts. Then basics physics about microfluidics is explained, followed by micro/nano manufacturing techniques and microfluidics components used. Finally, a review of microfluidics applications particularly microbioreactors, cell lysis and separation are presented.

The second chapter (Microbioreactor) entails design and fabrication of microbioreactor and proof of concept experiments to show the working in fed batch and continuous mode and finally optimization for growth of E. coli. This work has been submitted in special edition of Micro nano engineering journal.

The third chapter (Lysis and aqueous two-phase extraction) is based on downstream processing, describing the design and fabrication of lysis and liquid0liquid extraction module and determining conditions to lyse E. coli and partition GFP in aqueous two-phase system (ATPS).

The fourth chapter (Integrated device for continuous downstream processing) is concerned with design fabrication and working of integrated device where production of protein, lysis of cells and ATPs can be done on single chip. This work has been submitted to Separation and Purification Technology.

Finally, thesis ends with conclusion and potential for future work to further optimize the integrated device and its potential to screen conditions of further downstream processing.

2

Microbioreactor

2.1 Introduction:

This chapter focuses on design fabrication and optimization of microbioreactor for growing *E. coli*. Biomanufacturing process start with the production of biomolecule and microorganisms are widely used. Yield of the product and efficiency of the process depends on various factor like choice of strain, optimum growth conditions and medium used. Although upstream processing has been the focus of great advancements over the past few decades and had led to high titers of bioproduct but there is still need to develop platforms that can screen conditions faster and in economical way.

The applications of microfluidics have already been explored in the field of microbioreactor and advantages of microscale like low reagents consumption, high surface area to volume ratio, precise control over conditions like temperature and parallel processing have been exploited to develop microbioreactors. However, most of microbioreactors described in literature are operating in fed batch or batch mode and are primarily used to understand the growth rate dependence on various process factors with little to no focus on the impact on downstream processing. In order for the bioreactors to be integrated with downstream processing it must work in continuous mode with constant supply of cells and bioproducts.

In this work a microbioreactor is designed with a volume of 60 μL and have been operated both in fed batch and continuous mode for over 30 hours. The microbioreactor has been fabricated in PDMS which eliminated the need of separate channel to supply oxygen as PDMS is permeable to air. A peristaltic pump is used for constant circulation and mixing therefore there is no need of an external stirrer. As the proposed microbioreactor can be operated in continuous mode, it has the ability to be coupled with downstream processing which the main goal of this work.

2.2 Method and Materials:

2.2.1 Chemicals and biologicals:

E. coli bacterial strains BIVU0811 with the pMAB1-GFP-C-lytA plasmid were kindly supplied by Biomedal (Seville, Spain). LB broth cell culture medium was obtained from Nyztech. 1mg/mL fluorophore fluorescein isothiocyanate (FITC) was purchased from Sigma-Aldrich, St Louis, MO, USA. A Milli-Q® water purification system was used for water supply for all the experiments (Millipore, Bedford, MA, USA).

2.2.2 Design and Fabrication of Microfluidic device:

The microfluidic device is composed of two layers: fluid layer to grow cells and control layer with all the actuation component.

2.2.2.1 Fluid layer design:

Fluid layer design is based on growth parameters of *E. coli* and required output micro-chemostat. The goal of this work is to develop a microbioreactor which can be integrated to downstream processing modules like cell lysis and extraction, hence it should be able to grow *E. coli* with optimal cell concentration and produce product of interest. It should also provide an easy approach to change the growth conditions especially dilution rate in this case. The design must meet basic characteristics of the organism to grow. In case of *E. coli* temperature of 37°C is required and the doubling time of *E. coli* is about 30 minutes.

For *E. coli* critical dilution rate was calculated to be $0.77h^{-1}$ and it is not recommended to be operated at critical dilution rate as minute changes in dilution rate leads to high changes un growth. Using these parameters, the volume of microbioreactor was to found to be 60μL. Cross section of fluid layer was kept 1mm × 1mm. However, at the seat of valves the depth was kept 100μm because a PDMS membrane will be used to open and close the valve and deflecting a PDMS membrane by 1 mm require a high pressure that is beyond the operation limit of PDMS. The channels were of square shape except at valve location where round profile were used as PDMS membrane will deflect in round shape and will not be able to fully close a rectangular channel.

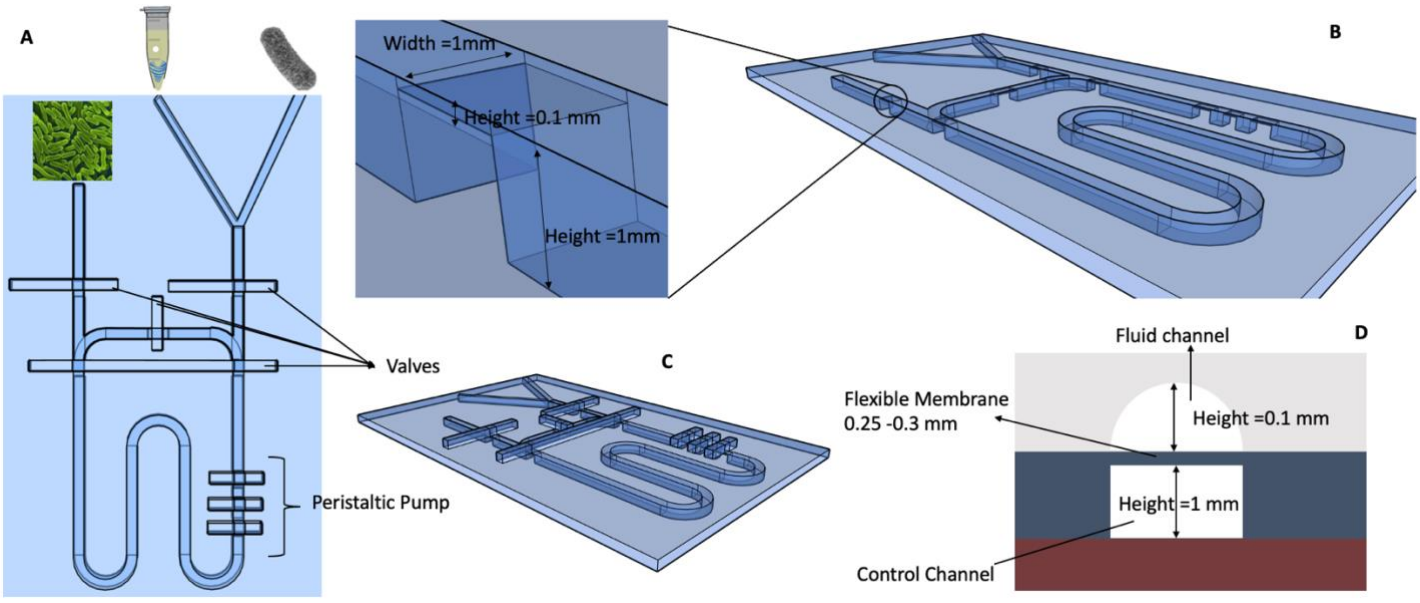

Figure 0.1: A) schematic of micro-chemostat; B) perspective view with critical dimensions; C) perspective view with fluid layer and control layer; D) showing the round profile in the fluid layer at valve/pump locations to ensure closing of valves by deflecting PDMS membrane.

2.2.2.2 Control layer design:

Though a chemostat requires continuous input and output the valve must be used to control the input and out of the fluid layer. Also, continuous circulation of medium and cells is required needing an integrated pump on chip. There have been many valves and pumps perpendicular channel separated by that have been designed for microfluidic devices but the simplest valves require two perpendicular channels separated by a PDMS membrane and is called Quake valve. This operation if the valve is quite simple, a positive pressure is applied on one side of the membrane which deflects the membrane and collapse the other channel thereby blocking the flow. When the pressure is released, the membrane goes back to its original position. The valve operation depends on factors like applied pressure, channels depts and membrane thickness and profile of the channel to be closed. In this work the channel depth was kept at 100μm with round cross section and membrane thickens of 250-300 μm was used with actuation pressure of 100-130KPa.

When three Quake valves are used together and actuated successively, they give rise to peristaltic motion. Three valves were used like that to make peristaltic pump. The valve channels were 1mm × 1mm square cross section.

2.2.2.3 Fabrication procedure:

Due to the mm-size dimensions of the channels, computerized numerical control (CNC) milling was used for fabricating mold out of PMMA using CAD file and later on PDMS structures were casted from PMMA mold. CNC milling has short turnaround time and channel with varying dimensions can be created in a single run because the diameter of tool and depth of cut can be changed during fabrication.

For the fluid layer micro milling technology (Minitech 3, Minitech machinery Corp., Norcross, GA, USA) was used to create a negative polymethylmethacrylate (PMMA) master mold. To create rectangular channels of 1mm×1mm a flat end mill of diameter 0.4 mm was used with depth increment of 0.3, 0.3, 0.3 and 0.1 mm. As previously described the round profiles were required at at valves location and for that purpose 1 mm round end mill was used but the depth was kept at 100 µm. Feed rate was varied between 150 mm/min to 600 mm/min at 16000 rpm. A negative master mold was fabricated to ensure round profiles. Then a positive polydimethylsiloxane (PDMS) countermold was fabricated by pouring PDMS and crosslinking agent in 5:1 weight ratio (Sylgard 184 silicone elastomer kit, Dow Corning, USA). It was baked at 70°C for 3 hours to increase the stiffens of the mold. Then PDMS was peeled and baked at 200°C for 1 hour which further increase the stiffness of PDMS mold. Finally, PDMS structures were fabricated from the PDMS countermold by pouring 10:1 ratio of PDMS to crosslinking agent and baking at 70°C for 90 minutes. The choice of processing parameters of positive PDMS mold (countermold) makes it easier to separate the two PDMS layers.

In case of the control layer, a positive PMMA mold was fabricated using flat endmill of diameter 0.4 mm to 2 mm. Feed rate was varied between 150 mm/min to 600 mm/min at 16000 rpm. Then PDMS structures were directly prepared using aforementioned protocol. The control layer was punched for access to both control channels and fluid channels using a 20-gauge needle. To complete the structure, first the control layer was sealed using oxygen plasma against a 250-300 µm thick PDMS membrane and then the fluid layer was sealed to the previously sealed structure. In order to improve sealing devices were kept at 70°C for 1 hour before using for experiments.

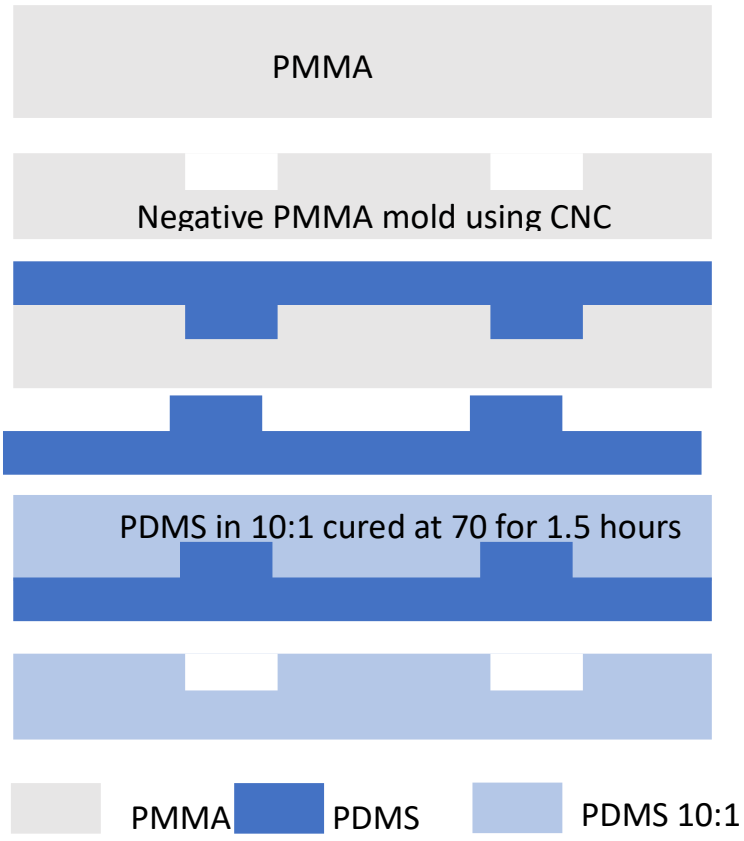

Figure 0.2: Fluid Layer fabrication process

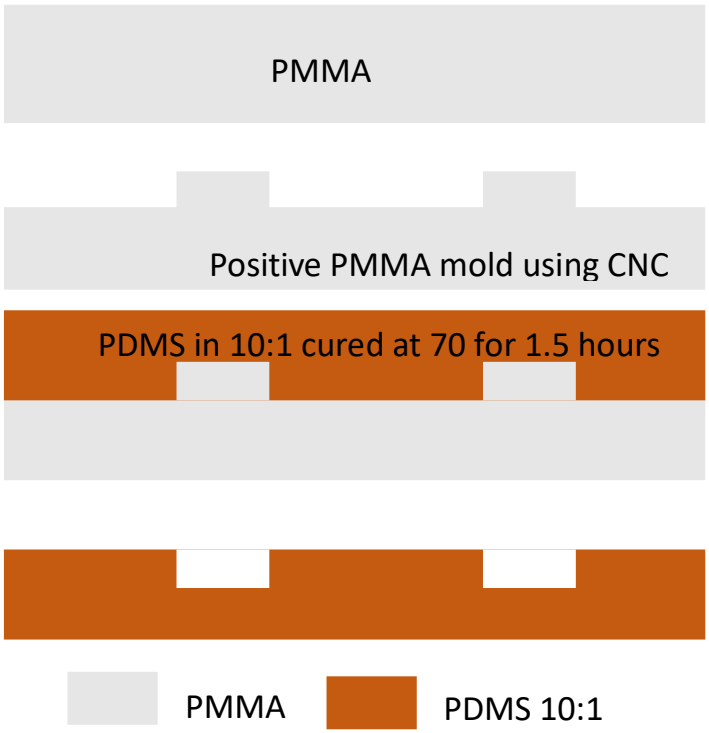

Figure 0.3: Control layer fabrication process

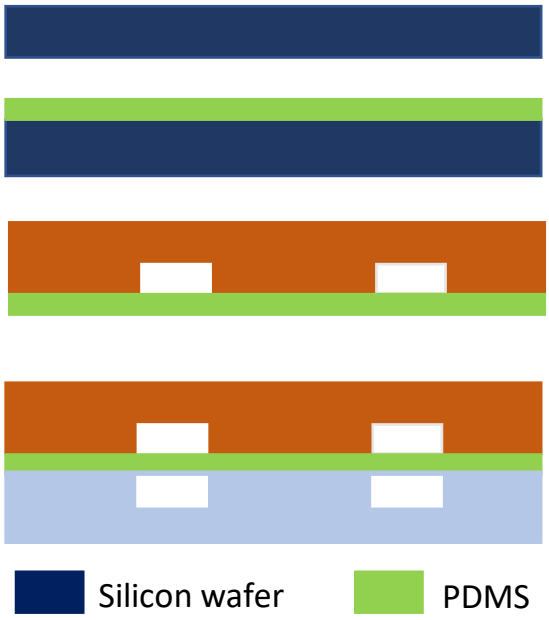

Figure 0.4: Complete Structure

2.2.3 Valve control:

Control channels were filled with milli Q water using capillary tubing and then connected to compressed air at 100 KPa for 1.5 hours. As the control channels has no output so this process removes the air in channels and water replaces that air. Then the valve operation is performed by pressuring that water. If this step is eliminated then the air from control layer will permeated through PDMS membrane causing air bubbles in the fluid layer and disrupting the flow. Two 4-port solenoid air controllers were used to control the airflow into the control layer. Each port was connected to a control channel through plastic tubing, and controlled through a custom circuit.

2.2.4 Fluorescence image acquisition:

Proof-of-concept experiments were performed with fluorophore fluorescein isothiocyanate (FITC) (FITC 90%, Sigma-Aldrich, St. Louis, MO, USA) (1 mg/mL) using an inverted fluorescence microscope coupled to a CCD colour camera with a filter cube with a band-pass excitation of 460-490 nm (blue) and a long-pass emission of 520 nm (green). All fluorescence images were acquired with a 1 s exposure time and zero gain and processed using ImageJ (National Institute of Health, Bethesda, MD, USA).

2.2.5 Cell counting:

In order to observe cell growth over time, a cell solution sample was extracted and inserted into a straight microfluidic channel (of section 350 µm ×100 µm). A grey scale image was acquired using exposure time of 10 ms and gain of 7.2 dB. The greyscale image was converted into binary image using a MATLAB script. Then that black and white image can either be processed using ImageJ (National Institute of Health, Bethesda, MD, USA) or MATLAB script. The use of each software was compared and the results were in agreement from two sources so MATLAB script was employed for image analysis for cell counting.

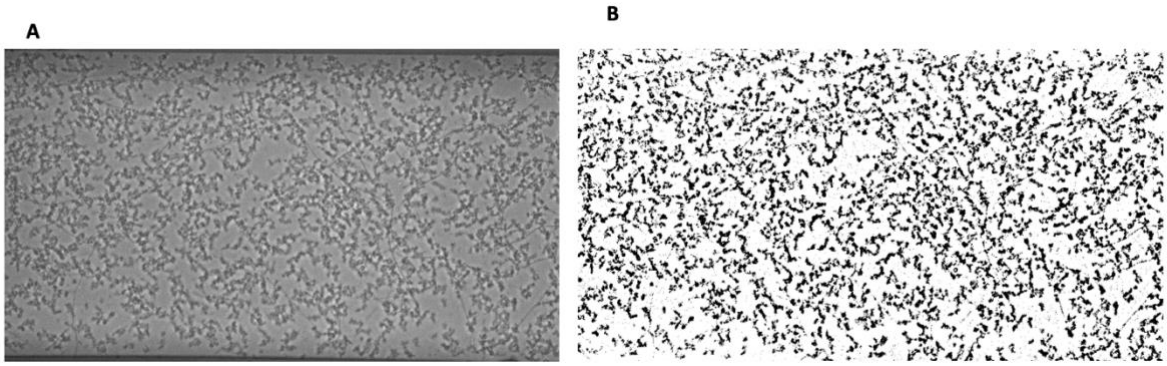

Figure 0.5: Conversion from (A) grey scale to (B) binary image

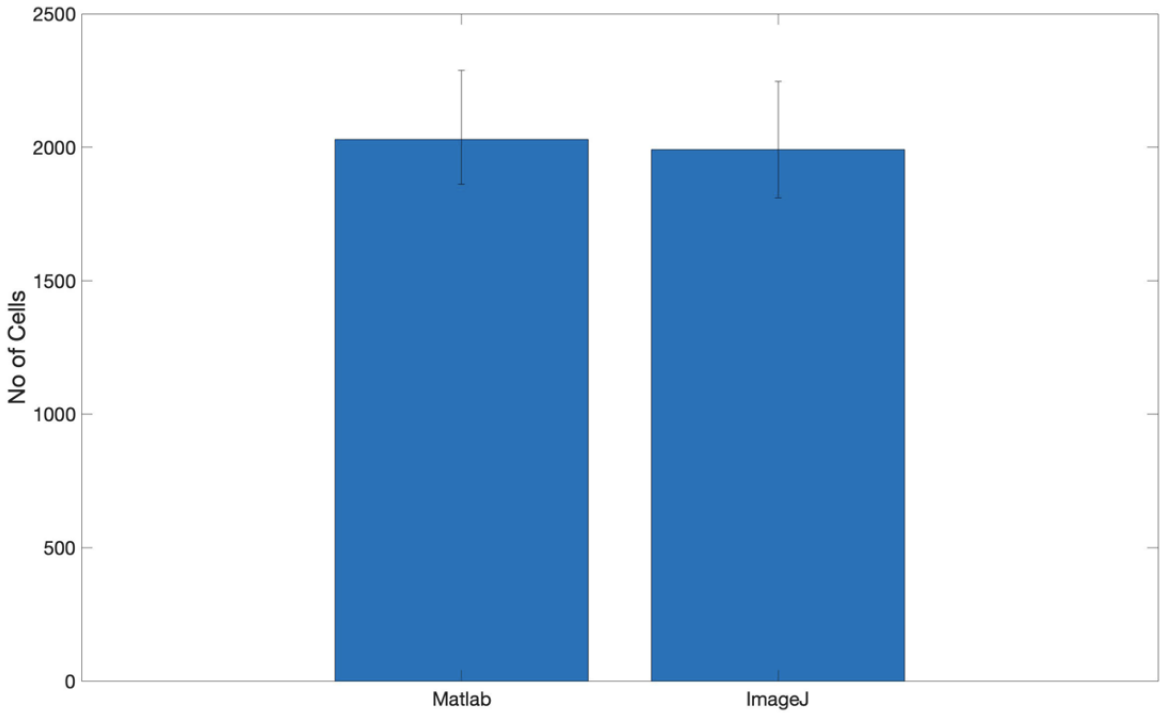

Figure 0.6: Comparison between Image J and MATLAB

2.2. 6 Operation of microbioreactor:

After sealing the device, connectors and tubing were sterilized using UV for 30 minutes. Then valve priming was done to get rid of all the air in control channels. Already autoclaved medium was used to completely fill the fluid channel and valve actuation and pump, medium was circulated at 37°C and then pre-inoculum was injected under continuous circulation.

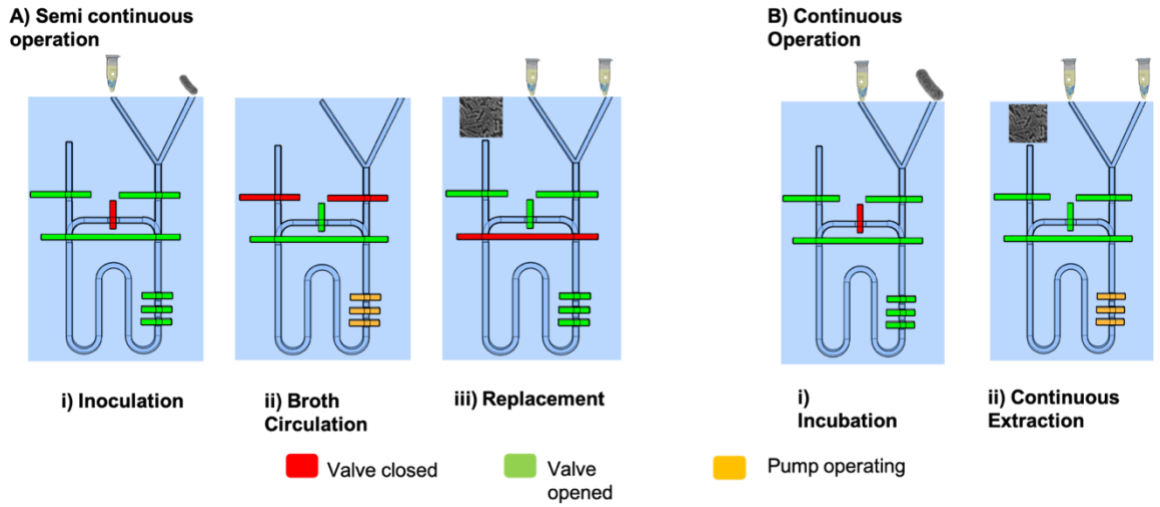

Figure 0.7: A) schematic of semi continuous operation: i) insertion of medium and cells; ii) closed circulation of medium using peristaltic pump to ensure cell growth; iii) extraction of cells with the volume being replaced with fresh medium; B) schematic of continuous mode operation: i) injection of cells and medium; ii) circulation using peristaltic pump and continuous injection of fresh medium and cell extraction

Figure 2.7 shows the schematic of operation. In case of semi continuous mode operation, first is the injection of medium and a known quantity of cells (Fig. 2.7A(i)). After that, the peristaltic pump is started which results in circulation of medium and cells in a closed loop (Fig. 2.7A(ii)). During this step cells start multiplying and enter the growth phase. After a certain period of time a section of the chemostat is isolated by closing the valve shown in Figure 2.7A(iii) and cells are replaced by medium in that portion.

In case of continuous mode operation as shown in Figure 2.7B, first step is similar to that of the batch mode where cells and medium are inserted (Fig. 2.7B(i)), however after that there is a continuous supply of medium and cells are extracted continuously (Fig. 2.7B(ii)).

2.3 Results and Discussion:

The micro-bioreactor fabricated is composed of a fluidic layer, containing the bioreactor channel, and a control layer, containing the actuation components, namely four Quake valves and one peristaltic pump, according to the design shown in Figure 1. This microbioreactor has been designed for cell growth and recombinant protein production, using as a model system *E. coli* cell harboring a pDNA encoding for the expression of the green fluoresce protein (GFP), aiming at continuous cell production and ultimately continuous GFP production.

2.3.1 Peristaltic pump flowrate:

In order to characterize the functioning of peristaltic pump, the flowrate of the pump was measured against actuation frequency. For this purpose, the reactor was filled with DI water mixed with colored dyes and the pump was operated at known frequency using Arduino programming and the time required for fluid to travel between two specific points in the channel was noted. As the channel dimensions are known the volume was calculated and flowrate was deduced by using the volume and time measured. For each pump frequency measurements were repeated several times to calculate the average flow rate. Figure 2.8A shows the sequence of valve actuation to operate the integrated peristaltic pump. Figure 2.8B shows the variation of flow rate with frequency. It can be seen the flow rate starts increasing with increasing frequency and after ~15 Hz it becomes constant. For further experiments a pump frequency of 10 Hz was used.

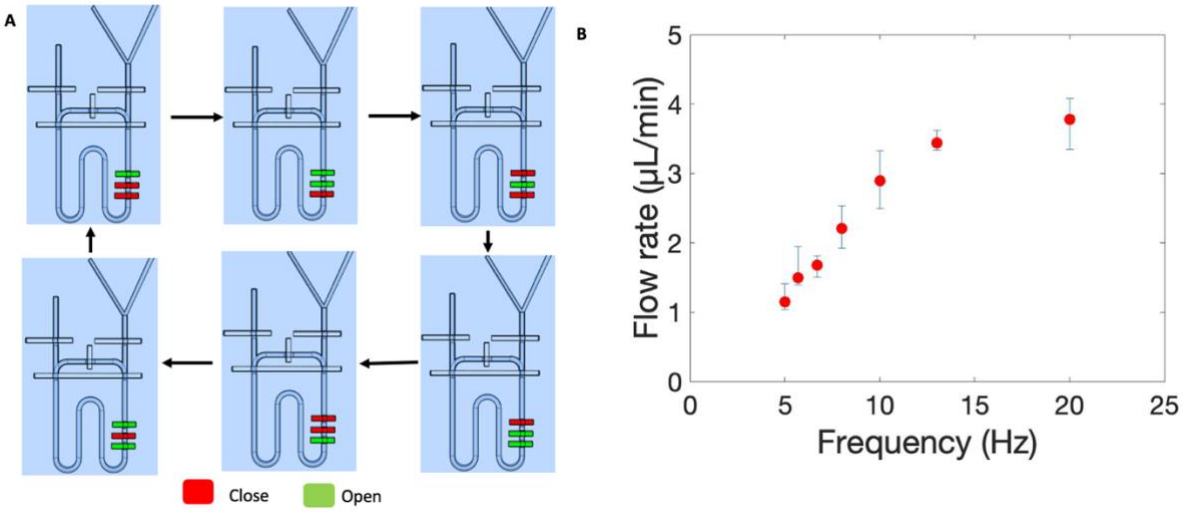

Figure 0.8: A) sequence of valve actuation to operate the peristaltic pump for circulation of fluid; B) flowrate of peristaltic pump vs frequency of actuation.

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2.3.2 Proof of concept:

In order to fully understand the working of microbioreactor both in semicontinuous mode and continuous mode, experiments were performed using fluorescein isothiocyanate (FITC) (FITC 90%, Sigma-Aldrich, St. Louis, MO, USA) (1 mg/mL) solution and fluorescence intensity was used to observe the flow paths.

2.3.2.1 Semi continuous mode:

In case of semicontinuous mode, the cell grows for a known period of time and then a known volume of cells is removed is replaced by fresh medium. In order to understand this first the fluid channel was completely filled with fluorophore solution the intensity was measured at 5 locations as shown in figure 2.9B. The average of these 5 values were taken and plotted in red in figure 2.9A with spatial variation.

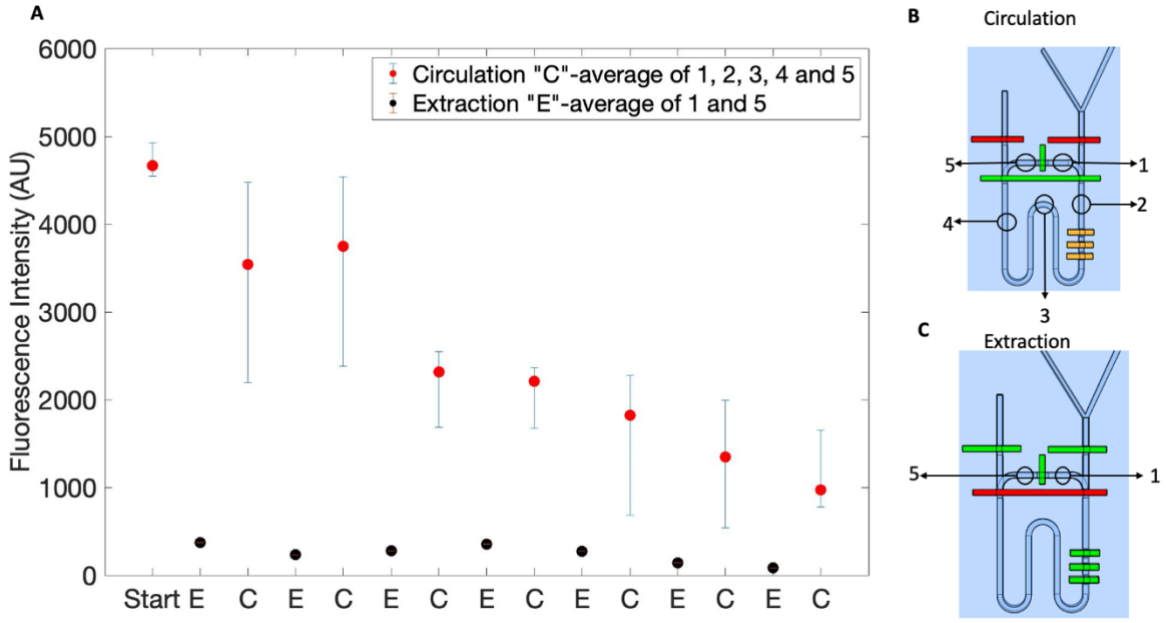

Figure 0.9: proof of concept experiment with fluorophore: at first injection of fluorophore solution followed by circulation shown in B to homogenize then extraction of small volume by isolating a portion of micro-chemostat shown in C and replacing it with milli-Q water. The process is repeated until all the fluorophore has been removed.

After that, a portion of micro chemostat is isolated as shown in Figure 2.9C and the fluorophore is replaced by milli-Q water, which results in very low florescence signal at points 1 and 5.

It is plotted in black in figure 2.9A. Then, the peristaltic pump is run again to circulate the fluid and after some time fluorophore redistributed in the channel and measurement is taken at 5 locations again and plotted with spatial variation. Then some fluorophore is removed again and so on. The process is repeated until most of the fluorophore is removed. This shows how microbioreactor will be operated in batch mode but in that case, when a volume of cell is replaced by medium, cell will multiply and the device will not run of out cells.

2.3.2.2 Continuous mode:

In case of batch mode as circulation is done with inlet and outlet valve closed so the flow pattern is defined, however in case of continuous mode both inlet and outlet valves are to be kept open and there should be continuous supply of medium which can result in medium flowing directly to the outlet thereby resulting is no cell multiplication whatsoever. Hence proof of concept experiment was designed with fluorophore fluorescein isothiocyanate (FITC) (FITC 90%, Sigma-Aldrich, St. Louis, MO, USA) (1 mg/mL) solution again.

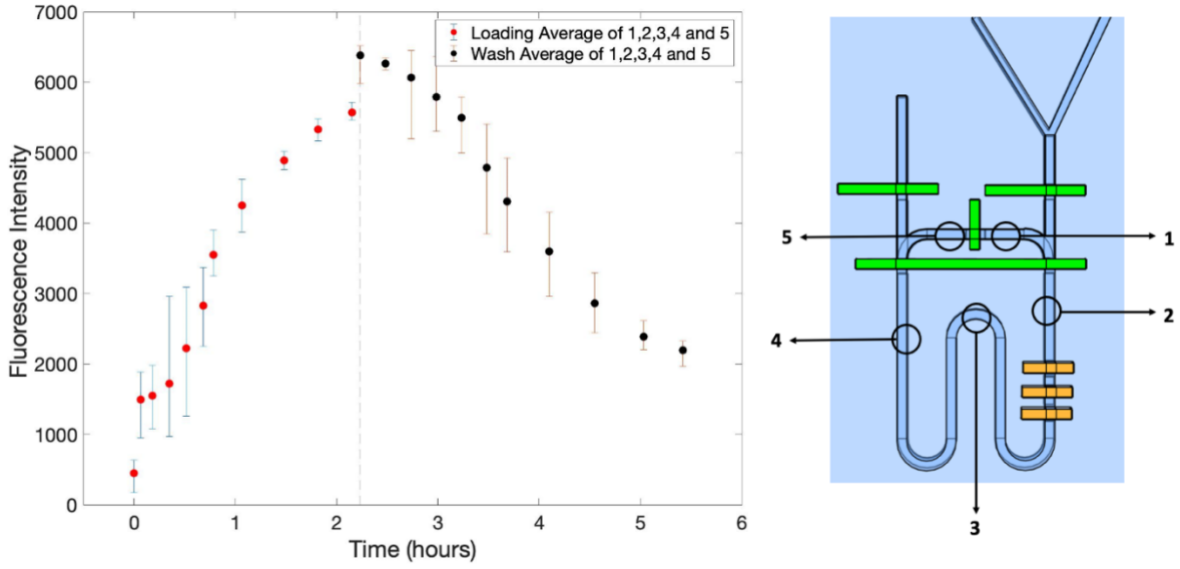

Figure 0.10: proof of concept experiment in continuous mode using fluorophore solution: initially fluorophore is loaded with continuous circulation and reduction in spatial variation is evidence that with pump operating fluid tends to go towards 2 rather than moving to the outlet and same trend is observed in washing (where milli Q water is used to flush out the fluorophore)

In this case the device was filled with milli Q water first and then fluorophore was injected with continuous circulation using peristaltic pump. Then fluorescence intensity was measured at location 1,2,3,4 and 5 and average was calculated and plotted with spatial

variation. As the time goes by the average intensity start increasing but the spatial variation was decreasing. As it can be seen in figure 10 that the ideal flow path is inlet-1-5-outlet. But if the fluorophore is flowing is taking that path, then average intensity will increase but the spatial variation will be high as there will be no fluorophore at location 2, 3 and 4. But with decreasing spatial variation it is evident that the injected fluorophore solution is moving sequentially from point 2 to point 3, to point 4, to point 5 and finally to point 1.After an hour of circulation is elapsed the fluorescence signal becomes more and more uniform which can be easily explained by the flow pattern. The suction created by the peristaltic pump is enough to direct flow into the channel towards point 2 rather than by passing it to outlet (i.e., towards point 1). So, most of the flow is recirculated into the channel and a portion of it goes to the outlet to compensate for the inflow.

The process was repeated by flowing milli Q water to replace fluorophore solution and the same trend was observed. Hence it was concluded from this experiment that microbioreactor can be used in continuous manner with continuous injection of medium and continuous extraction of cells.

2.3.3 Calibration of cell counting:

In order to quantify cell growth in microbioreactors the method described in section 2.2.5 was used. In order to check the validity of this method and whether it can distinguish between a very low concentration and high concentration a qualitative study was done. A sample of cells was diluted into 15 different concentrations starting 6.6% to 100% cells and labelled as sample number 1 to sample 15 with 1 being the most diluted and 15 being the 100%. Optical density (O.D) of these samples was measured using spectrophotometer (T70 UV/VIS Spectrophotometer, PG Instruments). Then the same samples were injected into the microchannel and cell were measured using image analysis described in 2.2.5.

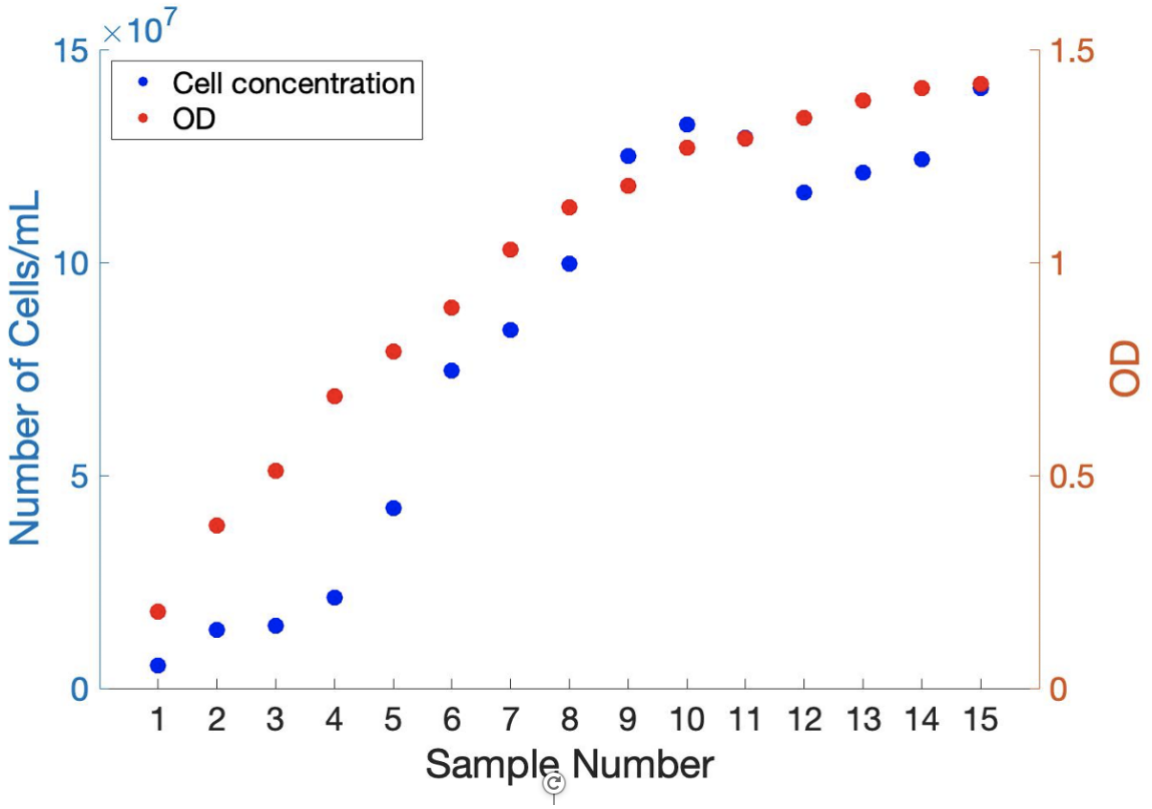

Figure 0.11: a sample of known OD is diluted and OD is measured using spectrophotometer (red dots) and then same sample are used for cell counting using MATLAB script (blue dots) to make sure cell counting is suitable for analysis.

As shown in figure 2.11, the results from conventional spectrophotometer and image analysis are qualitatively similar and hence making this analysis suitable for cell concentration measurement for microbioreactor experiments.

2.3.4 Cell growth in semi continuous and continuous mode:

The microbioreactor chip and all the tubing connectors and syringes were sterilized using UV for 1 hour. Then medium was injected into the channels and was recirculated to ensure that the peristaltic pump was working. A sample of cell solution incubated overnight in a shake flask (the inoculum) was injected afterwards using a syringe pump.

In semi-continuous operation mode, a cell sample corresponding to 15% of the bioreactor volume was taken out either every 1 hour or every 2 hours depending on the experimental runs. The sample is then used injected in straight channel and cell concentration is measured by image analysis. As shown in figure 2.12A, both batch extraction frequencies reached a steady cell density in 24 hours. However, it can be noticed that 1-hour sampling frequency had very low cell count in the start but after reaching steady state it had high cell

growth. This can be explained by the enhanced availability of nutrients, as the broth is replaced more frequently which means that fresh growth medium is available more frequently, thus resulting in higher growth rate. Indeed, for the 1-hour frequency, the replacement rate, and consequently the feeding rate, is double that of the 2-hour frequency, leading to a higher concentration of substrate and, according to Monod law, to a higher growth rate. However, in the case of the 2-hour replacement frequency, initially, the cell density is higher due to the availability of more time for cells to multiply before being removed, but later, the cell count is affected by the more limited supply of nutrients.

In case of continuous mode, the main variable is the flow rate with which medium is supplied and cells are extracted. Experimental runs using flow rates of 0.2, 0.5 and 1 µL/min all reached steady state as shown in figure 2.12B but the flow rate of 0.5 µL/min performed better than 0.2 and 1 µL/min. The explanation resides in the effect of the residence time and nutrient supply. Cell growth is hindered by the lower feeding rate and substrate availability, in the case of 0.2 µL/min, and by the low residence time, in case of 1 µL/min. At 0.5 µL/min, both residence time and nutrients availability are optimized hence a higher cell density is observed.

For *E. coli*, the critical dilution rate (D_{crit}) was calculated to be 0.77 h^{-1} [35]. Flow rates of 0.2, 0.5 and 1 µL/min correspond to dilution rates of 0.2, 0.5 and 1 h^{-1}. When the chemostat is operated at dilution rate higher than $D_{crit,}$ washout occurs. However, in this case there is cell adhesion to PDMS as previously reported by Zhang *et al.* that avoids complete washout as adhered cell still manage to multiply [26]. Optimum dilution rate (D_{opt}) is approximately equal to D_{crit} as well, but it is never recommended to operate at D_{crit} [35]. Hence maximum growth at 0.5 µL/min is expected as it is very close to optimum dilution rate.

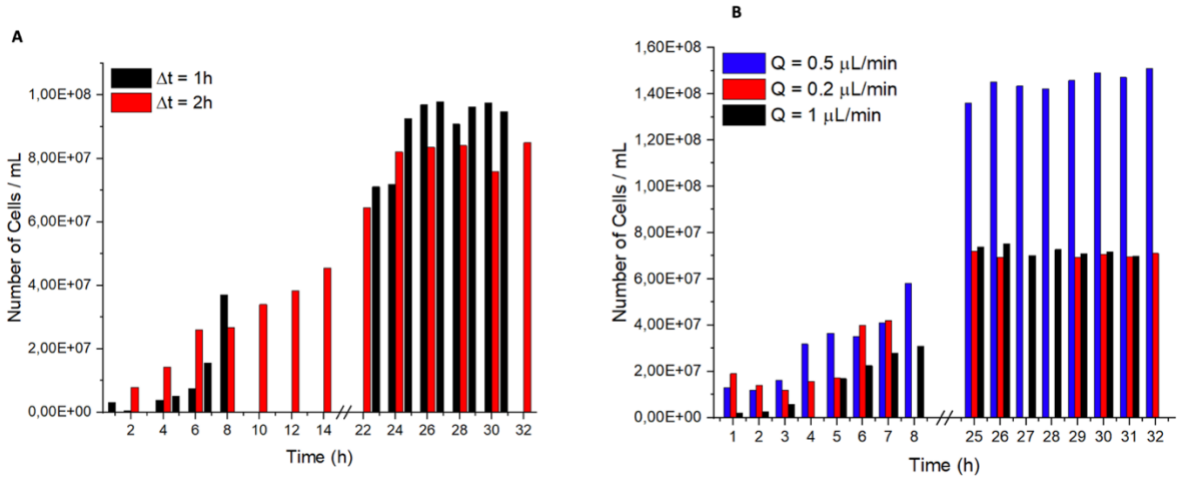

Figure 0.12: Cell growth over time: A) in fed batch mode at two extraction frequencies, both leading to a steady state cell growth over period of 24 hours; B) in continuous mode with two flow rate (for medium injection and cell removal), both leading to steady state.2.3.5 GFP production:

As bioprocessing involves production of a bio molecule usually a protein, GFP was chosen in this case for its fluorescence properties. As the plasmid of E. coli is already modified to produce the protein but inducers to start production. In this case medium with inducer (Sodium salicylate) was injected after 8-hour growth to ensure the production of protein. The experiment was kept running overnight and the sample was taken the next day.

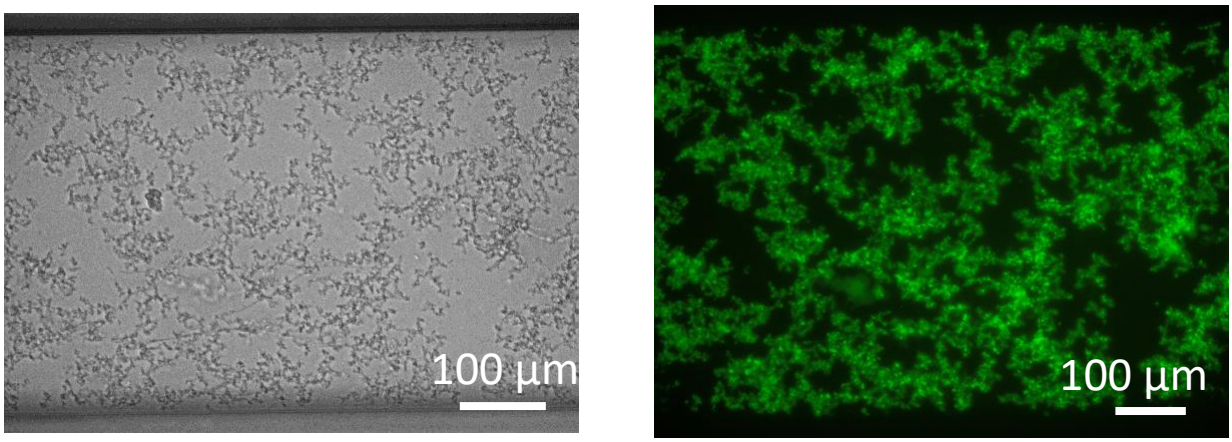

Figure 2.13: Greyscale image of cell sample and corresponding fluorescence image

Figure 2.13 shows an image of cell sample and corresponding fluorescence image taken at exposure time of 1s. By comparing these two images one thing is evident that cells were able to produce GFP and also there is no cross contamination as both images are quite consistent.

Just one such experiment was done here as proof of concept. Further results will be presented in coming chapters.

2.4 Conclusion:

This chapter focused on the design, fabrication and operation of a two layered structure microbioreactor, comprising a fluidic layer for cell grow and a control layer for actuation of valves and pump. The volume of the fluid layer was designed considering the growth characteristics of *E. coli*. The peristaltic pump was characterized for operation at various frequencies and then a frequency of 10 Hz was selected to be used in further experiments.

Proof of concept experiments were successfully performed with a fluorophore which allowed the demonstration of the working principle of the device in both feeding modes. It was also concluded that the suction created by the peristaltic pump was sufficient to maintain most of the flow in recirculation even when a syringe pump is continuously injecting fluid in channel. A cell counting method was also effectively developed using greyscale image processing and a MATLAB script and the validity of the method confirmed using samples of known cell concentration.

Finally, the device was operated for growing *E. coli* cells both in semi-continuous and continuous mode and it was observed that cell growth reaches a steady state after 24 h of operation. In the case of continuous mode operation, 0.5 μL/min (dilution rate of 0.5 h^{-1}) was found to be the most suitable flowrate allowing for the highest cell concertation. The device was kept running for a period of more than 30 hours which clearly shows that it can be coupled with further downstream processing. Lastly an experiment was performed using optimized conditions to demonstrate the production of GFP. Future step in this work would be to integrate micro-chemostat with further downstream processing so that the whole process can be optimized.

3

Lysis and aqueous two-phase extraction (ATPE)

3.1 Introduction:

Cell lysis is the next step in biomanufacturing process to release the product of interest. Several lysis methods exist on a large scale and most of them have been scaled down to be used in microscale. A brief review of the lysis techniques used on microscale is presented in section 1.8.2. Out of all the methods chemical lysis shows promising results on microscale due to its attributes like low cost, ease of handling and high efficiency [189]. Hence chemical lysis was selected to be done on chip for lysis of E. coli and it has been already used at microscale and the efficiency is compared for various conditions [199]. After lysis the next step is concentration of the product and ATPE is selected as the most appropriate technique be used on the modular chip as it allows integration of the upstream with the downstream process and continuous operation, besides having been studied widely in literature [192,193,200].

This chapter focuses on design, fabrication and operation of an integrated chip where cell lysis and extraction can be done continuously, envisaging integration of the DSP modular chip with the microbioreactor developed in chapter 2. A microfluidic chip is designed with two modules: a lysis module and ATPE module. Lysis module has two inlets one for the cell suspension and other for lysis solution and the outlet of the lysis module serve as inlet for the ATPE module where two other inlets are also used for injection of polymer and salt solution to form ATPE there by concentrating the protein of interest (GFP in this case) to one phase.

3.2 Methods and materials:

3.2.1 Chemicals and biologicals:

E. coli bacterial strains BIVU0811 with the pMAB1-GFP-C-lytA plasmid were supplied by Biomedal (Seville, Spain). LB broth cell culture medium was obtained from Nyztech. A Milli-Q® water purification system was used for water supply for all the experiments (Millipore, Bedford, MA, USA). BPER was purchased from thermo scientific. Sodium salicylate, PEG, and phosphate salts (K2HPO4 and KH2PO4) were purchased from Sigma-Aldrich.

3.2.2 Fluorescence image acquisition:

In order to observe cell lysis and partitioning of GFP an inverted fluorescence microscope coupled to a CCD colour camera with a filter cube with a band-pass excitation of 460-490 nm (blue) and a long-pass emission of 520 nm (green) was used. All fluorescence images were acquired with a 1 s exposure time and zero gain and processed using ImageJ (National Institute of Health, Bethesda, MD, USA).

3.2.3 Design and Fabrication of Microfluidic device:

An integrated device was designed for continuous lysis and extraction using ATPE, as shown in Figure 3.1. The lysis module exploits the difference between the diffusion coefficients of cells and GFP and the laminar flow profile which limits transversal mixing. The lysis module with two inlets one for cell suspension and the other for BPER (commercial lysis solution of E. coli). The channels in the lysis module are 30 cm long with square cross section of 50µm × 50µm and is based on optimization study done in literature [199]. The outlet of lysis module is then used as inlet for ATPE module with two other inlets one of PEG stock solution and other for phosphate stock solution. ATPE module has 30 cm long channel with rectangular cross section of 150µm × 50µm. In case of ATPE module the flowrate two stock solution and cell sample are adjusted to make sure the composition stays above the binodal curve and hence two phases are formed.

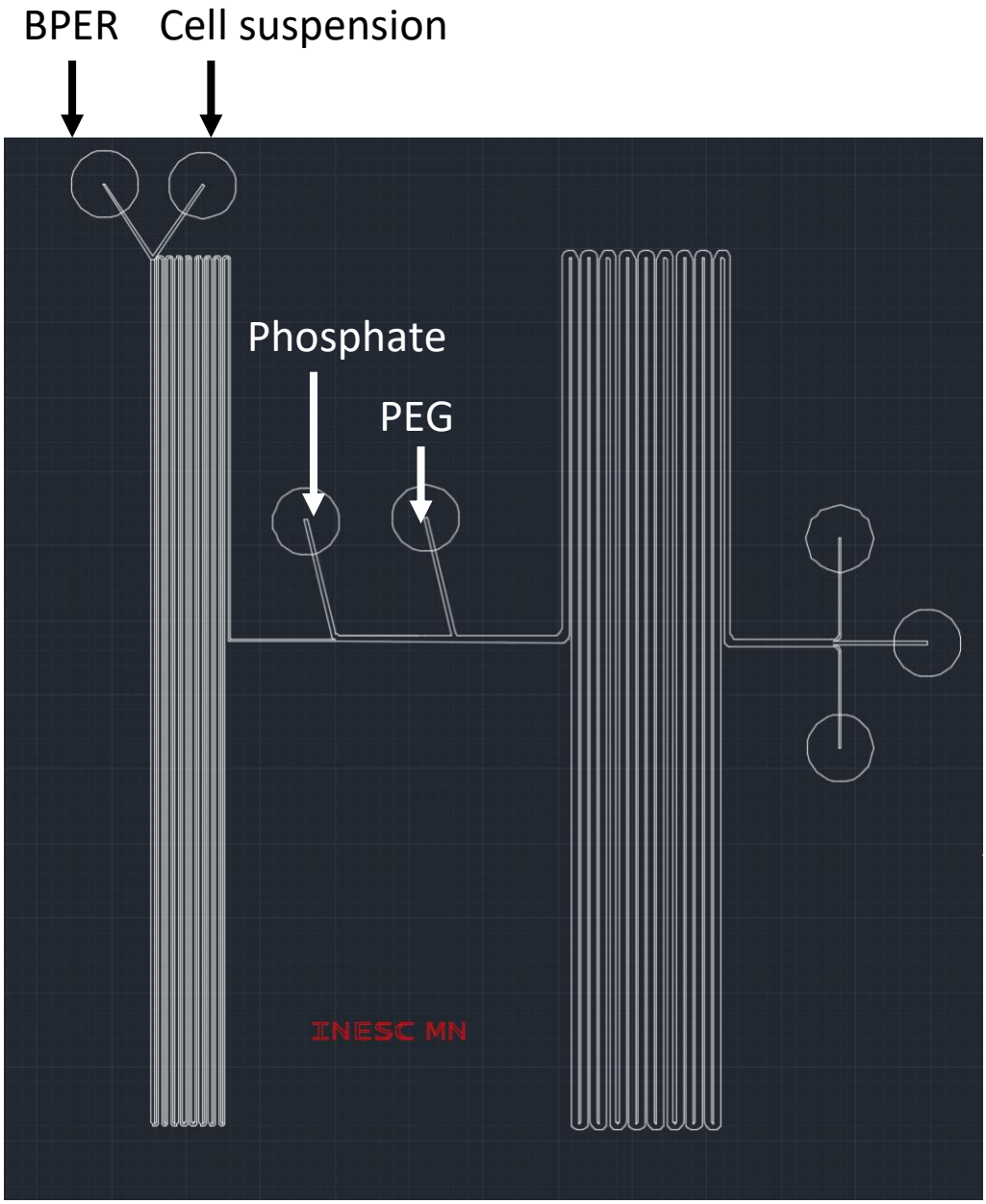

Figure 0.1: Schematic of device

Fabrication of the device:

Soft lithography was used for fabrication of the device. The process starts with preparing a 2-D CAD file. Fabrication process is done in 3 steps: A hard mask fabrication, an SU-8 mold fabrication and PDMS replicas.

For hard mask fabrication, 200 nm of Aluminum is deposited on corning eagle glass using sputtering (2000 Å; Nordiko 7000 magnetron sputtering system). After that a thin layer of photoresist (PFR7790G) is coated and pattered using direct write lithography system

(Heidleberg Instruments DWL II) and developed using developer solution. Finally, aluminum is etched using liquid etchant (TechniEtch Al80) and photoresist is stripped off.

The hard mask is used to fabricate SU-8 mold using photolithography. A 5 cm × 5 cm silicon substrate is first coated with SU-08 layer of thickness 50µm. Then this substrate is exposed to UV light (λ = 254 nm, 400 W) through previously fabricated hard mask. After development and hard bake a negative mold of the structure is obtained on silicon substrate.

Finally, SU-08 mold is used to fabricate several copies of the device using PDMS with curing ratio of 10:1 (Sylgard 184 silicon elastomer kit, Dow-Corning). The mixture is degassed and then poured on to the SU-08 mold and baked for 90 min at 70 $^{\circ}$C. The structure is the cut and inlets and outlets hole are punched using 20-guage needle. Then an oxygen plasma (Harrick Plasma PDF-002-CE) is used to seal the structure to glass slide.

3.2.4 Cell culture:

Recombinant, GFP producing E. coli strains were cultured according to the protocol of the supplier. Initially LB medium with concentration of 20g/L was autoclaved at 121°C for 20 minutes and then ampicillin (100mgL^{-1}) was added. A pre-inoculum was cultured overnight at 37°C with constant agitation at 250rpm. This culture was then used to inoculate 250mL of medium (already autoclaved and ampicillin added) at a starting OD600-0.1 at 37°C with constant agitation at 250rpm. When OD600=1 was achieved, gene expression was induced by addition of 1mM sodium salicylate and cultured for 5 hours at 20°C with constant agitation at 220rpm. Optical density was measured using a spectrophotometer (T70 UV/VIS Spectrophotometer, PG Instruments). The collected cell samples were centrifuged at 4000g for 15 min and then resuspended in 250mM Tris-HCL buffer of pH 7 and were used in microfluidic device later on.

3.3 Results and Discussion:

Lysis and ATPS modules were tested together where GFP was released from cells by chemical lysis and then concentrated/purified in ATPS module. The goal of this study is to observe the effect of lysis and ATPS module together on a single chip on GFP release, concentration and

purification. The overall goal of the thesis is to combine upstream (Cell culture and protein production) with the downstream (lysis and extraction) process, thus the single chip developed and tested in this chapter will be further integrated with the micro bioreactor developed in chapter 4.

3.3.1 Cell lysis module:

In downstream processing cell lysis becomes the first step if the product of interest in intercellular. As *E. coli* producing GFP is being used as a model system in this study hence cell lysis becomes the first step in downstream processing. Flow regime in the device is laminar which makes diffusion as dominant mode of mixing across the channel and the device exploits the diffusion coefficient between whole cells and free protein.

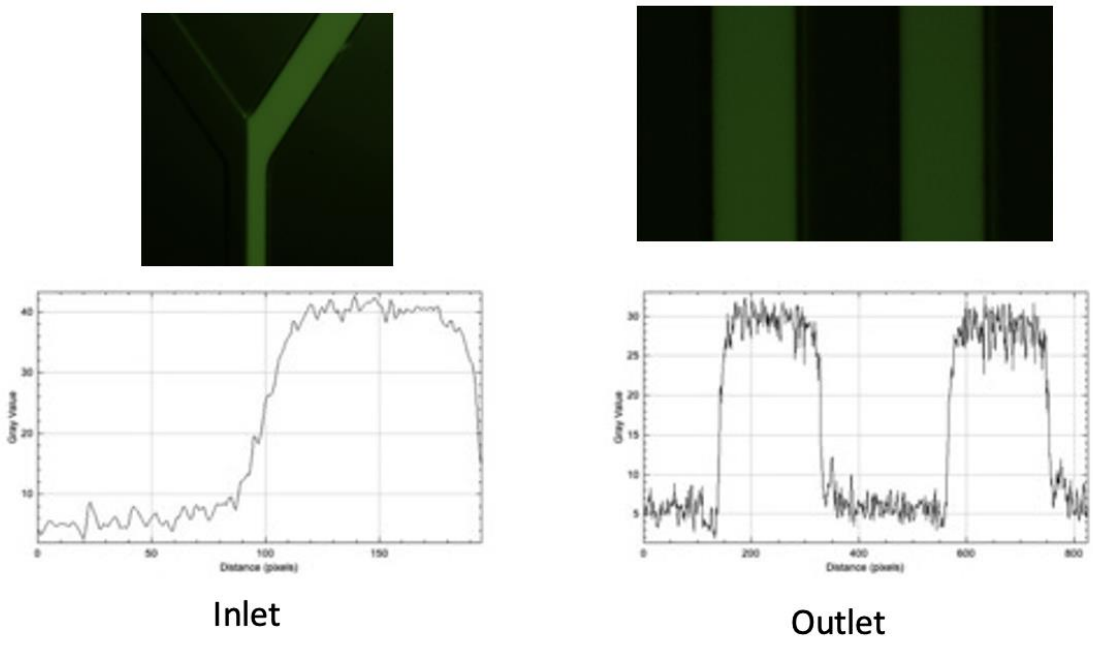

Figure 3.2:: Lysis: At inlet fluorescence on one side and outlet channel with a uniform profile which shows that free GFP has diffused throughout the channel

As shown in figure 3.2 at the inlet cell suspension is injected along with lysis buffer BPER. As the lysis buffer does not contain any GFP so there is no fluorescence on that side of channel while the other half of channel has florescence due to GFP present in cells. As the flow is laminar hence the dominant mode of mixing is diffusion. Due to diffusion across channel,

BPER starts disrupting the cells and free GFP is released. Now the free GFP start diffusing across the channel thereby giving a uniform fluorescence profile across the channel. The diffusion coefficient of E. coli is 2.5×10^{-9} cm^2s^{-1} while for GFP it is 8.7×10^{-7} cm^2s^{-1} and the diffusion length for E. coli is around 1300 cm and GFP to be 3.6 cm which clearly shows that channel length of 30 cm is enough for free GFP to diffuse but E. coli cannot diffuse fast enough. Hence the uniform profile at the end of lysis module is due to the fact that lysis took place resulting in free GFP. Fradique et al. [199] came to similar conclusion.

3.3.2 Aqueous two-phase extraction (ATPE):

Aqueous two-phase extraction is special case of liquid-liquid extraction in which both the phases used are aqueous making them very useful for biomolecule extraction. Aqueous two-phase system has been widely used for extraction of biomolecules. After the release of GFP by chemical lysis method the next step is to concentrate and purify GFP to one of the phases. Stock solution of PEG and phosphate was injected and the flowrate were adjusted so that the composition is above the binodal curve which favors the formation of two phases. As the GFP produced in this work is tagged with Lytag and thereby have the tendency to partition towards PEG rich phase. Micrographs were acquired both in bright field and using fluorescence microscope to observe the partition. In figure 3.4 a brightfield image of the channel is presented and it can be seen that there are two phases present with an interface. Salt rich phase is on right side while PEG rich phase is the left side. Figure 3.4 b is the same image taken under fluorescence microscope and it can be seen that the fluorescence is only on the left side and there is no fluorescence on the right-hand side. It was expected as all of the free GFP partitions to PEG rich phase leaving no GFP in salt rich phase.

In order to verify the results, the experiments were repeated with green dye mixed with DI water and is it is well known the green dye will be portioning to the PEG rich phase. When the experiment was repeated with same flowrate condition with green dye and it was seen that two phases formed with green dye portioning to PEG rich phase as shown in figure 3.3. In figure 3.3 it can be seen that there are two phases present with an interface between them. As expected, the green dye partitions to PEG rich phase while salt rich phase has no dye present. If figure 3.3 and 3.4 are considered together (as the experiments were done at same flow rate hence similar volume ratio is expected for the phases), it can be concluded that PRG rich phase has high volume than salt rich phase and all of the GFP is in PEG rich phase.

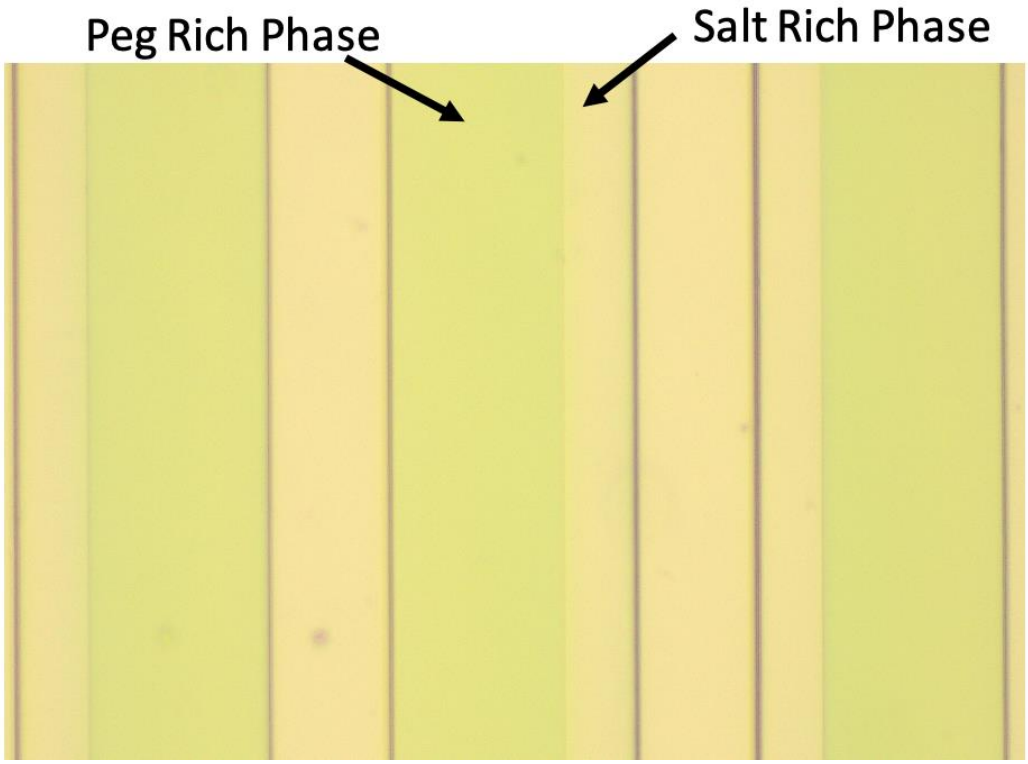

Figure 3.3: Identification of phases

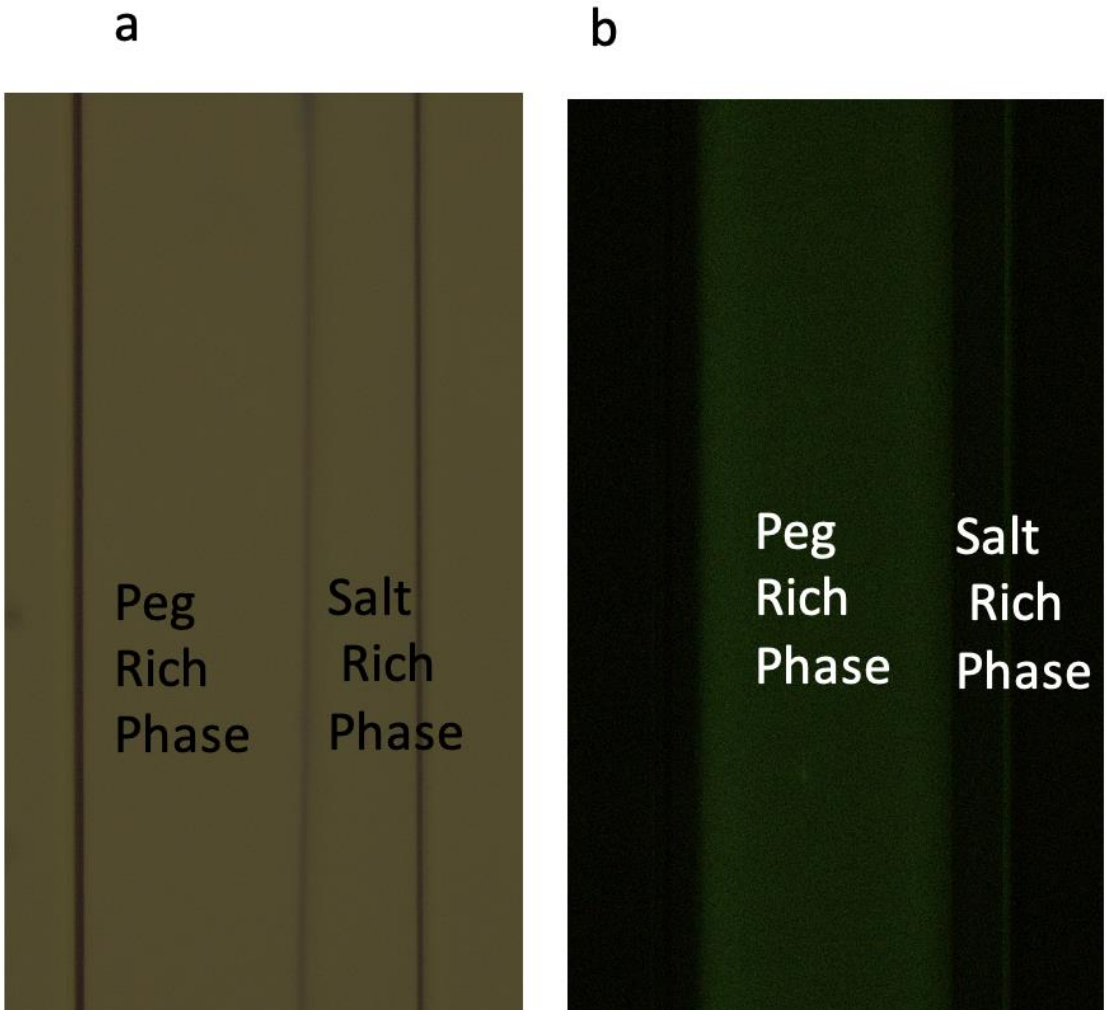

Figure 3.4: ATPE (a) brightfield image showing two phases (b) Fluorescence image showing GFP in PEG rich phase

3.3.3 Composition calculation:

In order to make sure the phases observed in microfluidic have same composition as suggested by binodal curve, a mini study was performed. In this study conductivity and refractive index were used to calculate the composition of the two phases. Same composition was mixed in an Eppendorf and flowrates were adjusted to get the same composition in microfluidic device. Both the phases were separated and then the composition was calculated using following protocol. Firstly, a calibration curve was obtained for conductivity and Refractive index of Phosphate solution and Refractive index of PEG solution.

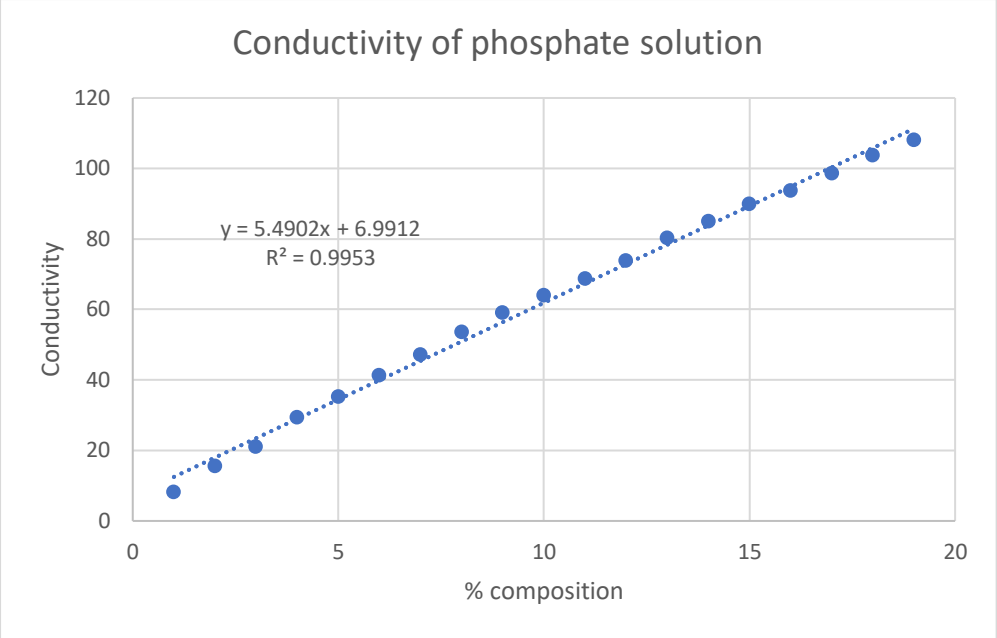

Figure 3.5: Calibration Curve for conductivity of phosphate solution

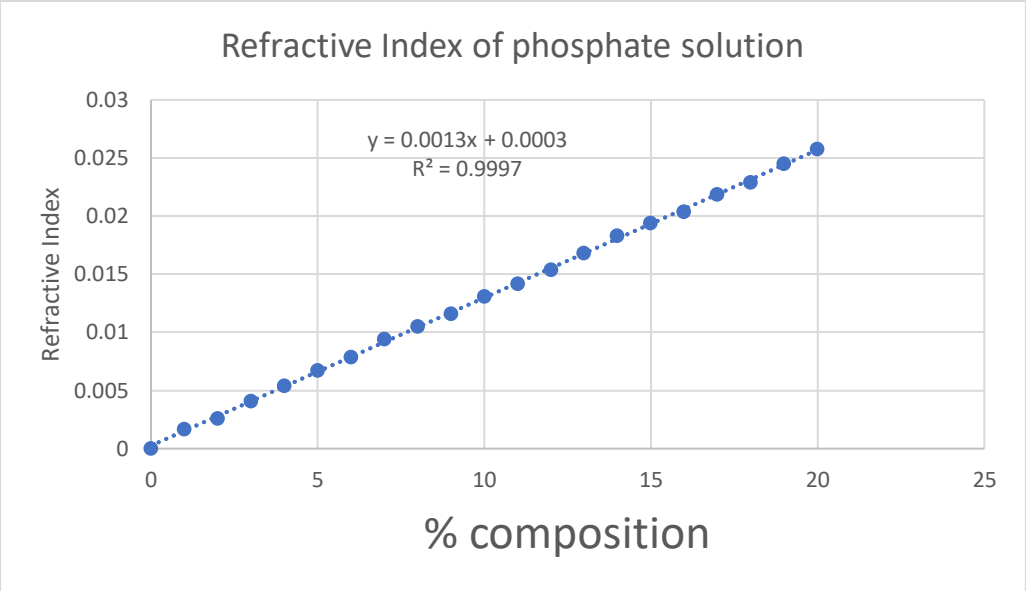

Figure 3.6: Calibration curve for refractive index of phosphate solution

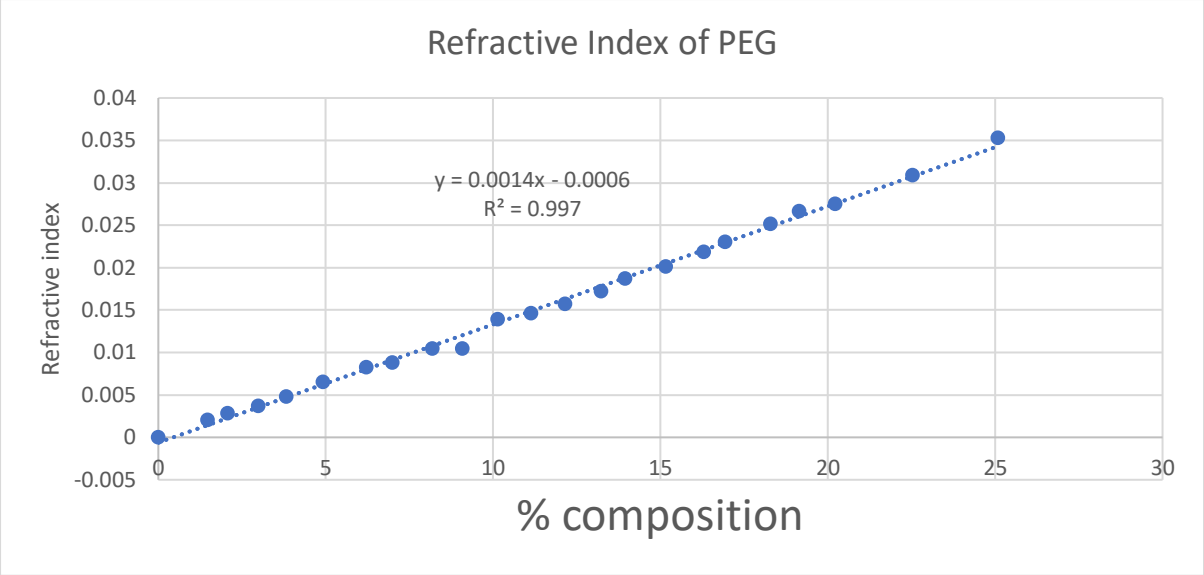

Figure 3.7: Calibration curve for the refractive index of PEG solution

After getting the calibration curve, conductivity and Refractive index of phases from batch and microfluidic experiments were measured. As conductivity of the phase is primarily due to present of ions of phosphate salt and PEG being a polymer has negligible conductivity so it can be assumed that the conductivity of phase is directly proportional to phosphate composition. So, conductivity measurement provided phosphate composition directly using calibration curve.

However, the refractive index is due to three components: water, salt and PEG. As water's refractive index is already subtracted for calibration curve same was done for the phases. From known salt composition the refractive index contribution was calculated and subtracted from the refractive index value of the phases thereby getting refractive index contribution of the PEG. And then using calibration curve of refractive index of PEG, PEG composition was determined.

Table 3.1: Composition Calculation

							Phases Concentration (%)	
	Conductivity	Salt Concentration	Refractive index contribution due to salt	Total Corrected Refractive index	Refractive index contribution due to polymer	Polymer concentration	PEG Rich	Salt Rich
Batch	10.44	0.62	0.0011	0.0064	0.0052	3.34	17.7	3.33
Microfluidics	11.9	0.89	0.0014	0.0061	0.0046	2.88	15.1	4.69

Table 3.1 summarizes the whole calculation process for composition calculation and as it can be seen that the values obtained both from batch and microfluidic are in agreement with very small error that can be contributed to the collection of phases in microfluidic as there can be cross contamination of phases at outlet.

3.4 Conclusion:

This chapter focused on design, fabrication and operation of an integrated lysis and ATPE module. GFP was produced using E. coli as host and then a chemical lysis solution BPER was used on chip for complete lysis of cells and release of GFP. Due to GFP's fluorescence property, fluorescence microscopy was used to observe the lysis and by comparing the diffusion coefficient of E. coli and GFP it was concluded that a uniform fluorescence profile at the end of lysis module is due to the diffusion of free GFP rather than the diffusion of E. coli.

The microfluidic device also allowed for the concentration/purification of free GFP in using ATPS. PEG-phosphate system was used in this device to partition GFP to the PEG rich phase. And the composition of both the phases were calculated in batch and in microfluidic device and were found to be consistent proving that microscale ATPS can be scaled up to be used for application is industry.

Maintaining the goal of process integration, the microfluidic device was developed as a modular component. Considering the continuous nature of this process and the manufacturing techniques used, it is possible to integrate these modules with upstream and downstream. For example, instead of having the cell culture in shake flask, the device designed in chapter 2 can be used to culture E. coli and its output fed directly into the lysis module. The goal of this study was to observe the effect of cascading two modules and finalize the optimum conditions that can be used while designing an integrated platform with microbioreactor, lysis and extraction module on same chip which will be presented in next chapter.

4

Integrated device for Continuous bioprocessing

4.1 Introduction:

Following the work from previous chapters, this chapter focuses on the integration of the previously described modules; micro-chemostat and lysis and extraction module. In this way it becomes possible to look at the whole process as a whole rather than screening and optimizing one module at a time. Though biomanufacturing process is viewed as combination of unit operations and each unit operation can have effect on the subsequent ones so that the optimal conditions for each unit operation may not necessarily correlate with the optimal conditions of the whole process.

As micro-chemostat was designed to work in continuous mode providing a continuous flow of cell culture, it is possible to combine all the three modules on a single chip. In this chapter the focus has been on continuous integrated processing and therefore an integrated microfluidic device is designed with three modules. A PDMS-based microfluidic device is realized which consists of a chemostat with integrated peristaltic pump for circulation of medium, a 30 cm channel for chemical lysis and an aqueous two-phase extraction module for purification and concentration of the desired protein to one phase. The device has been successfully operated for over a week with continuous extraction of cells producing Green fluorescent protein (GFP), that are subsequently lysed to recover GFP and finally purify and concentrate GFP to a PEG-rich phase using a PEG-phosphate ATP system for aqueous two-phase system.

4.2 Methods and materials:

4.2.1 Chemicals and biologicals:

E. coli bacterial strains BIVU0811 with the pMAB1-GFP-C-lytA plasmid were supplied by Biomedal (Seville, Spain). LB broth cell culture medium was obtained from Nyztech. A Milli-Q® water purification system was used for water supply for all the experiments (Millipore, Bedford, MA, USA). Bacterial Protein Extraction Reagent (B-PER ®) was purchased from Thermo scientific. Sodium salicylate, PEG, and phosphate salts (K_2HPO_4 and KH_2PO_4) were purchased from Sigma-Aldrich.

4.2.2 Fluorescence image acquisition:

In order to observe cell lysis and partitioning of GFP an inverted fluorescence microscope coupled to a CCD colour camera with a filter cube with a band-pass excitation of 460-490 nm (blue) and a long-pass emission of 520 nm (green) was used. All fluorescence images were acquired with a 1 s and 250 ms exposure time and zero gain and processed using ImageJ (National Institute of Health, Bethesda, MD, USA).

4.2.3 Design and fabrication of integrated microfluidic bioprocessing platform:

The integrated platform consists of three modules: a microbioreactor to culture E. coli producing Green fluorescent protein (GFP); a lysis module to release the protein; and finally, an extraction module to concentrate GFP to one phase using aqueous two-phase system (Figure 4.1(a) and 4.1(b)). The microbioreactor is composed of two layers: a fluid layer containing the bioreactor channel and a control layer containing the pneumatic actuation components. The fluid layer was composed of 1 mm× 1 mm square section channel with a total volume of 60 µL. The control layer contains channels of 1 mm× 1 mm square section (Figures 4.1(b) and (c)). The depth of fluid channel is 1 mm everywhere except at the points where valves are used. As valves are to be closed by deflecting a PDMS membrane the depth of channels at valves was kept at 100 µm. Moreover, the profile of the channel at the valve locations was also kept round because the membrane would not be able to completely close a rectangular cross-section channel (Figure 4.1(d)). The outlet of microbioreactor is directed

into lysis module. The lysis module exploits the difference between the diffusion coefficients of cells and GFP and the laminar flow profile which limits transversal mixing. The optimized dimensions of the channel were obtained using scale out from previous study reported by Fradique *et al* [199]. A scale out factor of 4 was used since the minimum dimension in the micro milling system is limited to 100 μm. So, the lysis channel has a square cross section of 200 μm × 200 μm and a length of 30 cm. After cell lysis, the next step is the extraction module where an aqueous two-phase system (ATPs) is used. The extraction module, has a total of 3 inlets, one coming from the lysis module and two more for generating an aqueous two-phase system (ATPs) with a 30 cm long channel having a cross section of 600 μm × 200 μm.

Computerized numerical control (CNC) milling was used for mold fabrication. For the fluid layer micro milling technology (Minitech 3, Minitech machinery Corp., Norcross, GA, USA) was used to create a negative polymethylmethacrylate (PMMA) master mold. For rectangular channels of micro-chemostat a flat end mill of diameter 0.4 mm was used and for round profile a 1 mm round end mill was used. For the lysis and ATPs modules a flat end mill of diameter 0.2 mm was used. Feed rate was kept around 150 mm/min at 16000 rpm. For the control layer a negative PMMA mold was fabricated using a flat end mill of diameter 0.5 mm with feed rate of 150 mm/min at 16000 rpm. Then, a positive polydimethylsiloxane (PDMS) counter mold for both fluid and control layer was fabricated by pouring PDMS and crosslinking agent in 5:1 weight ratio (Sylgard 184 silicone elastomer kit, Dow Corning, USA), baking at 70°C for 3 hours and, after removing from the PMMA mold, baking the PDMS counter mold at 200°C for 1 hour. The PDMS structures were fabricated using 10:1 ratio of PDMS to cross linking agent and baking at 70°C for 90 minutes. Inlet access were punched in the control layer using 20-gauge needle and finally the control layer was first sealed against a PDMS membrane of thickness between 0.25-0.3 mm and then the fluid layer was sealed to already sealed

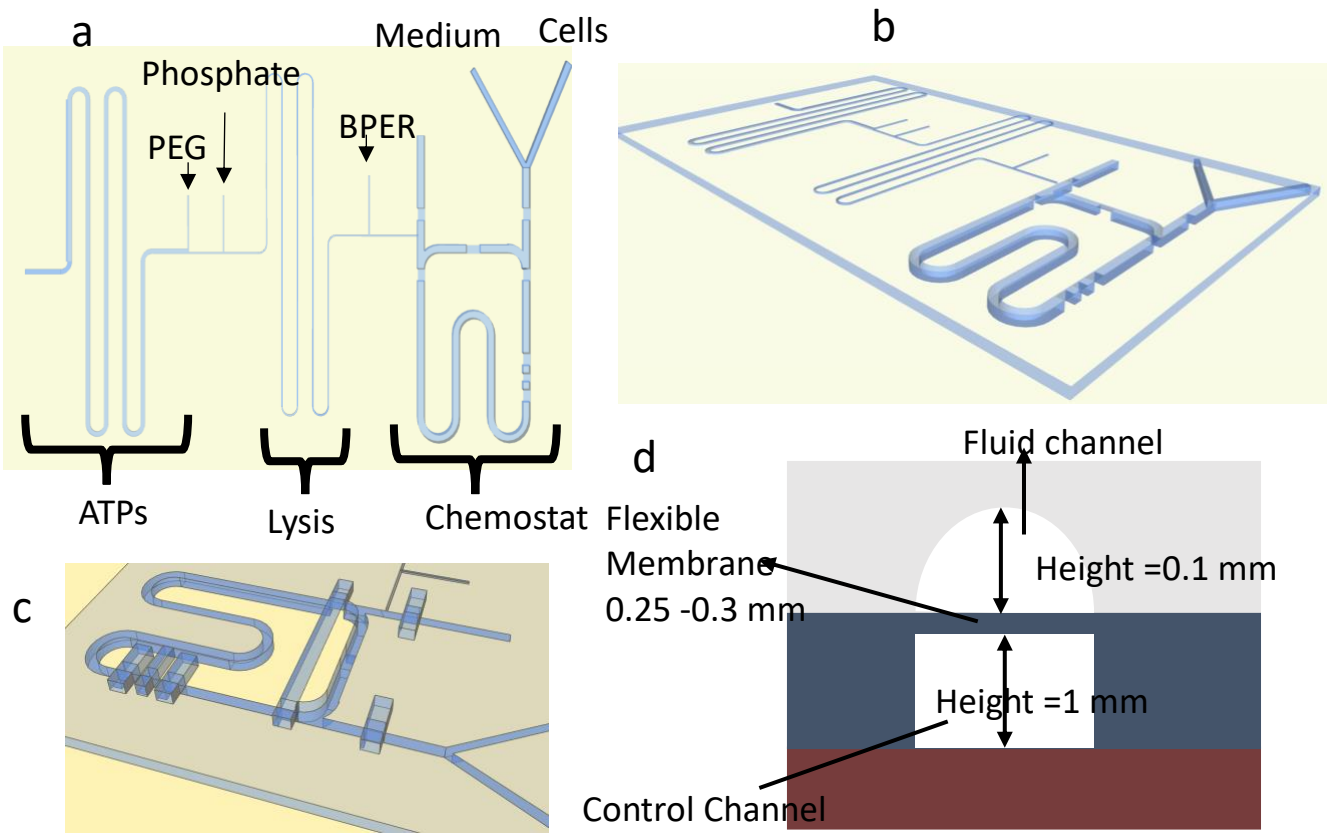

Figure 0.1:(a) Layout of integrated device with micro-chemostat, lysis module and ATPs module. (b) Perspective view. C) Perspective view with fluid layer and control layer: D) showing the round profile of the fluid layer to ensure closing of valves by deflecting PDMS membrane

structure. Connections to the fluid layer was made by punching through the membrane using same 20-gauge needle.

4.2.4 Microfluidic operation and image acquisition and processing:

Syringe pumps (New Era Pump Systems, NE-300) were used to inject medium, BPER and phosphate and PEG stock solutions. Fluorescence images were acquired with 1 s exposure time and 250ms exposure time in micro-chemostat and 1s exposure in lysis and ATPS module and processed using ImageJ (National Institute of Health, Bethesda, MD, USA). In the micro-chemostat images were acquired at different locations and the fluorescence intensity was taken to monitor the GFP production for over a week. In the lysis, images were acquired right after injection of BPER and just before injection of PEG and Phosphate and in ATPS images were acquired right after injection of PEG and phosphate and then at equidistant throughout the channel and then at the outlet. In the lysis assays, images the were analyzed by plotting a fluorescence profile across the microchannel. In ATPS assays average fluorescence intensity

in PEG rich and phosphate rich phase and the calculating partition coefficient by ratio of fluorescence intensity in PEG rich to phosphate rich phase.

4.3 Results and Discussion:

This objective of this work is to develop an integrated microfluidic device for simulating the continuous production of a biological at a micro-scale, allowing the analysis of how the cascading effects that changes in each operation affects the final process performance. To demonstrate the concept, three modules were designed and optimized individually and then combined in an integrated chip, as described below.

4.3.1 GFP production using E. coli as host using a micro-chemostat module:

Microbioreactor can be operated in semi continuous and continuous mode both as described in chapter 2, however to allow easy integration with downstream processing it must operate continuously. The microbioreactor was optimized for continuous production of E. coli cells as described in chapter 2 and it was observed that a flowrate of 0.5 µL/min of medium at the inlet of the micro-chemostat (and, in steady-state, the same flowrate of cell solution at the outlet of the micro-chemostat) provide the maximum cell concentration because the maximum growth rate and optimum dilution rate for E. coli are close to 0.5 h^{-1}. For these experiments the microbioreactor was incubated with medium and 10 µL of pre-inoculum and peristaltic pump was run for circulation for a period of 8 hours at 37°C. Then medium with 2mM sodium salicylate (inducer) was injected continuously at 0.5 µL/min which prompts E. coli to start producing GFP at 30°C. This step was continued overnight. As the experiments were run for an extended period of time micrographs were taken at various portion of chemostat to see the evolution of GFP production over several days.

Figure 4.2 shows the average fluorescence profile over time which is constant. Initially images were acquired using exposure time for 1s and it was observed that fluorescence signal is quite uniform. In order to make sure that uniformity is not due to saturation, exposure time of 200 ms was also used and it gave the same results. Constant fluorescence profile shows that cell concentration and GFP production remains constant throughout and there is availability of constant cell concertation for downstream processing throughout the operation.

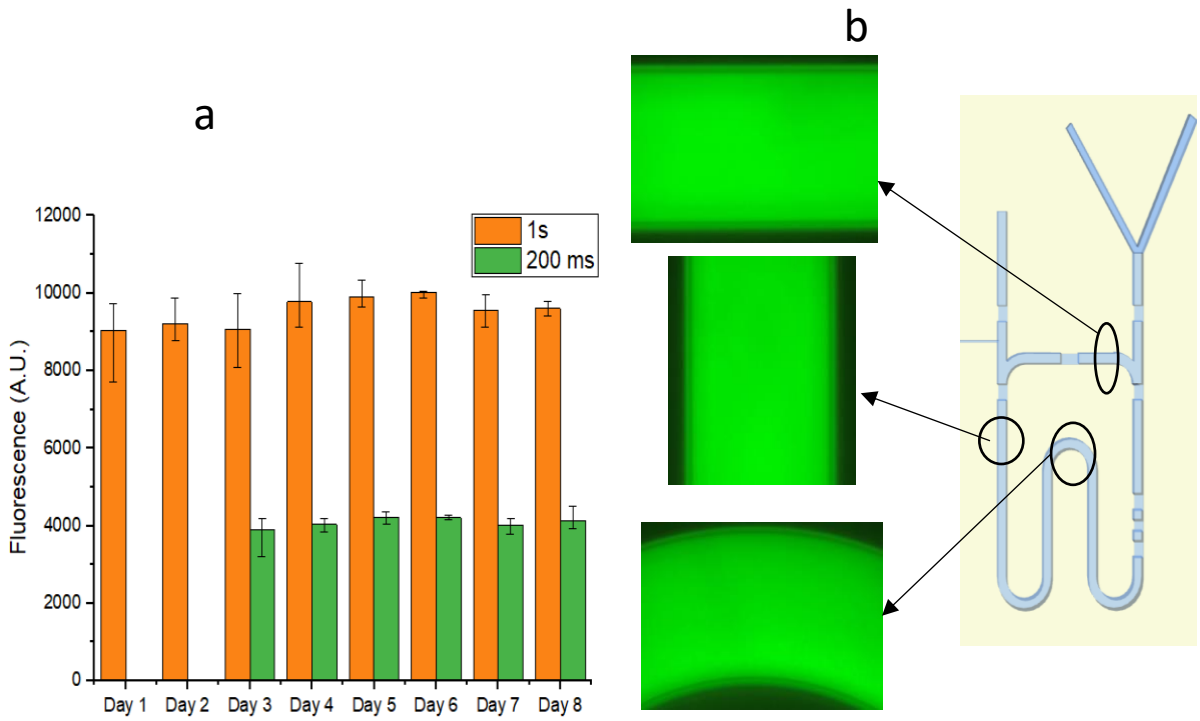

Figure 0.2: (a) Evolution of GFP production shown by florescence profiles in chemostat over time with two different exposure times. (b) Micrographs taken from chemostat at various locations for 1s exposure time

After optimizing the cell growth and GFP production, the role of the next module was to achieve lysis to recover the GFP.

4.3.2 Lysis microfluidic module

If the desired product is intercellular the second step in bioprocessing is cell lysis to extract the product of interest. Various cell lysis procedures can be used on chip but chemical lysis is one that is most suitable for high throughput. Fradique *et al.* [199] have previously demonstrated the efficacy of using chemical lysis on chip where various chemicals and conditions were tested and it was concluded that B-PER®, a commercially available lysis solution, provides the highest lysis efficiency and a 1:1 ratio of the flowrate of lysing agent to cell suspension was the best condition. Hence these optimized conditions were used in this work. Output from the microbioreactor was directed into lysis module where B-PER®, a commercially available lysis solution, was injected. Flowrate of BPER was kept at 0.5 µL/min to match the chemostat output flowrate. It can be seen in Figure 4.3 that the fluorescence profile is only in one half of the channel where cell suspension is flowing, and no

fluorescence can be seen in the other half where BPER is flowing just after the BPER injection. But as the flow progresses BPER diffuses to the E. coli solution stream and starts lysing the E. coli and the resulting free GFP starts diffusing to the other half of channel and thereby making the uniform fluorescence. The diffusion coefficient of E. coli is 2.5×10^{-9} cm^2s^{-1} while for GFP it is 8.7×10^{-7} cm^2s^{-1} and the diffusion length for E. coli is around 832 cm and GFP to be 2.4 cm which clearly shows that channel length of 30 cm is enough for free GFP to diffuse but E. coli cannot diffuse fast enough.

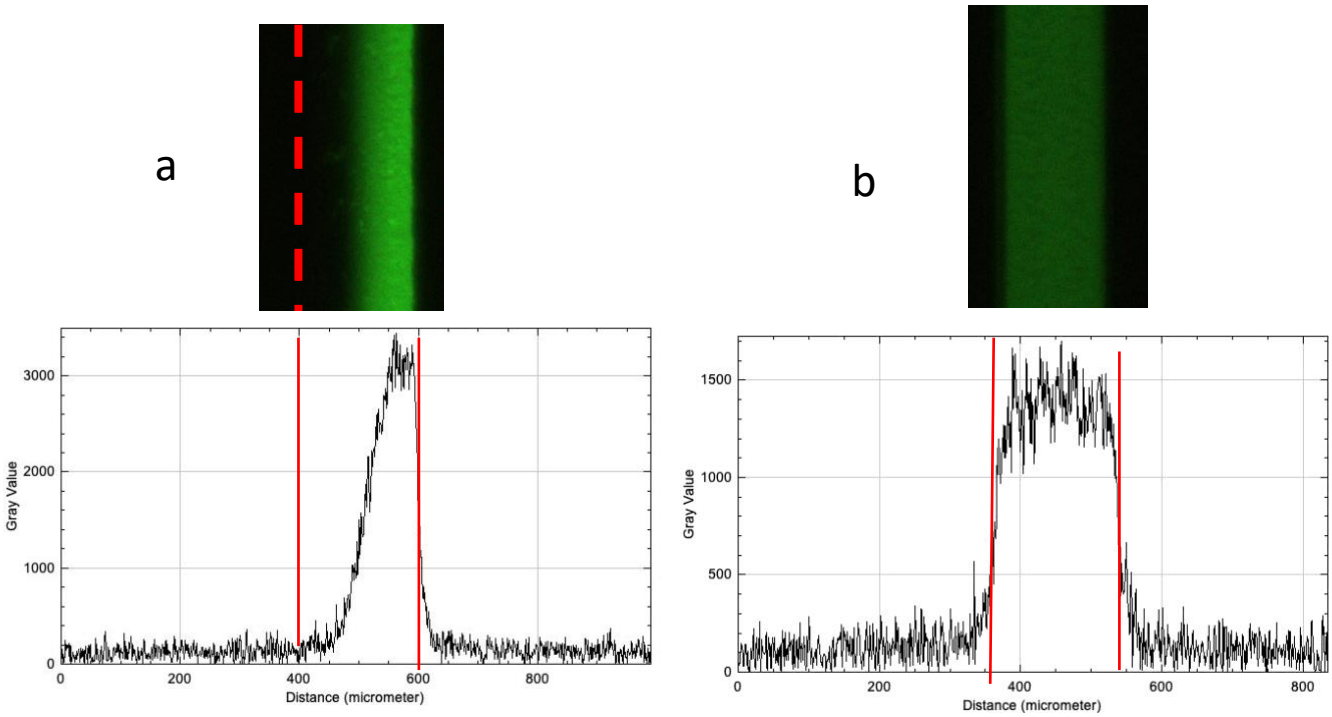

Figure 0.3:Lysis (a) start of lysis channel with fluorescence on one side (b) end of lysis channel with a uniform profile which shows that free GFP has diffused throughout the channel

4.3.3 Aqueous two-phase extraction (ATPE) microfluidic module

ATPE was used for concentrating the protein to one phase. As the GFP produced here is tagged with Lytag which has tendency to partition to PEG rich phase, a PEG-phosphate ATP system was used. In ATPs module there are three inlets, one from output of lysis module, one for 40% stock solution of phosphate and the third for 50% stock solution of PEG. The ATPS channel has a cross section of 600 μm × 200 μm and is 30 cm long. Channel dimensions were based on previous work [200] scaled with a factor of 4 in cross section to allow for the use of micro milling as the fabrication technology. In previous studies, microfluidic ATPS was used for binodal curve determination and screening of conditions, however in this study only the

optimal conditions are used to demonstrate the integrated process [200].By adjusting the flowrate, it was ensured that the composition is above the binodal curve where two phases are formed. As shown in Figure 4.4, initially all the fluorescence is on phosphate side but as the flow progresses free GFP start diffusing to PEG rich phase and at the end of channel all the GFP has been partitioned to PEG rich phase as evidenced by the fluorescence. The partition coefficient (K_p), defined as the ratio between the fluorescence in the PEG-rich phase to the fluorescence at the salt-rich phase, was determined to be 2, showing the affinity of GFP to the PEG-rich phase under the experimental conditions selected (13% PEG, 15% phosphate pH 7.0).

$$K_p = \frac{Flour_{PRP}}{Flour_{SRP}} = 2$$

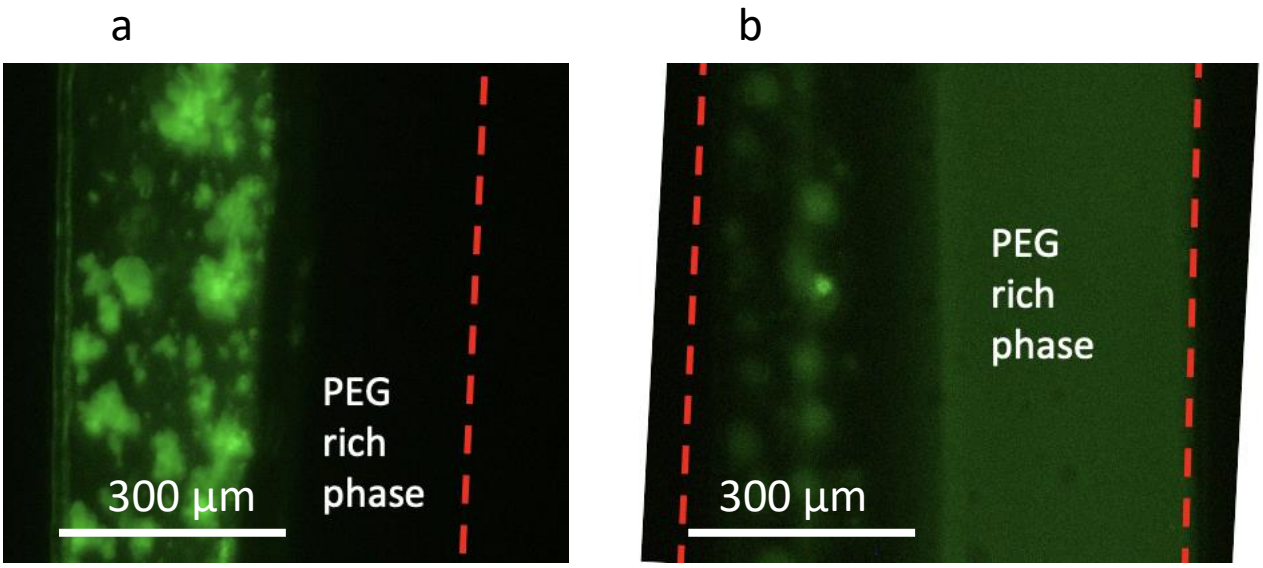

Figure 0.4:a) start of ATPs channel and (b) end of ATPs channel where most of GFP has moved to PEG rich phase

4.3.4 Integrated continuous bioprocessing microsystem

The overall aim of this work was to have an integrated chip starting with cell and medium as input and product as the output. In section 4.3.1-4.3.3 each individual module has been described individually.

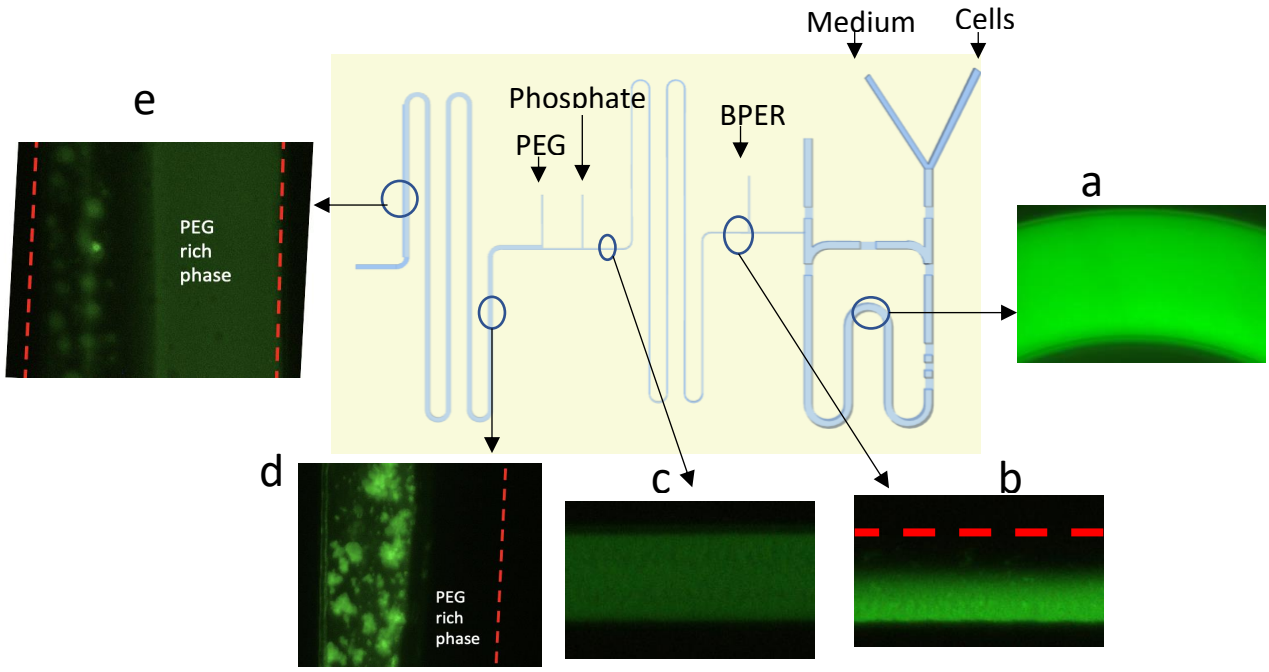

Figure 0.5: Integrated device operation showing: (a) GFP production in a microbioreactor (b, c) lysis; and (d, e) ATPE.

Figure 4.5 shows how the integrated device is operating. Initially GFP is being produced continuously in the micro-chemostat and as the chemostat reaches steady state the amount of GFP being produced is uniform as shown in figure 4.2. Then cells enter lysis module where BPER disrupts the cell wall and free GFP is released which then diffuses in channel thereby giving a uniform fluorescence profile as seen in figure 4.5(c). Then by adding potassium phosphate and PEG solution an ATPs is produced in the final module. As GFP being produced is tagged with Lytag which has affinity towards PEG so GFP goes to PEG rich phase while cell debris stays in phosphate rich phase as depicted in figure 4.5(d) and 4.5(e).

4.4 Conclusion:

In this chapter an integrated microfluidic platform is developed to mimic the bioprocessing of a target protein, allowing process design and optimization. The integrated chip consists of a production module for the GFP model protein, followed by a lysis module to release the intracellular protein using chemical lysis, and finally an ATPS module to concentrate the target protein into one phase using liquid-liquid extraction. After initial incubation and induction to produce GFP, the device was kept running for up to 8 days and the fluorescence intensity was measured regularly at different parts of the microfluidic

bioprocessing system and was found to be uniform. BPER was used to disrupt the cells and release protein and then it was successfully concentrated in the PEG rich phase.

The multimodular microfluidic device developed allows for the screening and optimization of continuous production, lysis and separation conditions. By combining multiple unit operations in a single device, it would be possible to screen not only for the optimal conditions for each operation, but to account for the combined effect of each on the overall process

In this way, the microfluidic device can deliver a rapid process screening and optimization, with very low reagent consumption, and using a simple fluorescence microscope for data collection. It should also be noted that it may be possible to combine other modules to this system, such as other downstream processing operations for further testing or product purification, and that the concept could be applied to other biologicals. Furthermore, sensors like OD sensor for micro-chemostat, and fluorescence sensors for lysis and ATPS module can be used to measure the lysis efficiency and partition coefficient in an automated way. A bifurcation channel at the end can be employed to sperate both the phases (though there will be some cross contamination) for further analyzing the process yield and purity.

5

Conclusion and Future work

5.1 Outlook and Conclusions:

This thesis described the development of a continuous microfluidic platform capable of guiding process development, integrating upstream with downstream process combining multiple unit operation on a single chip to consider the holistic approach in process development.

A microbioreactor was developed to emulate the upstream process in bio manufacturing. This work has successfully addressed the design, fabrication and operation of a two layered structure microbioreactor, comprising a fluidic layer for cell grow and a control layer for actuation of valves and pump. The volume of the fluid layer was designed considering the growth characteristics of *E. coli*. The peristaltic pump was characterized for operation at various frequencies and then a frequency of 10 Hz was selected to be used in further experiments. Proof of concept experiments were successfully performed with a fluorophore which allowed the demonstration of the working principle of the device in both feeding modes. It was also concluded that the suction created by the peristaltic pump was sufficient to maintain most of the flow in recirculation even when a syringe pump is continuously injecting fluid in channel. A cell counting method was also effectively developed using greyscale image processing and a MATLAB script and the validity of the method confirmed using samples of known cell concentration. Finally, the device was operated for growing *E. coli* cells both in semi-continuous and continuous mode and it was observed that cell growth reaches a steady state after 24 h of operation. In the case of continuous mode operation, 0.5 μL/min (dilution rate of 0.5 h^{-1}) was found to be the most suitable flowrate allowing for the highest cell concertation. The device was kept running for a period of more than 30 h, clearly showing that further integration of downstream processing would be possible, thus opening the door for integrated continuous biomanufacturing

An integrated platform, combining cell lysis and product extraction using ATPS was successfully developed envisaging screening of downstream bioprocess conditions. Chemical lysis being the most optimum method of lysis for scale down operation was used with BPER as lysis solution. Cell samples being collected from a bench top reactor were lysed on a chip and by comparing the diffusion coefficient of GFP and E. coli that the uniform fluorescence profile at the end of the channel was due to lysis and diffusion of free GFP as E. coli needed much longer channel to diffuse which was consistent with previous work done. After lysis module comes ATPS where PEG-Phosphate system was used to concentrate free GFP into PEG rich phase.

An integrated microfluidic platform is developed to mimic the bioprocessing of a target protein, allowing process design and optimization. The integrated chip consists of a production module for the GFP model protein, followed by a lysis module to release the intracellular protein using chemical lysis, and finally an ATPS module to concentrate the target protein into one phase using liquid-liquid extraction. After initial incubation and induction to produce GFP, the device was kept running for up to 8 days and the fluorescence intensity was measured regularly at different parts of the microfluidic bioprocessing system and was found to be uniform. BPER was used to disrupt the cells and release protein and then it was successfully concentrated in the PEG rich phase. The multimodular microfluidic device developed allows for the screening and optimization of continuous production, lysis and separation conditions. By combining multiple unit operations in a single device, it would be possible to screen not only for the optimal conditions for each operation, but to account for the combined effect of each on the overall process. In this way, the microfluidic device can deliver a rapid process screening and optimization, with very low reagent consumption, and using a simple fluorescence microscope for data collection.

5.2 Future work:

5.2.1 Microbioreactor operation and integration of sensors:

One of the first step in manufacturing of biological products is the selection of the organism to be used and what type of plasmid modifications are required and then to screen the optimum conditions to increase the yield of the desired product at the same time limiting

the contaminations and impurities that might hinder the downstream processing. Current strategies in terms of screening of strains and the conditions are limited due to time and resource constraints. Micro-chemostat developed in this work can be helpful in overcoming those constraint as reagents requirement here is very low. In this way several strains and conditions can be screened very effectively using less resources.

Another more important modification can be the integration of sensors not only for O.D measurement but measuring various other parameters like pH and oxygen will be very helpful. This will not only result in online measurement of growth but will be very useful in rapid screening of growth parameters. As PDMS is known to have adsorption and absorption issue surface modifications of the channels to decrease or remove this adhesion and absorption of the molecules will be another improvement that can result is making the microbioreactor closer to the bench top or industrial scale bioreactor.

In this work microbioreactor is integrated with downstream processing and a similar approach can be the combination of this device with other modules of upstream processing like microfluidic cell factory to create and test the microorganisms' libraries which can accelerate the selection process of new strains for manufacturing of new products. Another use modification can be automated operation where feedback loop can be used to prompt the change of flowrate of medium or removal of the waste from the microbioreactor.

5.2.2 Screening of downstream conditions:

Microfluidic devices had been at the core of development of bioprocessing and diagnostics due to automated and parallel processing capabilities. Most of the devices developed in literature are based in single processes with little to no automation capabilities. So apart from integrated approach that is done is this work, there is continuous need for integration of data acquisition and control systems that can result in automated operation. Data science is at the fore front of the scientific development in every field and bioprocessing is not an exception. Many models are being developed but in order for models to predict the outcome of an experiment accurately more and more data is required and use of data acquisition system in these platforms can be very helpful for gathering a large amount of data in very short time by utilizing a very small amount of consumables.

For an automated platforms one would start with the use of multiple sensors and feedback loops to an actuation mechanism that can then change the input conditions to provide the required output. In action it would combine fluid control components like syringe pumps and pneumatic valves to automatically characterize the formation of ATPS or change the flowrate of lysis solution depending on the lysis efficiency. Though several works are being done to develop these kinds of sensors but there is still room for improvement and their integration to these downstream processing devices rather than just proof of concept experiments performed in very controlled environment. By using these sensors in downstream processing, it will be possible to accelerate the process development and to derive optimal conditions for purification of bioproducts.

5.2.3 Integrated device:

In the future it should be possible to improve the integrated device in multiple ways. As flowrate is a very important input parameter for the working of whole device whether it is the cell concentration or lysis efficiency or even the formation of ATPS, a control unit for this input will be very helpful in screening various conditions faster. Not only an OD sensor in the microbioreactor but also the integration of sensors at the end of lysis and ATPS module would be a step towards fully automated platform and will be really save time spent on image analysis and will result in faster output method so that the required conditions can be screened more effectively. A bifurcation channel at the output will be quite useful to obtain parameter like yield of the process and purity of the product. A successful integration of three modules on a chip its paves the way for integrating further downstream processing modules and finally able to develop end to end process on single chip.

5.3: Future of bioprocess development:

Potential of microfluidics have been explored in medical diagnostics for quite some time with the development of ELISA chips, and now the focus is on the development of new bioprocesses. Many devices initially developed for sample treatment are now being adapted for bioprocess development and microfluidic chips with the ability to create, sort and evaluate cell libraries in a very short time are being developed. The use of microfluidic platform for

studying the metabolic pathways of microorganisms with as low resolution as single cell are also paving the way for use of new substrates. By combining microbioreactor with the cell factory devices the development of biopharmaceuticals can be greatly accelerated. With the creations of variety of modified microorganisms and the capability of microbioreactors to test multitude of strains and growth conditions can become a powerful tool in bioprocess development. With the huge data generated in these types of devices, more reliable models can be developed that will be able to predict the more accurate outcome of the experiments without even performing those experiments thereby saving time and money and streamline the development of new drugs.

In case of downstream processing microfluidic devices have been developed which are miniaturized versions of large-scale methods and have been used for optimization of the process. The optimized parameters at small scale have been used at large scale as good correlation exist between small- and large-scale results. Whether its precipitation, ATPS, or chromatography, microfluidic devices have been able to identify range of optimal conditions so that there is no need to test all the conditions at large scale and resulted in saving cost and time. Future of use of microfluidics in process development is in further integration and automation where these devices can be used in combination with current development frameworks used in companies for gathering large amount of data to guide the development stage. Microfluidic devices will be very helpful in generating large amount of data from parallel experiments in a very short time and in very cost-effective manner.

Another area where integrated microfluidic devices can be very helpful is personalized medicine. The current advances in the field are clear indication that it is not far in the future where whole bioprocess will be integrated on a single chip. This will reduce the cost associated with development of new drugs and at the same time with the development of organ on a chip platform the testing of these drugs will be streamlined as well making this a reality in near future.

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A

Publications and other work

The work described in this thesis has led to some publications and scientific presentations. These are described in this section, followed by some relevant scientific activities.

A.01 Publications:

The work described in chapter 2 has already been converted into paper form and is submitted to special edition Micro nano engineering journal.

Design and fabrication of a microbioreactor for E. coli production

Malik Abdul Wahab, Ana M. Azevedo, Virginia Chu, M.R. Aires-Barros, Joao Pedro Conde.

Abstract: Microscale process techniques provides effective tools for expediating bioprocess design in a cost-effective manner. These miniaturized platforms, together with bioprocess models, can be used to predict industrial scale performance. The first step in bioprocess design is bioproduct production and in order to be able to evaluate not only the conditions that effect production but also purification, it was important to develop an integrated device. In order to integrate upstream with downstream processing, the microbioreactors must work in a continuous mode. Hence the focus of this work is to design and fabricate a micro-chemostat that can work in a continuous mode. A micro-bioreactor of volume 60 µL has been developed using a two-layer PDMS structure. The bottom layer comprises the reactor channel while the top layer is the control layer which can be actuated to operate the peristaltic pump and the valves. The micro-bioreactor working in both semi continuous and continuous mode for over 30 hours has been successfully demonstrated and a steady state cell production was recorded after a settling time for various operational conditions.

The work described in chapter 4 has already been converted into paper form and is submitted to Separation and Purification Technology

An integrated microfluidic device for continuous bioprocessing

Malik Abdul Wahab, Ana M. Azevedo, Virginia Chu, M.R. Aires-Barros, Joao Pedro Conde.

Abstract: Microfluidic devices have been increasingly used as tools to accelerate the development of new biomanufacturing methods, given their ability to test a large set of variables in a short time, with minimal reagent consumption. However, while being able to expedite a bioprocess design, the modular aspect of these microfluidic devices has not been systematically explored, with most applications focusing on single unitary operations.
Integrated continuous bioprocessing has the potential to reduce cost, optimize yield and reduce footprint as compared to batch operation. In this work an integrated microfluidic device is designed to continuously produce, concentrate and purify proteins.
The integrated system consists of a micro-chemostat to produce the protein continuously, a chemical cell lysis module for protein release and a module to purify and concentrate the protein using aqueous two-phase systems. In this way, it is possible to screen multiple conditions for each operation and evaluate their combined effect on the final product.
A recombinant *Escherichia coli* (*E. coli*) strain producing green fluorescent protein (GFP) was used as a model system, which allows for GFP production, lysis efficiency and partition coefficient to be evaluated by fluorescence microscopy. Experiments were done for over a week by continuously supplying medium and monitoring the GFP production by fluorescence intensity. A chemical lysis solution B-PER® was used for continuous lysis and then the extracted GFP was clarified and concentrated in a PEG-rich phase with a partition coefficient of 2. As the integrated device is working in continuous mode it can be integrated to further downstream processing modules.

A.02 Conference proceedings:

Oral presentation:

"Design and Fabrication of Microfluidic devices for cell-lysis and separation of protein using aqueous two-phase system", SCBN 21 virtual.

Poster presentation:

"Design and fabrication of a micro-chemostat for E. coli production", MNE 2022 Sept 19-23 2022