Towards Extracellular Release of Recombinant Protein from *Escherichia coli* During Fermentation

Utilisation of Antisense Technology in Bioprocesses

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DOCTOR OF PHILOSOPHY

By

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Declaration

I, Shahin Heshmatifar confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract:

Biopharmaceutical and biotechnology businesses use *Escherichia coli* to make recombinant proteins for medicinal, diagnostic, and analytical uses. Early downstream processing and recovery follow upstream production and processing. Cell lysis procedures damage cellular integrity and release specific periplasmic products. These releases include host cell proteins (HCP) and DNA that will challenge late downstream processing and purification, leading to time-consuming and complex unit operating steps, and an expensive bioprocess and output.

Antisense technology can be used to target and suppress the synthesis of indigenous proteins, in this example an outer membrane protein, to promote the release of the recombinant product out of the cell during fermentation with the inner membrane intact, removing the need for a cell lysis phase.

This study will investigate the effect of antisense-RNAs that suppress the synthesis of an outer membrane protein (Omp), murein lipoprotein (Lpp), on *Escherichia coli* fermentation.

The bacterium strain was transformed with a model product and antisense-DNA plasmids. This experiment studied recombinant alpha-amylase and fragmented antibodies. Using 0.7 and 4-litre working volumes, reproducible and robust fermentations were achieved. Influential factors affecting product release percentages were identified and studied. Extracellular product levels in the medium reached 60%, up from 10% in initial studies.

After high release levels, the industry can adopt the approach to eliminate cell lysis processes and minimize the overall number of recovery and purifying procedures, saving time and costs. This project's success will attract the industry and lead to a new bioprocess strategy.

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Impact statement:

This project was successful in applying innovative approaches in bioprocessing, which have tremendous potential for enhancing the manufacturing bioprocess of recombinant proteins. Having demonstrated the methodology's proof of concept and that it is both reproducible and robust, it is necessary to do additional research to investigate a wider range of methods that make use of more sophisticated synthetic biology approaches. Further research may be conducted that will build on top of this project, including twofold induction systems and the comparison of results with gene knock-out approaches.

The next steps of this project for industrial bioprocesses relating to the production of therapeutic and non-therapeutic recombinant proteins can be investigated by formation of collaborative initiatives between the private sector and academic institutions. Developing an efficient extracellular release method often involves engineering the host organism to express specific secretion mechanisms or tags. Understanding and optimizing these mechanisms can be a focus of research and development.

Extracellular release can simplify the downstream purification process. Since the product is secreted into the fermentation broth, it may be easier to separate from the cellular biomass and other impurities, leading to higher product purity. The elimination of cell disruption steps in a bioprocess, a significant reduction in levels of impurities such as HCP and DNA, a reduction in proteolytic activity in the culture medium, a reduction in the number of unit operations in the bioprocessing, and, as a result, a reduction in the process running cost and time, all have the potential to be significant for the industry. The development and implementation of extracellular release methods may involve additional costs related to genetic engineering, fermentation process optimization, and monitoring. However, these costs may be justified by the benefits in terms of product quality and yield.

This work opens doors to novel applications of technologies to address industrial bottlenecks and challenges. Employing a tool that allows cheaper and less laborious and consuming bioprocessing also has positive environmental impacts, such that the reduced number of consumables, energy and reagents used to manufacture a recombinant product and meet the targets will be a further step towards sustainability.

With the successful industrial adoption of antisense technology building on the foundations laid by this study, therapeutic recombinant proteins that can be used by the general public at a lower cost could be made available to them.

In summary, having a method for the extracellular release of recombinant products during fermentation can offer several advantages in terms of product quality, yield, and process control. However, it also requires careful planning, engineering, and optimization to achieve the desired results and may come with associated costs and regulatory considerations.

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Chapter 1: Introduction (Literature Review)

1.1 Overview

Bioprocessing and biotechnology have evolved significantly over the last fifty years. With vast advancements in molecular biology and research in microbial biology, production and manufacturing of recombinant proteins has reached new levels of optimisation. Molecular modifications in microbial genome allow further optimisation in overall productivity of a production process. Having knowledge of microbial and in particular bacterial metabolic and production pathways within the cell allows us to exploit its microbiology in favour of our production process productivity.

Many therapeutic or non-therapeutic proteins that are produced in a gram-negative bacterium such as *Escherichia coli*, must be expressed in the native host cell. This is because the active form of the product possesses a unique 3-dimensional structure that is achieved after translation and in a particular location, either cytoplasm or periplasm. In this process Disulphide bridges are formed and the protein folds into its active form. Therefore, it is important to note that bypassing this process will not produce an active desired product.

It is intended to explore a novel approach in exploiting bacterial microbiology in order to increase productivity of an *Escherichia coli* recombinant protein production process. This novel approach aims to aid and enhance complete secretion of expressed protein into the growth media, thus removing the homogenisation steps and reducing the number of primary recovery and purification steps in the overall process.

Extracellular secretion of recombinant protein by *Escherichia coli* offers many considerable advantages such as reduction in complexity of the bioprocess engineering and also in terms of the quality of the product, the secreted product will possess a greater quality, this is because the medium outside of the cell offers a better environment for

folding and it is also free from chemical and enzymes that are present in the cell and may affect the recombinant product negatively, such as degradation due to cell associated proteolytic reactions (Ni et al., 2009).

Numerous laboratory strains of *Escherichia coli* exist that carry the potential to secret their recombinant product into the growth medium. Research has been going on studying these potentials and optimising the performance the organism such that it would become appealing for an industrial application (Ni et al., 2009). *Escherichia coli*

expression systems are widely used in the biotechnology industry for manufacturing of many therapeutic recombinant proteins. In fact, almost 40% of all available recombinant therapeutic proteins are manufactured by *Escherichia coli* expression systems (Langer, 2009). Nearly all *Escherichia coli* expression processes currently adapted in the biotechnology industry are designed such that many cell lysis, primary recovery and purification steps are included to recover the periplasmic or intracellular recombinant products. Many companies will be interested in a novel approach to eliminate several of the traditional unit operation steps. Success in providing a novel methodology for complete secretion of bacterial recombinant product will draw huge investments in order to exploit the technique in manufacturing protein of interest (Ni et al., 2009).

Extracellular secretion by *Escherichia coli* may have applications other than that of manufacturing therapeutic proteins. Recombinant enzymes that are made via directed evolution are easier to be detected if they were secreted into the medium such that lysing cells and removing cell debris wouldn't be necessary (Nisole et al. 2006)

1.2 *Escherichia coli*

Escherichia coli is a well-known gram-negative bacterium. Gram-negative bacteria have a thin peptidoglycan layer (1-2nm) compared with gram-positive bacteria and an outer membrane. Its outer membrane consists of unique components that allow it to survive harsh environments. The space between the outer membrane and inner cell membrane is referred to as the periplasmic region. (polissi et al , 2014)

Outer membrane proteins (Omp) are shed during growth. A study on *Escherichia coli* W3110, plain and ones expressing periplasmic products has shown that during growth prominent proteins are found in the media. Following detailed analysis, it was found that the proteins were present in the media despite no cell lysis. Further analysis revealed that the Omp were shed during growth (Nandakumar, 2006). The mechanism of this occurrence should be studied in order to reveal any possible potential for exploitation in biotechnology.

1.2.1 Gram negative bacterial cell membrane components and characteristics

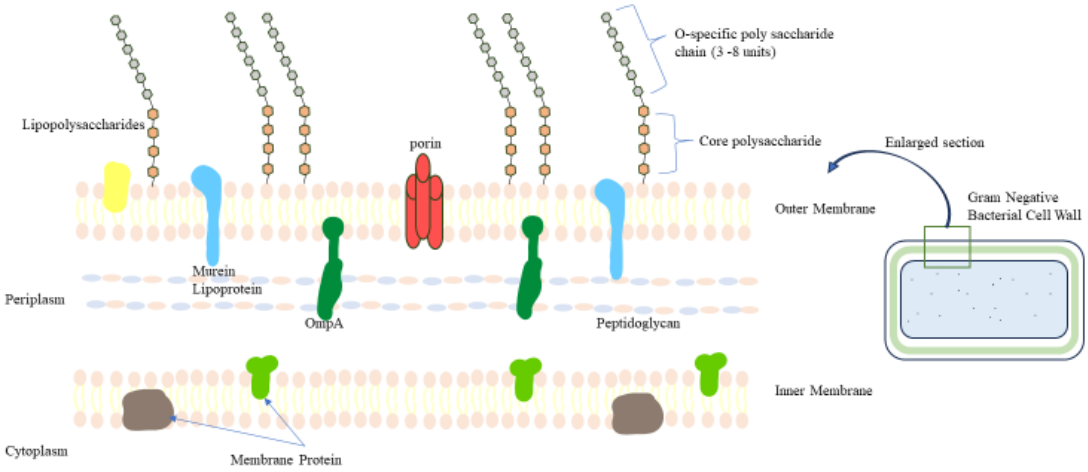


Figure 1- An image of gram-negative bacterium with emphasis on the periplasmic region and prominent membrane proteins. (Image digitally drawn by Shahin Heshmatifar, inspired by Willey et al., 2022)

The permeability or traffic of molecules especially larger proteins is more controlled by membrane proteins. There are many different kinds of these membrane proteins and exist in both inner and outer membrane of an *Escherichia coli*. Some proteins in the outer membrane are more abundant than others such as murein lipoprotein (Lpp) that exists in over 0.7 million replicates of the molecule in every cell (Typas et al., 2012). It has an approximate size of 8.3kD (Ni et al, 2009)

The figure below outlines some of the most important membrane proteins. Some of which may be possible to be exploited in producing genetically modified (GM) *Escherichia coli*

to secrete its recombinant product into the media. The amino acid sequence is also listed next to each named membrane protein.

Signal sequences	Amino acid sequences
PelB (pectate lyase B) from *Erwinia carotovora*	**MKY**LLPTAAAGLLLLAAQPAMA
OmpA (outer-membrane protein A)	**MKKT**AIAIAVALAGFATVAQA
StII (heat-stable enterotoxin 2)	**MKK**NIAFLLASMFVFSIATNAYA
Endoxylanase from *Bacillus* sp.	**MFKFKKK**FLVGLTAAFMSISMFSATASA
PhoA (alkaline phosphatase)	**MKQS**TIALALLPLLFTPVTKA
OmpF (outer-membrane protein F)	**MMKR**NILAVIVPALLVAGTANA
PhoE (outer-membrane pore protein E)	**MKKS**TLALVVMGIVASASVQA
MalE (maltose-binding protein)	**MKIKT**GARILALSALTTMMFSASALA
OmpC (outer-membrane protein C)	**MKVK**VLSLLVPALLVAGAANA
Lpp (murein lipoprotein)	**MKATK**LVLGAVILGSTLLAG
LamB (λ receptor protein)	**MMITLRK**LPLAVAVAAGVMSAQAMA
OmpT (protease VII)	**MRAK**LLGIVLTTPIAISSFA
LTB (heat-labile enterotoxin subunit B)	**MNKVK**CYVLFTALLSSLYAHG

Figure 2 – Representative signal sequences used for the secretory production of recombinant proteins in *Escherichia coli.*

The signal sequence is composed of N-, H- and C- domains. The N- domains of signal sequences are shown in bold and the C- domains are underlined. (Choi et al., 2004)

E. coli strains possess either common or conjugative fimbriae (sex pili). Common fimbriae (100–1000 per cell) are primarily made of fimbrin, an acidic hydrophobic protein. Based on the amino acid sequence of its primary fimbrin, common fimbriae are divided into seven distinct groupings. Fimbriae are extremely antigenic and contain several F antigens. Notably, *E. coli* strain K12 possesses only type 1 common fimbriae, and it can alternate between the fimbriated and nonfimbriated states, a process known as phase variation. This may be because the presence of common fimbriae permits organisms to connect to epithelial cells during their initial attempts to colonise their host. Turning off the creation of common fimbriae throughout the body may reduce the likelihood of organisms being phagocytosed by white blood cells.
Plasmids like as F or R encode the sex fimbriae (often referred to as pili, with one to several copies per cell). These structures allow for the transfer of DNA during conjugation by bringing donor and recipient bacteria into contact (Liu, 2014).

Five to ten flagella per cell are responsible for *E. coli*'s movement. The flagella are typically 5–10 mm in length and are peritrichously distributed around the cell surface. *E.*

coli flagella are composed of a long filament, a hook, and a basal body, with flagellin, a 55 kDa N-methyl-lysine-rich protein, being the primary structural component. Approximately 20,000 subunits of this protein form the flagellar filament. In vitro, flagellin self-assembles into filamentous cylindrical lattices with hexagonal packing that resemble flagella (Liu, 2014).

The flagellar genetic system of *E. coli* consists of around 40 genes organised into five sections. These genes contribute to structure, function, assembly, and control. Flagella are highly antigenic, containing a significant number of H antigens, with highly conserved N- and C-termini of diverse H antigens (Liu, 2014).

The Embden–Meyerhof–Parnas pathway, the pentose pathway, the tricarboxylic acid cycle, and the Entner–Doudoroff pathway all contribute to *E. coli*'s central metabolism (for the metabolism of gluconate). *E. coli*, a facultative anaerobe, satisfies its energy requirements via either respiratory or fermentative pathways. Under anaerobic conditions, the principal byproducts are formate, acetate, lactate, succinate, ethanol, 2,3-butanediol, carbon dioxide, and hydrogen. It meets its demand for biosynthetic building blocks by producing 12 precursor metabolites shared by all bacteria. The periplasm of *E. coli* has an inducible alkaline phosphatase for the transport and use of organic phosphates (Liu, 2014).

E. coli is a chemoheterotroph with the ability to grow on a variety of sugars and amino acids supplied singly or in combination. Some naturally occurring strains, like as thiamin, have a single auxotrophic need. The presence of individual amino acids such as serine, valine, or cysteine inhibits the growth of several strains. *E. coli* multiplies quicker with glucose than with any other single carbon and energy source, and its doubling time under well-oxygenated conditions at 37 degrees Celsius is 50 minutes. Using an externally regulated continuous culture system or the addition of a metabolic mimic and its antagonist in the correct proportions will result in slow growth rates. At 37 degrees Celsius, doubling times for *E. coli* in nutrient-rich broths (including amino acids, nucleosides, sugars, and vitamin precursors, etc.) are 20 minutes. *E. coli* may grow between 8 to 48 degrees Celsius, depending on the strain and nutrient media. It thrives at a temperature of 39 degrees Celsius. *E. coli* cannot grow in conditions with a NaCl content

exceeding approximately 0.65 M. *E. coli* increases its concentration of ions, particularly K+ and glutamate, in response to changes in the osmotic pressure of the medium. The optimal pH range for growth is between pH 6.0 and pH 8.0, however growth is achievable at values around 1 pH unit above and below this range (Liu, 2014).

The genome of *E. coli* strain MG1655 (a derivation of laboratory strain K-12) consists of a 4.64 Mb circular DNA molecule including 4288 protein-coding genes (arranged into 2584 operons), seven ribosomal RNA (rRNA) operons, and 86 transfer RNA (tRNA) genes. In addition, it contains a considerable amount of transposable genetic elements, repeat elements, cryptic prophages, and bacteriophage remnants. The genomes of certain pathogenic *E. coli* strains are approximately 1 Mb larger than that of the commensal K-12 strain, as exemplified by enterohaemorrhagic *E. coli* strains O157:H7 Sakai (5.50 Mb), enteroaggregative *E. coli* strain O42 (5.36 Mb), and UPEC isolates CFT073 (5.23 Mb), 536 (4.94 Mb), UTI89 (4. (5.07 Mb). The additional DNA segments were likely obtained by horizontal gene transfer (Liu, 2014).

Comparative investigations of the *E. coli* and *Shigella* genomes reveal that the anticipated pan-genome has 15,741 gene families, of which just 993 (6% of the total) are present in every genome, constituting the core genome. The variable or 'accessory' genes constitute greater than 90 percent of the pan-genome and around 80 percent of the average genome. Two genetic configurations, virulence-related plasmids and chromosomal pathogenicity islands, are largely responsible for the plasticity of the *E. coli* genome. Despite the fact that many strains (e.g., strain MG1655) do not include plasmid, some strains may contain between 1 and 5 plasmids. Each of the seven groups of diarrhoeagenic *E. coli* carries at least one plasmid-based virulence-related feature. Frequently prevalent in EIEC, EHEC, EAEC, and EPEC is a member of a highly conserved plasmid family. The plasmid is often big (>60 megadalton or MDa), has a low copy number, and is either conjugative or of transmissible incompatibility group. It encodes several virulence factors. While plasmids and pathogenicity islands encode clusters of virulence traits, individual traits (such as shiga toxin) may be encoded by transposons (Liu, 2014).

Similar to other bacterial genomes, the *E. coli* genome is compacted into a nucleoid-like structure. Nucleoid functions like as transcription, replication, recombination, and repair are affected by the nucleoid's unique structural characteristics and conformations. The nucleoid of *E. coli* is a heavily lobulated intracytoplasmic area located generally in the cell's centre. In this region, the DNA concentration is between 2 and 5 percent (w/v). In vivo, DNA is negatively supercoiled into around fifty distinct domains. As the nucleoid forms a considerable barrier to the diffusion of many macromolecules, transcription takes place at the nucleoid–cytoplasm interface. The very uneven structure of the nucleoid may contribute to the transcriptional accessibility of genes (Liu, 2014).

At least four DNA-binding proteins with low molecular weight are implicated in transcription, recombination, and replication. The molecular weight of these nucleoid-associated proteins ranges from 9.2 to 15.4 kDa. Twenty to fifty thousand monomers of the proteins HU and IHF are present in each *E. coli* cell (Liu, 2014).

The commencement of DNA replication occurs at a specific origin site, oriC, and is governed by the DnaA protein, which is highly conserved among bacteria. Once began, DNA replication occurs at an almost constant rate in moderately fast and rapidly expanding *E. coli* until it achieves its conclusion. Segregation of the nucleoids occurs with a high degree of accuracy; hence, it cannot be the consequence of random partitioning into daughter cells. Recently replicated (hemimethylated) origin DNA binds with high selectivity to the membrane in vitro (Liu, 2014).

Lpp (murein lipoprotein), present in the wall of some gram-negative bacteria, known to be amongst the most abundant outer membrane proteins; it has a molecular weight of 7.2 kDa. It is bound at its C-terminal end by a covalent bond to the peptidoglycan layer and is embedded in the outer membrane by its hydrophobic head (Seltmann & Holst, 2002).

Representative signal sequences used for the secretory production of recombinant proteins in *Escherichia coli*. The signal sequence is composed of N-, H- and C-domains. The N-domains of signal sequences are shown in bold while the C-domains are underlined. The signal sequence Lpp (murein lipoprotein) and the amino acid sequence is **MKATK**LVLGAVILGST<u>LLAG.</u> (Choi & Lee, 2004)

Lpp is one of the most abundant proteins in *E. coli*, with more than 700,000 copies of the protein per cell (Ni et al., 2007). Whilst most Lpp is free in the membrane, around a third is bound to the peptidoglycan (Braun and Sieglin, 1970), covalently linked by the carboxyl-terminal lysine; virtually all are present only in the outer membrane area (Lee and Inouye, 1974). Mutants lacking this lipoprotein have been generated, spontaneously forming blebs upon their surfaces, resulting in the leakage of periplasmic enzymes outside the cells into the supernatant, without necessarily damaging cell viability, although hypersensitivity to toxins is observed (Inouye et al., 1977).

Before transport, Lpp is expressed as prolipoprotein with a 20 amino acid signal sequence extending from its amino terminus (Inouye et al., 1977). The amino terminus cysteine residue is modified during translocation across the cytoplasmic membrane, before cleavage of the signal peptide extension (Sankaran and Wu, 1994). After subsequent N-acylation the lipoprotein may then be covalently bound to the peptidoglycan.

The Gram-negative bacterium *Escherichia coli*'s secretory production of recombinant proteins has many advantages over intracellular production as inclusion bodies. Targeting the protein to the periplasmic space or the culture medium in most cases promotes downstream processing, folding, and in vivo stability, allowing soluble and biologically active proteins to be produced at a reduced rate (Mergulhão et al., 2005).

Strategies used to boost the permeability of the outer membrane have includes mechanical (ultrasound), chemical (addition of magnesium, calcium, EDTA, glycine, and Triton X-100), and enzymatic (lysozyme) treatments (Choi and Lee, 2004; Shokri et al.,2003). Extracellular release can also be enhanced by variation of physical and chemical parameters (temperature, culture medium composition, pH, or aeration), or by taking advantage of the growth-coupled effects on membrane components (Rinas and Hoffmann,2004; Shokri et al., 2003).

The *Escherichia coli* microorganism is widely used to manufacture recombinant proteins. Despite many advantageous features such as rapid growth and high protein yields, its inability to secrete recombinant proteins into the extracellular medium easily remains a disadvantage for processes of industrial production. A multitude of approaches have been

developed in recent years to increase the extracellular yield and the secretion efficiency of recombinant proteins to address this limitation (Kleiner-Grote et al., 2018).

The current accepted technique for cell disintegration prior to the extraction of cytosolic and periplasmic protein from *E. coli* is high-pressure homogenization. However, its primary flaw is limited selectivity, which leads to a heavy burden of host cell contaminants. The outer membrane may be selectively permeable via a pulsed electric field (PEF) treatment. PEF, also known as electroporation, is a technique that may create holes inside cell membranes. In a study by Schottroff et al., (2021) PEF was employed selectively extract recombinant Protein A from the periplasm of *E. coli*, It was discovered that raising the energy input levels led to a maximum product release of 89 %, and a significant reduction of HCP (40%), DNA (96%), and ET loads (43%) compared with HPH. The rate of cell death likewise steadily rose. PEF may have detrimental effects on product quality to varying degrees. Furthermore, in terms of scalability and feasibility of application, there may be considerable obstacles. having 60% of HCP released is not ideal. This methodology will require further optimization and demonstration of scalability and wide applicability to various large-scale manufacturing of recombinant proteins.

Extraction of recombinant periplasmic proteins under industrially relevant process conditions. It is desired to find a technique that may be used commercially for the selective extraction of periplasmic endogenously produced proteins. In order to do this, Schimek et al., (2020) employed an expression strategy that enables the simultaneous production of two fluorescent proteins, each of which has a clear preference for the cytoplasm or periplasm. They tested the capacity to preferentially extract periplasmic proteins rather than cytoplasmic proteins using a variety of scalable lysis techniques, including high-pressure homogenization, osmotic shock treatments, extraction with ethylenediaminetetraacetic acid, and extraction with deoxycholate. Their key finding was that none of the examined lysis conditions were preferential for periplasmic protein over cytoplasmic protein, despite the fact that they had established industrially scalable lysis conditions that considerably boosted the starting purity for further purification. Furthermore, they showed that the amount of total protein in the cell had a significant impact on how well the produced recombinant proteins were extracted. They concluded that selectivity and yield strongly depend on protein titer and methodology.

Recently, the periplasmic excretion methodologies have shifted the emphasis to genomic engineering techniques in strain growth. There are many methods available for selective gene knockout; methods of phage transduction, recombinase-based systems, and, more recently, clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 mediated mutations have been investigated (Kleiner-Grote et al., 2018).

There are several methods available to facilitate the product's selective release from the periplasm. However, the majority of these investigations do not distinguish between leakiness and lysis since cells are taken before they are re-suspended in permeabilization agent. Wurm et al., (2016) experimented with and contrasted various leakiness-inducing methods. They carried out these trials during culture, in contrast to prior investigations, and measured both leakiness and lysis. In conclusion, they suggest a several-hour incubation with 350 mM TRIS at a steady pH followed by a gentle heat treatment up to 38°C to induce leakiness with hardly detectable lysis. Their research provides a comparative overview of several methods for causing *E. coli* leakiness and lays the groundwork for further studies in this area. By the developed strategy, about 30% of the periplasmic content containing the target product was released without significant cell lysis (<5%).

Although it is known that fermentation conditions might influence cell membrane permeability, it is yet unclear how osmotic shock, a method for recovering periplasmic proteins, can physically impact the proteins. Ramalakshmi et al., (2021) show how changes in fermentation conditions and osmotic shock modify membrane characteristics, which then affect the selective release of particular periplasmic proteins. Their findings imply that the Young's modulus and initial pore size of cells are greatly influenced by the fermentation temperature and harvesting phase, respectively. The initial pore size and the expression of soluble proteins both have an impact on the selective protein release during a typical osmotic shock when 20% sucrose was used, especially in cells with a Young's modulus of less than 1 MPa. The dependence of the selective protein release on the initial pore size decreases as the sucrose concentration was increased further to 30%.

The main desired objective is to be able to selectively support the release of the recombinant proteins to a significate proportion of the total produced product from the periplasmic region without compromising the cell integrity, cell growth and morphology,

titres and the quality of the product whilst having minimal contaminants such HCP and DNA released during the separation method after fermentation. Furthermore, the method must be scalable and feasible at industrial scale and applicable to a variety of product characteristics such that the technology for the extracellular release may be transferred and applied to an already established large scale manufacturing process. In this study these factors were considered in developing a methodology for extracellular release of periplasmic proteins during fermentation by utilizing antisense technology.

1.2.2 Bacterial protein secretion systems

Multiple strategies are employed by bacterial infections to invade mammalian hosts, damage tissue locations, and prevent the immune system from responding. Secretion of proteins through phospholipid membranes is a crucial component of these techniques for a number of bacterial diseases. Multiple roles can be played by secreted proteins in boosting bacterial pathogenicity, including strengthening adhesion to eukaryotic cells, scavenging nutrients in an environmental niche, and directly intoxicated and altering the functioning of target cells. Numerous pathogens use specialised protein secretion systems to secrete virulence factors from the bacterial cytoplasm into host cells or the host environment. On the basis of their shapes, activities, and specialisation, protein secretion apparatuses in bacteria can be categorised into distinct types. Some systems are prevalent in all bacterial classes and produce a diverse array of substrates, whilst others are restricted to a small number of bacterial species and/or are exclusive to a few proteins. In this chapter, we examine the canonical characteristics of various prevalent bacterial protein secretion systems and their functions in enhancing the pathogenicity of bacterial pathogens. In addition, we discuss new discoveries indicating that the host's innate immune system can recognise and respond to the existence of protein secretion systems during infection in mammals.

Protein secretion is a fundamental bacterial cell function that involves the movement of proteins from the cytoplasm to different compartments of the cell, the environment, and/or other bacteria or eukaryotic cells. Prokaryotes have evolved several methods for transferring protein cargo between places, the majority of which rely on protein secretion systems. Protein secretion systems are necessary for bacterial growth and are utilised in

a variety of functions. Others have been identified in only a small number of bacterial species or are dedicated to the secretion of just a few proteins. In certain instances, bacterial infections exploit these specialised secretion systems to influence the host and establish a niche for reproduction. Occasionally, they are necessary to exploit an environmental niche, perhaps by secreting proteins that aid bacteria in competing with neighbouring microbes. There are numerous classes of bacterial secretion systems, and their designs can vary depending on whether their protein substrates cross a single phospholipid membrane, two membranes, or even three membranes, two of which are bacterial and one of which is host.

This overview provides a concise introduction to a variety of protein secretion systems in order to emphasise their structural and functional similarities and distinctions. The discussions will centre on the canonical characteristics of each system rather than the myriad variations of each.

Secretion Apparatus	Secretion Signal	Steps in Secretion	Folded Substrates?	Number of Membranes	Gram (+) or Gram (−)
Sec	N-terminus	1	No	1	Both
Tat	N-terminus	1	Yes	1	Both
T1SS	C-terminus	1	No	2	Gram (−)
T2SS	N-terminus	2	Yes	1	Gram (−)
T3SS	N-terminus	1–2	No	2–3	Gram (−)
T4SS	C-terminus	1	No	2–3	Gram (−)
T5SS	N-terminus	2	No	1	Gram (−)
T6SS	No known secretion signal	1	Unknown	2–3	Gram (−)
SecA2	N-terminus	1	No	1	Gram (+)
Sortase	N-terminus (Sec) C-terimnus (cws)	2	Yes	1	Gram (+)
Injectosome	N-terminus	2	Yes	1	Gram (+)
T7SS	C-terminus	1	Yes	1–3	Gram (+)

Table 1 Classes of bacterial protein secretion systems

The utilisation of secretion systems to move proteins from bacterial cells into the environment or into destination cells is addressed. Proteins can also be secreted from the bacterial cytoplasmic compartment into other compartments of the cell, specifically into or across the cytoplasmic membrane. The Sec and Tat pathways are the bacterial secretion systems most frequently used to move proteins across the cytoplasmic membrane (Natale et al., 2008). The Sec and Tat routes are the most highly conserved protein secretion processes, and have been identified in all three domains of life (bacteria, archaea, eukaryotes) (Papanikou et al., 2007). The majority of proteins transported through the Sec and Tat routes remain in the periplasm or inner membrane of the cell. In Gram-negative

bacteria, however, proteins supplied to the cytoplasmic membrane or periplasm by the Sec or Tat routes can either remain in those compartments or be exported from the cell via another secretion mechanism. Despite the fact that the Sec and Tat systems share a number of components, they transport proteins through fundamentally distinct processes.

1.2.2.1 The Sec Secretion Pathway

Unfolded proteins are typically translocated via the Sec pathway. This system is comprised of three components: a protein targeting component, a motor protein, and a membrane-integrated conducting channel known as the SecYEG translocase (Papanikou et al., 2007). In addition, numerous Gram-positive bacteria develop Sec accessory proteins that play crucial roles in the secretion of certain proteins. While proteins secreted by the Sec apparatus can have a variety of functions, this pathway transports a number of proteins that improve the pathogenicity of bacterial infections. Gram-negative bacteria *Vibrio cholerae*, *Klebsiella pneumoniae*, and *Yersinia enterocolitica* are pathogens that utilise Sec-dependent secretion to transport virulence components across the cytoplasmic membrane. *Staphylococcus aureus* and *Listeria monocytogenes* are examples of Gram-positive bacteria that utilise Sec accessory systems (Korotkov et al., 2012).

Export by the Sec pathway depends on a hydrophobic signal sequence at the N-terminus of the secreted protein, which is generally 20 amino acids long and consists of three regions: a positively charged amino terminal, a hydrophobic core, and a polar carboxyl-terminus (Papanikou et al., 2007). Proteins destined to be secreted into the periplasm or outside of the cell by the Sec route have SecB-specific signal sequences, whereas proteins destined to remain within the inner membrane contain an SRP-specific signal sequence.

1.2.2.2 The Tat Secretion Pathway

The Tat route, unlike the Sec pathway, largely secretes folded proteins. This process is essential because not all unfolded proteins can be secreted, as certain cytoplasmically produced proteins, such as redox factors, have post-translational modifications. Since the resources required for these alterations are unavailable extracellularly or in the periplasm,

these proteins must be folded and changed in the cytoplasm prior to their 3-dimensional secretion.

The Tat pathway of protein secretion consists of 2–3 subunits: TatA, TatB, and TatC (TatA and TatB are joined into a multifunctional protein in Gram-positive bacteria). TatB and TatC bind the signal peptide of Tat-secreted proteins in *Escherichia coli* before recruiting TatA, which forms the membrane-spanning channel The Tat signal sequence at the N-terminus of the folded protein contains a pair of "twin" arginines in the motif S-R-R. (Müller, 2005). In contrast to Gram-positive bacteria, where the majority of Tat-secreted proteins are released extracellularly, Tat-secreted proteins in Gram-negative bacteria can either remain periplasmic or be carried out of the cell by the Type II Secretion System.

While the Tat pathway is essential for the physiology and survival of both pathogenic and non-pathogenic bacteria, a number of pathogenic bacteria, such as *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, and *Escherichia coli* O157:H7, require a functional Tat pathway for full virulence in animal infection models. Phospholipase C enzymes are a prominent example of Tat-secreted proteins that serve as virulence factors for a variety of pathogens, such as *P. aeruginosa*, *Legionella pneumophila*, and *Mycobacterium* TB. These enzymes, which cleave phospholipids just prior to their phosphate group, can perform a range of purposes during infection, including reduction of immune cell activity and support of intracellular survival (Songer, 1997).

A number of Gram-negative bacteria use specialised secretion systems to convey virulence proteins outside of the cell and, in some instances, straight into the cytoplasm of a eukaryotic or prokaryotic target cell. Extracellular protein secretion can be difficult for Gram-negative bacteria, as these proteins must traverse two (and in some cases three) phospholipid membranes to reach their destination. Some secreted proteins in Gram-negative bacteria traverse these membranes in two distinct processes, first being delivered to the periplasm by the Sec or Tat secretion systems, as described in the previous section, and then being transported across the outer membrane by a second transport system. This method is referred to as Sec- or Tat-dependent protein secretion. In addition, many

additional proteins are secreted through channels that span both the inner and outer membranes of bacteria, a process known as Sec- or Tat-independent protein secretion. Gram-negative bacteria have Type I through Type VI dedicated secretion systems, with each system conveying a distinct group of proteins. These systems rely on β-barrel channels that form a ring in the outer membrane of the bacterial cell, but their shapes and molecular functions vary considerably.

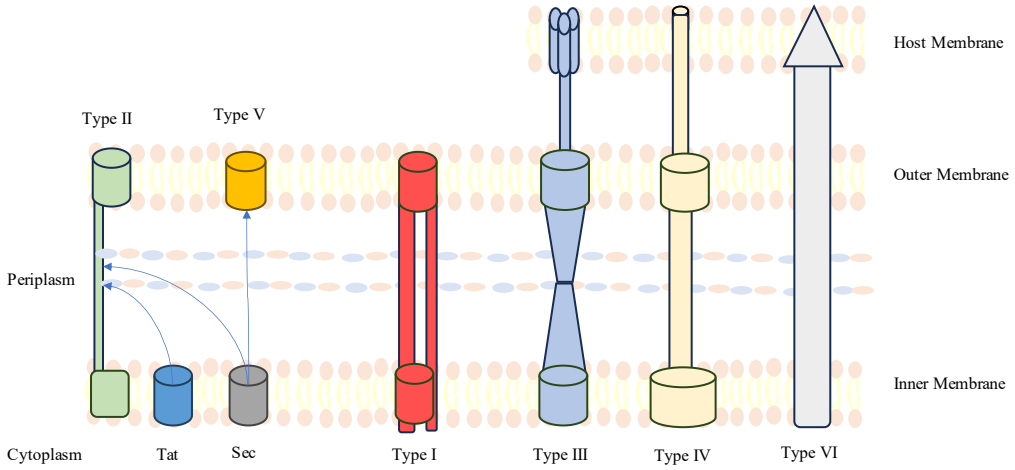


Figure 3 Secretion systems in Gram-negative bacteria (Image digitally drawn by Shahin Heshmatifar, inspired by Green & Mecsas, 2016)

Gram-negative bacteria use multiple protein secretion mechanisms to transfer proteins across one, two, or three phospholipid membranes. Some proteins are secreted by a two-step process depending on Sec or Tat. These proteins are carried over the outer membrane by a second secretion mechanism after crossing the inner membrane via the Sec or Tat secretion pathways. This is how the T2SSs and T5SSs secrete proteins. T2SS translocates Tat or Sec pathway-initially delivered proteins because it secretes folded substrates (where Sec substrates are folded in the periplasm). In contrast, T5SS autotransporters must be unfolded prior to outer membrane transport and must therefore be secreted via the Sec pathway across the inner membrane. In addition, a number of Gram-negative protein secretion systems transport their substrates across both bacterial membranes in a single step, independent of Sec or Tat. This consists of T1SSs, T3SSs, T4SSs, and T6SSs. All of these pathways have periplasm-spanning channels and secrete proteins from the cytoplasm to the exterior of the cell; nevertheless, their protein secretion processes are highly distinct. Three of these secretion systems, the T3SS, T4SS, and T6SS, are also

capable of transporting proteins over an extra host cell membrane and delivering them straight to the cytoplasm of a target cell.

1.2.2.3 Type I Secretion System

Type I secretion systems (T1SSs) have been identified in a wide number of Gram-negative bacteria, including plant and animal diseases, where they transport their substrates in a single step through both the inner and outer bacterial membranes (as seen in Figure 3. T1SSs resemble a broad family of ATP-binding cassette (ABC) transporters that export tiny compounds such as antibiotics and poisons from the cell (26). Some bacteria may have many T1SSs, each of which is responsible for transporting one or a small number of unfolded substrates. In addition to digestive enzymes like as proteases and lipases, these substrates also include adhesins, heme-binding proteins, and proteins containing repeats-in-toxins (RTX) motifs. T1SS substrates are typically Sec-independent and contain a C-terminal signal sequence that is recognised by T1SS and remains intact. (Thomas et al., 2014)

T1SSs contain three key structural components: an ABC transporter protein in the inner membrane, a membrane fusion protein (MFP) that bridges the inner membrane to the outer membrane factor (OMF), and a membrane fusion protein (MFP) that crosses the inner membrane. It catalyses ATP to provide energy for substrate transport, interacts with the MFP, and plays a role in substrate recognition. The MFP connects with the ABC transporter in the inner membrane and traverses the periplasm to associate with the OMF . In addition, the cytoplasmically positioned N-terminus of the MFP is considered to have a role in substrate selection. The OMF forms a pore in the outer membrane, through which the substrate travels in an unfolded state. Notably, T1SSs frequently utilise the multifunctional protein TolC as their OMF. This pore-forming protein is also utilised to export molecules and other substances, and is recruited to the MFP once the ABC transporter and MFP have come into contact with a substrate (Thomas et al., 2014)

Based on their N-terminal sequences, the T1SS ABC transporters have been subdivided into three families. A C39 peptidase domain, which is a structural motif of the papain superfamily, is present in one class of ABC transporters. ABC-transporters containing C39-peptidase are essential for detecting and cleaving the N-termini of substrates. Colicin

V of *E. coli* is an example of a T1SS substrate having a C39 peptidase domain. A second class of ABC transporters comprises a C39-like peptidase domain (CLD) that lacks proteolytic activity and, consequently, does not cleave its specific substrates. The substrates of CLD-containing ABC transporters typically include RTX motifs and are significantly bigger than those of C39-containing peptidase ABC transporters. Extracellular calcium is bound by RTX motifs, but not intracellular calcium. These big substrates are able to remain unfolded within the cell because calcium binding stimulates the folding of these proteins. The N-terminal domain of a third class of T1SS ABC transporters is devoid of any extra sequences. Their substrates may or may not contain RTX motifs, but they are smaller than substrates transported by CLD-containing ABC transporters and possess C-terminal secretion signals. (Lecher et al., 2012)

1.2.2.4 Type II Secretion System

T2SSs, which transport folded proteins from the periplasm to the extracellular environment, are conserved in the majority of Gram-negative bacteria. Since the T2SS channel is only found in the outer membrane, proteins secreted by this apparatus must first be transported to the periplasm via the Sec or Tat secretion routes, which move protein substrates across the inner membrane, as detailed before in this chapter. This secretion system was initially referred to as the primary terminal branch of the Sec secretion pathway because to its ability to export proteins carried across the inner membrane by the Sec secretion pathway. Nonetheless, this nomenclature has been changed to T2SS to highlight the capacity of these secretion systems to transport Tat-secreted proteins as well. Since proteins destined for secretion by the T2SS apparatus must first pass via the Sec or Tat inner membrane transporters, T2SS substrates must include a Sec- or Tat-type cleavable signal sequence at their N termini. Moreover, since the T2SS secretes folded substrates, proteins carried across the cytoplasmic membrane by the Sec route must be folded in the periplasm prior to export through the T2SS. (Korotkov et al., 2012)

T2SSs are capable of secreting a varied array of substrates outside the bacterial cell, some of which contribute to the pathogenicity of bacterial pathogens. In some bacterial species,

the T2SS is necessary for the secretion of numerous substrates, whereas in others, it is used to transport only a single protein. These secreted proteins have a variety of biological roles, but the majority of them are enzymes, such as proteases, lipases, and phosphatases, as well as many proteins that process carbohydrates.

The outer-membrane complex, the inner-membrane platform, the secretion ATPase, and the pseudopilus are the four subassemblies that make up T2SSs. As its name suggests, the outer-membrane complex sits in the outer membrane, where it functions as the translocation route for folded periplasmic T2SS substrates. This channel is made up of the multimeric protein known as secretin. It is assumed that the secretin's N terminus extends all the way to the periplasm in order to interact with other T2SS proteins in the inner membrane. The inner membrane platform, formed of multiple copies of at least four proteins, is implanted in the inner membrane and extends into the periplasm, where it contacts the secretin. This platform coordinates the export of substrates by connecting with the secretin, pseudopilus, and the ATPase. The ATPase is found in the cytoplasm and supplies the system with energy. As its name suggests, the T2SS pseudopilus is evolutionarily linked to and structurally comparable to the proteins that form type IV pili on bacterial cell surfaces and certain bacterial competence systems. Consequently, one hypothesis for secretion via the T2SS channel posits that these pseudopili retract to force the folded T2SS substrate through the outer membrane channel. In this "piston" scenario, the periplasmic region of the secretin is in touch with "secretion-competent" proteins. It is assumed that this contact stimulates the cytoplasmic ATPase to accelerate the retraction of the T2SS pseudopili, which propel proteins into the secretin channel. (Korotkov et al., 2012)

1.2.2.5 Type III Secretion System

Large numbers of Gram-negative bacterial pathogens and symbionts possess Type III secretion systems (T3SSs). Due to their structure, T3SSs have been referred to as "injectisomes" and "needle and syringe-like devices." They produce many proteinaceous substrates across the inner and outer bacterial membranes. In addition, the majority of T3SSs transport substrates into the membrane of the target eukaryotic cell in the same step, and so transport proteins across three membranes. In general, it is believed that T3SS

substrates are secreted in a single step; however, *Yersinia* has lately cast doubt on this assumption (discussed below). The generic term for T3SS substrates is effector proteins. Pathogens may release only a few effector proteins, as with *Pseudomonas* and *Yersinia*, or several dozen, as with *Shigella* and EHEC. Secretion signals are incorporated into the N-termini of T3SS substrates and are not cleaved. Not all T3SS effectors are guided to the T3SS base by chaperones, where they are released in an ATP-dependent, unfolded state. (Troisfontaines & Cornelis, 2005)

The core of the T3SS consists of nine proteins that are substantially conserved across all known systems. They are evolutionarily related to flagellin and share eight of these proteins with the flagellar machinery present in many bacteria. In addition to these nine core proteins, T3SSs contain 10 to 20 other proteins that are either required or critical to their function. The structural components of T3SSs are normally encoded in a small number of operons, which are typically located on plasmids or in pathogenicity islands on the bacterial chromosome. Due to the fact that T3SSs are often acquired horizontally, bacteria with divergent evolutionary histories may possess closely related systems and vice versa. For instance, the genomes of Shigella and *E. coli* are substantially identical, although the Shigella T3SS is more related to the Salmonella T3SS than to the *E. coli* diseases EHEC and EPEC. On the basis of the homology of their extracellularly developed needles, tips, and translocons, seven families of T3SSs have been postulated. (Troisfontaines & Cornelis, 2005)

The T3SS consists of three major components: a base complex or basal body, a needle component, and a translocon. The base complex consists of cytoplasmic components and crosses the inner and outer membranes to produce a structure resembling a socket with many rings and a central rod. In the majority of systems, it consists of at least 15 proteins. A filament called the needle is encased by and emerging from this socket and rod-like structure; it extends through the secretin and into the extracellular area. The T3SS needle features an interior hollow core that is sufficiently broad to accommodate an unfolded effector. Excitingly, recent research has observed a 'trapped' effector protein using cryo-EM and single particle analysis, supporting the hypothesis that substrates can traverse the needle. (Akopyan et al., 2011)

The T3SS tip complex, which sits at the outer tip of the needle, is essential for detecting host cell contact and controlling effector secretion. It is also required for translocon insertion into host cell membranes. The T3SS translocon is required for the transit of effectors through the membranes of host cells, but not for the secretion of effectors outside of the bacterium. Upon contact with host cells, translocons combine to produce a pore that is crucial for effector distribution. Recently, however, an alternate two-step model of Type 3 effector translocation has been proposed, in which effectors and translocon components are produced prior to host cell interaction and stay linked with the bacterium, possibly in the form of lipid vesicles. The translocon and tip proteins establish a pore through which the effectors can pass after sensing contact with host cells, possibly via the needle. To determine the mechanism of translocation, additional tests are required. (Akopyan et al., 2011)

1.2.2.6 Type IV Secretion System

Type IV secretion systems (T4SSs) are ancestrally related to bacterial DNA conjugation systems and are capable of secreting a variety of substrates, such as single proteins, protein-protein and DNA-protein complexes. T4SSs release substrates into numerous target cells, including bacteria (of the same or other species) and eukaryotic cells. These macromolecular complexes transport substrates across both the inner and outer membranes of Gram-negative bacteria. T4SSs, like T3SSs, can bridge an extra host cell membrane, allowing for the direct transfer of substrates into the recipient cell's cytoplasm. Due to the fact that T4SSs are capable of transporting both DNA and proteins, they can perform a range of functions, including conjugative transfer of DNA, DNA absorption and release, and translocation of effector proteins or DNA/protein complexes directly into recipient cells. (Cascales & Christie, 2003)

Despite the diversity of their substrates and roles, all T4SSs are evolutionary linked, sharing shared components and working in a comparable fashion. This section will therefore concentrate on the VirB/D system of Agrobacterium tumeficans as a model of Type IV Secretion. A. tumeficans employs its T4SS to transport oncogenic T-DNA into plant cells, and its T4SS has used as a model for researching the assembly and function of T4SS. The VirB/D T4SS comprises 12 proteins with the designations VirB1-VirB11 and VirD4. The majority of these proteins are membrane-bound and multi-copy,

interacting with one another. The periplasm, inner and outer membranes include the VirB6-10 proteins, which constitute the secretion channel and its auxiliary proteins. VirB4, VirB11, and VirD4 localise to the inner membrane and function as the system's ATPases. VirD4 also serves as a coupling protein, binding proteins prior to channel-mediated secretion. T4SSs typically have an extracellular pilus made up of a main (VirB2) and minor (VirB5) subunit. (Cascales & Christie, 2003)

The process of substrate secretion by the T4SS apparatus is still a subject of current research. Nevertheless, it is hypothesised that substrate DNA or protein initially interacts with VirD4, a molecular "gate" at the base of the secretion apparatus. The substrate is then transferred by VirD4 to VirB11, which delivers it to the inner membrane channel complex. Finally, the substrate is transported from the periplasm to the protein complex of the outer membrane. It is yet unknown what function the T4SS pilus performs in the process of secretion. Some believe the pilus serves only as an attachment mechanism, allowing bacteria to get into close contact with their target cells. Others, however, hypothesise that the pilus may serve as the conduit for substrate translocation, particularly into target cells. Work is currently being done to identify which of these two models is correct. (Backert & Meyer, 2006)

T4SSs play crucial roles in the pathogenesis of numerous bacterial species. Neisseria gonorrhoeae, which uses its T4SS to mediate DNA uptake (which promotes virulence gene acquisition), and L. pneumophila, Brucella suis, and Helicobacter pylori, which use their T4SSs to translocate effector proteins into host cells during infection to disrupt their defence strategies, are notable examples of bacterial pathogens that use T4SSs for virulence. These effector proteins serve a variety of purposes. For instance, the intracellular pathogen L. pneumophila employs its T4SS to translocate over 200 effector proteins into the host cell, where they play crucial roles in altering the host cell architecture to generate a vacuole suited for bacterial reproduction. (Backert & Meyer, 2006)

1.2.2.7 Type V Secretion System

In contrast to other secreted substrates, which traverse the bacterial membrane with the aid of a specific secretion apparatus or membrane channel, Type V secretion system

(T5SS) substrates secrete themselves. These proteins or protein complexes contain their own -barrel domain, which inserts into the outer membrane and produces a channel via which the remainder of the protein or another protein is transported. Because T5SSs only secrete proteins from their outer membrane, the Sec apparatus must first translocate these proteins in an unfolded condition across the inner membrane and into the periplasm. Consequently, T5SS proteins have an N-terminal Sec signal sequence that is removed upon entry into the periplasm. (van Ulsen et al., 2014)

The majority of identified T5SS substrates are virulence proteins, which function as poisons and receptor-binding proteins. T5SS substrates that play crucial roles in pathogenesis include the immunoglobulin A protease of N. gonorrhoeae, which cleaves host antibodies, the IcsA protein of Shigella flexneri, which promotes actin-based intracellular motility and also serves as an adhesin, and YadA of Y. enterocolitica, which helps to promote translocation of T3SS substrates into host cells, and aids in T5SSs can be divided into three types based on the number of proteins involved in the process of secretion. These classes consist of autotransporter, two-partner, and chaperone-usher secretions.(van Ulsen et al., 2014)

1.2.2.7.1 Autotransporter secretion

The autotransporter system is the type of Type V secretion that is the simplest. Autotransporters have components that allow them to secrete themselves, as their name suggests. Autotransporters contain 3–4 domains: a translocator domain at the C-terminus that forms the outer membrane channel, a linker domain, a passenger domain that contains the functional portion of the autotransporter protein, and sometimes a protease domain that cleaves off the passenger domain once it passes through the channel.

Following the secretion of the unfolded autotransporter protein through the inner membrane, the translocator domain forms a 12-stranded -barrel in the outer membrane, typically with the assistance of a variety of auxiliary proteins, such as the periplasmic chaperone Skp and the Bam complex. The flexible linker domain then guides the passenger domain out of the cell through the channel. Once the transporter domain has

reached the cell's exterior, it is either released by its protease domain or remains connected to the translocator domain and protrudes from the cell. (van Ulsen et al., 2014)

1.2.2.7.2 Dual-participant secretion

Despite the fact that the majority of T5SS substrates are secreted via the autotransporter mechanism, a few rely on other polypeptides for transport outside of the cell. In two-partner secretion, a pair of proteins participate in the secretion process, with one protein carrying the -barrel domain and the other serving as the secreted protein. Two-partner secretion is chiefly responsible for conveying large virulence proteins, such as the filamentous haemagglutinin of Bordetella pertussis and the high-molecular-weight adhesins HWM1 and HWM2 of *Haemophilus influenzae* (Henderson et al., 2004).

1.2.2.7.3 Chaperone-usher glandular secretion

A third subcategory of T5SSs comprises proteins secreted with the assistance of two other proteins: the usher protein, which forms the β-barrel channel in the outer membrane, and the chaperone, a periplasmic protein that aids in the folding of the secreted protein prior to its delivery to the channel. Typically, chaperone-usher systems are employed to assemble pilins on the surface of Gram-negative bacteria, such as the P pilus of uropathogenic *E. coli* (Waksman & Hultgren, 2009).

1.2.2.8 Type VI Secretion System

Type VI secretion systems (T6SSs) are the most recently discovered bacterial secretion systems, and consequently, much remains to be learned about their structure and functions. T6SSs translocate proteins into eukaryotic cell targets and, more frequently, other bacteria. These systems are relatively conserved among Gram-negative bacterial species, with approximately a quarter of sequenced genomes harbouring genes for T6SS components. T6SSs are capable of delivering effector proteins from one bacterium to another in a contact-dependent manner, which is thought to play a role in bacterial communication and environmental interactions (Russell et al., 2014).

T6SSs are extremely big, encoding up to 21 proteins within a single gene cluster. Thirteen of these proteins are thought to perform a structural role in the secretion system and are conserved in all T6SSs. T6SSs share structural similarities with phage tails, and it has been postulated that T6SSs originated from inverted phage tails that eject proteins outside

of the bacterial cell rather than injecting them into the cell. Some structural components of the T6SS apparatus have been postulated to serve as effector proteins, however other T6SS effector proteins have been found. Many of these effectors are directed against the bacterial cell wall and membrane, suggesting that this secretion mechanism may play a role in encouraging interspecies bacterial competition. Many T6SS effectors are encoded alongside a gene that confers immunity to the effector, hence avoiding self-intoxication, lending support to this theory (Russell et al., 2014) (Russell et al., 2011).

It is postulated that T6SSs contribute to the pathogenicity of certain bacterial infections by delivering protein substrates to host cells and by secreting substrates into nearby bacteria that may be competing for a specific host niche. While it is known that several bacterial pathogens, including *P. aeruginosa*, *V. cholerae*, and *S. marcescens*, can utilise their T6SSs under laboratory settings, it is unknown how these T6SSs contribute to survival in the environment (and in mammalian infection) (Russell et al., 2011) (Russell et al., 2014).

1.2.2.9 Biotechnology industrial applications of secretion systems

Wacker Biotech is a company with particular interest in recombinant protein expression in *Escherichia coli* and they develop an expression system that secretes the product in its native conformation into the culture broth. This will simplify the primary recovery and purification processes significantly and offers a more cost-efficient process for manufacturing a product. The Wacker secretion system operates in two-step export mechanism. Firstly, path of product from the cytoplasm into the periplasm via the sec-pathway during which the signal peptide is cleaved off. The second step which is unique to Wacker secretion strain is designed to carry the product through the outer membrane and into the broth. The outer membrane has been modified in their *Escherichia coli* K12 derivative. They have showed high yields and purity. The Wacker secretion system has been successfully utilised in manufacturing of various enzymes, proteins, Fabs and peptides (Wacker. 2012a).

Biopharmaceutical companies are getting more involved with novel secretion systems such as the patented Wacker ESETEC technology. XL-protein and Wacker Biotech have

signed a long-term agreement to utilise the ESETEC secretion system to manufacture PASylated human growth hormones and it has been done successfully in high yields (Wacker. 2012b). ESETEC® is the trade name of an expression and secretion system developed and patented by WACKER. It is based on an *Escherichia coli* K12 strain and a series of highly expressive plasmids. PASylation is a technology that extends the half-life of proteins by applying a natively disordered amino acid chain as a biological alternative to PEG, which is a chemical conjugate to small-sized biopharmaceuticals. (Wacker. 2012b)

The twin arginine translocation (Tat) pathway of bacteria regulates the transportation of proteins from the cytoplasm to the periplasm. The genetic analysis of this pathway and its functionality was studied, and it has been shown that this pathway can be altered and modified as desired to an extent (Delisa et al, 2002). This possibility allows over delivery of product to the periplasm and further modification of the outer membrane can be researched in combination with Tat optimisation to achieve high secretion of protein into the broth.

A prominent difference between the Tat system and Sec system is that Sec transports the protein (product) from the cytoplasm to the periplasm in an unfolded state whereas the Tat system transports the product in a folded state. This system tends to transport proteins in its correctly folded state. However, it has its limitations such as inability to transport proteins that have disulphide bonds as these are usually acquired in the periplasm (Matos et al., 2013). A study has been carried out to investigate novel strains that oxidise disulphide bonds in the cytoplasm and allows Tat system to transport correctly folded disulphide bonded proteins to the periplasm (Matos et al., 2013). This technology allows higher yields to be achieved from primary recovery steps. However, cell lysis is still required as the products are in the periplasm. Complete secretion of the recombinant product into the medium would still be very attractive, although combined technologies (Tat and antisense) may lead to very high levels of complete secretion.

Export of recombinant products to the peirplasm in *Escherichia coli* is often preferred. Previous studies have shown that the Tat secretion system is capable to transport high

levels of recombinant product into the periplasm (Albiniak et al., 2013). In a study a novel approach was investigated, *Escherichia coli* expressing Bacilus subtilis TatAdCd system was studied and it was shown that during fermentation the mature proteins are mostly found in the medium. It proven that the outer membrane became leaky during fermentation and that the cells were intact (Albiniak et al., 2013). This novel approach offers a methodology with great potentials to harvest recombinant products directly from medium skipping cell lysis steps and thus avoiding associated problems following cell lysis.

Tat secretion pathway was studied to see whether it can offer the same results in industrial-type fermentations. Tat secreted products have relatively easier downstream processing and purification (Matos et al., 2012). However, its capacity and robustness under industrial conditions must be proven and this study has shown great potentials and industrial application.

The native capacity of tat transportation of proteins to the periplasm is very limited. However, overexpression of Tat pathway is possible. Over accumulation of recombinant product in the periplasm that have been transported by the tat system may have an impact on the cell integrity and primary recovery processes. A study was carried out to show that the enhanced strains of *Escherichia coli* can undergo the primary recovery steps and show similar robustness to traditional strains and thus offer the advantages of the tat system over Sec system (Branston et al., 2012).

Outer membrane of the bacteria can be engineered such that its function will be enhanced to our desire. Bacterial outer membrane vesicles have been engineered in *Escherichia coli* that can be characterised and utilised for various functions in biotechnology and clinical studies. In biotechnology it offers the ability to display proteins on the surface and it can also be utilised in delivery of biologics (Kim et al., 2008). This again is a novel methodology in manipulating native paths and patterns of produced proteins in order to be exploited in manufacturing.

There are numerous methodologies to express recombinant proteins extracellularly and strains of *Escherichia coli* with these particular properties exist in many laboratories. However their efficiency and industrial application is still premature. A universal genetic assay has been developed to screen libraries of these GMOs that are engineered for extracellular protein expression. This assay explores secretion pathways and gives a higher understanding of the events occurring in secretion. FlAsH-tetracysteine-based genetic assay provides a convenient, high-throughput tool that can be applied generally to diverse secretory pathways. It eliminates poorly understood events in secretion and aids development of more efficient strains of *Escherichia coli* that secret their product into the broth (Haitjema et al., 2013)

A study on *Escherichia coli* (K12) was carried out and secretion mechanism of this organism was investigated with an concentration on YebF which is a small, soluble protein. It was shown that recombinant proteins that were fused to YebF were successfully secreted to the extracellular environment. The function of YebF is not fully understood, normally it is not secreted outside of the cell, however it can act a potential carrier protein that aids transport of recombinant proteins across the membrane and into the medium(Zhang et al., 2006).

The study above leads to a potential research subject such that in case of high efficiency, it will provide a useful tool towards complete secretion of recombinant products in to the medium.

Extracellular secretion of recombinant protein by *Escherichia coli* offers many considerable advantages such as reduction in complexity of the bioprocess engineering and also in terms of the quality of the product, the secreted product will possess a greater quality, this is because the medium outside of the cell offers a better environment for folding and it is also free from chemical and enzymes that are present in the cell and may affect the recombinant product negatively, such as degradation due to cell associated proteolytic reactions (Ni et al., 2009).

Escherichia coli BL21(DE3) is adapted in the biotechnology industry to produce an enzyme used in the textile industry. This enzyme is secreted extracellularly, and the Type II secretion system is utilised in this production (Su et al., 2012). It was found that some of the product is accumulated in the periplasm (Su et al., 2012). Another secretion system was studied to see whether it can replace the described process. Alpha-hemolysin secretion system was explored and compared with Type II secretion system (Su et al., 2012). It was shown that under the same fermentation conditions the alpha-hemolysin system secreted 2.5 times more product into the medium compared with the original Type II secretion system (Su et al., 2012). Other secretion systems can be explored to show potential off higher yields specific to some products or strains of *Escherichia coli*.

1.2.3 Expression Systems and plasmids

Escherichia coli is one of the preferred organisms for recombinant protein synthesis. Its application as a cell factory is well-established, and it has become the most often used expression platform. For this reason, there are several molecular tools and methods available for the high-level generation of heterologous proteins, including a broad catalogue of expression plasmids, a large number of modified strains, and numerous culturing techniques.

The creation of recombinant proteins in microbial systems has undoubtedly altered biochemistry. Almost gone are the days when kilos of animal and plant tissues or enormous volumes of biological fluids were required to purify modest quantities of a specific protein. Every scientist who begins a new project requiring pure protein instantly considers how to obtain it in recombinant form. The ability to generate and purify the desired recombinant protein in high quantities permits its biochemical evaluation, application in industrial processes, and development of commercial products.

Theoretically, the methods required to produce a recombinant protein are rather basic. The gene of interest is cloned into an expression vector, transformed into the host of choice, induced, and then the protein is ready for purification and characterisation. In actuality, though, a multitude of issues can arise. Down the pipeline, it is common to

encounter issues such as poor host growth, inclusion body (IB) formation, protein inactivity, and even the inability to get any protein.

Well-known are the benefits of utilising *E. coli* as the host organism. Its growth kinetics are unprecedentedly rapid. Its doubling period in glucose-salts medium under optimal environmental conditions is approximately 20 minutes (Sezonov et al., 2007). Therefore, a culture injected with a 1/100 dilution of a saturated starter culture may achieve stationary phase within a few hours. It should be emphasised, however, that the production of a recombinant protein may impose a metabolic load on the microorganism, resulting in a significant reduction in generation time (Bentley et al., 1990). It is simple to cultivate cultures with a high cell density. The theoretical density limit for an *E. coli* liquid culture is predicted to be approximately 200 g dry cell weight/l, or approximately 1 1013 viable bacteria/ml (Lee, 1996; Shiloach and Fass, 2005). Nonetheless, exponential development in complex medium results in densities that are nowhere near this value. In the simplest laboratory setup (i.e., batch cultivation of *E. coli* at 37°C using LB medium), the upper limit may be less than 0.1 percent of the theoretical maximum (Sezonov et al., 2007). In order to improve *E. coli* growth, even when creating recombinant proteins, high cell-density culture techniques were developed (Choi et al., 2006). Being a workhorse organism, these tactics emerged because to an abundance of physiological knowledge. Rich complex media can be produced from easily available and inexpensive components. Transformation using exogenous DNA is quick and straightforward. *E. coli* plasmid transformation can be done in as little as 5 minutes (Pope and Kent, 1996).

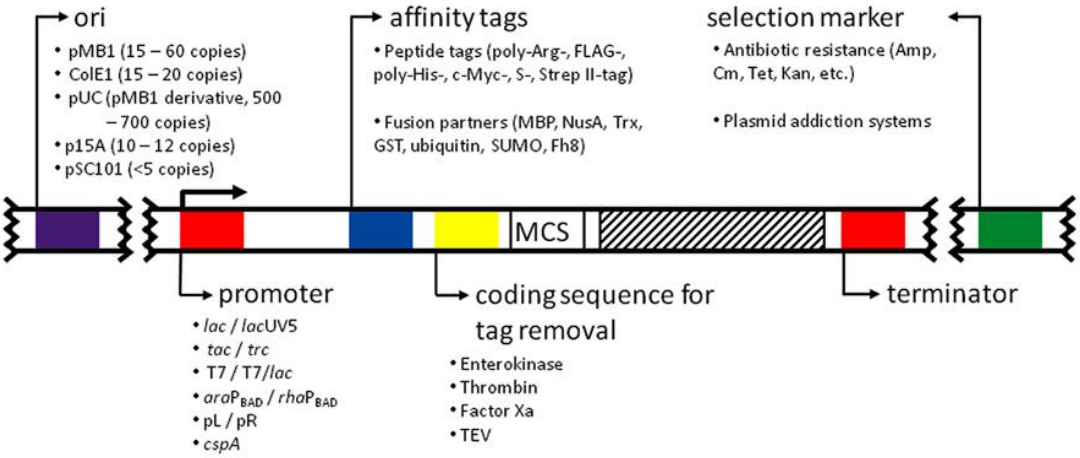


Figure 4 Anatomy of an expression vector. the figure illustrates the primary features of common expression vectors (adopted from Rosano & Ceccarelli, (2014))

Numerous combinations of replicons, promoters, selection markers, multiple cloning sites, and fusion protein/fusion protein removal techniques produce the most prevalent expression plasmids in use today (Figure 4). Because of this, the catalogue of accessible expression vectors is vast, and it is simple to become confused when selecting one. To make a well-informed decision, these characteristics must be carefully analysed based on the individual's requirements.

1.2.3.1 Replicon

Replicons are present in genetic components that replicate as autonomous units, such as plasmids. It is composed of a single origin of replication and its associated trans-acting regulatory components. Copy number is an important factor to consider while selecting an appropriate vector. The replicon contains the control of copy number. As there are several expression units in the cell, it is logical to assume that a high plasmid dosage corresponds to a higher recombinant protein production. However, a large number of plasmids may impose a metabolic load that slows bacterial growth and may cause plasmid instability, hence reducing the number of healthy organisms for protein synthesis. Therefore, the use of plasmids with a high copy number for protein expression does not imply an increase in production yields (Rosano & Ceccarelli, 2014).

The pET series contains the pMB1 origin (ColE1-derivative, 15–60 copies per cell; Bolivar et al., 1977), whereas the pUC series contains a modified form of the pMB1 origin (500–700 copies per cell; Minton, 1984). The ColE1 wild-type origin (15–20 copies per

cell; present in pQE vectors) (Qiagen). They are all members of the same incompatibility group, hence they cannot be propagated in the same cell since they compete for the replication machinery. For the dual expression of recombinant proteins employing two plasmids, p15A ori-containing systems are available (plasmid series pACYC and pBAD, 10–12 copies per cell). Using the plasmid pSC101, triple expression can be achieved, but rarely. This plasmid is subject to tight replication control, resulting in a low copy number (5 copies per cell). The use of plasmids containing this replicon can be advantageous in situations when the presence of a high concentration of a cloned gene or its product has a detrimental effect on the cell. Alternatively, the Duet vectors (Novagen) simplify dual expression by enabling the cloning of two genes on the same plasmid. Each of the Duet plasmids contains two multiple cloning sites that are preceded by a T7 promoter, a lac operator, and a ribosome binding site. By mixing multiple compatible Duet vectors, four expression plasmids can yield up to eight recombinant proteins (Rosano & Ceccarelli, 2014).

1.2.3.2 Promoter

Lac promoter, a crucial component of the lac operon, is without a doubt the cornerstone of bacterial promoter research. The gathered understanding of the system's operation allows for its expansion into expression vectors. Lactose induces the system, and this sugar can be utilised for protein synthesis. However, induction is difficult when readily metabolizable carbon sources are present (such as glucose present in rich media). In the presence of lactose and glucose, the lac promoter is not fully activated until all glucose has been consumed. At this stage (low glucose), cyclic adenosine monophosphate (cAMP) is created, which is required for lac operon activation. This positive regulation of gene expression is referred to as catabolite suppression. Accordingly, cAMP levels are low in cells growing in lac operon-repressing carbohydrates, and this coincides with reduced lac operon expression. In addition, glucose prevents lactose uptake since lactose permease is inactive in its presence. To achieve expression in the presence of glucose, a mutant lacUV5 promoter was added that decreases (but does not eliminate) sensitivity to catabolite control. Due to titration of the low levels of the lac promoter repressor protein LacI from the single chromosomal copy of its gene, both promoters present in multicopy plasmids suffer from the disadvantage of sometimes exhibiting unacceptable levels of

expression in the absence of inducer (also known as "leakiness") (about 10 molecules per cell). By introducing a mutant promoter of the lacI gene termed lacIQ, which results to about 10-fold greater levels of LacI expression, it is possible to modulate basal expression. The lac promoter and its derivative lacUV5 are somewhat ineffective for recombinant protein synthesis. There exist synthetic hybrids that combine the strengths of different promoters with the benefits of the lac promoter. The tac promoter, for instance, is composed of the -35 area of the trp (tryptophan) promoter and the -10 region of the lac promoter. This promoter is roughly 10 times more powerful than lacUV5. The pUC series (lacUV5 promoter, Thermo Scientific) and pMAL series of vectors are notable examples of commercial plasmids that utilise the lac or tac promoters to induce protein production (tac promoter, NEB) (Rosano & Ceccarelli, 2014).

For recombinant protein expression, the T7 promoter system included in pET vectors (pMB1 ori, medium copy number, Novagen) is highly popular. In successful circumstances, the target protein can account for up to fifty percent of the total cell protein. The target gene is cloned in this technique behind a promoter recognised by the phage T7 RNA polymerase (T7 RNAP). This extremely active polymerase must be supplied by a separate plasmid or, more typically, it is integrated into the bacterial genome in a prophage (DE3) encoding for the T7 RNAP under the transcriptional control of a lacUV5 promoter. Thus, lactose or its non-hydrolyzable counterpart isopropyl β-D-1-thiogalactopyranoside can trigger the system (IPTG). Co-expression of T7 lysozyme and lacIQ can both regulate basal expression. T7 lysozyme suppresses transcription initiation from the T7 promoter by binding to T7 RNAP. T7 lysozyme will efficiently limit undesired expression of foreign genes put under the T7 promoter if only tiny amounts of T7 RNAP are generated due to leaky expression of its gene. A suitable plasmid contains the T7 lysozyme gene (pLysS or pLysE). The amount of T7 RNAP produced after induction exceeds the level of polymerase that T7 lysozyme can block. Thus, the "free" T7 RNAP is able to transcribe the recombinant gene. The insertion of a lacO operator downstream of the T7 promoter, creating a hybrid T7/*lac* promoter, provides an additional degree of control. All three strategies (strict repression of the lac-inducible T7 RNAP gene by $lacI^{Q}$, inhibition of T7 RNAP by T7 lysozyme, and inclusion of a *lacO* operator

following the T7 promoter) make the system optimal for avoiding basal expression (Rosano & Ceccarelli, 2014).

The problem of leaky expression is a result of the *lac* promoter's negative control. Background expression levels should be reduced for promoters that rely on positive control. This is the situation with the araP_{BAD} promoter found in pBAD vectors. The AraC protein serves as both a repressor and an activator. In the absence of an arabinose inducer, AraC inhibits translation by binding to two DNA locations. The protein–DNA combination effectively prevents RNA polymerase from attaching to the promoter by forming a loop. Upon addition of the inducer, AraC enters "activation mode" and enhances ara promoter transcription. In this manner, arabinose is required for induction(Rosano & Ceccarelli, 2014).

Placing a gene under the control of a controlled phage promoter is a regularly utilised alternative method. Expression of early lytic genes is directed by the strong leftward promoter (pL) of phage lambda. During lysogenic growth, the promoter is strongly inhibited by the cI repressor protein, which binds to the operator sequences. When the host's SOS response is triggered by DNA damage, the production of the protein RecA is increased, which catalyses the self-cleavage of cI and enables transcription of pL-controlled genes. This approach is utilised in pL promoter-containing expression vectors. The SOS reaction (and recombinant protein expression) can be induced by adding the DNA gyrase inhibitor nalidixic acid. Controlling cI synthesis by subjecting its gene to the influence of another promoter is another method for activating the promoter. This two-stage control scheme for T7 promoter/T7 RNAP-based vectors has already been reported. Under the control of the trp promoter, the cI repressor gene was inserted into the bacterial chromosome in the pLEX family of vectors (Life Technologies). This promoter is always "on" in the absence of tryptophan, and cI is continuously generated. The addition of tryptophan results in the formation of a tryptophan-TrpR repressor complex that binds strongly to the trp operator, hence inhibiting λcI repressor production. Following this, the desired gene is expressed under the pL promoter (Rosano & Ceccarelli, 2014).

All previously discussed promoters activate transcription in response to chemical stimuli. Also accessible are systems that respond to physical cues (such as temperature or pH). The pL promoter is an illustration. A mutant λcI repressor protein (λcI^{857}) is unstable at temperatures over 37°C and is temperature-sensitive. First, *E. coli* host strains harbouring the λcI^{857} protein (integrated into the chromosome or into a vector) are grown to the required density at 28–30°C, and then protein expression is stimulated by a temperature shift to 40–42°C. The industrial advantage of this technique stems in part from the fact that heat is typically created during fermentation, and it is simple to increase the temperature in high density cultures. In contrast, genes under the control of the cold-inducible promoter cspA are activated when the temperature drops to 15°C. This temperature is optimal for the expression of challenging proteins, as will be discussed in the following section. The plasmids of the pCold family feature a pUC118 backbone (a variant of pUC18) with the cspA promoter. Twenty to forty percent of the total expressed proteins were successfully expressed for over thirty recombinant proteins derived from various sources in the original study. In a number of instances, however, the target proteins were acquired in an insoluble state (Rosano & Ceccarelli, 2014).

1.2.3.3 Selection Indicator

Adding a resistance marker to the plasmid backbone inhibits the development of plasmid-free cells. The *E. coli* system routinely employs antibiotic resistance genes for this purpose. The *bla* gene, whose product is a periplasmic enzyme that inactivates the β-lactam ring of β-lactam antibiotics, confers resistance to ampicillin. As the β-lactamase is continuously released, however, breakdown of the antibiotic proceeds, and within a few hours, ampicillin is nearly depleted. In this instance, cells lacking the plasmid are permitted to proliferate during cultivation. Although not experimentally confirmed, it is possible that selective agents whose resistance is dependent on degradation, such as chloramphenicol and kanamycin, are also susceptible to this issue. Because resistance relies on the active efflux of the antibiotic from resistant cells, tetracycline has been demonstrated to be exceptionally durable during cultivation (Rosano & Ceccarelli, 2014).

Antibiotic costs and the spread of antibiotic resistance are key considerations for initiatives involving large-scale cultures. Antibiotic-free plasmid systems have been

developed with significant effort. These systems are based on the concept of plasmid addiction, a phenomenon that arises when cells lacking plasmids are unable to grow or survive. A gene can be removed from the bacterial genome and then put on a plasmid, for instance. Consequently, plasmid-less bacteria perish following cell division. According to their function principle, there are three subtypes of plasmid-addiction systems: (i) toxin/antitoxin-based systems, (ii) metabolism-based systems, and (iii) operator repressor titration systems. Despite the fact that this promising technique has been demonstrated to be effective in large-scale fermenters, expression systems based on plasmid addiction are not yet widely deployed (Rosano & Ceccarelli, 2014).

The T7 system is the most widely used method for generating recombinant protein in *Escherichia coli* production. The target gene is cloned into an expression vector downstream of the T7 promoter and then injected into a T7 expression host. T7 expression hosts, such as DE3 prophage strains and T7 Express strains, carry a copy of the phage T7 RNA polymerase gene on their chromosomes. T7 RNA polymerase is expressed and committed to target gene transcription when an inducer is introduced. T7 expression is frequently quite robust, while DE3 strains may suffer from undesired basal (non-induced) transcription. By co-expressing the lac repressor from a plasmid or a host-encoded *lacI* gene and T7 lysozyme, the natural inhibitor of T7 RNA polymerase activity, IPTG-induced T7 expression is regulated. T7 lysozyme can be expressed from the plasmids pLysS or pLysE, or a variant T7 lysozyme can be expressed from the *lysY* gene found in many NEB protein expression strains. The *lysY* gene product loses amidase (lysozyme) activity against the *Escherichia coli* cell wall, but retains the capacity to decrease the basal activity of T7 RNA polymerase (NEB, 2015).

Strains of *Escherichia coli* expressing the T7 RNA polymerase gene are particularly advantageous for robust overexpression of recombinant protein. The NEB T7 Express strain is a descendant of BL21 with various distinctive characteristics. Importantly, the T7 RNA polymerase gene is expressed from the wild-type lac promoter, resulting in a lower baseline expression of the target protein than if it were expressed from a promoter of the T7 RNA polymerase gene.

Strains containing the lambda DE3 prophage in which the expression of T7 RNA polymerase is regulated by lacUV5. However, T7 expression of recombinant protein is frequently enhanced by the co-expression of T7 lysozyme, which binds to and inhibits the action of T7 RNA polymerase until the point of induction. T7 Express variants enable the expression of more challenging proteins (NEB, 2015).

On a mini-F plasmid, a single copy of a T7 lysozyme gene (*lysY*), *lacIq* gene, or both (*lysY/lq*) was produced. Mini-F plasmids are maintained in a stable state without antibiotic selection. The lysY gene encodes the T7 lysozyme variant K128Y, which lacks amidase function but nevertheless inhibits T7 RNA polymerase. The T7 Express lysY/Iq strain is less sensitive to lysis than the pLysS and pLysE strains when an inner membrane protein is over-expressed. Strains harbouring the lysY gene suppress T7 expression completely in the absence of an inducer molecule. Nevertheless, a time course study reveals that T7 expression is rapidly (within 30 minutes) induced upon induction. Consequently, the T7 Express *lysY* strains express the appropriate amount of lysozyme for maximum regulation of T7-mediated hazardous protein expression. (NEB, 2015)

1.2.4 Periplasm and Cell Wall

K antigens coat the outer membrane of certain *E. coli* strains with a polysaccharide capsule. M antigens (colonic acids, which are polymers of glucose, galactose, fucose, and galacturonic acid) are formed in conditions of high osmolarity, low temperature, and low humidity. Additionally, *E. coli* has a glycolipid called the enterobacterial common antigen attached to the outside leaflet of its outer membrane (ECA). The outer membrane of *E. coli* is composed of a lipid bilayer with a phospholipid-rich inner leaflet and a lipopolysaccharide-rich outside leaflet (LPS). There are multiple types of membrane proteins interspersed (Liu, 2014).

The murein lipoprotein, a membrane protein with a molecular weight of 7.2 kDa, is present in 7105 copies per cell. This protein comprises three fatty acid residues that serve to anchor it to the inner leaflet of the outer membrane, while the remainder of the molecule resides in the periplasm. Approximately one-third of the molecules are covalently attached to the cell-wall peptidoglycan. The primary outer-membrane proteins include the

porins Omp C, Omp F, and Pho E, which produce pores. These porins are found in around 105 copies per cell. Their sizes range between 36.7 and 38.3 kDa. The pore widths for Omp F and Pho E are 1.16 nm and 1.08 nm for Omp C. Omp F synthesis is repressed by high osmotic conditions or high temperature, Omp C synthesis is repressed by high osmotic conditions, and Pho E synthesis occurs when cells are phosphate-starved (Liu, 2014).

Special transport proteins carry substances that are too big to diffuse through *E. coli* porin channels over the outer membrane. Maltose oligosaccharides, nucleosides, different iron chelates, and vitamin B12 are among these molecules. Involved proteins, in addition to porins, serve as receptors for the attachment of bacteriophages and colicins (Liu, 2014).

E. coli's periplasm (the space between the inner and outer membranes) comprises 20–40% of the cell's volume. This compartment is osmotically active in part because it includes substantial amounts of membrane-derived oligosaccharides (8–10 linked glucose residues with 1-phosphoglycerol and O-succinyl esters replaced). The periplasm of *E. coli* contains more than 60 known proteins, including binding proteins for amino acids, sugars, vitamins, and ions; degradative enzymes (phosphatases, proteases, and endonucleases); and antibiotic detoxifying enzymes (β-lactamases, alkyl sulfodehydrogenases, and aminoglycoside phosphorylating enzymes). The periplasmic environment is oxidising while the cytoplasmic environment is decreasing. This explains why certain secretory proteins whose activity depends on disulfide links are inactive in the cytoplasm. *E. coli*'s cell wall is composed of a layer of peptidoglycan responsible for cell shape and rigidity. One or a few molecules thick, the peptidoglycan layer is anchored to the outer membrane at approximately 400 000 locations by covalent linkages to the main membrane lipoprotein and noncovalent bonds to porins. 200–400 adhesion zones cross the periplasm between the outer membrane and the cytoplasmic membrane. These appear to be the sites of attachment and export of outer-membrane proteins and lipopolysaccharide by certain bacteriophages. In addition to these apparently random junctions, the two membranes appear to be linked at defined periseptal annuli, which are ring-shaped adhesion zones near the cytoplasmic membrane (Schmidt & Liu, 2019).

E. coli's cytoplasmic membrane is composed of around 200 different proteins and four types of phospholipids. Proteins account for approximately 70% of the structure's mass. Under aerobic circumstances, the cytoplasmic membrane of *E. coli* contains a variety of dehydrogenases (including NADH-, D- and L-lactate, and succinate dehydrogenases), pyruvate oxidase, cytochromes (of the o and d complexes), and quinones (mainly 8-ubiquinone). Other dehydrogenases (such as formate and glycerol-3-phosphate dehydrogenases) and enzymes involved in anaerobic respiration may be present in anaerobically grown *E. coli* (nitrate and fumarate reductases). Adenosine triphosphate (ATP) is produced at the cytoplasmic membrane. It can develop in relatively dilute nutrient solutions because the cytoplasmic membrane systems involved in the transport of solutes are highly efficient. Over 20 proteins are involved in diverse aspects of peptidoglycan production, cell wall elongation, and cell division in the cytoplasmic membrane of *E. coli*. Approximately 10 of these proteins have been identified based on their capacity to bind -lactam antibiotics covalently. Some of these penicillin-binding proteins have been demonstrated to be directly engaged in cell-wall formation (Schmidt & Liu, 2019).

The genome of *E. coli* strain MG1655 (a derivation of laboratory strain K-12) consists of a 4.64 Mb circular DNA molecule including 4288 protein-coding genes (arranged into 2584 operons), seven ribosomal RNA (rRNA) operons, and 86 transfer RNA (tRNA) genes. In addition, it contains a considerable amount of transposable genetic elements, repeat elements, cryptic prophages, and bacteriophage remnants. The genomes of certain pathogenic *E. coli* strains are approximately 1 Mb larger than that of the commensal K-12 strain, as exemplified by enterohaemorrhagic *E. coli* strains O157:H7 Sakai (5.50 Mb), enteroaggregative *E. coli* strain O42 (5.36 Mb), and UPEC isolates CFT073 (5.23 Mb), 536 (4.94 Mb), UTI89 (4. (5.07 Mb). The additional DNA segments were likely obtained by horizontal gene transfer (Liu, 2014).

Comparative investigations of the *E. coli* and Shigella genomes reveal that the anticipated pan-genome has 15,741 gene families, of which just 993 (6% of the total) are present in every genome, constituting the core genome. The variable or 'accessory' genes constitute greater than 90 percent of the pan-genome and around 80 percent of the average genome.

Two genetic configurations, virulence-related plasmids and chromosomal pathogenicity islands, are largely responsible for the plasticity of the *E. coli* genome. Despite the fact that many strains (e.g., strain MG1655) do not include plasmid, some strains may contain between 1 and 5 plasmids. Each of the seven groups of diarrhoeagenic *E. coli* carries at least one plasmid-based virulence-related feature. Frequently prevalent in EIEC, EHEC, EAEC, and EPEC is a member of a highly conserved plasmid family. The plasmid is often big (>60 megadalton or MDa), has a low copy number, and is either conjugative or of transmissible incompatibility group. It encodes several virulence factors. While plasmids and pathogenicity islands encode clusters of virulence traits, individual traits (such as shiga toxin) may be encoded by transposons (Liu, 2014).

Similar to other bacterial genomes, the *E. coli* genome is compacted into a nucleoid-like structure. Nucleoid functions like as transcription, replication, recombination, and repair are affected by the nucleoid's unique structural characteristics and conformations. The nucleoid of *E. coli* is a heavily lobulated intracytoplasmic area located generally in the cell's centre. In this region, the DNA concentration is between 2 and 5 percent (w/v). In vivo, DNA is negatively supercoiled into around fifty distinct domains. As the nucleoid forms a considerable barrier to the diffusion of many macromolecules, transcription takes place at the nucleoid–cytoplasm interface. The very uneven structure of the nucleoid may contribute to the transcriptional accessibility of genes (Liu, 2014).

At least four DNA-binding proteins with low molecular weight are implicated in transcription, recombination, and replication. The molecular weight of these nucleoid-associated proteins ranges from 9.2 to 15.4 kDa. Twenty to fifty thousand monomers of the proteins HU and IHF are present in each *E. coli* cell (Liu, 2014).

The commencement of DNA replication occurs at a specific origin site, oriC, and is governed by the DnaA protein, which is highly conserved among bacteria. Once began, DNA replication occurs at an almost constant rate in moderately fast and rapidly expanding *E. coli* until it achieves its conclusion. Segregation of the nucleoids occurs with a high degree of accuracy; hence, it cannot be the consequence of random partitioning into daughter cells. Recently replicated (hemimethylated) origin DNA binds with high selectivity to the membrane in vitro (Liu, 2014).

1.2.5 Bacterial cell culture growth phases

In the natural world, bacteria do not experience optimal development conditions. Consequently, the species inhabiting a habitat vary with time. However, ideal conditions can be achieved in the laboratory by cultivating bacteria in a closed culture environment. Under these circumstances, the growth curve pattern of bacteria can be observed.

The bacterial growth curve depicts the number of living cells in a population of bacteria over time.

This first phase (lag) is marked by cellular activity but no growth. A limited number of cells are placed in a nutrient-rich medium that permits them to manufacture the proteins and other molecules required for replication. During this phase, these cells increase in size, but there is no cell division.

Exponential (Log) Phase: Bacterial cells enter the exponential or log phase following the lag phase. After each generation time, this is when the cells divide via binary fission and double in number. During the division of DNA, RNA, cell wall components, and other chemicals essential for growth, metabolic activity is strong. Antibiotics and disinfectants are most effective during this growth phase because they target the bacterial cell wall or the protein synthesis processes of DNA transcription and RNA translation.

Stationary Phase: Eventually, as available nutrients are expended and waste begins to build, the population expansion experienced during the log phase begins to wane. The stationary phase of bacterial cell growth occurs when the number of dividing cells equals the number of dying cells. This leads in negligible population growth overall. Under less favourable conditions, competition for nutrients increases and metabolic activity of cells decreases. During this phase, spore-forming bacteria make endospores, and pathogenic bacteria begin to manufacture chemicals (virulence factors) that enable them survive in hard environments and cause disease.

As resources become scarcer and waste products multiply, the number of dying cells continues to increase. During the dying phase, the number of living cells drops

exponentially, and population growth plummets dramatically. As dying cells lyse or rupture, their contents are released into the surrounding environment, making these nutrients accessible to other bacteria. This aids spore-producing bacteria in surviving long enough to produce spores. Spores are able to survive the harsh conditions of the death phase and transform into developing bacteria when placed in a life-supporting environment.

1.3 Upstream bioprocess engineering principles

1.3.1 Bioreactor design

Modern bioprocesses are characterised by highly specialised designs that serve a wide variety of customers and products. There has been an increase in platform technologies as a result of specialisation, beginning with the cell line or organism and extending through equipment design. Regardless of the breadth and diversity of equipment designs, all equipment must be scalable and meet the needs of all involved parties, from the scientist/engineer to the maintenance staff. This chapter introduces and discusses in depth, from an engineering perspective, the needs of upstream bioprocessing equipment, with the bioreactor as the focal point, and provides examples of available bioreactor technology (Clapp et al., 2018).

Significant obstacles accompany the maintenance of an aseptic environment and sanitary design using autoclavable, sterilize-in-place (SIP), or chemically sanitizable systems. Single-use equipment establishes a new and active branch of bioprocess equipment that is mostly unburdened by sanitary design. Flow routes and elements that are disposable allow the same flexibility for equipment that cannot be made entirely disposable. Components, automation, and design considerations must advance rapidly as the applications proliferate and the benefits of single-use technology continue to be sought after. In the past, dedicated manufacturing facilities that utilised reusable equipment were the standard; however, modern biomanufacturers require greater agility and adaptability. The expectation is that new, modern facilities will be more efficient and effective, capable of handling many products and adjusting production volumes as the market dictates. One observation is that a genuine integration of upstream and downstream unit operations is

now conceivable and becoming prevalent as never before. Numerous gains in capital equipment procurement, data management, and the deployment of single-use technology underpin this integration. In contrast to the past, when capital equipment acquisitions were separated along the upstream and downstream sectors, disposable assemblies and real, specialised single-use equipment necessitate a study of the entire bioprocess. Regardless of the buyer and seller of bioprocess equipment, this larger perspective creates an opportunity for the biopharmaceutical business. Not just the use and integration of technology presents an opportunity. The possibility will enable the biggest range of medicines to be designed and manufactured at the lowest cost and with the highest quality (Clapp et al., 2018).

The components that engage the process include vessels and tanks, piping and fittings, tubing and hoses, filters and filter housings, valves, diaphragms, seals, and gaskets, and probes and sensors. These will be presented and debated in the parts that follow (Clapp et al., 2018).

Components that serve to store, mix, or otherwise hold a large volume of bioprocess fluid are referred to interchangeably as vessels and tanks. Depending on their function and the conditions of the bioprocess, vessels can be built from a variety of materials. The most commonly utilised materials are stainless steel alloys, specifically 304 and 316. Additionally, glass-lined and smaller all-glass jars are utilised. On a larger scale and where pressurisation is required, stainless steel vessels continue to be the most popular option. For the biomanufacturing culture step (bioreactors) as well as for storage and mixing, 100 to 2500 L single-use suitable vessels are currently the norm. The vessel's complexity is determined by the bioprocess function it fulfils. The simplest definition of a vessel is a storage container. If mixing is necessary, the size, kind, and placement of the mixer are design concerns. Other needs that affect the design of the tank include gassing, temperature control, pH control, and liquid addition and withdrawal. Various design choices exist to suit those needs. As an example, one can adjust the temperature of the process liquid in a vessel by (Clapp et al., 2018):

- A jacketed vessel with a heat transfer surface that is welded to the primary vessel. Within the jacket, a temperature control fluid circulates, achieving the required temperature control in isolation from the process fluid.
- A blanket or wrap that serves as an electrically resistive heating element and is physically placed to the exterior surface of a vessel.
- An immersion heat exchanger in which a temperature-controlling fluid runs through a heat exchanger to modify the temperature within a vessel.

Various sizes of stainless steel tanks and some tiny glass jars are routinely jacketed. Heating blankets can be found at pilot scale, however they are most typically utilised in small scale, such as in process development with glass bioreactors for small-scale use. In such circumstances, jacketed vessels and submerged heaters are unfeasible, and heat blankets offer a simpler alternative. Immersion heaters, despite being effective for some big and small scale applications, add complication to equipment installation and maintenance (Clapp et al., 2018).

Rapid and precise temperature management requires the optimization of heat transmission between the heating element and process liquid. A sufficient contact area between the process liquid and the heating element is required. In jacketed vessels, the design of the jacket may be open, half-pipe, or dimpled; each is a compromise between production cost and effectiveness. In this instance, the heat transfer contact area can encompass a substantial portion of the vessel's surface, including the sides and bottom. Before heat reaches the process liquid, indirect contact, such as when a heating blanket is attached to the exterior surface of a vessel, might result in heat transfer losses across the different surfaces. As a result, control can become sluggish, for instance during the process's ramp up. In tanks, the vessel area and the vessel volume do not scale linearly, which is a consideration for jacketed vessels and heating blankets. Consequently, a bigger scale system requires a heat exchanger or heat element with a greater capacity than a smaller size system. If the vessel is utilised at a very low capacity, temperature control limitations may also occur. In certain instances, jacketed containers and heating blankets may not provide enough surface area to appropriately regulate temperature. A last remark is that a heating blanket is normally built to merely heat and has no cooling capability,

but a jacketed vessel can heat and cool. A lack of cooling increases the risk of potential temperature overshoots and slows system responsiveness in the event of a biphasic process with a step change in temperature. In the case of single-use rocking-type bioreactors, where a plastic culture bag is put on an incorporated heating element of the rocking unit, indirect temperature control also exists (Clapp et al., 2018).

There are various bioreactor designs. The format and size of a bioreactor will be determined by its performance and capacity requirements in research and development, pilot, or production contexts. For cell culture and fermentation, cylindrical stirred-tank bioreactors with a long history are available. Prior to the development of single-use bioreactor technology, glass and stainless steel were the materials of choice for bench-scale and larger bioreactors, respectively. However, single-use bioreactor systems with sizes ranging from less than 500 mL to 2,000 L are currently prevalent in cell culture. From 25 to 500 L, single-use fermentors are also available (Clapp et al., 2018).

The shape of the reactor has a significant impact on its performance, since it influences the power transmission and fluid dynamics that drive heat and mass transport inside the system. The height-to-diameter ratio H/D, or H:D, is a crucial design characteristic associated with the form of a reactor. The extended liquid residence periods of gas bubbles sparged from the bottom of tall, thin reactors with H:D > 3:1 allow for an efficient gas-to-liquid mass transfer. Nonetheless, in tall and slender reactor designs, the headspace surface area is restricted, which might result in restrictions in the associated partial pressure driving forces. In contrast, a short and wide reactor with H:D 1:1 has a brief gas bubble residence time and a huge headspace surface area. When gas flow rates are high and mixing is strong, as in microbial fermentations, vessels with a H:D ratio of 3:1 are usual. In cell culture, when gas flow rates are lower and mixing is moderate, the typical H:D ratio range is 1.5:1–2.1:1. Current designs for single-use vessels only include bottom heads, whereas autoclavable and sterilize-in-place bioreactors are constructed with top and bottom heads. Top and bottom heads can have a variety of profiles, which influence the system's mixing properties (Clapp et al., 2018).

The mass transfer in the reactor is dependent on the flow pattern of the process liquid, which is influenced by the interior architecture of the reactor and the agitation system. Mass transfer is larger during turbulent flow than during laminar flow. However, excessive turbulence can hinder delicate bioprocesses. There are numerous techniques to introduce turbulence, including the design of the agitation system and the use of internal baffles. Baffles generate fluid resistance and disrupt laminar flow. Common agitation system orientations include center-drive and offset by 15 degrees. Typically, baffles are equally spaced throughout the vertical walls of the main vessel cylinder when the center-drive configuration is utilised. The specifications of the baffle in terms of height, width, thickness, and offset distance from the vessel wall are frequently standardised relative to the H:D ratio of the vessel. When shear pressures must be limited, such as in some cell culture applications, 15-degree offset agitators produce sufficient turbulent flow without the need for baffles. When higher turbulence is required, such as in microbial fermentation, baffled tanks are used (Clapp et al., 2018).

Location and orientation of the agitation system are other bioreactor design concerns. The available options for agitation are either top-drive or bottom-drive. Motor, gearbox, mechanical seal, shaft, and impellers are typical components for agitation. With the H:D ratios given in this document, both top- and bottom-driven systems can be utilised without the requirement for shafts with excessively large diameters or shafts with extra internal supports. The motor is external to the reactor, and the interface between the drive motor and agitator shaft must convert the motor's driving force into fluid motion. Whether it should be directly associated with a static or rotating mechanical seal, or indirectly coupled with a magnetic assembly, is a related factor. The ability of direct, mechanically coupled systems to convey the motive power of the motor to the process fluid is almost limitless, but the torque limit of magnetically coupled systems is determined by the strength of the mating magnets (Clapp et al., 2018).

Using impellers to move the fluid within the vessel and create mixing. There are numerous impeller choices that maximise energy transfer while balancing the system's shear forces. Based on the fluid pumping characteristics, impellers fall into two basic categories: axial and radial or tangential flow. However, there are numerous impeller

types, and different impellers can be coupled to provide a specific effect. All types of impellers have a corresponding power number, Np (or P0), which can be used as a scaling factor. For microbial fermentations, Rushton impellers are the most popular choice. Axial and radial fluid flow is produced using Rushton impellers. Impellers with angled blades or those resemble marine propellers are most prevalent in cell culture. Impellers with angled blades primarily generate axial flow, relying on vessel geometry to support flow in other directions, such as radially. Combining Rushton and axial flow impellers has been utilised in both fermentation and cell culture to enhance mixing, minimise power requirements, and modify shear force properties. Depending on the application, the number and location of impellers along the shaft are important considerations (Clapp et al., 2018).

1.3.2 Modes of Fermentation

Any bioprocess requires a substrate, even if it is merely mineral salts, light, and CO2 for algae. It's usually in the form of sugar, which is either introduced at the start of the process or gradually increased over time. The method to utilise will be determined by the organism, application, and aim.

Batch, in which no additional feeding is needed from the start to the finish of the process. Fed-batch, in which feeding with substrate and supplements can lengthen the culture time for larger cell densities or alter metabolism to make recombinant proteins, for example. Continuous culture, in which the feed rate of a growth-limiting substance keeps cell density constant (a chemostat) or the feed rate of the substrate is determined by cell density (turbidostat). Another extremely productive method is cell retention (perfusion). The pace of entering feed corresponds to the rate of harvest removal. The balanced nature of the feeding allows for the establishment of a stable state that can continue for days to months. This condition is ideal for research into microbial metabolism or long-term production. Repeated Fed-batch, in which all but a minimal residual of a finished (fed) batch is harvested and the leftover cells are used as an inoculum for the following batch. In a bioreactor, organisms may be cultivated in a variety of methods. One of the most sensitive and productive methods is to change in the feed composition and feed rates. Modern bioreactors have feed addition controls that allow for all major feeding methods

and fine control for the approach chosen. batch culture can be adopted for a quick, restricted culture processes. Fed-batch is used for high-density, flexible productivity applications. Continuous culture is used for reliable, long-term investigations and regulated manufacturing in small quantities.

1.3.2.1 Batch Process

In a batch process, all nutrients are provided at the start of the growth, with no further nutrients added during the fermentation. There are no extra nutrients introduced throughout the bioprocess; only control components such as gases, acids, and bases are added; it is a closed system. The bioprocess continues until all of the nutrients have been utilized. This method is ideal for quick investigations like strain characterisation or nutrition media optimization. The biomass and product yields are restricted, which is a downside of this simple approach. Microorganisms do not stay in the exponential growth phase for very long since the carbon source and/or oxygen transfer are generally the limiting factors. The oxygen transfer rate must be enhanced to boost the availability of dissolved oxygen. This can be accomplished by raising the stirring speed, gas flow, oxygen fraction in the gas mix, or pressure (if the bioprocess takes place in a steel bioreactor). Because the goal of combining the different characteristics is to improve concentration, comprehensive management and monitoring of these parameters is required. Cascades are a type of process that may be customised to fit the needs of a certain application. The controller has one or more parameters that are designed to be utilised for changing the dissolved oxygen concentration. Variation of the first parameter (for example, stirrer speed) within the given range is the first step toward achieving the goal value. If it doesn't work, the next step is to experiment with other parameters until the Dissolved oxygen set point value is sustained. Only the biomass or medium is collected and suitably treated to generate the desired product after a batch bioprocess run. The process is constantly halted by cleaning and sterilising operations in the bioreactor, and biomass is only generated in phases. Batch methods have a higher risk of substrate or product inhibition, in addition to the poor biomass yield. The latter refers to the interruption with enzyme activity caused by high concentrations of substrate or product, which can result in metabolic feedback that reduces yield dramatically. (Allman, 2020)

Advantageous of a batch process include:

- The duration is relatively brief.
- Because no nutrients are supplied, there is a lower risk of contamination.
- Batch material separation for traceability
- It's easier to manage.

Disadvantageous of a batch process include:

- Nutrients, reagents, cell debris, and toxins are all mixed together with the product.
- Time spent productively is reduced.
- Batch storage for downstream processing is possible.

1.3.2.2 Fed-batch process

Constantly supplying nutrients throughout cultivation is one approach to avoid them becoming a limiting factor. A fed-batch process, which is a partially open system, is what this is termed. The benefit of feeding during cultivation is that it allows for increased total product amounts. Microorganisms and/or cells regularly double under specified growth circumstances, resulting in an exponential growth curve. As a result, the feed rate should also rise exponentially. A silicone tube is used to pump the substrate from the supply bottle into the culture vessel. The user may manually adjust the feed at any moment (linear, exponential, pulse-wise) or supply nutrients when circumstances are satisfied, such as when a certain biomass concentration is achieved, or a nutrient is exhausted. The fed-batch method allows for a variety of control schemes and is also appropriate for highly specialised applications. However, it may lengthen the processing time and raise the risk of inhibition by accumulating hazardous by-products. To do so, the scientist will also require a more in-depth grasp of bioprocesses, which should not be viewed as a drawback. A fed-batch process is a semi-continuous process, whereas a batch process is a discontinuous process. Excessively high substrate concentrations (in this case glucose) were found to impede growth, mostly through the generation of ethanol, during research at the turn of the century with the goal of producing as much biomass as possible from baker's yeast in a batch process. This feature of baker's yeast, on the other hand, may be exploited to make ethanol. The Crabtree effect happens when glucose concentrations are high and there is enough dissolved oxygen in the medium for alcoholic fermentation to

occur. This phenomenon is exploited in various yeast-based food manufacturing techniques. (Allman, 2020)

Fed-batch techniques are widely employed in all sectors of biotechnological production, particularly for the creation of recombinant proteins and antibiotics, due to their benefits.

Advantageous of a fed-batch process include:

- Extends the productive lifespan of a culture.
- By modifying the substrate, it is possible to turn genes on or off.
- Using various feeding tactics, it may be managed for optimal productivity.

Disadvantageous of a fed-batch process include:

- Allows inhibitory agents and poisons to accumulate.
- Provides a new entry point for contaminants.
- May result in high cell density and product yields that are difficult to manage in downstream, creating bottlenecks throughout the whole process.

1.3.2.3 Continuous culture

After a batch growth phase, an equilibrium with regard to a specific component is achieved (also called steady state). As much new culture media is added as it is withdrawn under these circumstances (chemostat). Continuous cultures are a type of bioprocess that is particularly useful when an overabundance of nutrients would cause inhibition owing to toxin build up or excessive heating. Reduced product inhibition and enhanced space-time yield are two more advantages of this approach. Because cells are collected when the medium is withdrawn, the inflow and outflow rates must be smaller than the microorganisms' doubling time.

Perfusion, on the other hand, is a method of retaining cells in a number of ways (for example, in a spin filter). In comparison to a fed-batch method, the bioreactor's space-time yield may be enhanced much more in a continuous process. The lengthy cultivation time, on the other hand, raises the possibility of contamination and long-term cultural changes. Furthermore, because all process parameters stay constant when the system is

working properly, continuous processes are good instruments for acquiring a deeper knowledge of the process.

Examples of continuous cultures include:

- Chemostat: Cell multiplication is controlled by the rate at which a single growth-limiting substrate is added.
- Turbidostat: Addition and withdrawal of liquid are controlled by an indirect assessment of cell counts (turbidity or optical density). This requires an extra sensor, but it is powered by real-time feedback.
- Perfusion: In this form of continuous bioprocessing mode, the cells are either kept in the bioreactor or recycled back to the bioreactor. At the same time, fresh medium is supplied, and cell-free supernatant is withdrawn.

Advantageous of a continuous culture include:

- Allows for optimal efficiency.
- Cleaning, sterilising, and vessel handling time are all decreased.
- When numerous components sum to zero, it creates a stable state for metabolic investigations.

Disadvantageous of a continuous culture include:

- It's challenging to maintain a consistent population density over lengthy periods of time.
- A continuous process's outputs cannot be cleanly divided into batches for traceability.
- Contamination and/or genetic alterations are more likely.

1.3.2.4 Repeated Fed-batch / Semi-continuous Culture

In addition to fed-batch or continuous culture bioprocess, there are hybrid approaches that can be advantageous when running a. Harvesting all but a minimal residual of a completed (fed) batch and using the remaining cells as an inoculum for the next filling of the vessel, for example. This feeding technique fills the gap between fed-batch and continuous culture systems, as well as addressing fundamental concerns with continuous culture in

biopharmaceutical and other manufacturing contexts. This application is for the manufacture of Single Cell Protein (SCP), as well as the fermentation of lipids, fatty acids, and penicillin. (Allman, 2020)

Repeated Fed-batch a simple concept; after a time of batch culture, a considerable portion of the bioreactor working volume (between 25 and 75 percent) is withdrawn and replaced with fresh medium, including the carbon substrate. The bioreactor's residual suspension culture serves as an inoculum for the following batch. This cycle is repeated numerous times, with the length of time defined by the growth rate and substate use profile. The procedure is usually ended after a few days.

The feed rate is critical in a fed-batch to actively control growth. Almost all repeated-processes, on the other hand, strive to accumulate substrate, implying that no active effect on growth is feasible. The procedure may also be defined as a recurring batch, as a result, multiple techniques and terminology can be found in the literature. Another similar term is "semi-continuous culture."

Advantageous of a continuous culture include:

- The medium exchange system prevents the buildup of poisons and undesirable metabolites.
- Culture density will not escalate to the point where concerns such as oxygen transfer or cooling capacity develop as a result of increased biomass.
- Across cycles, biomass and protein yields stay constant.
- Because the culture effluent is less diluted, downstream processing needs are reduced.
- Allows for product division into sub-batches organised by time, which helps with quality control and troubleshooting.
- Rather than weeks or months, the overall duration of repeated fed-batch cultures is closer to fed-batch, i.e. several days.

1.3.3 Gas hold up

Gas hold is defined by the volumetric flow rate of gas per volume of liquid (VVM)

$$\boldsymbol{VVM} = \frac{\boldsymbol{LPM}}{\boldsymbol{V_W}} \text{ (L L}^{-1}\text{ s}^{-1}\text{)}$$

Equation 1

1.3.4 Superficial gas velocity

Superficial gas velocity is defined by the volumetric gas flow rate per cross-sectional area (m s^{-1}). An interpretation can be an approximate average vertical speed of bubbles.

$$\boldsymbol{V_s} = \frac{\boldsymbol{LPM}}{\boldsymbol{60.A}} \text{ (mm s}^{-1}\text{)}$$

Equation 2

1.3.5 Mixing

A bioreactor's mixing time is an important aspect. It specifies how long and at what speed a liquid must be churned to achieve an ideal condition of homogeneity. A colouring approach is frequently used to calculate the duration: A starch solution is combined with a 25 °C tempered iodine-potassium-iodine solution. The sodium thiosulfate is then added to this solution mixture, and the reaction time is monitored until the solution turns clear. The time it takes to reach a homogeneous solution is determined by this. The greater the impeller's mixing characteristics and the balance of shear force inside the bioreactor, the shorter the duration. When compared to microbial fermentations, cell culture procedures often have longer mixing durations. Slower speeds and the usage of various impeller types generate less turbulence, this is due to Slower speeds and the usage of various impeller types generate less turbulence.

Mixing times can be evaluated from different correlations available in the literature. The equation below is an example. (Nienow, 1998)

$$\boldsymbol{\theta_m = 5.9 \left(\frac{P}{\rho V_W}\right)^{-\frac{1}{3}} (d/T)^{-1/3} T^{2/3}}$$

Equation 3

θ_m = mixing time (s) T= tank diameter (m)

In all available correlations, a common characteristic is that mixing time is inversely proportional to $(\frac{P}{V_W})^{113}$. In other words, technology transfer or scale up at lower power input per unit volume will result in longer mixing times.

1.3.6 k_La and OTR and OUR

Determination of the volumetric oxygen mass transfer coefficient (k_La O_2) is regarded as a standard benchmarking method for assessing the ability of the bioreactor system to transfer oxygen from the gas to liquid phase. Oxygen mass transfer (OTR) is critical for aerobic cultures and can be challenging, as oxygen is the least soluble and most quickly consumed nutrient by cells, therefore this typically becomes the rate limiting factor in high cell density cultures. Oxygen transfer rate should be equal or above oxygen uptake rate (OUR).

$$\boldsymbol{OTR = k_L a(C^* - CL)}$$

Equation 4

$$\boldsymbol{OUR = xq_{O_2}}$$

Equation 5

C* = equilibrium oxygen concentration in the liquid phase (mgL^{-1})

C_L= oxygen concentration in the liquid phase

OTR= oxygen transfer rate (mmol L^{-1} h^{-1})

OUR= oxygen uptake rate (mmol L^{-1} h^{-1})

X= cell concentration (cells L^{-1})

q_{O_2}= specific oxygen consumption rate (mmol $cell^{-1}$ h^{-1})

k_La= volumetric oxygen mass transfer coefficient (h^{-1})

The volumetric oxygen mass transfer coefficient k_La is an agglomeration of two components $k_{L,}$, Liquid mass transfer coefficient (m s^{-1}) and 'a', the specific surface area (m^2 m^{-3}). k_La depends on medium composition, the sparger design and agitation rate; because of their impact on the size of bubble and liquid mass transfer coefficient.

For any bioreactor design the Van't Riet correlation can be determined. That correlation links the k_La to the Power input per unit volume and superficial gas velocity, meaning scale up or technology transfer with constant k_La can be achieved by maintaining these two parameters.

$$\boldsymbol{k_L a = A.\left(\frac{P}{V}\right)^{\alpha}.(v_s)^{\beta}}$$

Equation 6

α & β and A= regression coefficients determined from experimental measurements

Published data shows that α & β are usually in the range of 0.5± 0.1 (Nienow, 2006). However, the value A is susceptible to the medium composition. Antifoam and Pluronic F68 modify A and k_La dramatically. Therefore, measured k_La values from different experimental methods and material including media composition and protocol should not be directly compared. Moreover, multiple approaches exist to derive k_La values from experimental measurements like k_La_{max} and kLa_{2080}, these methods give different results, thus its important to know which method was used when comparing two sets of k_La data.

1.3.7 Impeller

The impeller, commonly referred to as the agitator, is an important part of the bioreactor. Within the vessel, it is responsible for mixing, aeration, heat, and mass transport. The are many properties of different types of impellers, as well as the significance of their designs.

The impeller's principal job is to continually swirl the contents of the vessel, providing homogeneous mixing and hence consistent access to the nutrients therein for the cells. Certain impellers work by increasing energy transfer while balancing shear forces in the bioreactor (Wong & Zhong, 2007). The position and size of the impeller are determined by the bioreactor's size. In general, bioreactors with a significant height-to-diameter ratio will employ many impellers to ensure adequate aeration and agitation for the cells/microorganisms contained within the vessel.

1.3.7.1 Types of impellers

The flow direction in which the fluid is mixed can be used to classify impellers. Axial flow, radial flow, mixed flow, and dispersed flow are the four classes that allow for up or down regulated flow direction. There many different designs of impeller and a few examples are described below.

Axial flow is generated by pitch-blade or marine impellers, which are extensively employed in cell culture operations. This happens when the cell suspension is pushed in one direction and sucked parallel to the axis shaft from the other side. At low impeller tip speeds, marine impellers are noted for their shear sensitivity and effective mixing.

Rushton impellers, for example, pump the fluid radially outwards and suck it in in an axial flow direction from both sides of the impeller. Microbial fermentation systems frequently use this style of impeller.

Angled pitch-blade impellers provide a mixed flow, in which the liquid is moved in both axial and radial directions by the vertically angled blades.

Helicoidal impellers, which generate an evenly distributed shear plane as well as gradients in all directions, are used to create distributed flow. Solid substrate or high viscosity fermentations typically require impellers of this type (Peker et al., 2008)

Impeller	Rushton Turbine	Pitched blade/marine	Helical	Angled pitched-blade
Impeller image	 Adopted from (Rushton-Type, 2021)	 Adopted from (Pitched-Blade Impeller, 2021)	 Adopted from (Nino et al., 2018)	 Adopted from (EKATO, 2021)
Application	Radialflow, Microbial applications	Axial flow, Shear sensitive applications	Distributed flow, solid substrate and/or high viscosity	Mixed flow, solid substrate and/or high viscosity

Table 2 examples different impeller types and their application

The impeller must thoroughly swirl the substrate, cells, and oxygen mixture to ensure proper cell development. Furthermore, selecting the appropriate impeller type is critical for achieving a high output and excellent product quality. This isn't always an easy choice to make. The important parameters to consider are mentioned below.

When choosing an impeller for an application, the impeller must have a certain off-bottom clearance and have the right size in relation to the vessel size to provide homogeneous and shear sensitive mixing. Furthermore, the impeller's performance is influenced by the position and kind of sparger. The impeller's performance is also influenced by the ratio of liquid height to vessel diameter. As a result, bigger bioreactors will employ multiple impellers to provide uniform mixing and aeration across the whole bioreactor (Wang & Zhong, 2007). Power input and power number can be extremely important parameters to consider when determining the suitable dimensions of the impeller based on the vessel size.

1.3.8 Power

The torque acting on the impeller shaft while it rotates may be used to calculate power input. Because of its relationship with shear pressures, the power input is a critical aspect to consider during scale-up procedures. The power input is important since the impeller can impact the quality of the product as well as cell development. Other approaches, such as electrical power draw or calorimetric methods, are available. The formulae and explanations for key variables for determining power input are listed below.

$$P = 2 \,.\pi\,.N\,.T_{eff} = 2\,.\pi\,.N\,.(T_L - T_D)$$

Equation 7

P= power (W) T_{eff} = effective torque (Nm) T_D= torque in empty vessel (Nm)

T= torque (Nm) T_L= torque in liquid (Nm) N= agitation rate (s^{-1})

The torque, particularly the effective torque, which incorporates losses incurred during agitation (bearings, seals, and motor), may be used to compute the power input. The difference between the torque recorded in the empty vessel and the torque measured in the liquid vessel is the effective torque (Kaiser et al., 2018)

1.3.9 Power number

The density of the liquid and the impeller diameter are used to get the dimensionless power number. It may be used to compare various agitators (Kaiser et al., 2018)

$$P_0 = \frac{P}{\rho\,.N^3.\;d^5} = \frac{2\,.\pi\,.T_{eff}}{\rho\,.N^2.\;d^5}$$

Equation 8

P= power (W) P_0 = Power number ρ = density (kg m^{-3})

T_{eff}= effective torque (Nm) N= agitation rate (s^{-1}) d= impeller diameter (m)

The power number, which becomes constant in totally turbulent conditions, is a function of the Reynolds number (Kaiser et al., 2018)

$$Re = \frac{N.d^{2}.\rho}{\eta}$$

Equation 9

Re= Reynolds number ρ = density (kg m^{-3})

η = viscosity (kg m^{-1} s^{-1}) N= agitation rate (s^{-1})

1.3.10 Power input per unit volume

Power input per unit volume (P/V, Wm^{-3}): Power input can be identified as impeller energy transfer rate into the cell culture media. This will be a function of the design, diameter, and agitation rate of the impeller.

$$\frac{\boldsymbol{P}}{\boldsymbol{V}} = \frac{\boldsymbol{P_0}.\boldsymbol{\rho}.\boldsymbol{N^3}.\boldsymbol{d^5}}{\boldsymbol{V_W}} \ (\boldsymbol{Wm^{-3}})$$

Equation 10

P= power (W) V_W = working volume (m^3) P_0= power number

ρ= density (kg m^{-3}) d= impeller diameter (m) N= agitation rate (s^{-1})

1.3.11 Tip speed

The impeller tip speed (V_{tip}) is the linear velocity at the impeller's tip and also the highest velocity into the bioreactor:

$$\mathbf{V_{tip}} = \boldsymbol{\pi}\mathbf{Nd} \ (ms^{-1})$$

Equation 11

1.3.12 Scale up and scale down

In scaling up a fermentation or a cell culture process, one must consider the physical environment and engineering parameters that impact the said environment. There are scalable and non-scalable parameters that are considered for a scale up operation. Agitation speed and gas flow rate must be calculated for each scale with respect to an appropriately selected parameter including tip speed and power input per unit volume that are a result of agitation, and gas hold up and superficial gas velocity that are a result of gas flow rate. Depending on the cell culture characteristics and demands the appropriate constant parameter is selected and the respective agitation and gas flow rate is calculated for each scale. The resultant parameters of the decisions including mixing time, Kolmogorov scale, shear stress, OTR (k_La), CO_2 stripping, and foam production must be considered in the scale up operation. Different cells have different demands and grow optimally in particular conditions. Microbial fermentations such as *Escherichia coli*

have high Oxygen demand and are less sensitive to shear compared with other cell types. Therefore OTR (k_La) or Power input per unit volume are typically the parameters that are kept constant in scaling up as well as applying a constant gas hold up in each scale. This will be an appropriate strategy in scaling up that would be able to deliver the oxygen demand as well as maintaining an acceptable mixing and homogeneity. In scaling down for the purpose of scale-down studies the same principle is applied. (Doran, 2013)

1.4 Fermentation, Growth and Metabolism

The medium in which cells grow plays a critical role in the overall productivity of the upstream process and fermentation. Different media compositions have been shown to result in higher or lower yields depending on the strain of *Escherichia coli*, expression system, type of product and the physical conditions of running the fermentation (Cunningham et al., 2007). Therefore it is important to select a defined medium composition for our fermentation that is optimum for the *Escherichia coli* strain in use, expression system adapted, product of interest and fermentation conditions such as temperature, min DOT, agitation rate and others selected (Cunningham et al., 2007).

During fermentation of *Escherichia coli* points of oxygen starvation may occur that are often localised due to poor mixing or aeration. A by-product of this starvation is acetate. This results in inhibition of growth and protein production. Strategies should be employed to minimise this effect (Han et al., 1992). Some strategies may include setting a higher minimum DOT or adapting a more sensitive control system. Sometimes these phenomena occur as result of metabolic events.

Oscillation in metabolism of *Escherichia coli* occurs in controlled fermentations. Cell growth is reduced during metabolic oscillation. It was found that these events are due to genetic and nutritional factors as well as a few others. These oscillations were eliminated by adjusting the engineering aspects of fermentation, nutritional optimisation, isoleucine feeding and repair of an ilvG frame shift mutation in *Escherichia coli* K-12 strains (Andersen et al., 2001).

Cultivation methods and conditions impact secretion destination. It was shown that batch fermentations a higher level of products were secreted in to medium whereas in fed-batch fermentation very limited amount was completely secreted (Dresler et al., 2006).

Recombinant asparaginase was secreted into the medium up to 75% due to cultivation methods (Khushoo et al., 2005). Optimising fermentation conditions can significantly aid complete secretion of recombinant products. It is noteworthy that some accumulation locations have seen to be time dependant. Different induction times have been studied and it as shown that initially the product was secrete into the medium and after a certain time it was only accumulated in the periplasm and finally it was only retained in the cytoplasm (papi et al., 2005). This can also be optimised in combination with fermentation conditions.

Growth and productivity of *Escherichia coli* producing Fab can be enhanced with cell engineering approaches in order to improve primary recovery performances. Cell engineered bacteria will have high molecular weight genome and thus produce very viscous homogenate. Nuclease-modified *Escherichia coli* was engineered such that it would co-express the nuclease and thus breaking down the DNA and this will reduce the

viscosity of the feed. It was shown that this approach will not compromise growth and productivity of the original strain (Nesbeth et al., 2012).

1.4.1 Induction systems

In order for a recombinant protein to be produced by the bacterium, an induction is carried out. This is done when the growth has reached its maximum, near end of log-phase. There are various systems available for induction, some are more efficient than others but may be rather expensive. Common promoters that are induced in HCDC fermentations include *lac* and *tac* promoter systems. The named promoters are stimulated by a chemical called IPTG (isopropyl-beta-D-thiogalactopyranoside).

Different concentrations of IPTG can be used and depending on the product and other culture conditions such as temperature, the yield of product varies. For example in Fab production, 0.1mM and 1mM showed similar yields and varying temperature from 37°C to 30°C resulted in significantly higher yields (Shibui and Nagahari, 1992).

When different recombinant products are desired to be co-expressed different inducers can be used to induce different promoters at desired times. Some promoter systems and associated recombinant product can be synthesised via molecular biology techniques.

1.4.2 Promotor

To discuss a variety of inducible promoter systems. The rhamnose-inducible promoter combines precise control with high expression levels. The T7 system requires specialised host cells that have a chromosomal copy of T7 polymerase under the control of the lac promoter. Induction of T7 polymerase by IPTG results in the transcription of any gene downstream of a T7 promoter. The IPTG-inducible T5 promoter is composed of a strong constitutive promoter flanked by lac operator sequences and is functional in all *Escherichia coli* strains. The PhoA promoter does not require costly or metabolizable inducers, but rather auto-induces once the phosphate in the media has been depleted by the cells.

Promoter	Inducer	Strain
T5	IPTG-inducible, Repressible with 2% glucose	Any *Escherichia coli*
T7	IPTG-inducible, Repressible with 2% glucose	BL21(DE3) or T7 Express
rham	Rhamnose-inducible, Tight regulation and tunability, Repressible with 2% glucose	Any *Escherichia coli*
phoA	Induction requires phosphate starvation, Repressible with 150µM phosphate. Excellent for low-cost autoinduction	Any *Escherichia coli*

Table 3 examples of promoters and their inducer and relevant *Escherichia coli* strain

The number of copies of the origin of replication might further regulate expression utilising a particular promoter. Particularly, expression levels of hazardous proteins can be decreased further by employing vectors with fewer copies.

In Gram negative bacteria, proteins tend to be poorly released and are generally directed to the periplasm. The periplasm provides a distinct environment to the cytoplasm, most critically the periplasm is oxidising and promotes disulfide bond production. Depending on the protein being expressed, the efficiency of a signal sequence in guiding the protein to the periplasm changes throughout bacterial protein expression.

This item explains the expression of genes by subordinating them to the RNA polymerase of the bacteriophage T7. T7 RNA polymerase is a highly active enzyme: it synthesises RNA at a rate several times that of *Escherichia coli* RNA polymerase and terminates transcription less frequently; in fact, its transcription can circumnavigate a plasmid, resulting in RNA that is many times the length of the plasmid. T7 RNA polymerase is also highly selective for initiation at its own promoter regions and resistant to *Escherichia coli* RNA polymerase-inhibiting drugs such as rifampin. The exclusive expression of genes under the control of a T7 RNA polymerase promoter (p(T7)) arises from the addition of rifampicin to cells that produce T7 RNA polymerase. Within a single *Escherichia coli* cell, two plasmids are maintained in the Basic Protocol. One (the expression vector) includes the p(T7) sequence upstream of the gene to be expressed. The second plasmid carries the T7 RNA polymerase gene controlled by a heat-inducible *Escherichia coli* promoter. T7 RNA polymerase is created in response to heat induction and initiates transcription on the expression vector, resulting in the expression of the gene(s) under the control of p. (T7). The gene products can be uniquely tagged, if desired, by doing the technique in minimum medium, adding rifampicin to block *Escherichia coli* RNA polymerase, and then labelling the proteins with [35S] methionine (Tabor, 1990).

1.4.3 Biomass- specific nutrient uptake rate

Calculating the biomass-specific nutrient uptake rate at a given time point is one technique to describe organisms' substrate consumption ($qs_{(t)}$). It shows how much substrate is utilized per grams of biomass per hour. If the nutrients are restricted and hence totally digested by the culture, the quantity of substrate consumed by the culture matches the amount of substrate put in. The feed pump rate can be used to adjust the nutrient delivery and hence the biomass specific substrate absorption rate. To calculate $qs_{(t)}$ the biomass concentration must be measured. (Kager et al., 2022)

$$qs_{(t)} = \frac{F_{(t)}C_s}{X_{(t)}V_{(t)}} \text{ (g. g}^{-1}\text{.h}^{-1}\text{)}$$

Equation 12

$qs_{(t)}$: Biomass specific substrate uptake rate (g/g) at time point (t)

$F_{(t)}$: Feed flow rate (L/h) at time (t)

C_s: Substrate concentration in feed (g/L)

$x_{(t)}$: Cell dry weight concentration (g/L) at time (t)

$V_{(t)}$: Bioreactor volume (L) at time (t)

1.4.4 Protein recovery from periplasm, methods and characteristics

In order to retrieve an intracellular microbial product from a microbial cell, the cell membrane must be sufficiently disrupted for product release. For the design of cell disruption systems and the prediction of their performance, it is essential to comprehend the location of the product in relation to the cell envelope, the effect of fluid forces on the cell wall, the physicochemical and enzymatic challenges to the cell wall, and the material properties of the cell wall. The latter is the subject of this section.

In general, microbial cell envelopes consist of one or more membranes and a structural cell wall. The cytoplasmic membrane controls concentration gradients between the intracellular and extracellular environments, forming the cell's biological boundary.

Typically, the cell wall has a polysaccharide structure that is cross-linked and provides structural strength [6]. In addition to protecting the cell and maintaining its shape, the cell wall stabilises the intracellular osmotic pressure and controls the cell's macromolecule permeability. For the recovery of periplasmic and wall-associated microbial products, it is not necessary to completely rupture the cell envelope. When a cytoplasmic product is present, the cell envelope must be ruptured. For the release of soluble cytoplasmic structures, point breaches are sufficient, whereas the release of granular inclusions or big molecules like DNA requires a complete disruption of the cell structure. When the product resides in an organelle, disruption of these substructures is also necessary.

Disruption of cells is vital to biotechnology and the subsequent processes involved in the production of biological products. The disruption of cells is required for the extraction and retrieval of desired products because cell disruption considerably improves the recovery of biological substances. The disruption of cells cannot be considered an isolated event because it modifies the physical properties of the cell slurry, hence indirectly affecting subsequent processes.

As biological products might be extracellular, intracellular, or periplasmic, there are a variety of cell disruption techniques. Methods for cell disruption can be classified as either mechanical or non-mechanical. There are two types of mechanical methods: solid

shear and liquid shear. Physical methods, chemical procedures, and enzymatic methods comprise the non-mechanical approaches.

In order to release the recombinant product from the periplasm, numerous techniques have been developed that can be categorised in to two general groups, mechanical and non-mechanical.

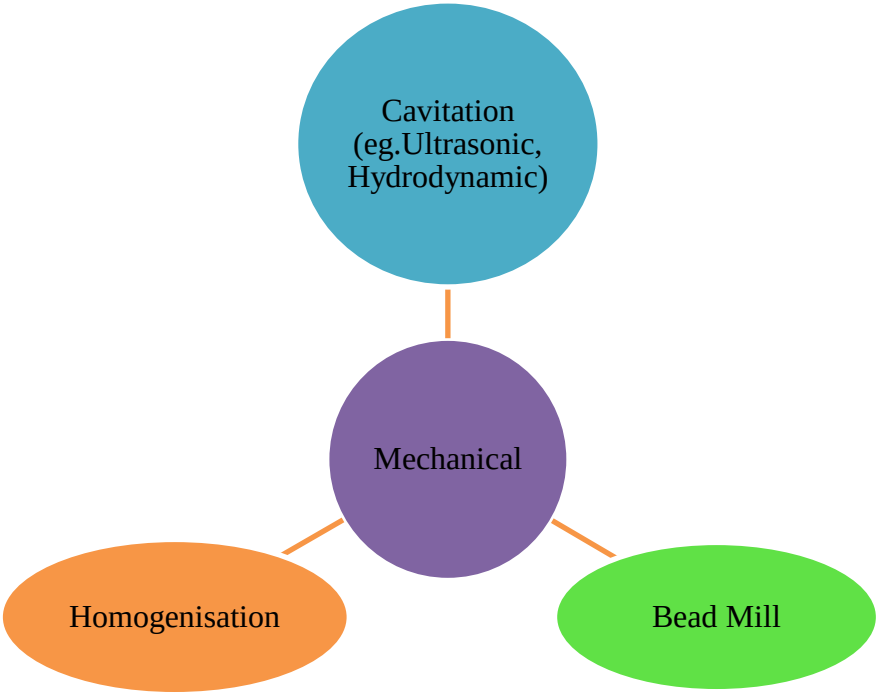


Figure 5 Mechanical Cell Disruption Methods

1.4.4.1 Mechanical methods:

Mechanical methods such as those named in the figure above offer a number of advantages and disadvantages. Advantages include high throughput and most importantly 100% product release. Prominent disadvantages must be considered. Cell breakage to fine and small particles creates great difficulties in recovery and purification of the product. DNA, host cell proteins and other undesired contaminants are very difficult to remove and prove that it has been removed. These problems lead to regulatory difficulties and validating process may become less possible (www.biopharma.co.uk. ,2014).

Methodologies that can be adapted to avoid homogenisation or mechanical cell disruption will offer vast advantages in manufacturing of recombinant products. This can be achieved if novel technologies lead to secretion of product into the broth with high yields.

1.4.4.1.1 Bead mill

Originally utilised in the paint industry, bead mills have been repurposed for both small- and large-scale cell disruption manufacturing. As several designs have been produced, it is an efficient method for disrupting diverse microbial cells. The key principle calls for a jacketed grinding chamber with a spinning shaft at its centre. The shaft is fitted with agitators that supply kinetic energy to the tiny beads present in the chamber. This results in the beads colliding with one another. The selection of bead size and weight is highly dependent on the cell type. In respect to the position of the desired enzyme, the diameter can influence the efficacy of cell disruption. Due to greater bead-to-bead interaction, the number of beads increases the degree of disruption. However, the increasing number of beads also influences heating and energy consumption. Between 80 and 85 percent of the free volume is regarded as the best condition for bead load. The discs rotate between 1500 and 2250 rpm. For yeast cells, glass beads with a diameter higher than 0.5 mm are optimal, while glass beads with a diameter less than 0.5 mm are optimal for bacterial cells. The process factors are agitator speed, proportion of beads, size of beads, cell suspension concentration, flow rate of cell suspension, and agitator disc design. Bead mills are plagued by excessive temperature increases as bead volume increases, limited scalability, and, most importantly, a high risk of contamination (Harrison, 2011).

1.4.4.1.2 Ultrasound

Ultrasonic vibrations with a wave velocity of around 20 kHz/s are responsible for ultrasonic disruption. Through a titanium probe immersed in the cell suspension, a transducer converts the electromagnetic waves into mechanical vibrations. This approach is used to disrupt both bacterial and fungal cells. Bacterial cells can be disturbed in 30 to 60 seconds, but yeast cells require between 2 and 10 minutes. This technique is typically employed in conjunction with a chemical technique (mostly lysis). On a small scale, sonication can be quite effective; but, scaling up is very ineffective. Due of noise, it has high energy consumption and health and safety concerns. It is not uninterrupted (Harrison, 2011). Ultrasound, defined as sound with a frequency greater than 15–20 kHz,

induces cavitation, or the creation of vapour cavities in low-pressure zones, in liquids. This has long been acknowledged as a tool for destroying microbial cells and is a common laboratory technique. Several variables influence the rupture of microbial cells by ultrasonication. These include power input per volume and suspension temperature. Typical acoustic power ranges are between 20 and 250 W at frequencies of 20 kHz and higher. Cell disruption decreases as volume increases and increases as input power increases. Over the temperature range of 17–30 degrees Celsius, a modest rise in disturbance is found. There is no effect of cell concentration over the ranges 3–20 g/L *E. coli* and 40–150 g/L yeast (dry mass). Ultrasound-induced cell lysis is adequately explained by first-order release kinetics. Since much of the ultrasonic energy is transferred to heat, it is necessary to maintain precise temperature control to prevent denaturation of proteins. result in the micronization of cell debris It is challenging to deliver enough electricity to a big volume of cell material. Sonication is most frequently employed in laboratories. It has been reported that adaptable focused acoustics (AFA) for the lysis of extremely small quantities (1.5 ml per sample) during 30–600 s facilitates microscale process development. AFA functions using a process similar to ultrasound, but at a higher frequency (102–105 kHz against 101–102 kHz for ultrasound). The Covaris E210 instrument has been used to demonstrate that AFA disrupts *S. cerevisiae* (Harrison, 2011).

1.4.4.1.3 high pressure homogeniser

In a French press or high pressure homogenization, the cell suspension is fed into the pump cylinder through a valve. Then, at pressure of up to 1500 bar, it is driven through a thin annular gap and discharge valve, where the pressure lowers to atmospheric. As a result of the discharge's abrupt drop in pressure, cells are destroyed and subsequently burst. This procedure is one of the most popular and well-known methods. It is typically employed for yeast cells. Milk homogenization is a crucial unit in the dairy production business. By operating the press at higher pressures, the necessary degree of disruption can be achieved with fewer passes of the slurry through it. However, the operating pressure may be constrained by the inactivation of heat-sensitive proteins, which may increase the number of needed crossings. Thus, protein release is dependent on a number of variables, including temperature, the intracellular location of the enzymes, the number of passes, and the operating pressure. The process is depending on the concentration of

biomass. The French press is a method for small-scale production, but the homogenizer can be used for mass production. Homogenisers are available in a variety of designs and can include up to fifty percent solids of the feed. The rate of heat production is very significant — 1.5oC/1000 psi. R is a first-order function of the number of passes (N). The dependency of protein release on operating pressure (P 400-600 bar) is expressed as an exponentiated function of the pressure (Harrison, 2011).

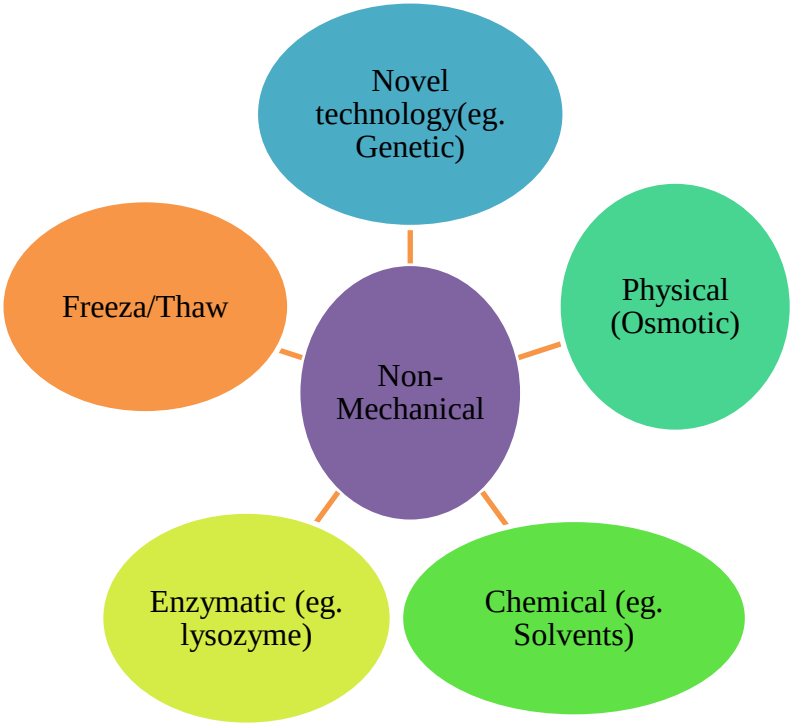


Figure 6 Non-mechanical Cell Disruption Methods

1.4.4.2 Non-mechanical methods:

Non-mechanical methods of disrupting cells in order to release its product are also used in the biotech industry. Examples of this category are illustrated in the figure above. An advantage of these methods is that the degree of cell disruption can be optimised such that only the outer membrane would break and therefore the periplasmic product would be released. These methods are not 100% efficient and they pose difficulties in primary recovery and purification in particular as it must be proven that the chemicals themselves

are removed and that they have not affected the Active Pharmaceutical Ingredient negatively. (Asenjo et al., 1990)

Being able to avoid non-mechanical cell disruption step would make the process much more efficient provided that most of the recombinant product is released in to the medium.

Genetic modification can also be carried out to lyse the cell such. A number of nucleases can be expressed and induced and lead to cell lysis (Berkmen et al., 1997). Induction systems can be utilised to control cell lysis in these methods. However, it does not seem to offer great applications in the biotechnology industry.

1.4.4.2.1 Thermolysis

Thermolysis has the potential to become increasingly prevalent in industrial production. When G (-) bacteria cells are heated to 50 degrees Celsius, periplasmic proteins are liberated. *E. coli* can release cytoplasmic proteins in 10 minutes at 90 degrees Celsius. Enhanced protein discharge has been produced after short exposures at high temperatures, as opposed to lengthy exposures to temperatures at lower levels. Unfortunately, the results are highly inaccurate because the solubility of proteins varies with temperature changes. Due to the production and melting of ice crystals, freezing and thawing a cell slurry can cause the cells to rupture. Gradual freezing, which results in the creation of bigger crystals, can cause significant cell damage. Combining this procedure with cell grinding has produced excellent results. However, it is prohibitively expensive and limited to small-scale facilities. Some findings also indicate a decline in enzyme activity (Harrison, 2011).

1.4.4.2.2 Osmotic shock

Where progressive alterations in osmotic pressure occur, microbial cells maintain a balanced osmotic pressure across the cell membrane by modifying their cytoplasmic composition. To induce lysis through osmotic shock, the cells are equilibrated under high osmotic pressure, which is commonly delivered by a mono- or disaccharide solution or a salt solution (1 M). Subsequently, rapid exposure to a solution with low osmotic pressure induces rapid water entry into the cell to eliminate the osmotic gradient. Increased internal pressure results in cell lysis. As a disruption tool, osmotic shock is limited to systems with compromised or nonexistent cell walls. Reportedly, it facilitates the release of proteins

from *E. coli* without causing cell rupture or reducing cell viability. Combining osmotic shock with mechanical disruption techniques, as described in Section 2.44.7, has been utilised in the past. Large-scale application of osmotic shock is constrained by the expense of the osmo-regulator, the increase in water consumption, and the possibility for unintended dilution of the process(Harrison, 2011).

1.4.4.2.3 Chemical

Chemical approaches for destroying microorganisms depend on the cell structure to be destroyed. pH extremes, particularly alkaline conditions, solvents, detergents, chelating agents, reducing agents, and chaotropic agents are typical agents. In this section, only the most widely applicable and often employed agents are described (Harrison, 2011).

Several bacterial systems have been shown to be susceptible to alkaline cell lysis at pH 10.5–12.5 for 30 s to 30 min. *Erwinia carotovora, E. coli*, and *C. necator* are examples of organisms that have been tested. For application, a stable-at-high-pH product is necessary, and the need for neutralisation will impair material stocks (Harrison, 2011).

It is possible to extract lipid components from the cell membrane using solvents, resulting in the release of intracellular components. Due to their combustibility and tendency to induce protein denaturation, they must be utilised with caution. Alcohols such as ethanol, isopropanol, and butanol (at concentrations of 10–80 percent), dimethyl sulfoxide, toluene (2 percent), and methyl ethyl ketone are utilised as solvents for the release of intracellular chemicals. It has been shown to be applicable to a wide variety of bacteria, including *E. coli, S. cerevisiae*, and *Kluveromyces* species. Although permeabilization happens at room temperature, an increase in release occurs at temperatures between 25 and 45 degrees Celsius (Harrison, 2011).

Detergent treatment is widely employed on a laboratory scale to lyse or permeabilize cells for the release of soluble components by disrupting protein–lipid connections via contact with the detergent's nonpolar hydrophobic tail and polar hydrophilic head. The product's stability in these systems must be ensured. According on the nature of the hydrophilic head, detergents are classed as anionic, cationic, or nonionic. Anionic detergents (e.g., sodium dodecyl sulphate, SDS) disorganize the cell membrane. It is believed that cationic detergents operate on the lipopolysaccharide component of the cell envelope in addition

to interacting with phospholipids. At concentrations between 0.02 and 0.4%, cetyltrimethylammonium bromide (CTAB) has been utilised for permeabilization of both yeast and bacteria. Nonionic detergents such as Triton X-100 and Pluronic F-68 partially solubilize proteins in the inner membrane structure, resulting in membrane permeabilization. Unless a combination of chemical methods is applied, the lipopolysaccharide component of the outer membrane confers resistance to the detergent. Triton X-100 has been shown to aid in the permeabilization of *E. coli, S. cerevisiae, P. pastoris, Nocardia rhodocrous,* and *Yarrowia lipolytica* at concentrations ranging from 0.1% to 4%. Chaotropic drugs mediate cell lysis by breaking H-bonding and changing hydrophobic interactions, hence decreasing cell wall cross-linking. The most common agents are guanidine hydrochloride and urea. With the addition of a chelator such as EDTA, the effects of chaotropic drugs and detergents can be amplified. This reduces membrane and lipopolysaccharide layer stability by chelating divalent ions (Harrison, 2011).

1.4.4.2.4 Enzymatic

Enzymatic cell lysis is a regulated, low-energy process that requires minimal capital expenditure. It can produce biological specificity and occurs under benign settings. It is possible to avoid harsh physical conditions, such as the high shear stress of mechanical disruption. For efficient lysis, the selection of an appropriate enzyme or enzyme system and the determination of specific reaction conditions are essential. There are three described approaches: autolysis, the inclusion of foreign lytic enzymes, and phage lysis. Due to the possibility of unintended infection, the latter is not preferred and is therefore not discussed here. Currently, the availability and cost of enzymes prevent the widespread application of enzymatic lysis (Harrison, 2011).

1.4.4.2.5 Selective Product Release (SPR)

It is common knowledge that the subsequent recovery and purification of this product is affected by other chemicals present in solution, i.e. the contaminated product load. Optimally, the product of interest should be delivered selectively into the suspending medium. The resulting reduction in contaminant load on SPR increases the adsorption capacity for the desired product where chromatographic separations are employed, reduces the purification factor required, prevents DNA release and its effect on viscosity,

and minimises the micronization of cell debris, thereby facilitating clarification of the product-containing solution or differential separation of the particulate product from the cell debris. The acquired selectivity can be classed either in terms of SPR, defined as the ratio of the product released to the pollutants released on a mass basis, or selectivity, defined as the ratio of the product released to the total soluble protein released on a mass basis. As is customary for the majority of liberation processes in process engineering, the degree of product liberation or release is typically raised at the expense of selectivity or purity. The upper-right section represents the optimal operation (Harrison, 2011).

To permit the release of products selectively, it is better to stockpile the product of interest in a well-defined place. Products located in the periplasm or cell wall are the most readily released. In both yeast and bacteria, these areas of the cell contain a restricted number of products (e.g., 4–11 percent of cellular proteins are periplasmic), facilitating selective release. In addition to the potential for selective release and the reduced intensity of disruption, additional advantages include the reduced presence of proteases (7 of 25 identified proteases are periplasmic) and the ability to protect the product from the extracellular milieu until the initiation of recovery. Heat-induced translocation of heterologous proteins to the periplasm has been hypothesised for proteins produced in *E. coli* due to changes in hydrophobicity (Harrison, 2011).

Using both enzymatic and chemical additions, the differential release of enzyme products by S. cerevisiae is reported. On isotonic digestion of the yeast cell wall with an enzyme from *O. xanthinolytica*, the cell-wall-associated invertase was liberated and spheroplasts were produced. After that, osmotic shock unleashed cytoplasmic enzymes such as alcohol dehydrogenase. Finally, detergent treatment released the mitochondrial fumarase (Harrison, 2011).

1.5 Antisense technology

Regulatory RNAs contribute to the regulation of gene expression in bacteria. Antisense RNAs (asRNA) are a type of regulatory RNAs that are transcribed from the complementary strands of their target genes. Typically, these untranslated transcripts bind to homologous mRNAs and immediately influence gene expression at the level following transcription. This article discusses asRNAs that affect bacterial fitness and enhance

pathogenicity. We chose examples that highlight the diversity observed in nature, such as plasmid- and chromosome-encoded asRNAs, a riboswitch-regulated asRNA, and asRNAs whose stability and activity depend on other RNAs or RNA-binding proteins. We investigate how asRNAs enhance bacterial fitness and pathogenicity by regulating plasmid acquisition and maintenance, transposon mobility, bacteriophage resistance, flagellar synthesis, and nutrition uptake. This section concludes with a brief overview of how this information is informing ongoing efforts to develop novel treatments (Millar & Raghavan, 2021).

1.5.1 History and background

The discovery of non-coding RNAs (ncRNAs) that affect gene expression but do not code for proteins was a major advance in biology. ncRNAs have crucial regulatory roles in every aspect of life. By binding to messenger RNAs (mRNAs), ncRNAs in bacteria influence gene expression at the post-transcriptional stage to govern several activities, including pathogenicity. Typically, ncRNAs that are encoded on the opposite strands of target genes (complementary to sense transcript) are known as cis-acting antisense RNAs (asRNAs), whereas ncRNAs that are encoded in a separate region of the genome in relation to their target mRNAs are known as trans-acting small RNAs (sRNAs). In general, regulatory RNAs have an advantage over regulatory proteins due to the fact that their synthesis requires less energy and they act more quickly. In addition, their co-degradation with target mRNAs permits precise regulation of regulatory circuits, which is essential for bacteria to rapidly adapt to the host's immune system. asRNAs are very effective for fast gene regulation because they form perfect complementarity with target mRNAs, whereas sRNAs frequently require chaperone proteins like as Hfq and ProQ for stability and activity. Initially, asRNAs were believed to be uncommon in bacteria, and the widespread antisense transcription reported in microarray-based research was thought to be the result of experimental errors. Low sequence coverage made it initially difficult to distinguish between authentic asRNAs and transcriptional noise, despite the introduction of high-throughput sequencing. Recent research has proven the presence of numerous asRNAs in bacteria and demonstrated that this is a phenomenon that spans the entire genome, as the sequencing resolution has increased. asRNAs have been demonstrated to affect bacterial pathogenicity by influencing the expression of virulence

genes or by directing metabolic processes that enhance bacterial fitness, hence enhancing virulence. There have been focuses on the latter option. In particular, cases where the mechanism of action is generally understood and the genomic sites of asRNAs have been established were discussed. Examples that highlight the diversity observed in nature were selected, such as short asRNAs and long asRNAs, asRNAs found in plasmids and asRNAs encoded on chromosomes, asRNAs that require binding stability from other RNAs or proteins, and asRNAs that collaborate with riboswitches. These examples are organised into the following sections depending on the principal functions asRNAs play in pathogenesis: 1) the acquisition and regulation of virulence plasmids, 2) the modulation of transposon mobility, 3) the enhancement of resistance against bacteriophages, 4) the regulation of flagellar production, and 5) the regulation of nutritional acquisition (Millar & Raghavan, 2021).

Noncoding RNAs have emerged as significant regulators of gene expression in recent years. Antisense RNAs (asRNAs) are notably prevalent among these RNAs; nonetheless, the function and mechanism of action of most asRNAs remain unknown. Here, we highlight a recently discovered paradigm known as the excludon, which characterises a genomic locus expressing an abnormally long asRNA that spans genes or operons with opposing roles. As these asRNAs can block the expression of one operon while functioning as mRNA for the next operon, they serve as regulatory switches in bacteria. (Sesto et al., 2012).

1.5.2 Applications of antisense technology

Since 1981, antisense RNA has been present naturally. This was first noticed when *Escherichia coli*'s protein synthesis was spontaneously downregulated. (Tamizawa, 1981). *Escherichia coli* has recently been found to have a large number of ncRNAs with antisense-like properties.(Park et al, 2014).

Traditional molecular biology methods, such as gene-knockout, which have drawbacks such complexity and high cost, have been repurposed to suppress the manufacture of specified proteins. Antisense technology provides a framework for controlling how bacteria regulate their metabolism and has benefits including quick turnaround, cheap cost, and simple operation. (Li et al., 2008).

Antisense technology is already beginning to deliver on its promise of targeting RNA to treat diseases. Nine single-stranded antisense oligonucleotide (ASO) medications representing four chemical classes, two modes of action, and four methods of administration have been approved for commercial usage, including nusinersen, the first RNA-targeted therapy to experience significant commercial success. Many of the ASOs in late- and middle-stage clinical research are designed to treat people with highly common diseases, despite the fact that all approved medications are for patients with rare disorders. As a result of developments in medicinal chemistry, the comprehension of molecular mechanisms, and targeted delivery, ASOs in development exhibit dramatic enhancements in potency and efficacy. In addition, the ASOs in development feature alternative modes of action and administration methods, including as aerosol and oral formulations (Crooke et al., 2021).

In a 1978 study, the scientists developed an oligonucleotide complementary to a short RNA region of the Rous sarcoma virus and demonstrated that it inhibited viral proliferation in tissue culture. Thus, the work pioneered the concept of considering a target RNA as a receptor for an antisense oligonucleotide (ASO) for which the Watson–Crick base pairing code that governs the affinity and specificity of oligonucleotide binding is known. The scientists also emphasised the broad potential of this method to limit the multiplication of other viruses and the creation of particular human proteins. The optimal length of planned oligonucleotides, potential chemical changes, and whether they may be single- or double-stranded were also not specified1. Thus, the notion encompassed both the single-stranded agents known as ASOs and the double-stranded agents known as small interfering RNAs (siRNAs) (Crooke et al., 2021).

However, efforts to translate the notion into a platform for drug development did not begin in earnest until the late 1980s, when many 'antisense' firms were founded. Despite the evident and intimidating obstacles, the potential benefits of the strategy were equally apparent and compelling. Watson–Crick base pairing, which determines to which receptor sequence an ASO may bind, was understood, making true rational design of ASO medications potentially possible for the first time; this could make the technology significantly more efficient than other drug development methods. Although the genomics revolution was still decades away, advances in molecular biology had already

demonstrated the need for drugs with greater target specificity than small molecules, and ASOs could theoretically be designed to interact with high specificity for their receptor sequences and to be effective against so-called "undruggable" receptors. Even though RNA sequences had never been termed "receptors" and ASOs were distinct from small molecules, it was possible to explain the behaviour of ASOs within the context of traditional receptor theory. As insignificant as the previous remark may appear, having a theoretical framework for issues and efforts to solve them has been essential to the development of technology (Crooke et al., 2021).

To transform the antisense concept from a naive dream into a robust therapeutic modality, each component of the platform had to be conceived and continuously improved. A conceptual framework was required to inform the difficulties, dangers, and possibilities, as well as outline the essential inventions. As antisense technology is chemically based, it was necessary to build novel medicinal chemistry for oligonucleotides and test hundreds of chemical alterations. Revolutionary analytical chemistry was required for product characterization and pharmacokinetic studies, as well as innovative process chemistry and production methods to substantially reduce the cost of synthesis and increase its scale. In the framework of molecular pharmacology, RNAs, their intermediary metabolism, their activities, and their properties required to be comprehended. It was necessary to identify the structure and function of the ribonucleoproteins that contain the RNA receptors for ASOs, as well as the factors that may influence access to the receptors and the potential post-RNA-binding pathways. In addition, since many RNAs contain several identically appealing ASO receptor sequences, it was crucial to comprehend the potential interactions between cognate sites present in a target RNA and how the ASO's efficacy varied as a function of the number of cognate receptors. Methods for evaluating pharmacokinetic behaviour and potential toxicity required to be developed, as well as rapid screening tools for identifying the best locations in RNAs where ASOs can bind. Finally, it was necessary to comprehend the causes of failure so that the lessons might be utilised to improve the performance of ASOs. Thanks to many years of study, we now have a strong understanding of these subjects, but there is still much to learn (Crooke et al., 2021).

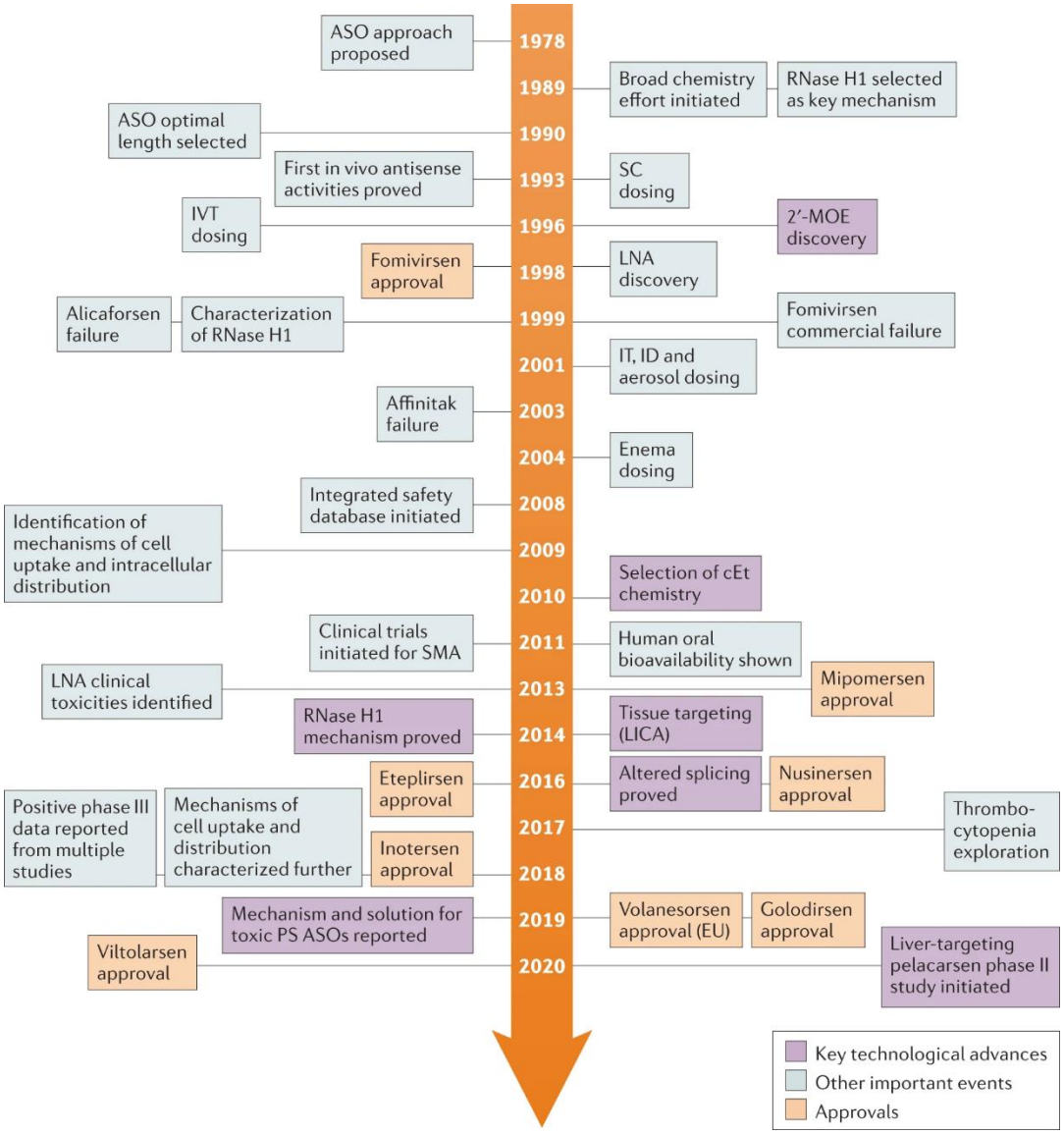


Figure 7 An overview of progress in antisense technology (adopted from (Crooke et al., 2021))

Significant technology advancements, medicine approvals, and other events and setbacks are emphasised. Some dates are approximations of the timing of events, and current developments are too frequent to fit on the diagram comfortably. As the efficacy of antisense oligonucleotides (ASOs) has increased, treatment prospects have expanded to include both rare and common disorders, as well as virtually every delivery method. ID, intradermal; IT, intrathecal; IVT, intravitreal; LICA, ligand-conjugated antisense; LNA, locked nucleic acid; PS, phosphorothioate; SC, subcutaneous; SMA, spinal muscular atrophy (Crooke et al., 2021).

1.5.3 Mechanism of antisense RNA inhibiting protein synthesis

The figure below illustrates how antisense RNA inhibits the syntheses of a target protein.

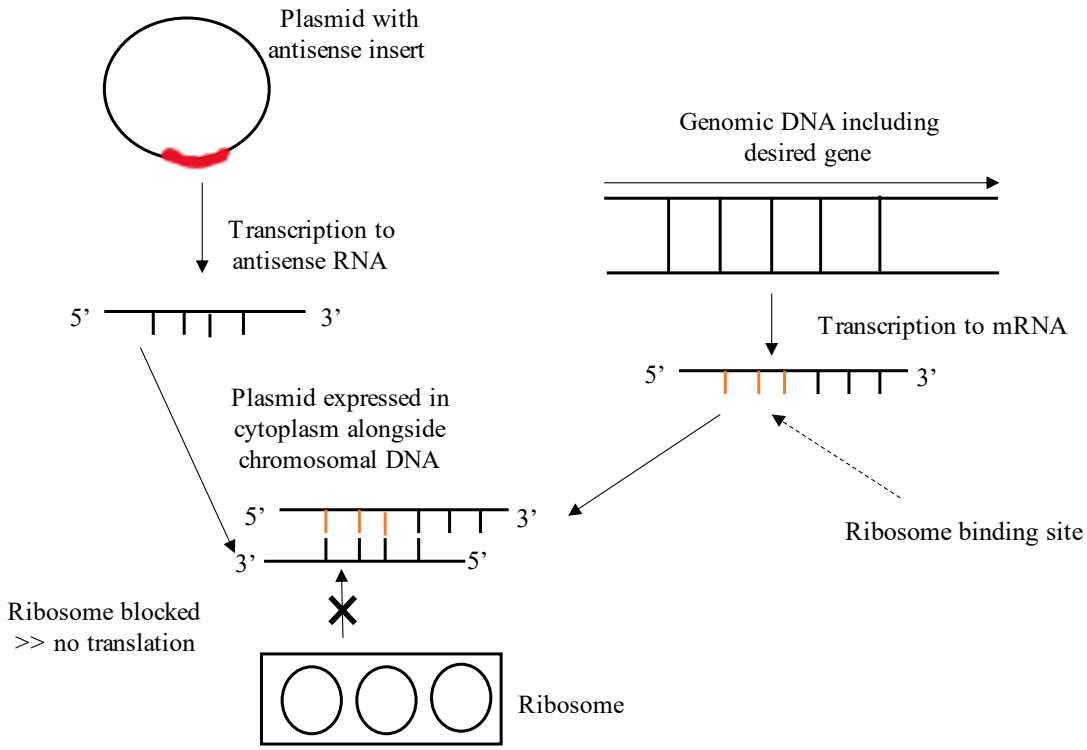


Figure 8 Illustration of antisense inhibition (image digitally drawn by Shahin Heshmatifar)

A plasmid that has been engineered to express a length of antisense RNA that is orientated in the opposite direction from the gene of interest can be seen depicted here in the form of a diagram. When it transcribes the RNA together with the sense mRNA, the two strands have the potential to form a duplex together. This would prevent the ribosome binding site from being accessed, hence preventing the gene from being translated.

As seen in the figure above, the antisense RNA that the cell produced after being transformed with a vector containing the DNA of the target protein binds to the target protein's mRNA and prevents it from being translated.

Non-coding RNA (ncRNA) called antisense is produced and subsequently binds to its intended complementary mRNA. This binds to the mRNA and prevents the ribosomes from translating it, indirectly suppressing the expression of the genes that produce it (Park et al , 2014).

Applications for antisense technology range from direct medicinal uses to the alteration of microorganisms and the improvement of bioprocess efficiency, which is the project's main objective.

A study was carried to investigate the impact of antisense RNA in down regulation of yidC in *Escherichia coli* in order to sensitize the bacterium to antibiotics. Lack of yidC, a protein that is crucial for the structure of cell membranes and reduces antibiotic resistance. Results revealed decreased growth and resistance, and treatment with eugenol/carvacrol and yidC silencing are suggestive of a possible antibacterial therapy. (Pati et al., 2013).
Antisense technology is also used directly for therapeutic purposes, such as in the treatment of cancer, where the suppression of mRNA targets the production of proteins vital for division (Devi et al., 2002)

Zamecnik and Stephenson discovered that antisense agents can be used for inhibition of viral replication in culture (Zamecnik et al., 1978). Theoretically, any disease or condition that is brought on by the expression of a gene, including viral infections, cancerous tumour development, and inflammatory diseases, can be treated with antisense technology. Despite the fact that practical implementation now faces several obstacles and limitations.

When compared to other traditional procedures, antisense technology effectively achieves gene functionalization and target validation with rapid development, cheap cost, high specificity, and a high probability of success (Bennet et al., 1999).

Antisense oligonucleotides (ASOs) are a family of specialised therapeutic medicines that decrease disease-associated gene products by altering the intermediate metabolism of messenger RNA (mRNA). ASOs exert their pharmacological effects by Watson-Crick

base-pairing with a particular target RNA. This event, if well planned, results in the recruitment of RNase H, the destruction of targeted mRNA or pre-mRNA, and the subsequent suppression of the synthesis of a certain protein. The capacity to specifically block targets that cannot be modified by conventional treatments, including as structural proteins, transcription factors, and lipoproteins, is a fundamental advantage of the technology. To provide an overview of antisense technology, then describe the status of lipoprotein-related genes that have been studied using the antisense platform, and then describe the general methodology required to design and evaluate the in vitro and in vivo efficacy of those drugs. (Crooke & Graham, 2013)

The use of antisense oligonucleotides as medicinal agents has aroused tremendous excitement among researchers and physicians. Antisense oligonucleotides were considered as therapeutic agents as early as the 1970s, when the antisense method was first developed. Nonetheless, it has taken over a quarter century to achieve this promise. Antisense technology is based on the sequence-specific binding of an antisense oligonucleotide to target mRNA, which inhibits gene translation. The specificity of Watson-Crick base pairing makes antisense oligonucleotides desirable as tools for targeted validation and functionalization, as well as treatments to selectively modulate the expression of genes involved in the development of illnesses. (Aboul-Fadl, 2005)

DNA and RNA were provided as potential medicinal agents prior to the development of effective transfection technologies and comprehension of molecular biological processes. For instance, DNA from many sources exhibited anticancer action, with the activity varying according to size, base makeup, and secondary structure. However, the molecular pathways through which DNA may generate an antitumor effect have never been described, and multiple investigations have failed to confirm DNA's anticancer properties. Compared to research on DNA as a medicinal agent, a great deal more research has been conducted on RNA and polyribonucleotides. Polyriboinosine: polyribocytidine is the polynucleotide that has been the subject of the most research in relation to its capacity to generate interferon (poly rI:poly rC). Poly rI:poly rC exhibited significant antiviral and anticancer properties in vitro and in vivo, which were related to

the production of interferon. However, the significant toxicity of this polyribonucleotide in both animals and humans severely restricts its usefulness. (Aboul-Fadl, 2005)

The other extensively investigated polyribonucleotide is ampligen, a mismatched poly rI: poly rC12U. It has been demonstrated that this polyribonucleotide induces interferon and activates 2'-5' adenosine synthase. Ampligen has similar features to poly rI:poly rC, but its activities are broader and its toxicity is lower, and it is still in development. (Aboul-Fadl, 2005)

Any desired protein with a role in the cell can be prevented from being translated using antisense technology. This method can block proteins that are either inner or outside membrane proteins. In order to fully release the recombinant product into the medium, research might examine antisense and membrane protein inhibition.

Blocking the 5' end of a gene, specifically the Shine-Dalgarno sequence and the ribosome binding site, is the most efficient method for inhibiting mRNA expression. These regions are cloneable. By reversing and balancing their direction, antisense orientation is achieved. Then, these sequences can be cloned onto a plasmid with an appropriate promoter, allowing the expression of antisense RNA to be activated during the growth of the cell culture by inducing the promoter. At this moment, the antisense RNA forms a duplex with the mRNA and prevents translation of the target gene. As with natural antisense RNA, adding GC-rich stem-loops to the 5' end of the antisense RNA can boost the construct's stability.

1.5.4 Limitations of antisense technology applications

Antisense approaches for achieving extracellular release of recombinant proteins from *Escherichia coli* during fermentation have both potential drawbacks and limitations.

In terms of efficiency and effectiveness, antisense approaches rely on the use of antisense oligonucleotides to inhibit the expression of target genes involved in protein secretion pathways. However, achieving efficient and effective inhibition can be challenging.

Antisense oligonucleotides may not always efficiently bind to their target mRNA, resulting in incomplete inhibition of the gene of interest. This can lead to suboptimal levels of protein secretion.

With respect to Specificity, antisense approaches typically target specific mRNA sequences, and off-target effects can occur. There is a possibility that the antisense oligonucleotides may bind to unintended mRNA sequences, leading to unintended gene silencing and potential disruption of other cellular processes. In terms of stability and delivery of antisense oligonucleotides, antisense oligonucleotides are susceptible to degradation by nucleases present in the cell, which can limit their stability and effectiveness. Additionally, efficient delivery of antisense oligonucleotides to the target cells can be challenging. Effective delivery methods, such as using cationic lipids or nanoparticles, may be required to ensure successful uptake and intracellular delivery of the antisense molecules.
Antisense approaches can potentially affect cell viability and growth. Inhibition of essential genes involved in protein secretion may result in cellular stress or perturbation of normal cellular processes, leading to reduced cell viability and growth rates. This can ultimately impact the overall protein yield and fermentation performance.

Antisense approaches may face scalability challenges when transitioning from laboratory-scale experiments to industrial-scale fermentation processes. Factors such as the cost of synthesizing antisense oligonucleotides, difficulties in optimizing large-scale delivery methods, and ensuring consistent and reproducible results in large bioreactors can pose significant challenges for industrial application. Designing effective antisense oligonucleotides and optimizing their concentration and delivery methods can be complex and time-consuming. Iterative experimentation and optimization may be necessary to achieve desired levels of protein secretion, which can increase the time and resources required for process development.

Despite these drawbacks and limitations, antisense approaches hold potential for enhancing protein secretion in *E. coli* during fermentation. Continued research and advancements in oligonucleotide design, delivery strategies, and optimization techniques

may help overcome these challenges and improve the efficiency and applicability of antisense approaches in biotechnological applications.

In summary:

- Antisense technology has shown promising capacity for inhibiting translation of a target protein, in this project Lpp.
- Lpp is an outer membrane protein (Omp) of *Escherichia coli* that has been chosen to be investigated in this project due to its abundance in the outer membrane and other suitable characteristics.
- Antisense making plasmid that codes for the antisense-RNA, will co-exist with another plasmid that contains the DNA sequence of the product of interest within the cell.

1.6 Aims of the project

This project's overarching objective was to evaluate the efficiency of antisense RNA in preventing the formation of cell membrane components during fermentation processes and to analyse how this would influence the release of target proteins that were being overexpressed by the cells. In order to do this, the periplasmic protein expression systems of *E. coli* were investigated. In particular, the downregulation of murein lipoprotein was investigated in BL21 and W3110 variants of *E. coli*, as well as the effects of this inhibition on the release of alkaline phosphatase, alpha-amylase, and Fab fragments. These effects were monitored with the help of assays that are used to determine the activity or quantity of the proteins in question. It was also important to make sure that these modifications did not result in the growth of the cells being hampered in any way, either in terms of the speed at which it occurred or the levels of biomass that it produced. The goal was for this process to be compatible with the production processes that are used in industrial settings, so this was an extremely important consideration throughout the process. In addition, it was important to measure the overall levels of protein that were being produced and released in order to ensure that any release of the target proteins was specific rather than providing a general release of many of the components of the cells. Measuring the overall levels of protein production and release was important.

This project aims to investigate a novel methodology and its effectiveness for extracellular release of recombinant protein by *Escherichia coli.* The methodology and approach being investigated will be utilisation of antisense technology to inhibit translation of selected outer membrane proteins.

One of the outer membrane proteins (Lpp) in the Figure 2 has been investigated in previous research projects (Gibbons, 2013) to explore its capacity for extracellular release, adopting antisense technology. The results were promising, and confirmatory research will be carried out as part of this project as well as improving and optimising the methodology. Other proteins in the outer or inner membrane will be subjected to study that has not yet been confirmed.

The proteins in the Figure 2 can be exploited in combination with antisense technology to aid optimal extracellular release of recombinant protein into the media. Antisense RNA complementary to the mRNA of a target protein will inhibit the translation and production of the target protein and therefore the membrane of the cell will lack the target protein. It is hoped that this will lead to leakage of the recombinant product into the media from the periplasm.

The experiments will be carried out using mainly two different strains of *Escherichia coli*, being W3310 and BL21(DE3) and specific plasmids in combination with the antisense construct. It is aimed to assess and enhance the effectiveness of antisense technology on a protein that has previously been researched on for the same application, this protein is Lpp. Other unexplored proteins in the membrane will also be investigated for the defined application.

Quantities of recombinant products in the periplasm, cytoplasm and supernatant will be measured using various assays and protocols. The products that will be produced in the cells under investigation will be alpha-amylase and Fab fragments.

Following success in driving the cells to completely secrete their product into the medium, the cells robustness in primary recovery operations such centrifugation will be assessed to show that the GM cells have the same physical attributes such that the yield and productivity of other unit operations will not be compromised when the antisense cells are used for manufacturing.

In order for the novel cells to be utilised in industry, it must be shown that all the advantages of the traditional strains are retained in the new cells. This issue will require more research following success in making cells with high extracellular secretion levels.

Furthermore, optimum conditions for maximum yield using the new cells will be investigated and established in both shake flask cultures and fermentation in bioreactors.

Following successful development of new strains of *Escherichia coli* with high capacity for extracellular release, the biotechnology industry may be very interested to invest and adopt the new methodology in manufacturing recombinant proteins.

In summary:

- Exploit antisense technology to achieve controlled and sustained extracellular release of recombinant protein during fermentation.
- Target selected outer membrane proteins(omp).
- Investigate the methodology on a variety of recombinant proteins including α-amylase and Fab
- Demonstrate reproducibility and robustness.
- Investigate the methodology in high cell densities.

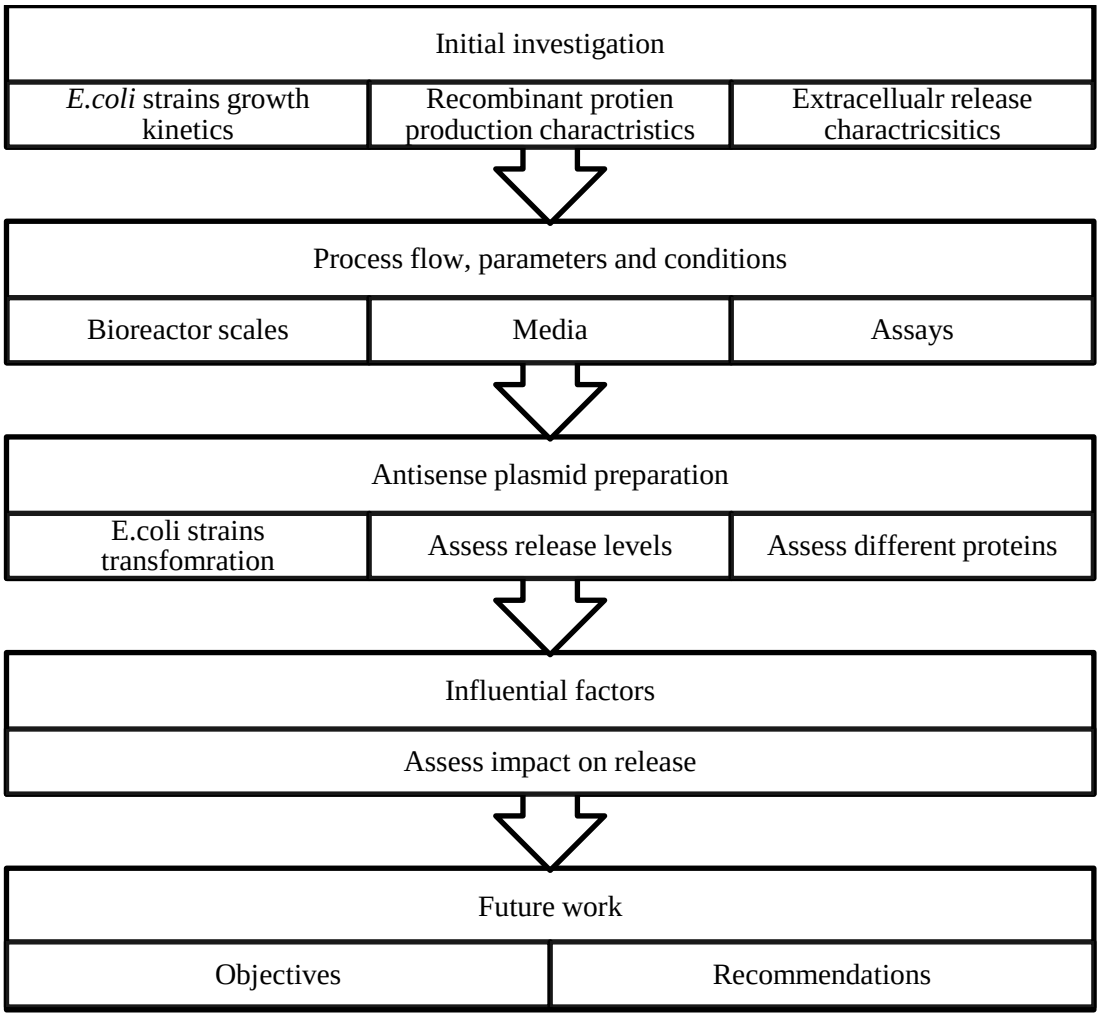


Figure 9 Project objectives and major steps

This thesis presents chapters and achieved goals as described below:

- Chapter 2: Methods and material followed and used in this research study including shake flask and bioreactor fermentations as well as assays and technical information regarding the plasmids and stains used/created
- Chapter 3: preparation of the strains of *E. coli* transformed is described here.
- Chapter 4: production and characterisation of alpha-amylase in various scales as well as its release level in the antisense fermentation
- Chapter 5: production and characterisation of fragmented antibody in various scales as well as its release level in the antisense fermentation. Preliminary investigation of factors that influences the release levels
- Chapter 6: future work and industrial applications

If the use of antisense RNA can successfully improve the release of proteins of interest from *E. coli*, then broader uses of the technology could be applied, both to further optimise the methods discussed in this thesis, as well as with for the modification of other microbes, and with other cell components. If the use of antisense RNA can successfully improve the release of proteins of interest from *E. coli*, then the use of antisense RNA can successfully improve the release of proteins of interest from *E. coli*.

Chapter 2 : Methods and Materials

2.1 Materials

Chemicals, enzymes, media and antibiotics were purchased form companies including Sigma-Aldrich, Invitrogen, VWR International, Fisher Scientific, Merck and New England BioLabs. Sterilisation of materials and media that required sterilisation was achieved through autoclaving at 121°C for 20 minutes, some solutions such as antibiotics were filtered through a 0.2µm sterile filters.

2.2 Bacterial strains and plasmids

The bacterium in use throughout the project will be *Escherichia coli*. Two main strains of the named bacterium will be utilised, these are BL21(DE3) and W3110.
The strains and plasmids that will be studied are outlined in the table below:

***Escherichia coli* strains**	**Details**	**Source**
BL21 (DE3)	Contains DE3, a λ prophage with T7 RNA polymerase and *lacl*^{q}	NEB Jeong et al., 2009
W3110	Non-proprietary. Similar to wild type *E. coli*	Blattner et al., 1997

Table 4 *Escherichia coli* Strains used

Since both strains are B strains, they lack Lon protease (cytoplasm) and OmpT protease (outer membrane). Consequently, B strains are favoured for recombinant protein expression. The DE3 designation indicates that the relevant strains include the λDE3 lysogen containing the T7 RNA polymerase gene under the control of the lacUV5 promoter. IPTG is necessary to maximally activate T7 RNA polymerase production in order to produce recombinant genes cloned downstream of a T7 promoter. BL21(DE3) is compatible with expression from a T7 or T7-lac promoter or other promoters recognised by *Escherichia coli* RNA polymerase, including as lac, tac, trc, ParaBAD, PrhaBAD, and the T5 promoter.

Note that BL21 lacks the gene for T7 RNA polymerase and is therefore only compatible with promoters recognised by *Escherichia coli* RNA polymerase, such as lac, tac, trc, ParaBAD, PrhaBAD, and the T5 promoter.

Plasmid	Details	Source/reference
pQR126	Contains *amy* gene and kanamycin resitant	Bahri and ward, 1990
pQR187	As above, but added *cer* fragment	John Ward, UCL
pTTOD A33 IGS2	coding for a 46 kDa antibody fragment (Fab') utilising a tac promoter and tetracycline resistant	UCB Pharma Ltd (Adams et al., 2009)
pMMB66EH_T5_Lpp	Contains RSF1010 replicon and ampicillin resistant Contains the antisense Lpp gene with T5 promotor	Furste et al., 1986
pMMB66EH_T7_Lpp	Contains RSF1010 replicon and ampicillin resistant Contains the antisense Lpp gene with T7 promotor	Furste et al., 1986

Table 5 Plasmids used for amylase and Fab' expression and antisense-Lpp plasmids

The plasmid pQR187, which is a derivative of pQR126. pQR126 itself is the ColE1-derived pBGS19 with the addition of pQR300 HindIII/PstI + alpha-amylase gene (amy) from Streptomyces thermoviolaceus CUB74 (Bahri and Ward, 1990). The plasmid encodes kanamycin resistance and has a reduced distance between the lacZ promoter of the vector and the translational starting site of the alpha-amylase gene (French et al., 1996). The alpha-amylase expressed by this gene has a MW of 47 kDa, and pQR126 itself is 7.8 kbp (French et al., 1996)

For Fab’ production An *Escherichia coli* w3110 strain (ATCC 27325) containing the plasmid pTTOD A33 IGS2, was kindly donated by UCB Pharma (Slough, UK), coding for a 46 kDa antibody fragment (Fab') utilising a *tac* promoter.

2.2.1 **pQR126 plasmid**

The named plasmid as outlined in table 2, codes for alpha-amylase and its linear map is shown in the figure below.

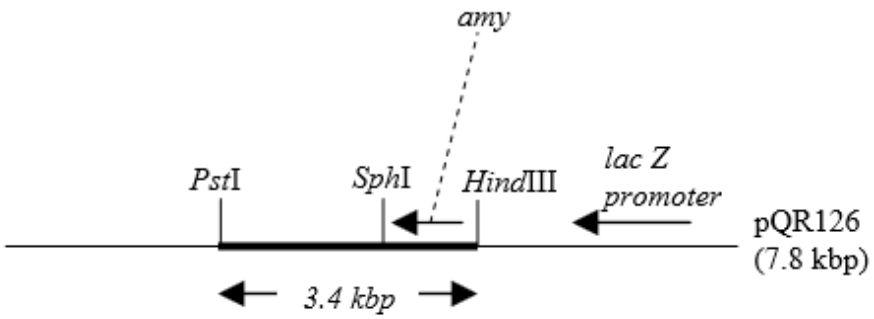


Figure 10 linear map of plasmid pQR126 (French et al., 1996)

Plasmid pQR187 was constructed by JMW and is a derivative of pQR126 (French et al., 1996; Pierce et al., 2002) containing a PstI/EcoRI 200 base pair *cer* fragment of pKS450 (Summers and Sherratt, 1984). The *cer* fragment imparts segregational stability to the plasmid (French and Ward, 1995) so that antibiotics are not needed to ensure stable carriage of the plasmid by the *Escherichia coli* strain. As with pQR126, this plasmid encodes kanamycin resistance.

2.2.2 Plasmid WFab'

Escherichia coli W3110 strain (ATCC 27325) containing the plasmid pTTOD A33 IGS2 (with specificity for human IL-17), was kindly donated by UCB Pharma (Slough, UK), coding for a 46 kDa antibody fragment (Fab') utilising a *tac* promoter. Strains of *Escherichia coli* transformed with the WFab' plasmid will be denoted as strain-WFab'. The plasmid was extracted and transformed into other strains the experiments of this project.

The plasmid also codes for tetracycline resistance and is shown in Figure 11

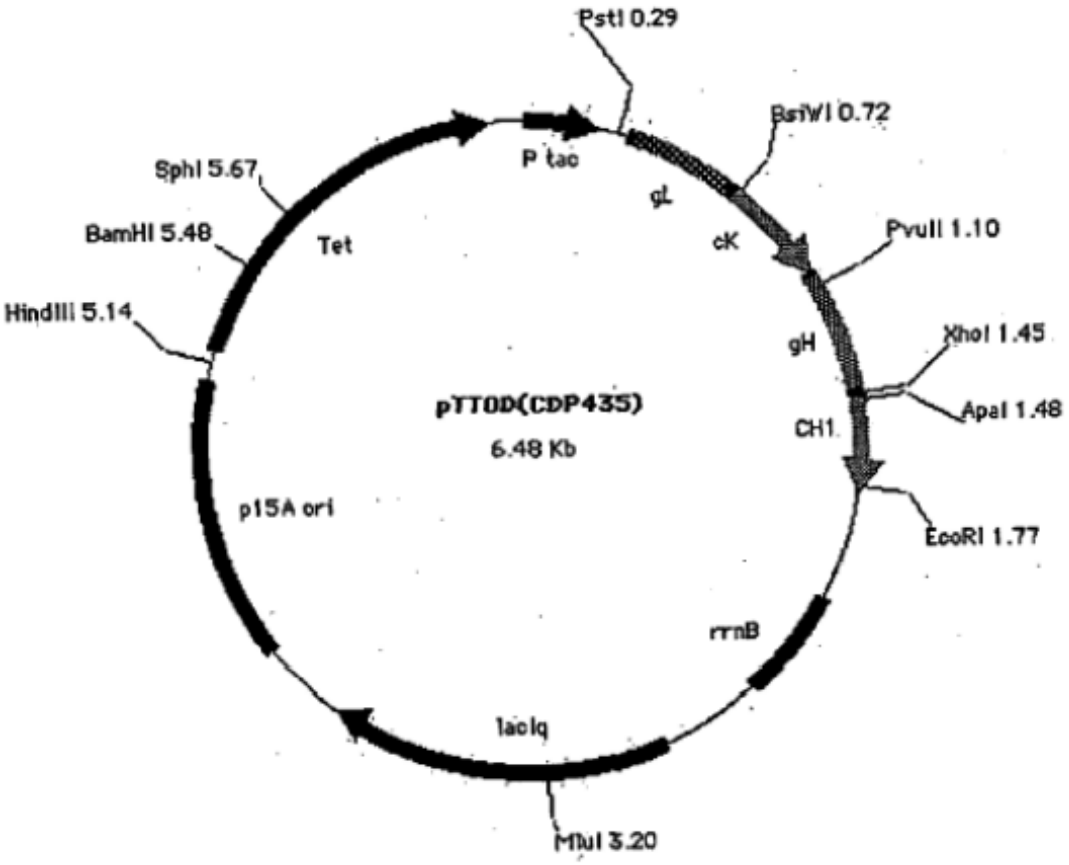


Figure 11 pTTOD A33 IGS2 plasmid used in *E. coli* w3110, coding for Fab' expression and tetracycline resistance, utilising a tac promoter (Adams et al., 2009)

2.2.3 **pMMB66EH Vector**

The named vector will be the vector that the antisense constructs will be inseted into and once combined with the antisense cunstrust, it will be transformed into the working starins. The figure below shows the map of the vector and its details.

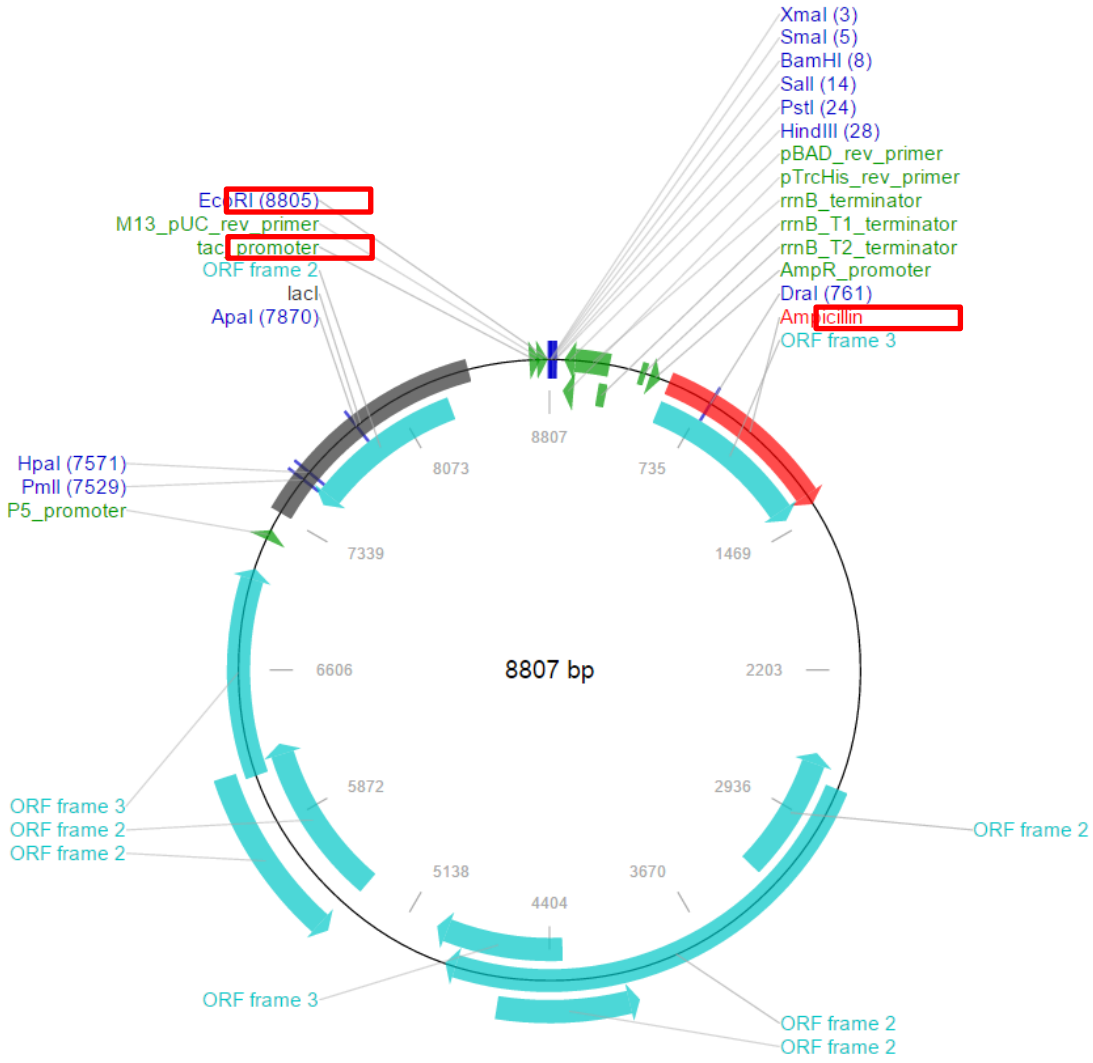


Figure 12 Map of Vector pMMB66EH (Image adapted from http://www.addgene.org/vector-database/3609/)

This vector is ampicillin resistant and can stably co-exist with other standard *Escherichia coli* cloning vectors, thus its transformation into an *Escherichia coli* with another vector is achievable. It contains the RSF1010 replicon allowing it to be used alongside plasmids

with other replicons such as the pQR plasmids used in this study. This vector has a low copy-number compared to ColE1 plasmids, at 10 – 15 copies per cell (Morales et al., 1991). The EH suffix of this plasmid refers to the orientation of the EcoRI and HindIII restriction sites in the multiple cloning site (MCS). Key features of the vector include the ampicillin promoter (482 – 510) and ampicillin bla gene (552 – 1412), lacIq repressor (8437 – 7347), tac promoter (8737 – 8765), multiple open reading frames (ORF) and the multiple cloning site, starting with EcoRI at 8805 and ending with HindIII at 28.

2.2.4 pJ411 vector

The Lpp antisense was ordered to be made by the company DNA 2.0, the Lpp antisense DNA was sent in vector pJ411. The figure below shows a map of this vector with Lpp antisense in it.

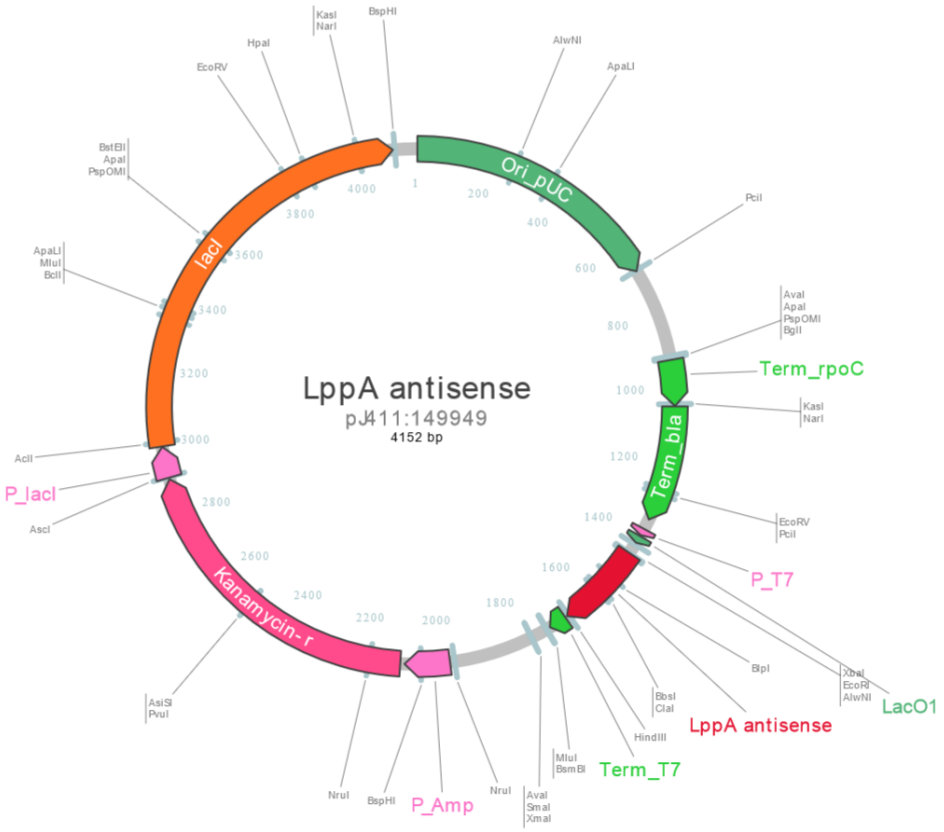


Figure 13 Map of pJ411 vector with Lpp antisense

It encodes for kanamycin resistance. The LppA antisense part of the plasmid will be dijested and transformed into pMMB66EH for its function and purpose of this study. Plasmid pJ411 has no further application in this project and simply served as a carrier of the LppA antisense from the manufacturing company to the university.

2.2.5 T7 Promotor systems

The T7 promoter is a sequence of DNA 18 base pairs long up to transcription start site at +1 (5' – TAATACGACTCACTATAG – 3') that is recognized by T7 RNA polymerase. The T7 promoter is commonly used to regulate gene expression of recombinant proteins, which can be subsequently used for a variety of downstream research applications (Merck, 2019). This promotor system was adopted in preparation of cell transformation in to the appropriate *Escherichia coli* strain (BL21(DE3)).

2.2.6 Induction

The cell biomass is induced at the appropriate time, i.e.. DOT spike. The fermentation temperature is then reduced as per protocol and the appropriate amount to achieve 1mM (final concentration) of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (from stock solution of 1 M solubilised in RO water and 0.2µm filter-sterilised) is added to the cell culture.

2.2.7 Cell Culture

Shake flask and benchtop bioreactor systems were used; Including 1.4L parallel infors Multifors bioreactor system and 7L Eppendorf BioFlo320 bioreactors.

The fermentations were run at 70% working volume and inoculated with 10% inoculum. Cascade control was utilized to maintain minimum DOT of 35% by controlling agitation (500-1200rpm) and a constant aeration (1vvm). pH was controlled at 7 and 1.72M phosphoric acid and 2M NaOH was used for this purpose. two temperature set points were implemented, 32ºC for growth and it was reduced to 27ºC post induction. Induction was carried out with IPTG to achieve a final concentration of 1mM. Fed-batch processes

having 80% glycerol as feed stock were conducted, added at 1.2% of working volume per hour via an external pump following DOT spike.

2.2.8 Plating bacterial strains

Plates contained 30ml LB-Agar with/without antibiotic.

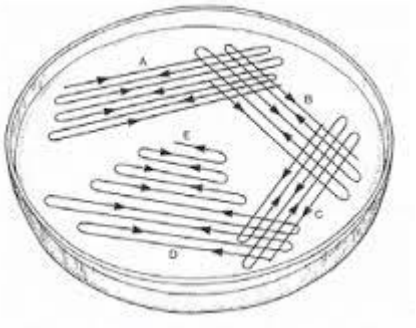


Figure 14 streaking plate

Figure above shows streaking technique adapted

2.2.9 Small volume overnight bacterial cultures

Cultures of 20ml +/- antibiotic plus a colony of desired strain incubated over night at 37°C and 250rpm in Falcon tubes. LB medium was used for this overnight culture.

LB media is also known as complex media and the one used contains 10g/L Tryptone, 5 g/L yeast extract and 5 g/L NaCl. This was purchased from Sigma-Aldrich. Lennox LB is a highly-referenced microbial growth medium used for the cultivation of *Escherichia coli*. This nutrient-rich microbial broth contains peptides, amino acids, water-soluble vitamins, and carbohydrates in a low-salt formulation. (Sigma-Aldrich, 2014)

Cultures of 100ml medium (LB) in 1L baffled flasks and cultures of up to 500 ml in 2L baffled flasks +/- antibiotic incubated at desired temperature and revolution for desired period of time.

2.2.10 Master cell bank and working cell bank production and storage

For preparation and stocking of master cell bank (MCB) and working cell bank (WCB) for each of the prepared *E. coli* strains containing the appropriate plasmid/s to be used in this research, a colony was picked and used to inoculate 50mL Falcon tubes containing 20ml of complex LB broth (h (10 g L^{-1} tryptone, 5 g L^{-1}yeast extract and 5 g L^{-1} NaCl) that were then incubated at 37°C and 250 rpm overnight, or until the optical density (OD_{600nm}) reached between 1.1 and 1.5, indicating that the cells were in exponential phase. To produce 10x MCB 0.5 mL of cell culture was mixed with 0.5. mL of 50 percent (v/v) glycerol solution and kept at -80°C in 1 mL cryovials. From 1 MCB vial of each strain, 30x WCB was produced by using 1 MCB vial to inoculate 100ml of LB Broth in a 500ml shake flask and incubated at 37°C for 5 hours or until the optical density (OD_{600nm}) reached between 1.1 and 1.5. 0.5 mL of cell culture was mixed with 0.5. mL of 50 percent (v/v) glycerol solution and kept at -80°C in 1 mL cryovials.

2.3 Cell culture technologies and bioprocess steps and parameters

Bioreactor systems were used, Including 1.4L parallel infors Multifors bioreactor system, 7L Eppendorf BioFlo320 fermenter. The fermentations were running at 70% and 50% working volumes and inoculated with 10% inoculum. Cascade control was utilized to maintain minimum DOT of 35% by controlling agitation and oxygen and a constant aeration (1vvm). pH was controlled at 7 and 1.72M phosphoric acid and 2M sodium hydroxide was used for this purpose. Two temperature set points were implemented, 32ºC for growth and it was reduced to 27ºC post induction. Towards the end of the exponential growth phase a dissolved oxygen spike and pH spike indicated that the culture had utilized all of the glycerol carbon source in the media. At this point, isopropylβ-D-1-thiogalactopyranoside (IPTG) was added to induce expression. A maximum of 2 mL of 100% polypropylene glycol (PPG) was added to the fermenter on demand to control foaming. Induction was carried out with IPTG to achieve a final concentration of 1mM. Fed-batch processes having 80% glycerol as feed stock were conducted. Fed at 0.13% of the working volume per hour via an external pump following dissolved oxygen spike. Up to 60 hours fermentations were carried out in this study.

2.3.1 Shake flask cultures and Seed train for bioreactor inoculum

The inoculum seed culture was prepared in two steps. First step in a 500mL shake flask, 100mL of Luria Broth Miller liquid microbial growth medium, 1 mL of the Working Cell Bank stock was added as well as the appropriate antibiotic. Incubated at 37ºC and agitation rate of 200 rpm overnight. The second step was in a 2L shake flask, adding 50 mL of the first step seed to 450mL of the defined fermentation media to the flask, incubated at 37ºC and agitation rate 150 rpm for 4 hours resulting in the inoculum for the fermentation. For shake flask fermentations the first step mentioned above was carried out.

2.3.2 Infors Multifors 4x 0.7L WV parallel fermentations

The bioreactors are 1L in volume and the system is shown in the figure below. The defined medium in which the growth and production takes place is detailed here. The running conditions are listed below the figure of the bioreactors system. The induction takes place at DOT spike and a shot of additional glycerol follows immediately; this is mentioned in the list below. deviations are mentioned appropriately.

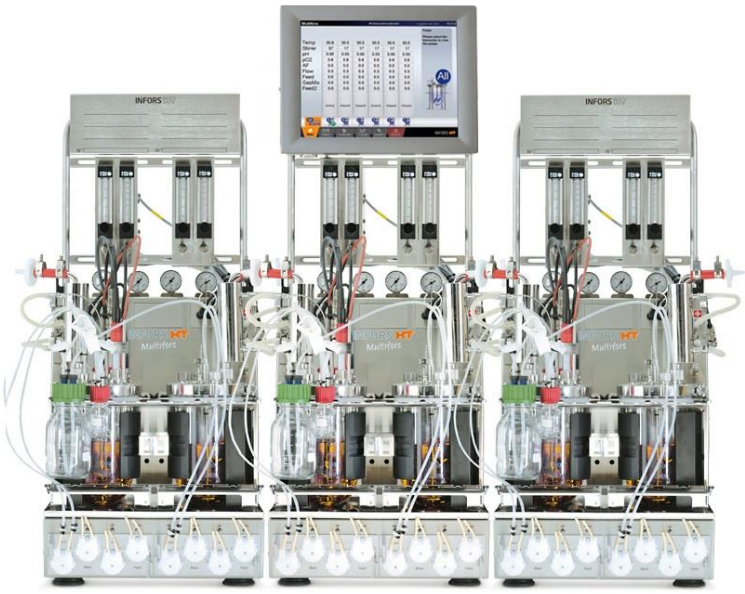


Figure 15 Infors HT Multifors parallel bioreactor and control system (image from SelectScience, (2022))

Defined media adopted from Bowering et al., 2002:

The defined medium used for the 0.7 L fermentations had the following composition (Media 1):

Chemical	Concentration (g/L)
$(NH_4)_2SO_4$	5
$NaH_2PO_4.H_2O$	3.3
KCl	3.87
$MgSO_4.7H_2O$	0.717
Citric acid. H_2O	5
Glycerol	30
PPG	0.2 ml /L
$CaCl_2.H_2O$	0.05
$ZnSO_4 \cdot 7H_2O$	0.0246
$MnSO_4 \cdot 4H_2O$	0.02
$CuSO_4 \cdot 5H_2O$	0.005
$CoSO_4 \cdot 7H_2O$	0.00427
$FeCl_3 \cdot 6H_2O$	0.0967
H_3BO_3,	0.0003
$NaMoO_4$	0.00024

Table 6 Defined media composition for fermentations – (Media 1)

Appropriate antibiotic, depending on the plasmids used was added after autoclave through a 0.2µm sterile filter into the bioreactor. The antibiotics used, and concentrations are as following: kanamycin, 20µg/ml, ampicillin 100µg/ml, tetracycline 10µg/ml

Media 2 (for High cell density fermentation) compositions are detailed in the tables below

Chemical	Concentration (g/L)
$(NH_4)_2SO_4$	5.2
$NaH_2PO_4.H_2O$	4.4
KCl	4
$MgSO_4.7H_2O$	1.04
SM6e Trace elements	10 ml
Citric acid. H_2O	4.2
Glycerol	141.12
PPG	0.2 ml
$CaCl_2.H_2O$	0.25
Citric acid	100
$CaCl_2.6H_2O$	5
$ZnSO_4.7H_2O$	2.46
$MnSO_4.4H_2O$	2
$CuSO_4.5H_2O$	0.5
$CoSO_4.7H_2O$	0.427
$FeCl_3.6H_2O$	9.67
H_3BO_3	0.03
$NaMoO_4.2H_2O$	0.024

Table 7 SM6Gc Defined Media - Formula for HCDF (Media 2)

2.3.3 4L WV fermentation in BioFlo320

The bioreactors are 7L in volume and the system is shown in the figure below. The defined medium in which the growth and production takes place is detailed here. The induction takes place at DOT spike.

Figure 16 BioFlo320 Bioreactor and control system (Eppendorf, 2021)

Defined media adopted from Bowering et al., 2002:

The defined medium used for the and 5 L fermentations had the following composition (Media 1):

Chemical	Concentration (g/L)
$(NH_4)_2SO_4$	5
$NaH_2PO_4.H_2O$	3.3
KCl	3.87
$MgSO_4.7H_2O$	0.717
Citric acid. H_2O	5
Glycerol	30
PPG	0.2 ml /L
$CaCl_2.H_2O$	0.05
$ZnSO_4 \cdot 7H_2O$	0.0246
$MnSO_4 \cdot 4H_2O$	0.02
$CuSO_4 \cdot 5H_2O$	0.005
$CoSO_4 \cdot 7H_2O$	0.00427
$FeCl_3 \cdot 6H_2O$	0.0967
H_3BO_3,	0.0003
$NaMoO_4$	0.00024

Table 8 Defined media composition for fermentations –(Media 1)

Appropriate antibiotic, depending on the plasmids used was added after autoclave through a 0.2µm sterile filter into the bioreactor. The antibiotics used, and concentrations are as following: kanamycin, 20µg/ml, ampicillin 100µg/ml, tetracycline 10µg/ml

Media 2 (for High cell density fermentation) compositions are detailed in the tables below

Chemical	Concentration (g/L)
$(NH_4)_2SO_4$	5.2
$NaH_2PO_4.H_2O$	4.4
KCl	4
$MgSO_4.7H_2O$	1.04
SM6e Trace elements	10 ml
Citric acid. H_2O	4.2
Glycerol	141.12
PPG	0.2 ml
$CaCl_2.H_2O$	0.25
Citric acid	100
$CaCl_2.6H_2O$	5
$ZnSO_4.7H_2O$	2.46
$MnSO_4.4H_2O$	2
$CuSO_4.5H_2O$	0.5
$CoSO_4.7H_2O$	0.427
$FeCl_3.6H_2O$	9.67
H_3BO_3	0.03
$NaMoO_4.2H_2O$	0.024

Table 9 SM6Gc Defined Media - Formula for HCDF (Media 2)

2.3.4 Process flow

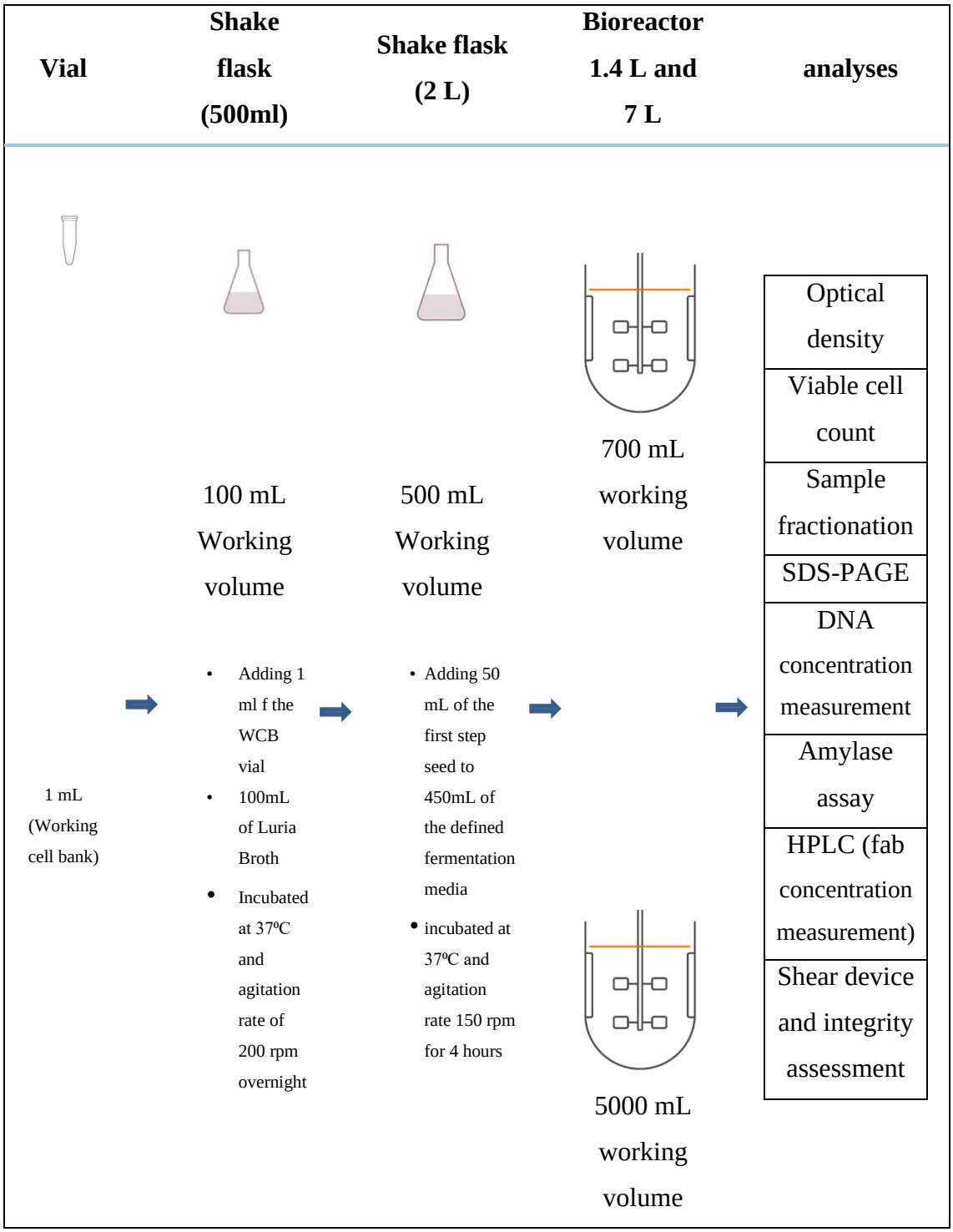


Figure 17 Process flow diagram

2.3.5 Technology Transfer, Scale up/down methodology

In order to provide a similar physical and chemical environment for the cells in different scales and deliver their nutrition and oxygen demand in a similar manner across different scales of bioreactor. A constant P/V and VVM was employed to calculate the respective agitation and aeration ranges and rates for the different scales used in this thesis. The methodology and information provided can be used to further scale up or to scale down for further studies. Table 10 and Table 11 outlines the bioreactor specifications and operating conditions and parameters for the 2 scale of bioreactors used in this study.

The power required to mix nonaerated fluids depends on stirrer speed, impeller shape and size, tank geometry, fluid density, and viscosity. Typically, the link between these factors is described using dimensionless numbers, such as the impeller Reynolds number Re and the power number. Experimentally determined relationships between Re and power number for a variety of impeller and tank types. After determining the value of the power number, the power is calculated. The overall relationship between power number and Reynolds number for a given impeller relies on the flow regime in the tank. The power number is independent of the Reynolds number and has a constant value in turbulent flow regimes. In turbulent regime, the power number for a Rushton turbine is roughly 5.0 (Doran, 2013). This is the value used in the technology transfer, scale-up/down calculations, and set point determinations for the various scales of bioreactors mentioned in this thesis.

Since Rushton turbines have been the standard impeller in the bioprocessing industry for many years, the agitation system in the majority of fermentation vessels is comprised of multiple Rushton turbines. If Rushton turbines are sufficiently separated in fluids with low viscosity, they each produce a radial discharge stream and construct independent large-scale circulation loops. In certain instances, vessels are fitted with two Rushton turbines. As if two independent stirred tanks were stacked one on top of the other, they are combined. The following equation can be used to predict the power required by multiple impellers under these conditions without gassing (Doran, 2013).

$$(P)_n = n\,(P)_1$$

where $(P)_n$ is the power required by n impellers and $(P)_1$ is the power required by a single impeller.

Given that all the bioreactor vessels used in this have 2 rushton turbines, the power number used for the P/V calculations is 10 (2 x 5 following the equation above.)

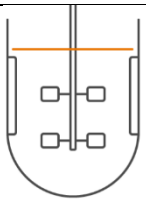
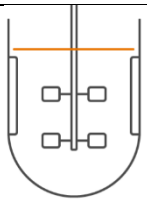
Bioreactor Specifications	 Multifors 2 (Infors HT, 2015)	 BioFlo 320 (Eppendorf, 2021)
Total Vessel Volume (mL)	1400	7000
Working volume (mL)	1000	4000
Vessel Diameter T (m)	0.09	0.185
Impeller diameter D_i (mm)	38	80
Power number per Rushton turbine (an estimation based on design and type from Doran, (2013).	5	5
Total power number	10	10

Table 10 Design specifications of bioreactor systems used

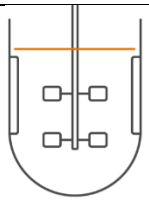
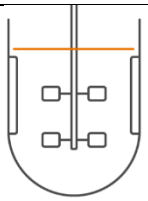
Bioreactor	 Multifors 2	 BioFlo 320
Total Vessel Volume (mL)	1400	7000
Working volume (mL)	700	4000
Process temperature – growth (℃)	32	32
Process temperature – Production (℃)	27	27
P/V range (kW/m^3)	0.65 - 8.9	0.65 - 8.9
Agitation range (rpm)	500 - 1200	260 -620
Minimum DO (%) Oxygen and agitation under the DO cascade control	35	35
Aeration – VVM (L/L/m)	1	1
Feed (% of working volume / hour)	0.13	0.13
pH	7	7
Acid for pH regulation	Acid = 1.72 M Phosphoric acid	
Base for pH regulation	Base = 2M Sodium Hydroxide	
Inoculum	10% of WV	
Induction	IPTG to the final concentration of 1mM	

Table 11 Operating parameters of the Bioreactor systems used

2.3.6 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis SDS-PAGE

SDS-PAGE was conducted on samples as per standard lab protocols for setting up polyacrylamide gels, with 10% acrylamide for resolution. Samples of protein were prepared by heating to 90°C in loading buffer for 10 minutes, before assembling the apparatus and pouring in 1x SDS running buffer into the reservoirs. Samples and 5 µL of marker were loaded into the wells, and the gels were run at 200 V for around 30 minutes. Gels were subsequently removed for staining and further visualisation, as described below.

The gels were washed for a short period of time with dH2O. They were then washed with gentle shaking for at least 10 minutes with fixing solution: 50% (v/v) ethanol containing 10% (v/v) acetic acid in dH2O. The gel was then was washed with dH2O three times for 5 minutes each and incubated for an hour in approximately 20 mL Coomassie stain solution: 0.1% (w/v) Coomassie blue R350, 20% (v/v) methanol and 10% (v/v) acetic acid. The gel was then washed several times with dH2O to remove the unbound Coomassie stain, before being documented with a scanner and the image recorded.

2.4 Cell growth measurements

Optical Density (OD) measurements:

To measure cell growth, the absorbance of 1 mL samples were measured in triplicates. For levels of growth more than 1 Abs the samples were diluted with distilled water. Fresh appropriate media was also used to provide a blank measurement. Optical density was measured at 600 nm using an Ultrospec 500 Pro spectrophotometer (Amersham Biosciences, Amersham, UK).

Dried Cell Weight (DCW) measurements:

Triplicate 1ml Eppendorf tubes were placed in 100°C oven for 24hrs to achieve maximum dryness, weight of the empty dried tubes were measured and recorded (tube). 1ml sample was added to the dried test tubes, centrifuged at max speed for 10 min. supernatant was

discarded and the tubes were placed in oven at 100°C for 24 hours, the weight of the this was measured and recorded (tube+cell). DCW = (tube+cell) – (tube)

2.5 Periplasmic release procedure (Cell fractionation)

A sample of 1ml was transferred to a large microfuge tube and centrifuged for 5 minutes at full speed. The supernatant was transferred into another tube and labelled ‘supernatant’. The pellet in the first tube was re-suspended in 200µl of extraction buffer and left at room temperature for 15minutes. 200µl of distilled water was added and left at room temperature for 15 minutes. This was centrifuged at full speed for 10 minutes. The supernatant was removed and transferred in to another tube and this was labelled ‘periplasm’. The pellet in the first tube was resuspended in 500µl of 50mM Tris.HCl and placed on ice. It was then sonicated using the small probe with 5 cycles of 10 seconds sonication 10 seconds cooling. It was then centrifuged at full speed for 10 minutes. The supernatant was transferred into a clean tube and labelled ‘cytoplasm’.

Extraction buffer = 20% sucrose, 1mM Na_2EDTA, 200mM Tris.HCl, 500□g/ml lysozyme

The ‘cytoplasm’ fraction is likely to be contaminated as ultracentrifugation was not carried out, this fraction can be regarded as partial spheroplast fraction. The HPLC analysis of the ‘cytoplasm’ fraction may be deemed unreliable as only HPLC assay was carried out in this project to quantify the produced fragmented antibody (Fab) in sample fractions and reducing SDS-PAGE was not carried out.

2.6 Assays

2.6.1 Amylase assay

α-Amylase activity was measured by monitoring the rate of decrease of a coloured starch/iodine complex using a modified version of the assay of Blanchin-Roland and Masson (1989). An appropriate dilution was made of the sample to be assayed in 0.5ml of 15mM sodium phosphate buffer (pH5.8). This was incubated at the assay temperature of 50^{o}C for 3 minutes to allow the solution to reach the correct temperature before the assay was commenced. The assay was started by adding 0.5ml of 0.5% (w/v) soluble starch in 15mM sodium phosphate buffer (pH5.8) into the enzyme solution. This solution had previously been heated to boiling point and filtered, whilst hot, through Whatman number 1 filter paper. It was kept at 50^{o}C during each set of assays and was freshly made up daily. Following addition of the starch solution, each assay tube was vortexed and immediately returned to the 50^{o}C water bath. 50µl aliquots were removed at various time points over a 20 minute period. These were immediately diluted with 1ml of iodine solution (freshly prepared by adding 200µl 2.2%I_2/4.4%KI (w/v) into 100ml of 2% (w/v) KI solution). The rate of decrease in absorbance over time was then measured at 600nm. One unit of enzyme activity corresponds to the hydrolysis of 143µg/minute of soluble starch at 50^{o}C.

2.6.2 Cell Viability – viable cell count

Serial dilutions of samples were prepared using the base media of the fermentation as diluent. 10^{-1} to 10^{-10} dilutions were prepared and tryptone soya agar (TSA) plates were used for spotting. Only 10^{-6} to 10^{-10} were spotted on the plates. For each dilution being spotted, 5 drops of 25µl was spotted on a plate. The average number of colonies on the 5 spots per plate were then calculated and the final viable cell counts were recorded as colony forming units (CFU)/ml .

2.6.3 Fab’ concentration measurement

Fab' concentrations in all fractions were measured using HPLC (Agilent 1200, Agilent Technologies, CA) utilising a 1 mL protein G column (HiTrap, GE Healthcare, Uppsala, Sweden).

HPLC running conditions for fab measurement was to bind at pH 7.4 and elute at pH 2.5 using 20 mM phosphate buffers; absorbance at 220 nm. HPLC calibration curve was done using serial dilutions of pure Fab’.

i.e.:

- Bind pH 7.4 and elute pH 2.5
- 20 mM phosphate buffers
- absorbance at 220 nm
- HPLC calibration curve was done using serial dilutions of pure and standard Fab’

2.6.4 Ultra scale-down centrifugation

Shear, such as that experienced in the feed zone of a large scale centrifuge or owing to solids discharge in centrifugation, and centrifugation stages were decoupled so that large scale centrifugation could be simulated. This was done and explained in full elsewhere (Chatel et al., 2013) (Chatel et al., 2013) (Voulgaris et al., 2016).

The samples were subjected to shear stress in an ultra scale-down (USD) rotary disc shear device (kompAsTM shear device, 20 mL stainless steel chamber of 50 mm internal diameter and 10 mm height, fitted with a stainless steel rotating disc of 40 mm diameter and 1 mm thickness, with disc speed 0-20,000 rpm) for 20 seconds. The shear stress was controlled by a custom designed power pack (UCL Mechanical Workshop) and was set at either 14,000 or 8,000 rpm (equivalent to 1.04×10^6 W kg^{-1} and 1.30×10^5 W kg^{-1} maximum energy dissipation rates (ε) respectively, determined by a computational fluid dynamics empirical correlation, as described by Chatel et al. (2013)) and adapted from Levy et al. (1999).

2.7 Molecular Biology

2.7.1 Competent cell preparation

An agar plate (with or with antibiotic) was streaked with the bacterium of choice from a glycerol stock. It was then placed in an incubator for 16-20 hours at 37°C. The next day a single colony was picked and transferred into a Falcon tube containing 25ml Lb media. This was then incubated at 37°C overnight. The next day 100ml of LB media was inoculated with 1ml of the saturated overnight culture. This was shaken in a 1L shake flask at 37°C and 250rpm for 2-3 hours. Around OD of 0.4-0.5, it was removed. It was then placed on ice for 10 minutes. Solutions, centrifuge, pippete tips and tubes were pre-cooled. The culture was transferred into two pre-cooled 50ml Falcon tube.it was then centrifuged at 2700X g for 10 minutes at 4°C. The medium was removed and the pellet was re-suspended with 1.6ml ice-cold 100mM CaCl2 by swirling on ice gently. It was then incubated on ice for 30 minutes. Then it was centrifuged at 2700X g for 10 minutes at 4°C. The supernatant was removed, and the cells were re-suspended in1.6ml ice-cold 100mM CaCl2 by swirling on ice gently. It was then incubated on ice for 20 minutes. The cells were combined to one tube and 0.5 ml ice-cold 80% glycerol was added and swirled to be mixed. This was now stored at -80°C.

2.7.2 Transformation of plasmids

Competent cells were thawed on ice (100µl). 2µl of DNA (20ng-100ng) was added to the cells and then incubated on ice for 30 minutes. The tube is placed in a water bath or a thermomixer at 42°C for 60 seconds. The cells were then placed back on ice for 5minutes. 900µl of LB was added and incubated for 2 hours at 37°C and 125rpm. In order to increase colony number, a concentration step was performed, the cells were centrifuged at 5000rpm for 45 seconds and then 600µ of the LB was removed. The cells were suspended in solution. Then 100µl of the transformed cells were streaked on an agar plate (with/without antibiotic). Plate was incubated at 37°C overnight.

2.7.3 Gel Electrophoresis

Electrophoresis was performed using 1 (w/v) agarose in Tris/Borate/EDTA buffer. TBE buffer was prepared from 10x stocks by dissolving 54 g Trizma base, 27.5 g boric acid and 3.75 g Na_2EDTA in 1L of RO water; the solution was stored at room temperature. Agarose powder was dissolved in TBE by boiling, then after cooling and addition of ethidium

bromide to a final concentration of 0.5 μgmL^{-1}, the gel was poured into a casting tray containing a comb which provided wells of the required size. After the gel had set, the comb was removed, and the gel submerged in TBE buffer. Electrophoresis was performed at 100 volts for one hour (+ 30 minutes). Gels were visualised using a short wave UV light transilluminator and photographed with UVB and software.

2.7.4 DNA concentration measurements

To determine the amount of nucleic acids present in samples containing plasmids or other sections of DNA, the Nanodrop (Thermo Scientific, Massachusetts, USA) spectrophotometer was used. The stage of the Nanodrop sampler was first washed with sterile dH2O, before another 2 µL of dH2O was added to the sampler to provide a blank baseline sample. The equipment measured the absorbance at 260 nm, and the software used the Beer-Lambert equation to convert this to nucleic acid concentration (Which could be configured to single or double stranded nucleic acids) in terms of ng/µL.

2.7.5 Plasmid extraction (miniprep)

For the Miniprep procedure, 20 mL of cells were grown in LB overnight (+/- antibiotic) at 37°C and 250rpm, next day the cells were pelleted by centrifugation. The standard Qiagen protocol was followed. 50 µL of plasmid DNA was produced from each miniprep.

2.7.6 Restriction digest

Restriction digests have been carried out to check for the presence of a specific piece of DNA, or to use the cut piece for ligation into other plasmids. Digests were performed with the following mixture:

- 2 µL DNA
- 1 µL Restriction enzyme (E.g. EcoRI)
- (Add 1 µL second restriction enzyme if necessary, e.g. HindIII)
- 1 µL 10x reaction buffer
- Up to 6 µL TE buffer

This gave a total volume of 10 µL. The mixture was then incubated for up to three hours at 37°C. The complete digests were then run fully on agarose gels, alongside a sample of the original uncut DNA and DNA ladder.

2.7.7 Ligation

Rapid DNA Ligation Kit from Thermo Scientific was used. 5X Rapid Ligation buffer was thoroughly mixed. The following were added to a microcentrifuge tube:

- Linearised DNA, 10-100ng
- Insert DNA at 3:1 molar excess over vector
- 5X Rapid Ligation buffer, 4µl
- T4 DNA Ligase (5U/µl), 1µl
- Nuclease free water, to 20µl

The mixture was vortexed and then incubated at 22°C for 5 minutes. 2-5µl of the ligation mixture was used for transformation.

Chapter 3 : Transformed *E. coli* Strains and Characterization

In this chapter the preparation and creation of all the strains used in this research will be described.

The antisense insert with two different promotors (T7_Lpp and T5_Lpp) were generated commercially by DNA 2.0 and it was delivered in vector pJ411(kanamycin resistant) in *E. coli* TOP10. This was streaked on selective agar and later grown in 50mL falcon tubes containing LB broth. It was then MAXIpreped to extract the DNA and then digested with enzymes and run on agarose gel, then the inserts were extracted to be ligated in to the pMMB66EH vector. The results was pMMB66EH_T7_Lpp and pMMB66EH_T5_Lpp. Competent cells of the selected strains were double transformed with the vector containing the recombinant product and the relevant antisense plasmid. This resulted in nine strains of antisense plus the product vectors. For control, each of the two strains of competent cells were transformed with the three recombinant plasmids, this resulted in six strains of control. The method for all the steps is described in chapter 2 and the summary of the steps and resultant strains are illustrated in Figure 18.

3.1 *Escherichia coli* Strains and plasmid preparation for transformations

For the purpose of investigating the impact of antisense-Lpp towards extracellular release. The pMMB66EH was selected to carry antisense-Lpp. Once transformed into an *Escherichia coli* strain containing another plasmid (eg. pQR187), and induced, it will inhibit translation of Lpp protein and thus facilitate extracellular release.

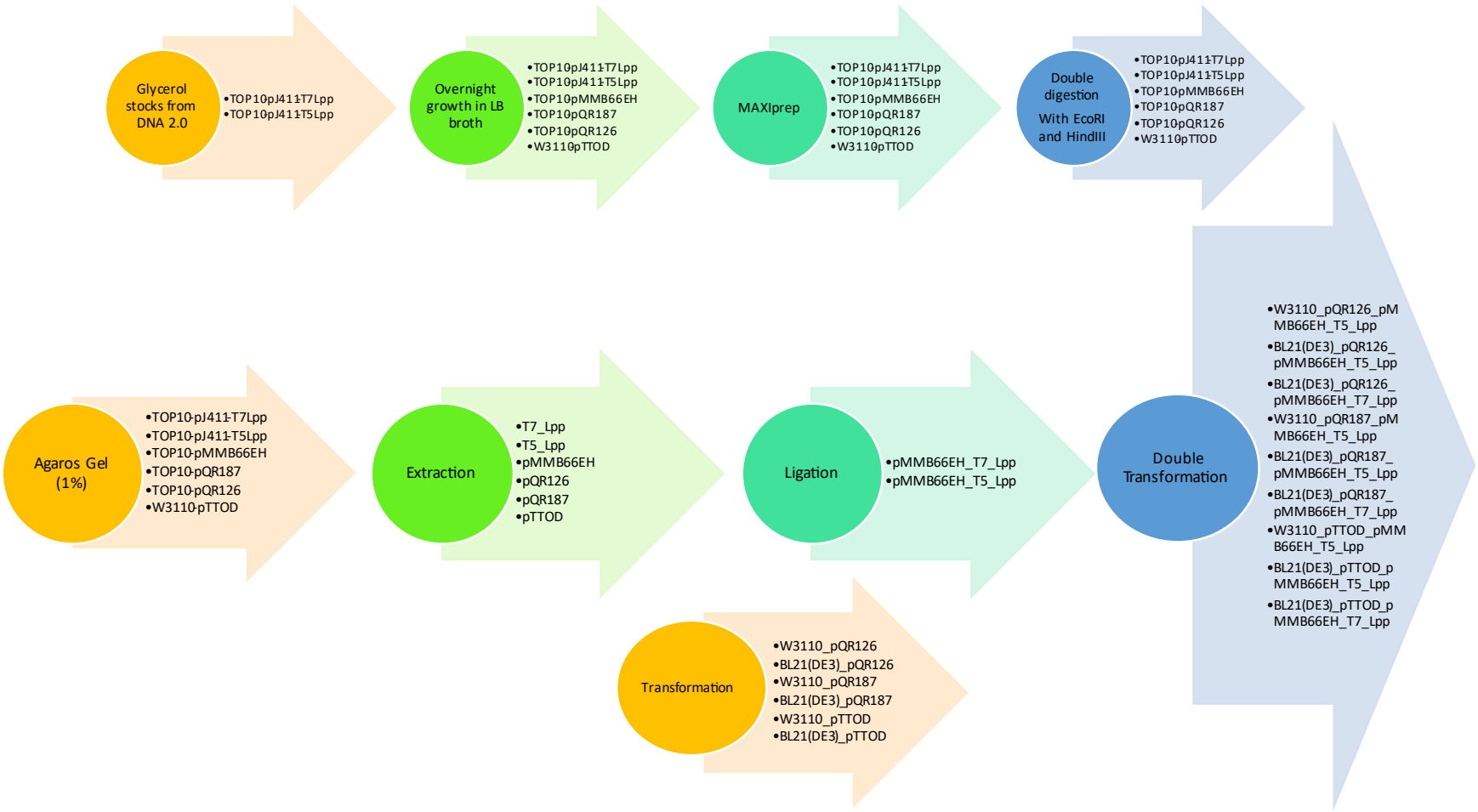


Figure 18 Illustration of the Prepartion of all of the transformed *Escherichia coli* starins used in this Thesis

3.2 Shake flasks fermentations

Shake flask fermentations were carried out with strains that were transformed for this study. They were control strains with only the recombinant product plasmid (without antisense plasmid) and strains with the antisense plasmid and recombinant product plasmid.

Control strains	With antisense strains
• W3110_pQR126	• W3110_pQR126_pMMB66EH_T5_Lpp
• BL21(DE3)_pQR126	• BL21(DE3)_pQR126_pMMB66EH_T5_Lpp
• W3110_pQR187	• BL21(DE3)_pQR126_pMMB66EH_T7_Lpp
• BL21(DE3)_pQR187	• W3110_pQR187_pMMB66EH_T5_Lpp
• W3110_pTTOD	• BL21(DE3)_pQR187_pMMB66EH_T5_Lpp
• BL21(DE3)_pTTOD	• BL21(DE3)_pQR187_pMMB66EH_T7_Lpp
	• W3110_pTTOD_pMMB66EH_T5_Lpp
	• BL21(DE3)_pTTOD_pMMB66EH_T5_Lpp
	• BL21(DE3)_pTTOD_pMMB66EH_T7_Lpp

Table 12 list of all strains transformed in this study

3.3 Non-induced shake flask short cultures

Growth profiles of the strains listed above were produced. These were in LB Broth and 5 hours of cell culture, 1ml of WCB vial in 100ml of LB Broth at 37°C and 200rpm. The results of the respective experiments are summarised in Figure 19 , Figure 20 and Figure 21.

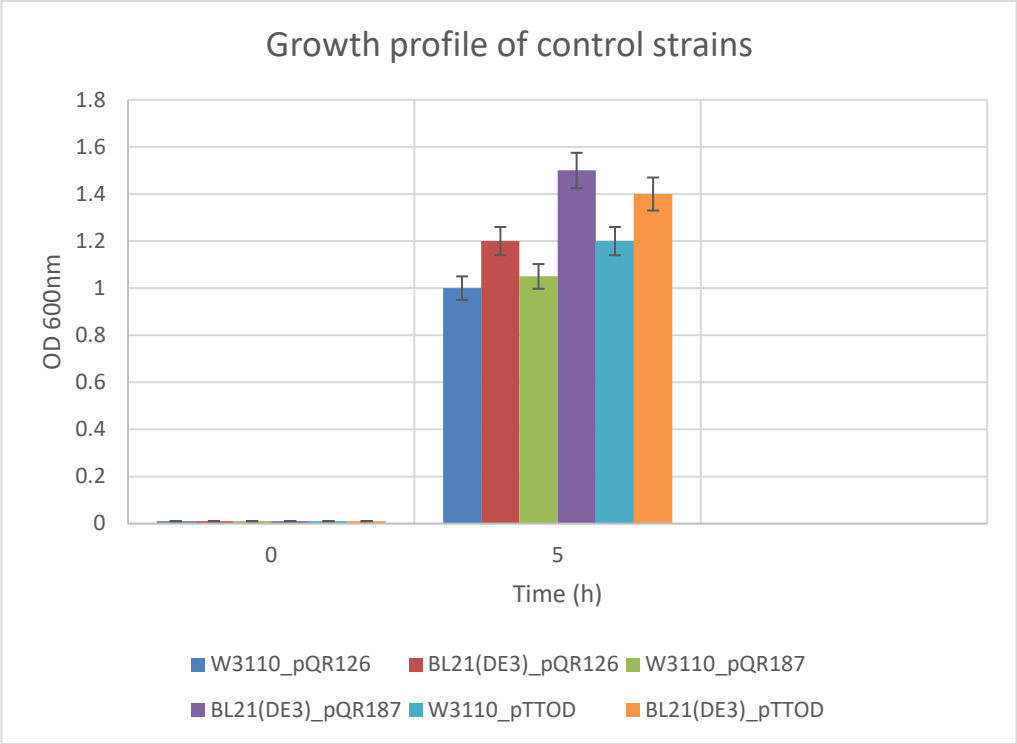


Figure 19 Growth profile of control strains- 1ml of WCB vial in 100ml of LB Broth at 37°C and 200rpm

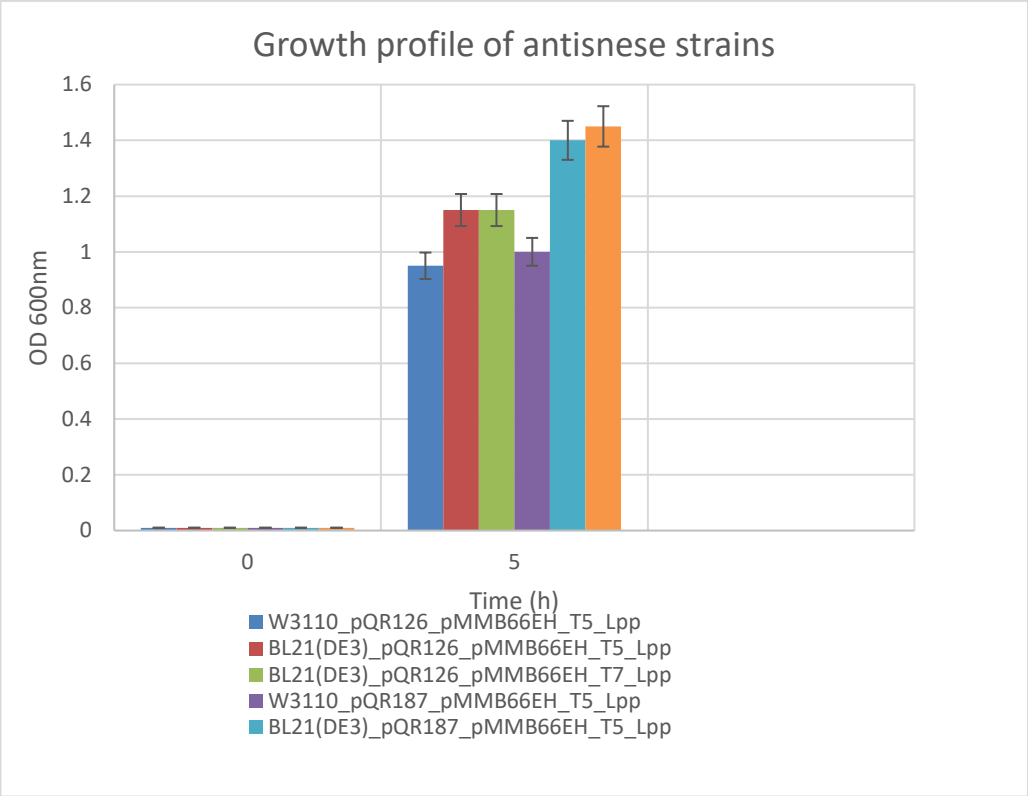


Figure 20 Growth profile of antisense strains- 1ml of WCB vial in 100ml of LB Broth at 37℃ and 200rpm

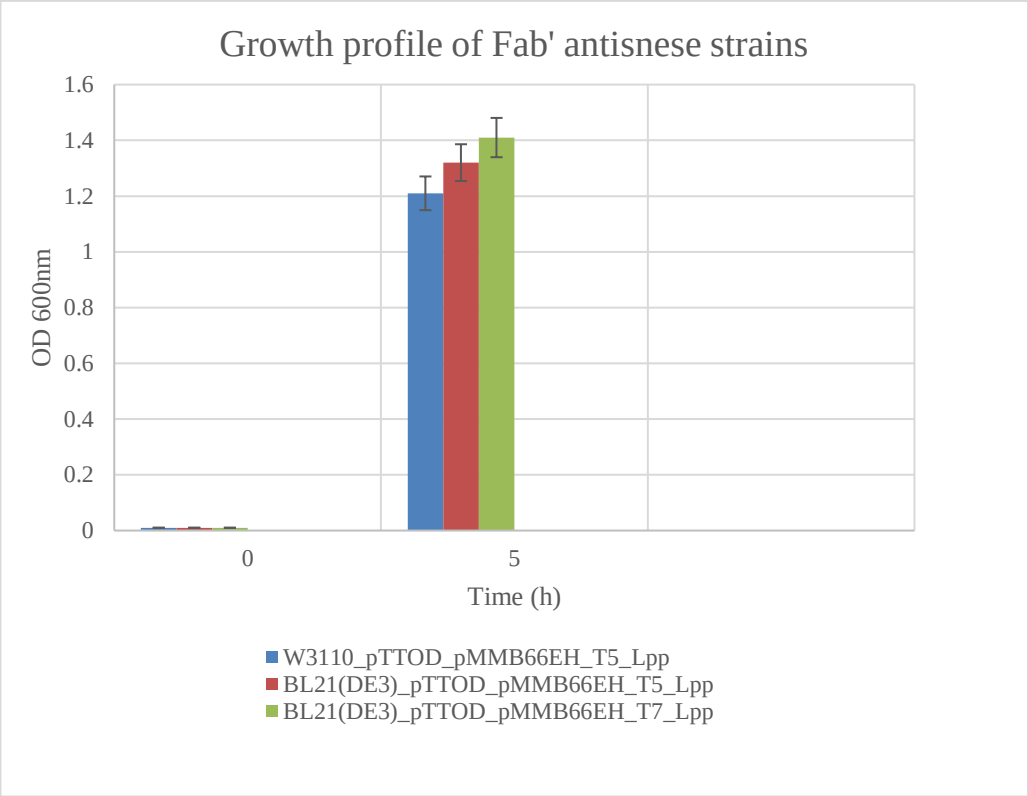


Figure 21 Growth profile of antisense Fab' strains- 1ml of WCB vial in 100ml of LB Broth at 37℃ and 200rpm

All the control and antisense strains in the figures above (19-21) show similar growth profiles, ODs ranging from 0.95 to 1.45 over 5 hours in 100ml of LB broth (with appropriate antibiotic) at 37℃ and 200rpm. The BL21(DE3) strain shows higher ODs in both controls and with antisense.

3.4 Induced shake flask short-cultures in LB broth

The strains listed Table 12 were cultured in LB Broth over 10 hours and sampled every 2 hours in triplicates (none-induced) and the experiments with all the mentioned strains were repeated three times with induction taking place at early, mid and late exponential, induction time indicated with an arrow in the relevant figures.

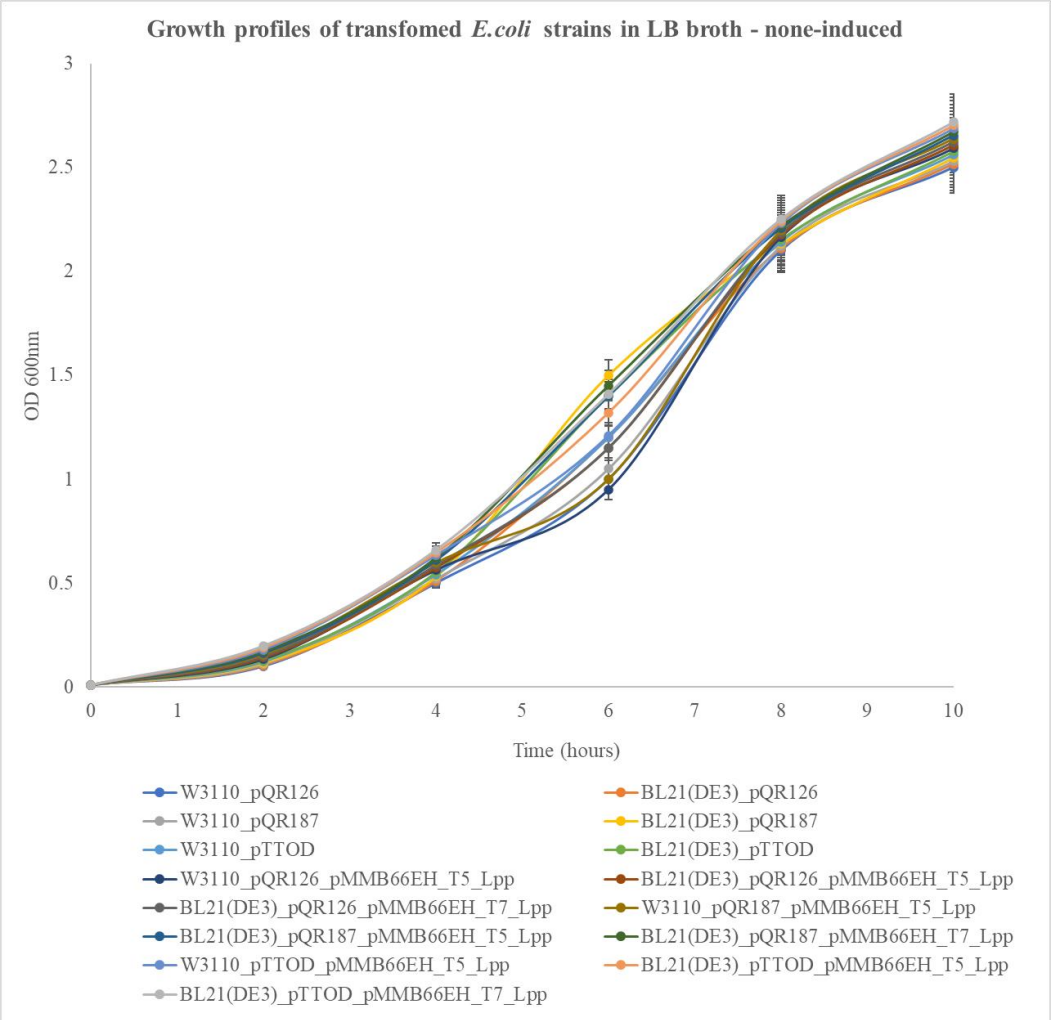


Figure 22 Comparative growth profiles of transformed *E. coli* stains (non-induced) in 100 mL LB broth grown in 500 mL shake flasks. Samples were taken at regular intervals n = 3 ± s.d

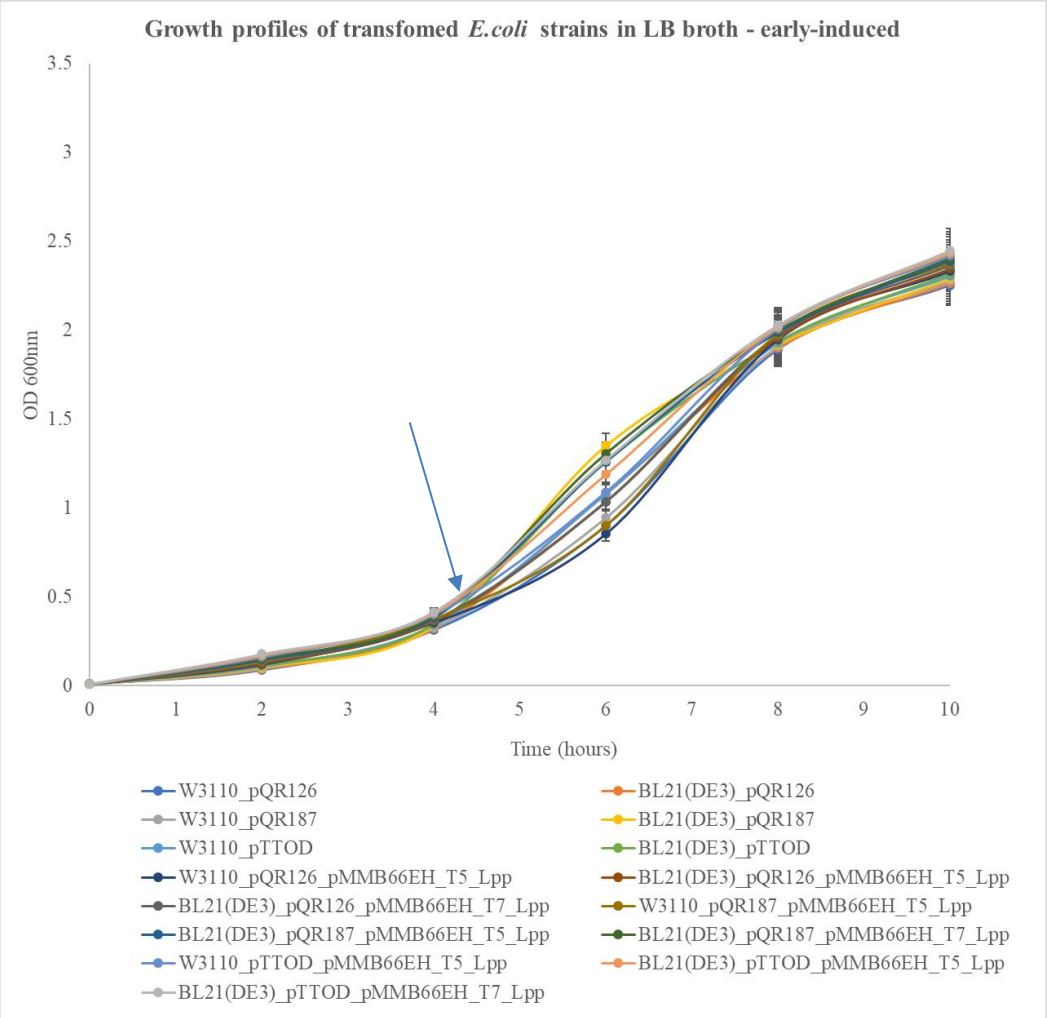


Figure 23 Comparative growth profiles of transformed *E. coli* stains (early-induced) in 100 mL LB broth grown in 500 mL shake flasks. Samples were taken at regular intervals n = 3 ± s.d

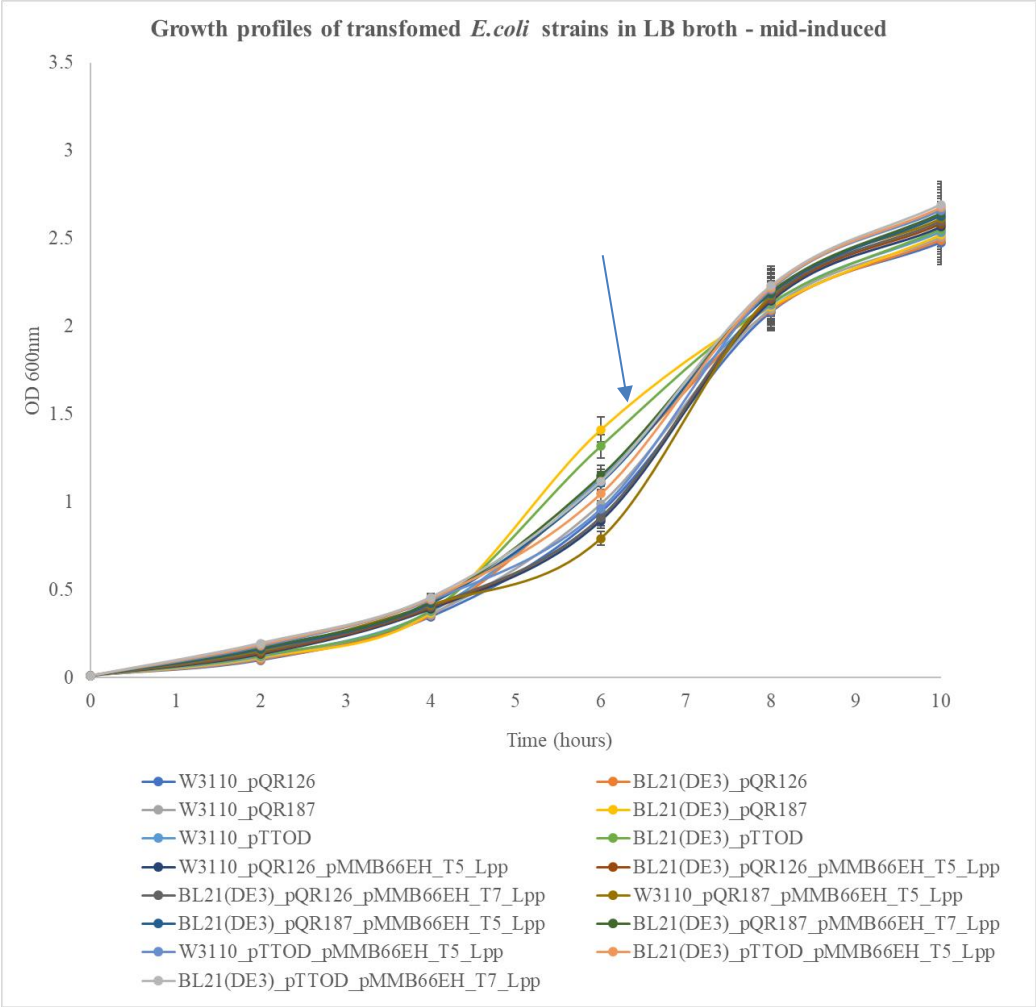


Figure 24 Comparative growth profiles of transformed *E. coli* stains (mid-induced) in 100 mL LB broth grown in 500 mL shake flasks. Samples were taken at regular intervals n = 3 ± s.d

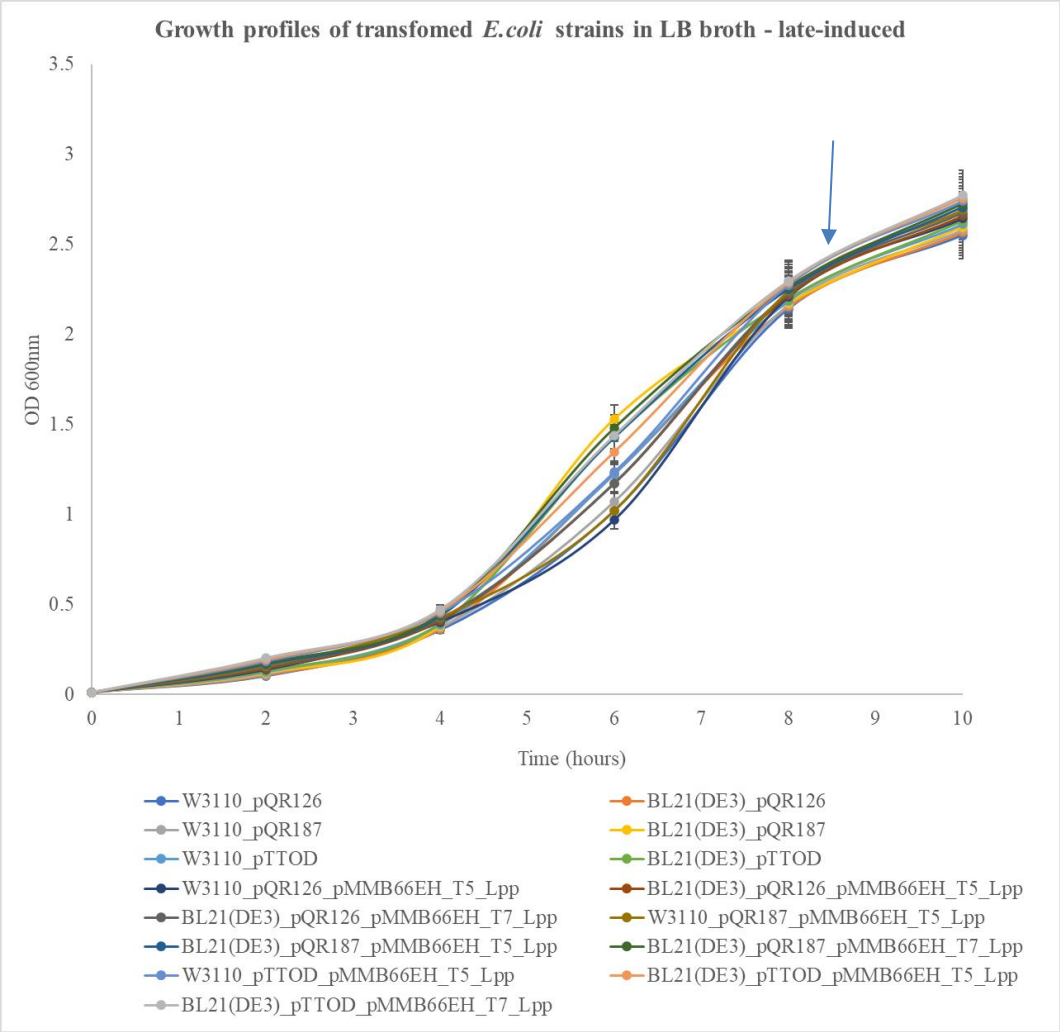


Figure 25 Comparative growth profiles of transformed *E. coli* stains (Late-induced) in 100 mL LB broth grown in 500 mL shake flasks. Samples were taken at regular intervals n = 3 ± s.d

The data illustrated in Figure 22, Figure 23, Figure 24 and Figure 25 show similar growth profiles with across all transformed strains. Optical density range of 2.2 to 2.8 was observed at the end of the cultures. BL21(DE3) strains show relatively better growth, not significantly different. The different induction times showed to have minimal effect on the maximum optical density. The early-induced experiments showed a lower optical density after 4 hours compared with the mid and late – induced experiments, not significantly different. The presence of the second plasmid and in the strains with the antisense plasmid had similar growth profiles to their respective control. In conclusion,

the growth of the transformed strains was not affected by either the time of induction or the presence of the second plasmid.

Final Samples of the experiments that were induced at late-exponential, Figure 25, were fractionated and the product titres and distributions were analysed.

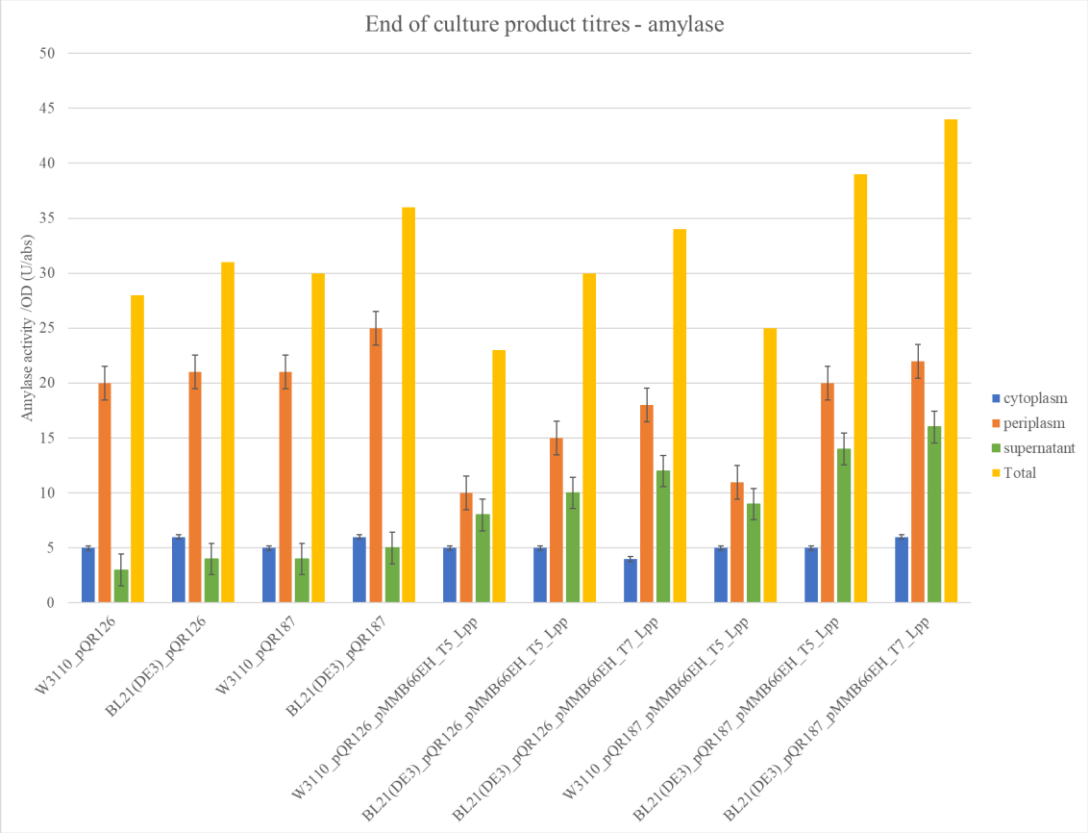


Figure 26 Comparative product titre (amylase) analysis of final sample of transformed *E. coli* stains (Late-induced) in 100 mL LB broth grown in 500 mL shake flasks. Samples were taken at regular intervals n = 3 ± s.d

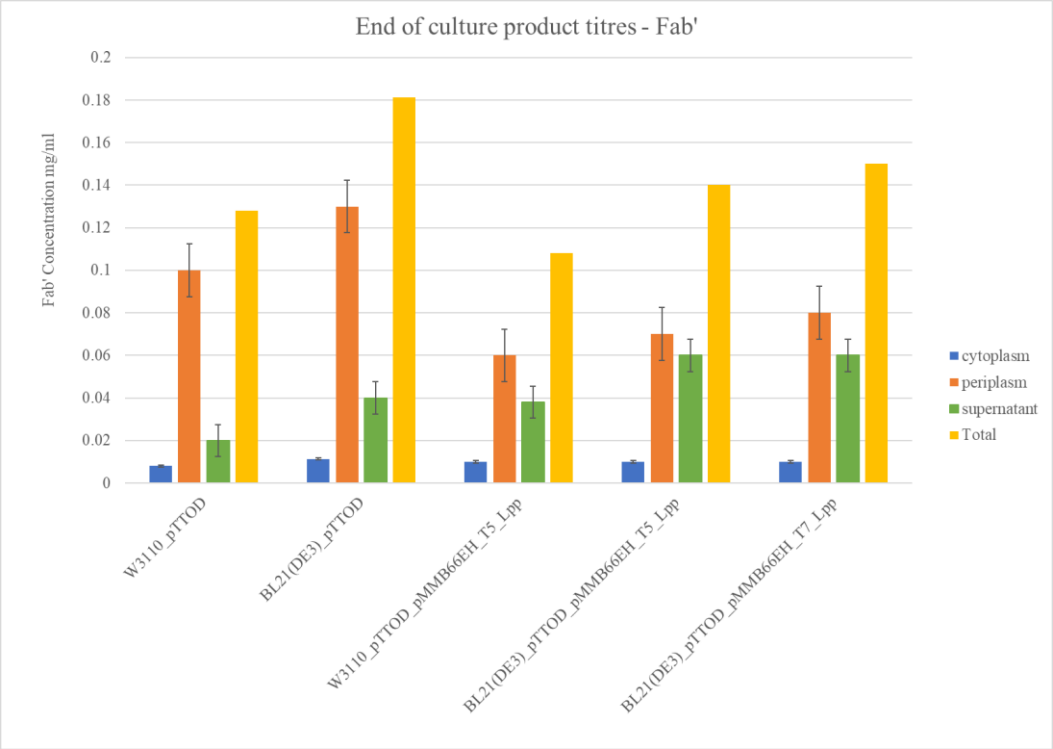


Figure 27 Comparative product (Fab') titre analysis of final sample of transformed *E. coli* stains (Late-induced) in 100 mL LB broth grown in 500 mL shake flasks. Samples were taken at regular intervals n = 3 ± s.d

The product tire analysis of the final sample of the late-induced shake flask studies of the transformed strains provides a comparative insight into the performance and capacity of the strains and plasmids as well as efficacy of the antisense technology for extracellular release of the product of interest. The amylase production with and without antisense are comparable across the different strains and plasmid compared with their respective controls (without antisense). In the strains with antisense, between 20 and 40 percent of total amylase were in the supernatant, and in their respective controls 10 to 15 percent of total amylase was in the supernatant, this is only 2 hours after induction, however the leaky expression of the product and the antisense prior to induction must be considered. Optimised and conditioned fermentations in bioreactors with an appropriate feeding and control regime can be implemented for improving the outcome. Proof of concept was established in the mentioned shake flask studies, demonstrating no negative impact in growth or production due to the antisense plasmid; furthermore, an increase level of product in the supernatant in the antisense strains was observed. The BL21(DE3) strain

with T7_Lpp antisense plasmid showed more promising results for both model recombinant proteins.

3.5 Shake flask seed train growth profiles

Seed train cultures for the fermentations in bioreactors were carried out generally for 9 hours prior to the bioreactor and an example of the growth profiles for the N-2 and N-1 shake flask fermentations are illustrated in the figures below.

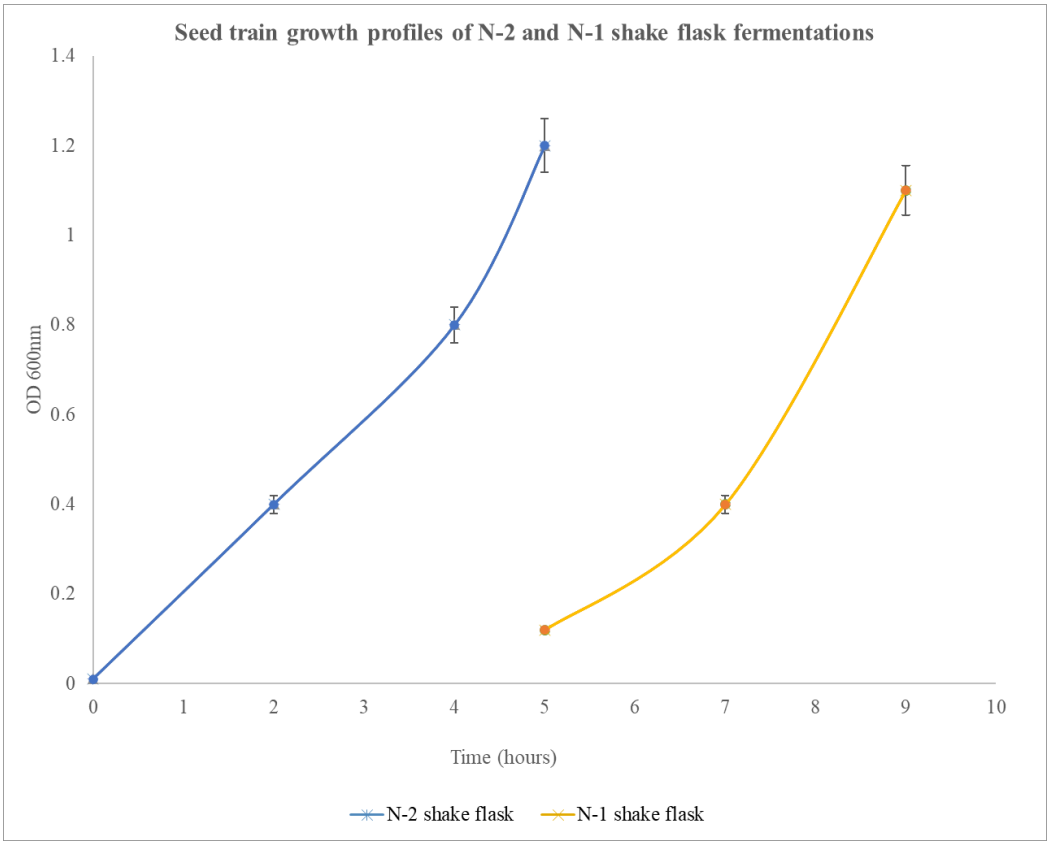


Figure 28 Average of all seed train shake flask fermentations OD values. n = 3 ± s.d

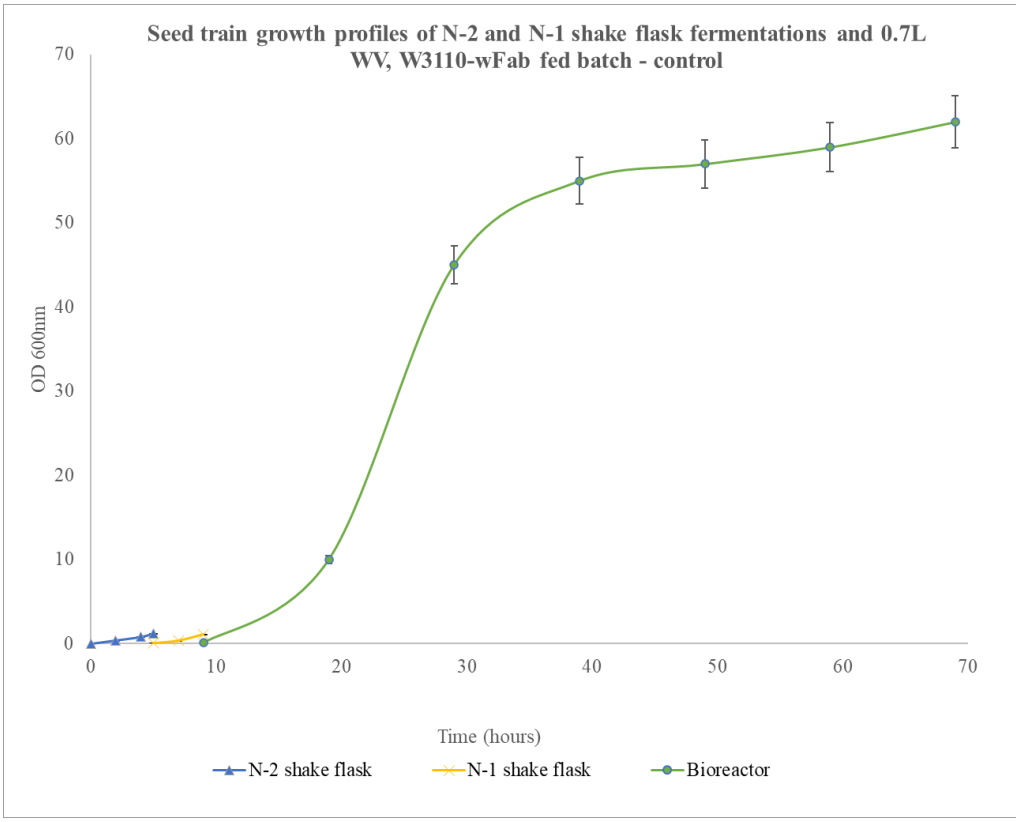


Figure 29 An example of Seed train and production bioreactor growth profiles and durations. n = 3 ± s.d

The figures above, Figure 28 and Figure 29, show the growth profiles and durations of the seed train shake flask fermentations and an example (Figure 45) of the growth and duration of fermentation in the bioreactor

Chapter 4 : Production and quantification of Amylase

4.1 Introduction

In this section growth selected *Escherichia coli* strains and production of amylase within the used strains will be explored. The selected *Escherichia coli* strains include BL21(DE3) and W3110. These strains with and without plasmids (pQR187and pQR126) were analysed in terms of growth and production during fermentation in 7L bioreactors (4WV). Shake flask cultures were also carried out for growth kinetics as well as seed training to prepare seed for the bioreactor systems.

4.2 *Escherichia coli* growth

The growth characteristics of BL21(DE3) and W3110 were determined by running 4L (working volume) fermentation of the two *Escherichia coli* strains without any transformed plasmids and antibiotic resistance.

A 50 ml complex media was inoculated with a colony of the strain from a petri dish and incubated overnight; this formed the inoculum for the next step. A 100 ml complex media was then inoculated at 10% inoculation and shaken in 1L shake flask for 3 hours; this formed the inoculum for the next step. Then 500ml defined media was inoculated at 10% inoculation and shaken in 2L shake flask and left overnight; this formed the inoculum for the next step. The bioreactors were inoculated the next day at 10% inoculation. More details regarding the methodology is given in section 2.3.

Samples were taken from the bioreactor at regular intervals and the OD was measured at 600nm. This allowed the growth profile to be determined. The figure below, Figure 30, illustrated the growth profile of W3110 as well as the logarithmic growth profile. The growth profiles of BL21(DE3) is also shown in Figure 31. Details regarding the running conditions and methodology are given in section 2.3.

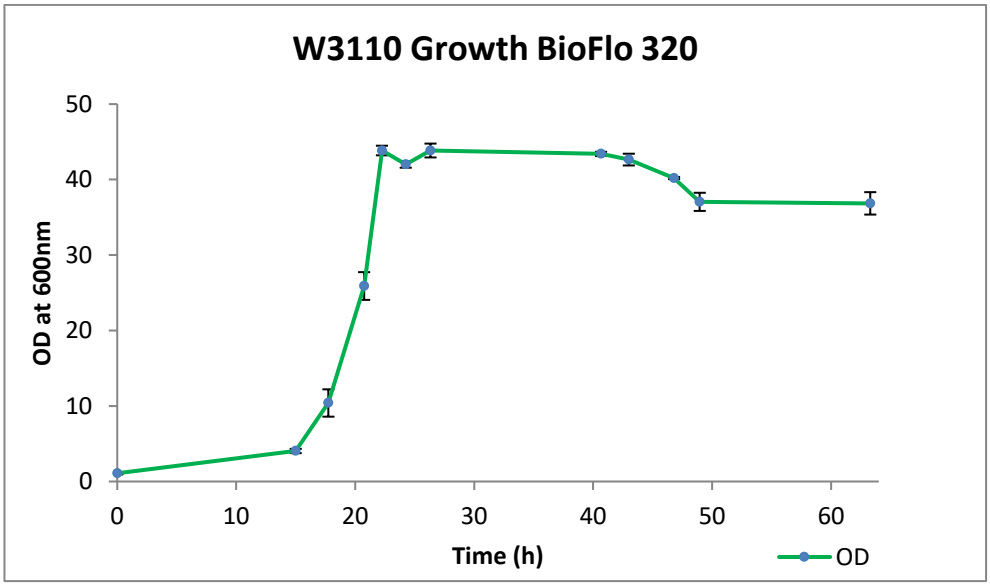


Figure 30 W3110 4L WV fermentation, 60 hours culture (Media 1)

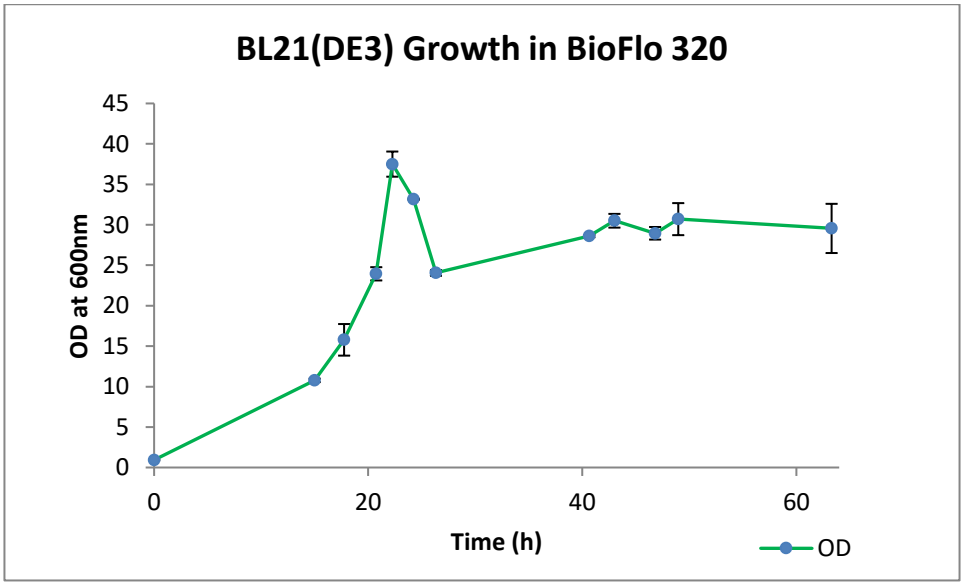


Figure 31 BL21(DE3) 4LWV fermentation, 60 hours culture (Media 1)

In general, the two strains exhibit similar growth profiles with minor differences. One prominent difference is the drop in cell density after the exponential phase. W3110 has an insignificant drop whilst in comparison, BL21(DE3)'s OD drops more dramatically. Both plateau between OD of 30 and 40, W3110 being higher. Both reach peak cell concentration at almost the same time, after ~20 hours. The slight increase of OD after the drop may be due to alternation of the type of nutrient. Carbone source being the initial

nutrient and later the bacteria started to synthesise enzymes in order to break down proteins and grow via utilising proteins that may be present after death of other cells.

4.3 Amylase expression in *Escherichia coli* W3110-pQR126 in 4L fermentation

The seed chain prior to inoculation of the bioreactor was similar to that described in the previous section (3.2). Only in this experiment the strains contain the named plasmid that once induced will express alpha amylase in the periplasm. The figures below, Figure 32 and Figure 33 show the growth profiles and DCW analysis of the W3110-pQR126. The point of induction is also denoted with an arrow on the graph which is after 24 hours of culture. More details regarding the methodology of the fermentation is given in section 2.3.3.

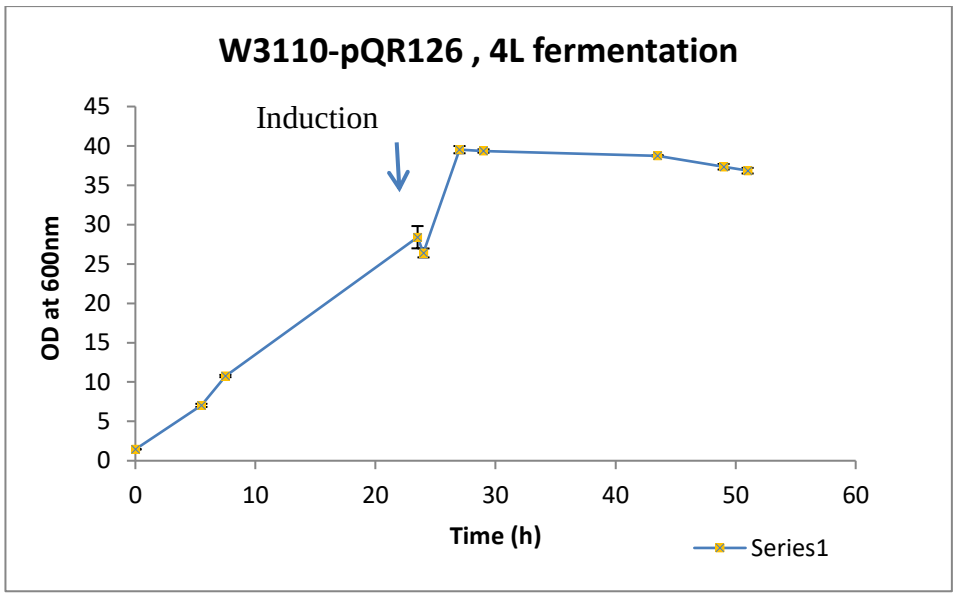


Figure 32 W3110-pQR126 4L fermentation, 50 hours culture, alpha-amylase as product

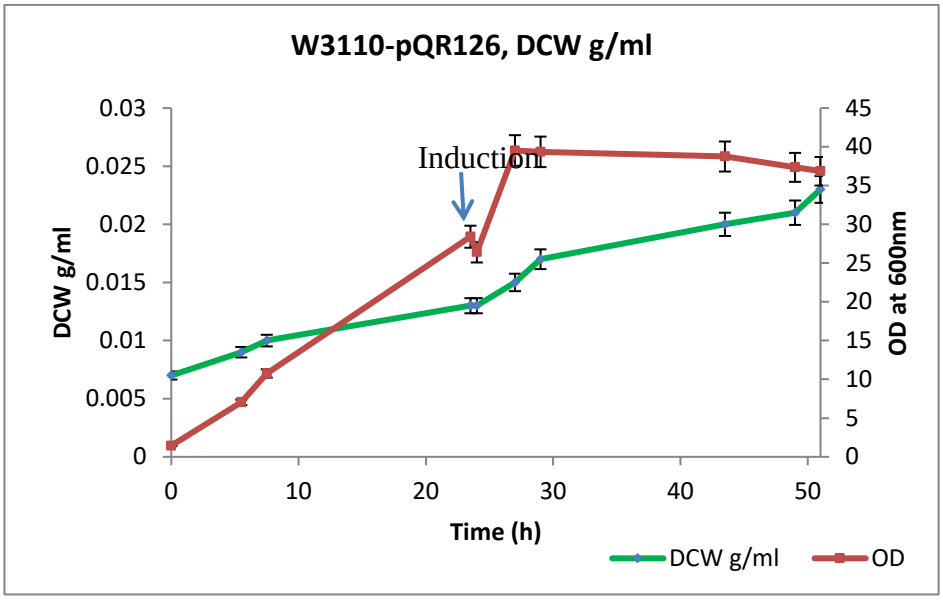


Figure 33 W3110-pQR126 4L fermentation, DCW analysis

As outlined before, the induction or addition of IPTG occurred at DOT spike which also indicates consumption of the entire carbon source. This point was selected for induction because with the given amount of nutrients in the media it represents the highest cell density that can be achieved. Therefore, in order for the metabolism to carry on and cells to be able to produce the desired protein after induction, a shot of glycerol was added to the bioreactor immediately after induction. Amount and details are given in 2.3.3.

Immediately after induction a dip and rise in the cell growth profile is prominent in the figures above. This is due to the events described above, running out of nutrient and reimbursement of nutrient after induction.

The growth profiles are similar to the W3110 without any plasmid, Figure 30. This shows that the addition of the named plasmid does not inhibit cell growth. This issue is more important when the second plasmid is added whose purpose is to inhibit Lpp translation and thus increase outer membrane permeability, allowing periplasmic products to be released in to the media during fermentation. The characteristic of extracellular release is desirable and attractive if it doesn't reduce cell density and production rate and capacity. Comparability to the results achieved at this stage without the antisense plasmid is key for further process development.

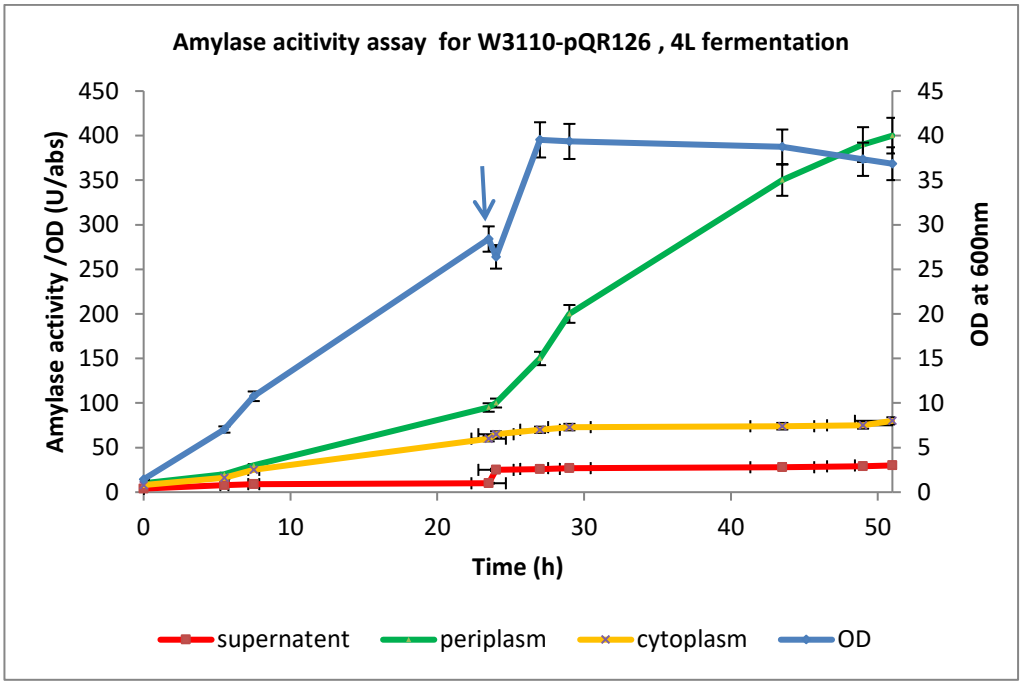


Figure 34 W3110-pQR126 amylase activity assay

In the figure above, it can be seen that most of the product accumulates in the periplasm and not much of the product is secreted into the medium.

Figure 34 is quite informative in a number of ways. The enzyme production profile with emphasis on the location that it mainly accumulates is clearly shown here. This is very important for the purposes of this project. It is absolutely key to be able to show that a periplasmically expressed protein accumulates in the periplasm, because any modification that aims to release the accumulated products in the periplasm can be confidently illustrated.

Several other notable events can be seen in Figure 34. Detection of amylase activity was achieved even prior to induction which shows that the plasmid is active from the beginning of the culture. The amount of amylase activity post induction in cytoplasm and periplasm remains relatively constant, however amylase activity in periplasm increases significantly and continues to rise.

4.4 Amylase expression in *Escherichia coli* W3110-pQR187 in 4L fermentation

The seed chain prior to inoculation of the bioreactor was similar to that described in the previous section (3.2). Only in this experiment the strains contain the named plasmid that

once induced will express alpha amylase in the periplasm. The figures below, Figure 35 and Figure 36Figure 33 show the growth profiles and DCW analysis of the W3110-pQR187. The point of induction is also denoted with an arrow on the graph which is after 24 hours of culture. More details regarding the methodology of the fermentation is given in section 2.3.3.

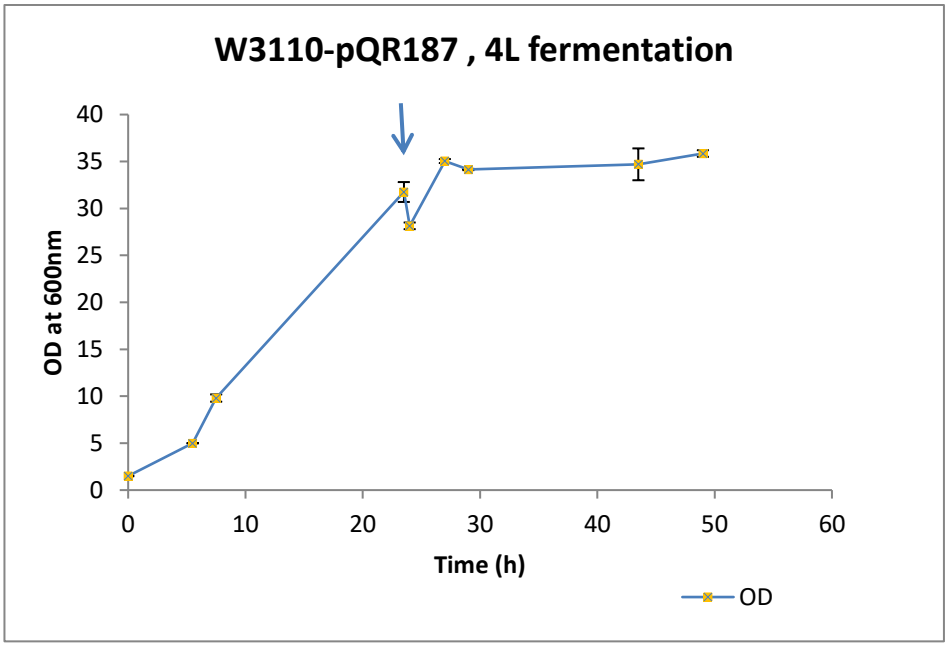


Figure 35 W3110-pQR187 4L fermentation, 50 hours culture, alpha-amylase as product

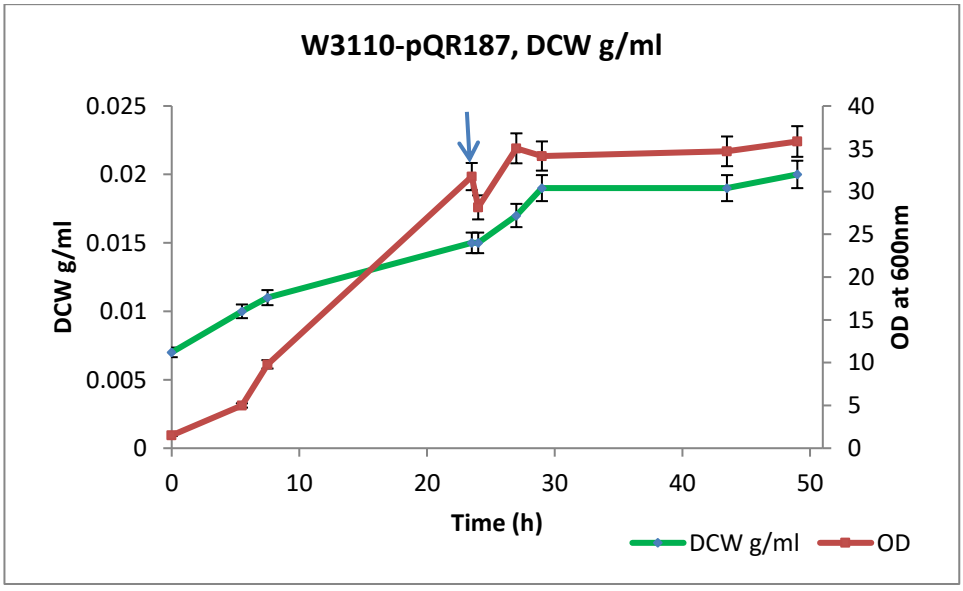


Figure 36 W3110-pQR187 4L fermentation, DCW analysis

The growth profiles of W3110-pQR187 shown above are very similar to that of W3110-pQR126. The same series of events in terms of dip and rise of OD after induction and addition of glycerol occur and this this is again due to the same reasons explained before in section 3.3.

One notable difference between the growth profiles of W3110-pQR187 and W3110-pQR126 is the growth profile after the hour 40. For W3110-pQR187 it is rather steady and continues with a slight upwards trend; however for W3110-pQR126 it is starting to continue downwards. This shows that W3110-pQR187 is a more stable strain post induction. Differences between the two plasmids are described in section 2.2.

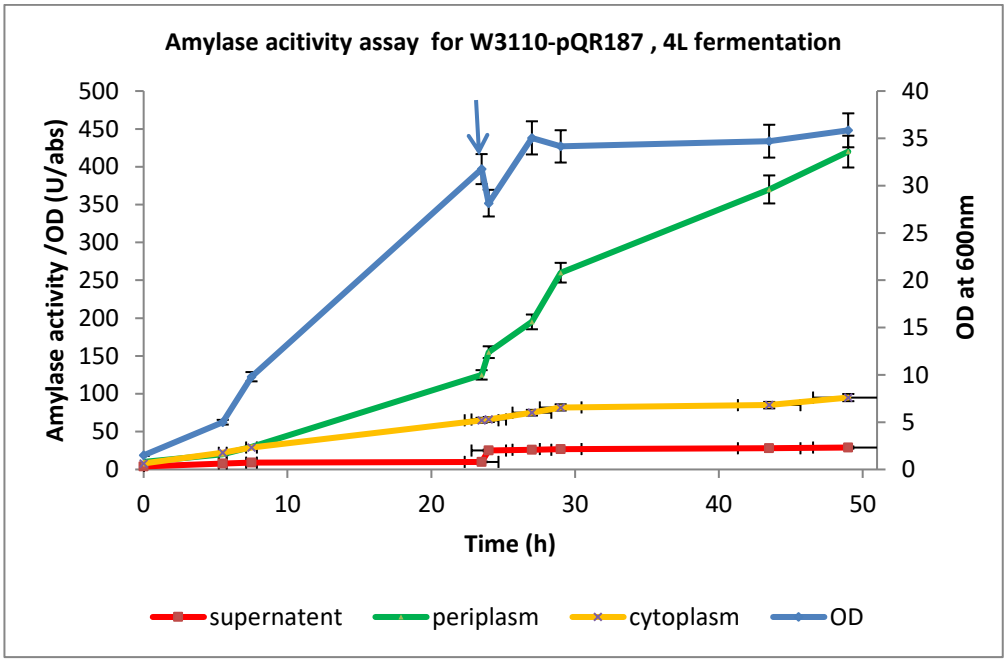


Figure 37 W3110-pQR187 amylase activity assay

In the figure above, it can be seen that most of the product accumulates in the periplasm and not much of the product is secreted into the medium.

Repetition of what was said for W3110-pQR126 is inevitable regarding Figure 37 due to prominent similarities.

Figure 37 is also quite informative in a number of ways. The enzyme production profile with emphasis on the location that it mainly accumulates is clearly shown here. This is very important for the purposes of this project. It is absolutely key to be able to show that a periplasmically expressed protein accumulates in the periplasm, because any

modification that aims to release the accumulated products in the periplasm can be confidently illustrated.

Several other notable events can be seen in Figure 37. Detection of amylase activity was achieved even prior to induction which shows that the plasmid is active from the beginning of the culture. The amount of amylase activity post induction in cytoplasm and periplasm remains relatively constant; however amylase activity in periplasm increases significantly and continues to rise.

4.5 Amylase expression in *Escherichia coli* BL21 (DE3) -pQR126 in 4L fermentation

The seed chain prior to inoculation of the bioreactor was similar to that described in the previous section (3.2). Only in this experiment the strains contain the named plasmid that once induced will express alpha amylase in the periplasm. The figures below, Figure 38 and Figure 40Figure 33 show the growth profiles and DCW analysis of the BL21(DE3)-pQR126. The point of induction is also denoted with an arrow on the graph which is after 24 hours of culture. More details regarding the methodology of the fermentation is given in section 2.3.3.

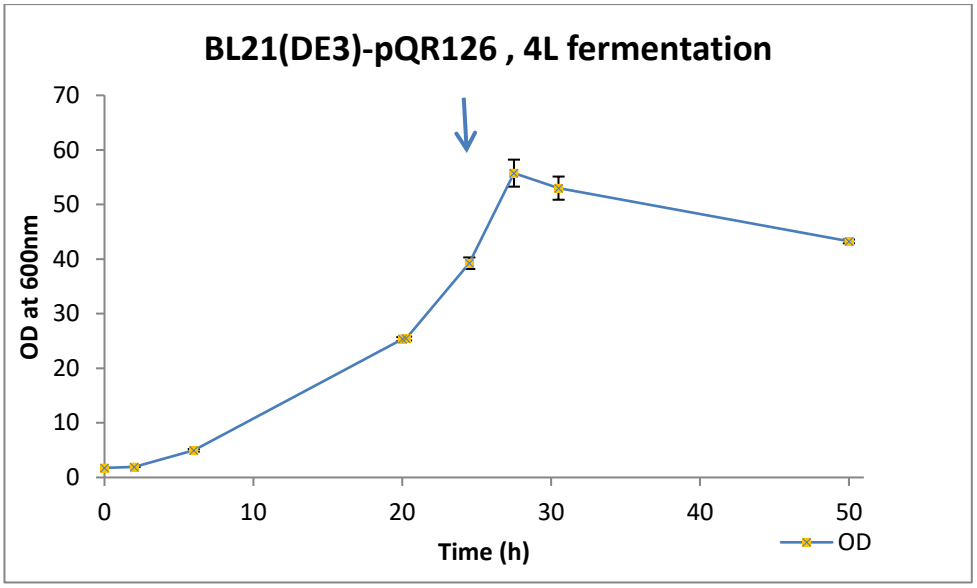


Figure 38 BL21(DE3)-pQR126 4L fermentation, 50 hours culture, alpha-amylase as product

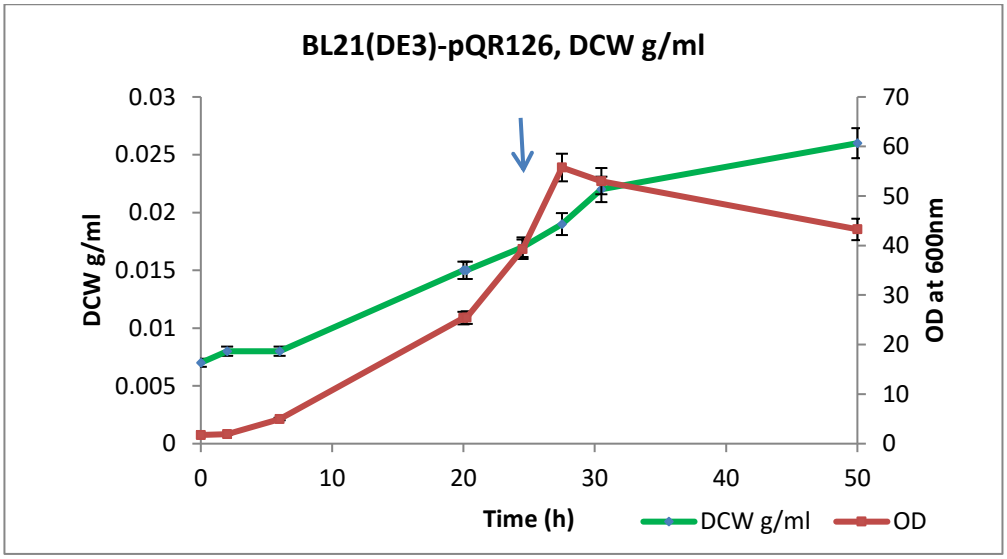


Figure 39 – BL21(DE3)-pQR126 4L fermentation, DCW analysis

As outlined before, the induction or addition of IPTG occurred at DOT spike (after 24 hours) which also indicates consumption of the entire initial carbon source. This point was selected for induction because with the given amount of nutrients in the media it represents the highest cell density that can be achieved. Therefore, in order for the

metabolism to carry on and cells to be able to produce the desired protein after induction, a shot of glycerol was added to the bioreactor immediately after induction. Amount and details are given in 2.3.3.

Notably there is no 'dip and rise' in the cell growth profile as was seen with the W3110 strains. This shows that the W3110 strain goes to stationary and death phase after running out of nutrients more alertly than BL21(DE3). BL21(DE3) takes longer time to switch mode when there is no more glycerol. Thus when more glycerol is added at induction/ DOT spike, no change in pattern of growth profile was notable. This conclusion is based on observation of the growth profiles discussed.

The growth profiles do not appear similar to the BL21(DE3) strain without any plasmid, Figure 31. The rate at which BL21(DE3)-pQR126 has reached peak cell density appears slower, the slop of the curve is not as steep as the BL21(DE3) growth profile. This shows that the plasmid pQR126 has altered the growth profile. However, BL21(DE3)-pQR126 was able to reach a much higher OD peak, ~58, whereas BL21(DE3) only reached a max OD of ~37.

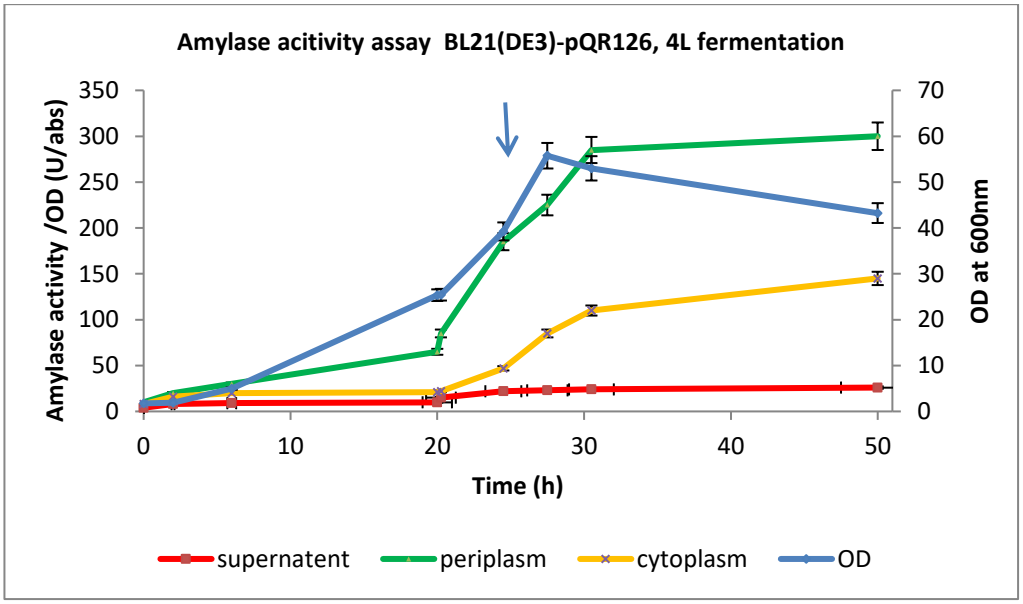


Figure 40 BL21(DE3)-pQR126 amylase activity assay

In the figure above, it can be seen that most of the product accumulates in the periplasm and not much of the product is secreted into the medium.

Inevitably, the previous comments regarding the previous amylase activity assays will be repeated as it was an identical experiment apart from the strain used. Figure 40 is quite informative in a number of ways. The enzyme production profile with emphasis on the location that it mainly accumulates is clearly shown here. This is very important for the purposes of this project. It is absolutely key to be able to show that a periplasmically expressed protein accumulates in the periplasm, because any modification that aims to release the accumulated products in the periplasm can be confidently illustrated.

Several other notable events can be seen in Figure 40. Detection of amylase activity was achieved even prior to induction which shows that the plasmid is active from the beginning of the culture. The amount of amylase activity post induction in cytoplasm and periplasm remains relatively constant with only a slight rise; however amylase activity in periplasm increases significantly and continues to rise.

The total amylase activity of BL21(DE3)-pQR126 and W3110-pQR126 are not significantly different, however the amylase activity in the cytoplasm of BL21(DE3)-pQR126 is notably higher than W3110-pQR126 and it shows an upward trend. This may be interpreted such that BL21(DE3)-pQR126 is more willing to allow periplasmically expressed products to escape into the medium. Only the next set of experiments in this project can prove this comment.

4.6 Amylase expression in *Escherichia coli* BL21 (DE3) -pQR187 in 4L fermentation

The seed chain prior to inoculation of the bioreactor was similar to that described in the previous section (3.2). Only in this experiment the strains contain the named plasmid that once induced will express alpha amylase in the periplasm. The figures below, Figure 41 and Figure 42 show the growth profiles and DCW analysis of the BL21(DE3)-pQR187. The point of induction is also denoted with an arrow on the graph which is after 24 hours of culture. More details regarding the methodology of the fermentation is given in section 2.3.3.

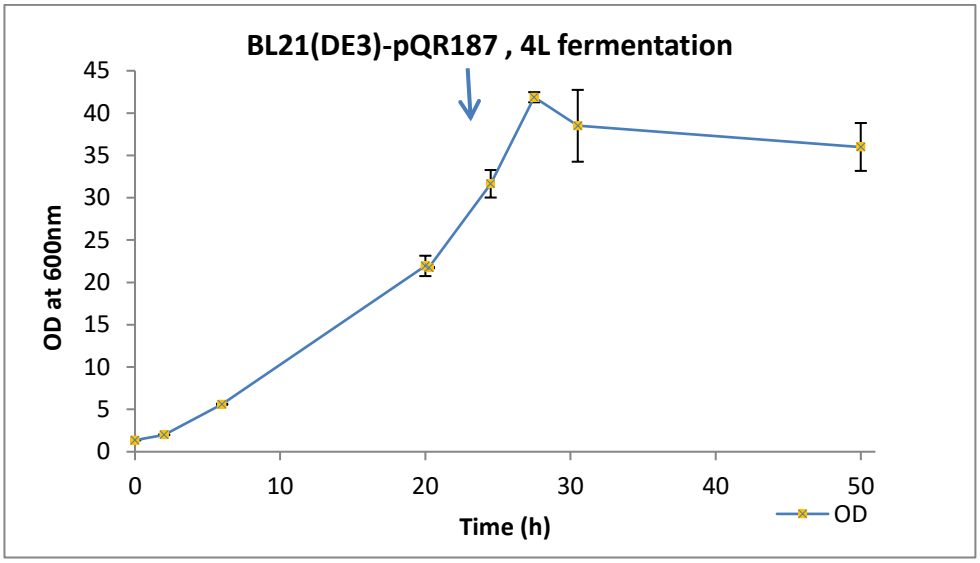


Figure 41 BL21(DE3)-pQR187 4L fermentation, 50 hours culture, alpha-amylase as product

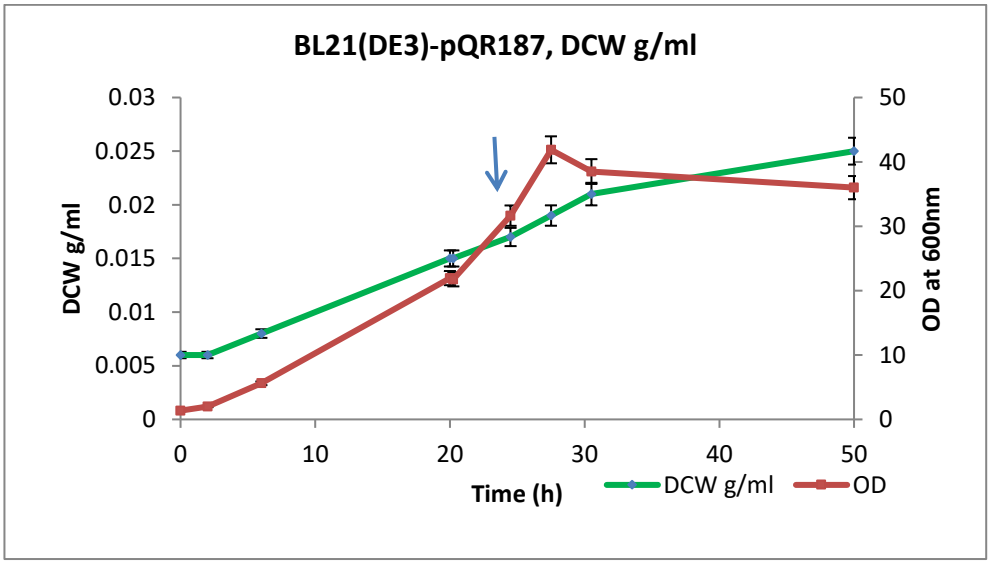


Figure 42-BL21(DE3)-pQR187 4L fermentation, DCW analysis

As outlined before, the induction or addition of IPTG occurred at DOT spike (after 24 hours) which also indicates consumption of the entire initial carbon source. This point was selected for induction because with the given amount of nutrients in the media it represents the highest cell density that can be achieved. Therefore in order for the metabolism to carry on and cells to be able to produce the desired protein after induction, a shot of glycerol was added to the bioreactor immediately after induction. Amount and details are given in 2.3.3.

Notably, same as BL21(DE3)-pQR126 there is no ‘dip and rise’ in the cell growth profile as was seen with the W3110 strains. The conclusion based on this observation is described above in section 3.5.

Again, the growth profiles do not appear similar to the BL21(DE3) strain without any plasmid, Figure 31. The rate at which BL21(DE3)-pQR126 has reached peak cell density appears slower, the slop of the curve is not as steep as the BL21(DE3) growth profile. This shows that the plasmid pQR126 has altered the growth profile. BL21(DE3)-pQR187 was able to reach a higher OD peak, ~43, whereas BL21(DE3) only reached a max OD of ~37. Compared with BL21(DE3)-pQR126 , BL21(DE3)-pQR187 has a lower max OD, otherwise relatively similar.

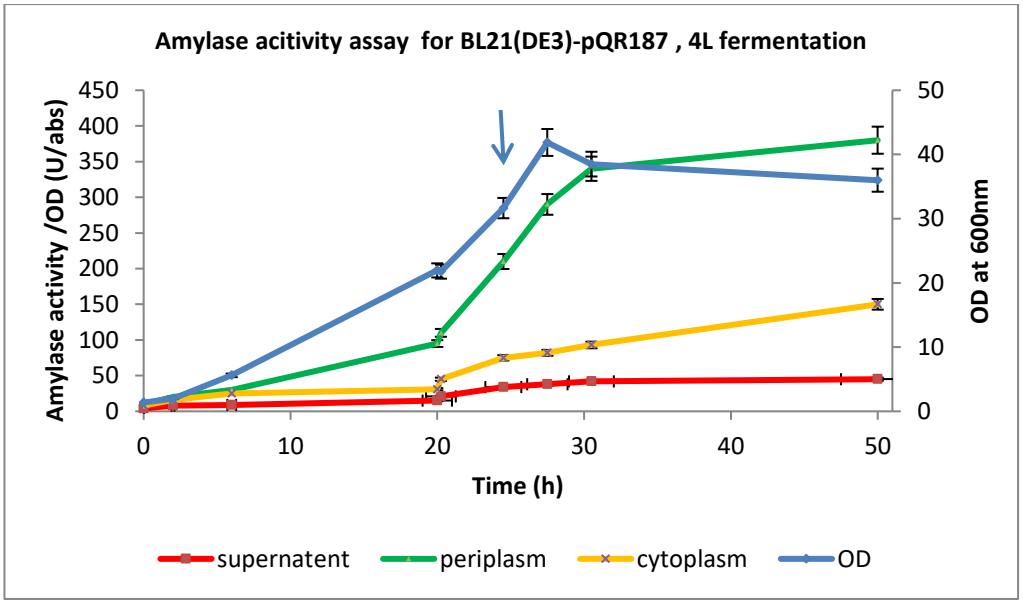


Figure 43 BL21(DE3)-pQR187 amylase activity assay

In the figure above, it can be seen that most of the product accumulates in the periplasm and not much of the product is secreted into the medium.

Inevitably, the previous comments regarding the previous amylase activity assays will be repeated as it was an identical experiment apart from the strain used. Figure 43 is quite informative in a number of ways. The enzyme production profile with emphasis on the location that it mainly accumulates is clearly shown here. This is very important for the purposes of this project. It is absolutely key to be able to show that a periplasmically

expressed protein accumulates in the periplasm, because any modification that aims to release the accumulated products in the periplasm can be confidently illustrated.

Several other notable events can be seen in Figure 43. Detection of amylase activity was achieved even prior to induction which shows that the plasmid is active from the beginning of the culture. The amount of amylase activity post induction in cytoplasm and periplasm remains relatively constant with only a slight rise; however amylase activity in periplasm increases significantly and continues to rise.

The amylase activity assay of BL21(DE3)-pQR126 and BL21(DE3)-pQR187 are very similar. Previous comments in section 3.5 applies here as well.

4.7 Amylase production with plasmids making antisense

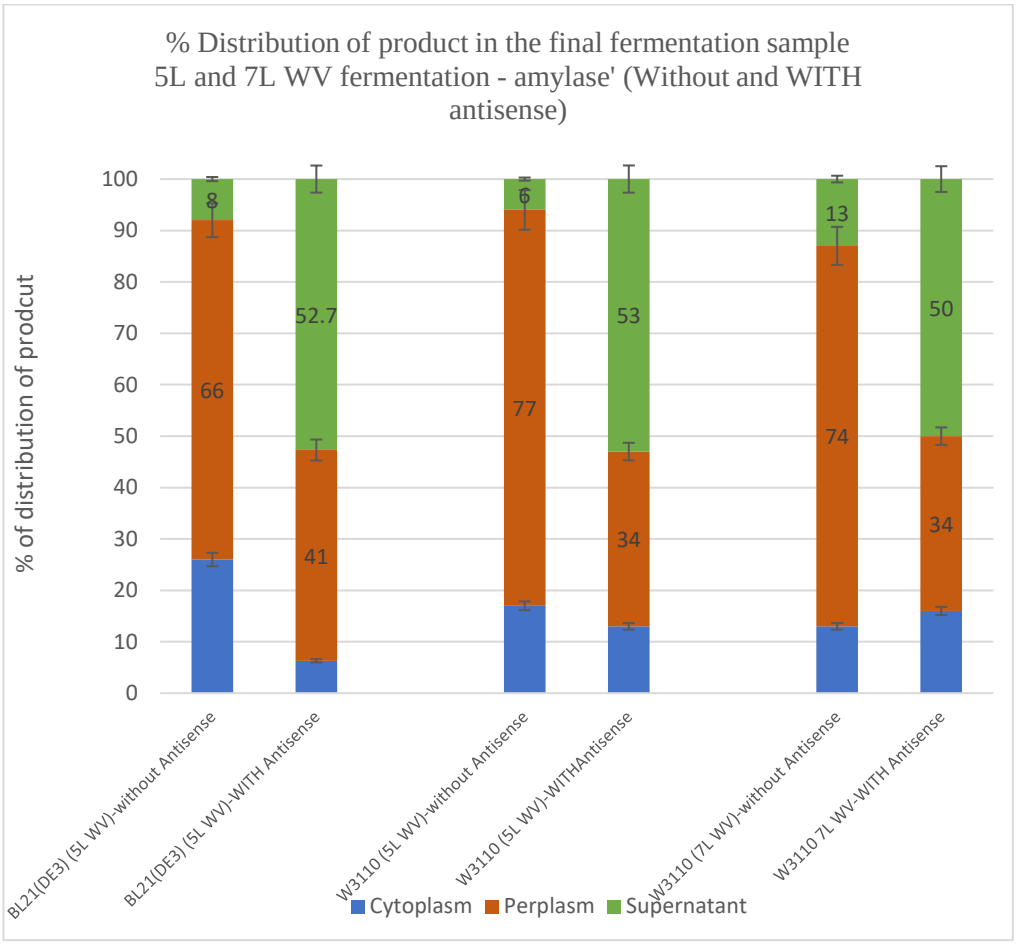


Figure 44 Product % distribution in the final sample fractions of fermentations Controls and With-antisense.

Fermentations of *E. coli* W3110 and BL21(DE3) strains contain amylase-producing plasmids in control cultures and an additional Antisense-RNA producing plasmid in Antisense cultures. 3 replicates of all fermentations were carried out and for each fermentation 3 replicates of the samples were analyzed. 66-77% of the product accumulated in the periplasm in the control experiment. A 5-fold increase in the release of the product, from 5-13% to 50-53% of the total production resulted in the Antisense fermentations. n = 3 ± s.d

No significant differences in amylase total titres of the above 6 fermentations.

In all cases of antisense utilisation – up to 40% of TOTAL titre has gone to the supernatant.

This work has focused upon the results obtained from fermentation cultures of *E. coli* W3110 pQR187 containing the antisense Lpp plasmid, comparing the data obtained to identical experiments where in the cell culture did not have the antisense plasmid. The results have shown that the amount of alpha-amylase released was increased from the low basal levels of around 10-13% in the controls to as much as 50-53% in the antisense cultures. The different strains of *E. coli* used in this controlled experiment demonstrates the applicability of the methodology to different strains of the host cell organism as well as robustness and reproducibility of results.

4.8 Conclusion

According to the findings, the periplasmic fraction of the cell contained the majority of the amylase activity, which was consistent with previous research. This is the main conclusion. This is quite clear across all four of the distinct strains that were investigated.

The findings were also consistent with those of other research that had a similar focus. For instance, W3110-pQR187 was grown in 2L bioreactors; however, the specified media and operating conditions were slightly different (Gibbons, 2012); in spite of these differences, the amylase activity assays were comparable to those obtained here. The similarities were found in the pattern of line progression during fermentation as well as the location of product accumulation. This demonstrates that the experiment is reasonably robust and reproducible, making it appropriate for investigations such as those that are planned to be carried out as part of this project.

The outcomes of the four different fermentations were very comparable, both in terms of growth and production. The subsequent step was to perform exactly the same four fermentations and assays, but this time with an additional vector containing the Lpp antisense that had been transformed into both of the strains. The goal of this step was to determine whether or not the amylase activity distribution in the cell fractions differed from that seen with the No antisense.

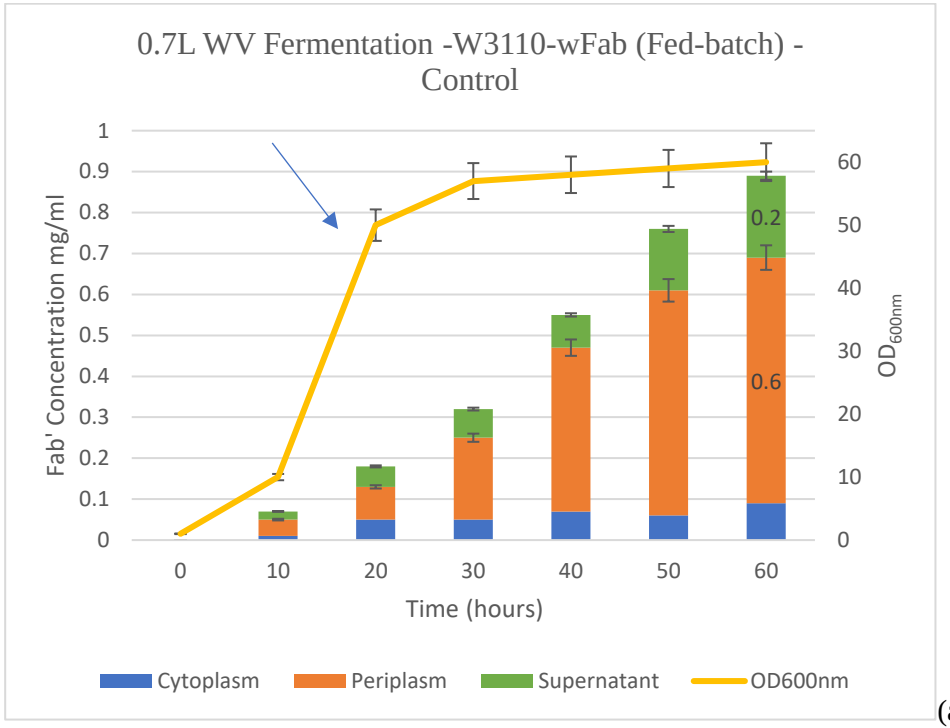
The proof of concept can be seen rather plainly in Figure 44 , which depicts a dramatic shift in the distribution of the recombinant products. With the implementation of antisense technology, the supernatant recovery rate increased from 5 to 13 percent to between 50 and 53 percent.

Chapter 5: Production and quantification of Fab’ fragments

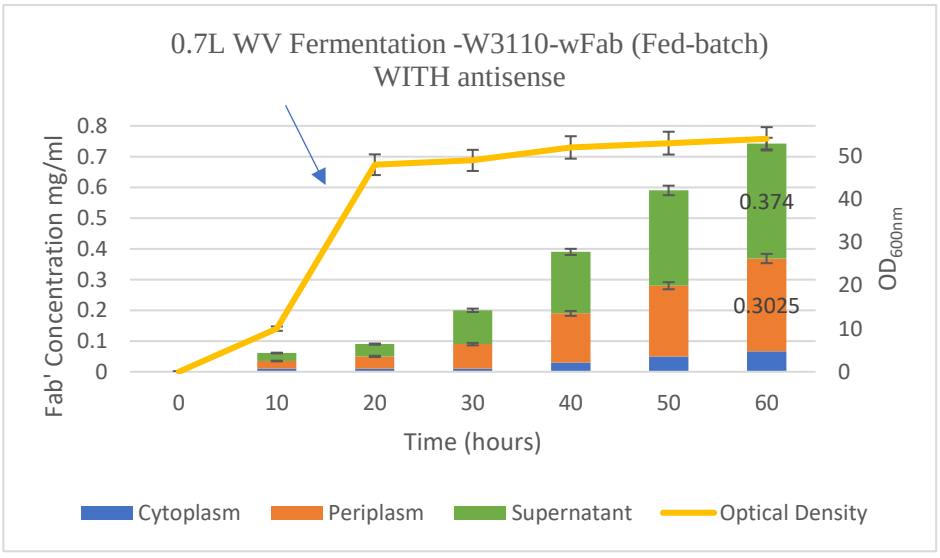
For the purpose of this chapter's investigation into the process, a model recombinant product of fragmented antibody Fab' was chosen to use. In addition to carrying out parallel fermentation at 0.7L WV scale and repeating the process three times in each scenario, we also carried out a control experiment in which there was no antisense, which was also carried out three times. *Escherichia coli* strains BEL21(DE3) and W3110 had the plasmid containing the antisense DNA and the product of the intertest respectively transformed into them in order to illustrate the wider use of the technology.

5.1 Fab' expression in *Escherichia coli* w3110 fed-batch WITH and without antisense

The fermentations in parallel were carried out for the W3110 strain with and without antisense and the sample were collected in set intervals and analysed accordingly.



(a)



(b)

Figure 45 fermentation (0.7L WV), W3110-wFab- fed batch ((a) Control, withOUT antisense) and ((b)-WITH antisense)

Control experiment above (a) indicates 0.85mg/ml total production, 80% in periplasm and 15% in supernatant. (b) (WITH antisense) a total titre of 0.75mg/ml production, 50% in periplasm and 45 % in supernatant is observed. A 3-fold increase from 15% to 45% in the released amount as percentage of the total production. Arrow indicating induction timepoint. n = 3 ± s.d.

Control experiment above (top) indicates 0.85mg/ml total, 80% in periplasm and 15% in supernatant. In the bottom (WITH antisense) a total titre of 0.75mg/ml, 50% in periplasm and 45 % in supernatant is observed.

$$\boldsymbol{yield}(\%) = \frac{\boldsymbol{concentarion\ of\ product\ in\ supernatant\ mg/ml}}{\boldsymbol{sum\ of\ concentarions\ of\ product\ in\ all\ fradctions\ \ mg/ml}}\ \boldsymbol{x100}$$

Yields of >45% were achieved reproducibly, an increase from 15%. In other words, a threefold increase in yields when adopting antisense technology was observed.

This is promising results clearly showing positive outcome due to the antisense utilisation and the effectiveness of inhibition of the selected outer membrane protein of Lpp.

1L fermentation – *Escherichia coli* w3110-Wfab' – **with antisense**			
Lane	Sample ID	Sample time	Fraction
1	MW	n/a	n/a
2	1	10	supernatant
3	2	20	supernatant
4	3	30	supernatant
5	4	40	supernatant
6	5	50	supernatant
7	6	60	supernatant
8	All fractions of sample 6	60	Neat (un-fractioned) sample 6

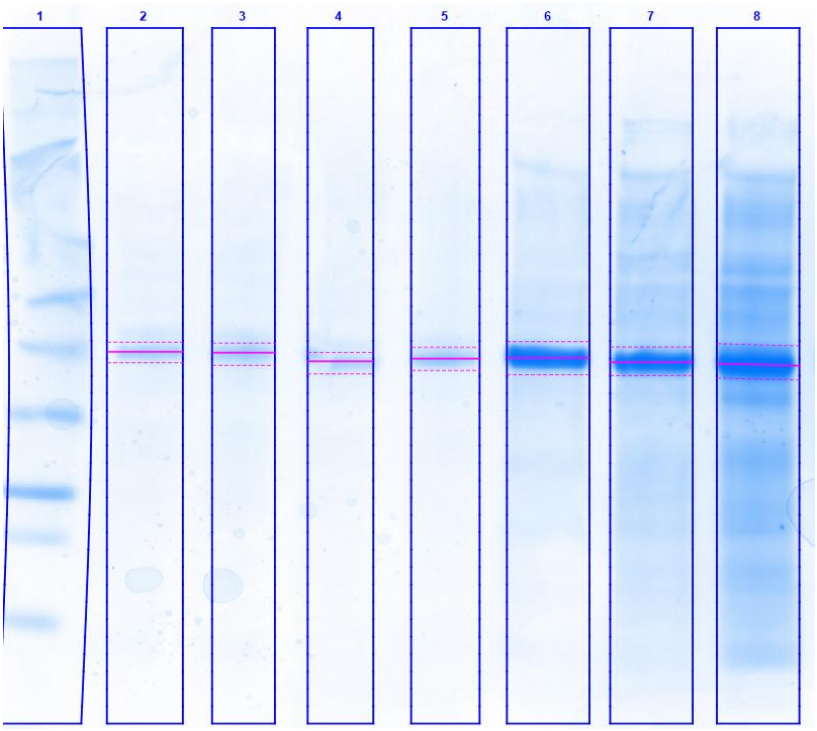


Figure 46 SDS-PAGE of 1L fermentation – *Escherichia coli* w3110-Wfab' – with antisense

Samples of the Figure above (0.7L WV) fermentations showing increase of amount of product in the supernatant as the fermentation goes forward. The product size is 46 kDa. The supernatant sample were gathered following centrifugation of the collected samples

respectively. The neat sample is the whole unprocessed sample at the end of the fermentation. Fab, Fragmented antibody.

The samples mentioned in the figure above were ran on an SDS-PAGE gel and qualitatively illustrate the release of the product of interest, in this case Fab' into the supernatant as the fermentation carries on. Lane 8 which is the whole of the last sample shows much background protein that is not present in the supernatant fraction off the same sample, i.e.. Lane 7. Therefore showing that the impurities were contained by the inner membrane.

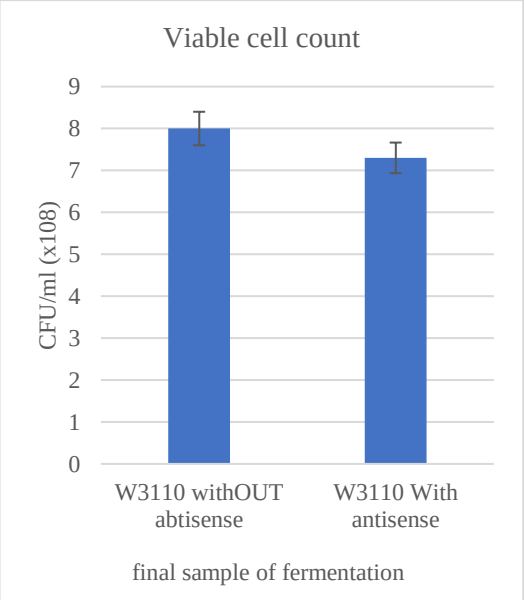


Figure 47 viable cell count of final sample of Figure 45 fermentations.

The viable cell count data shows no significant differences between the samples with and without antisense. It can be concluded that the addition of antisense making plasmid and the resultant extracellular release of recombinant product does not impact cell viability. CFU, Colony forming units

An important factor to consider includes cell viability and any caused difference due to the antisense utilisation and the figure above clearly shows no negative impact.

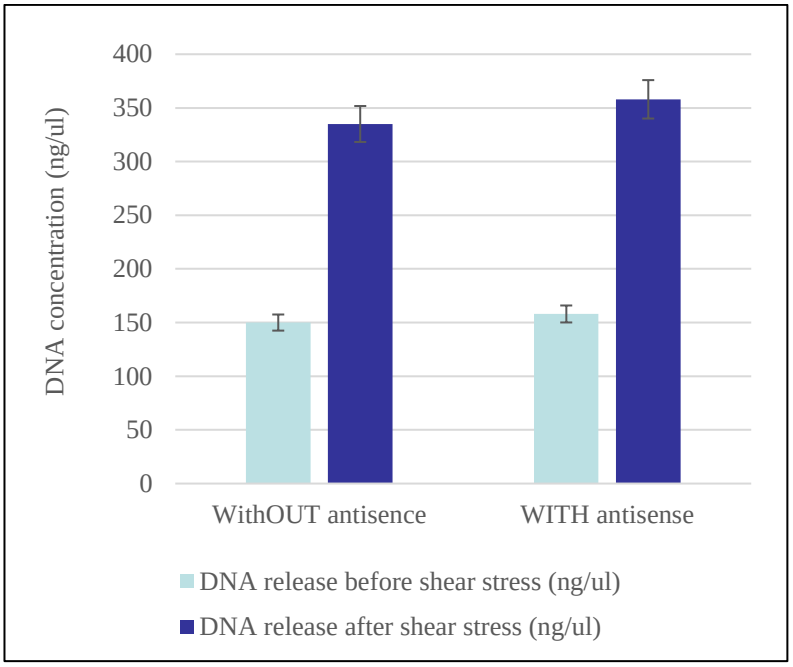


Figure 48 Robustness assessment and DNA release assays of the final sample of Figure 45 0.7L WV fermentations

with antisense and without antisense(control). An ultra-scale-down (USD) shear device developed by UCL to mimic the shear stress that cells are exposed to in a disk-stack centrifugation was utilized to assess robustness of the modified cells in subsequent Down Stream Processing. No significant difference in robustness between the Control and the antisense samples is demonstrated. DNA, Deoxyribonucleic acid.

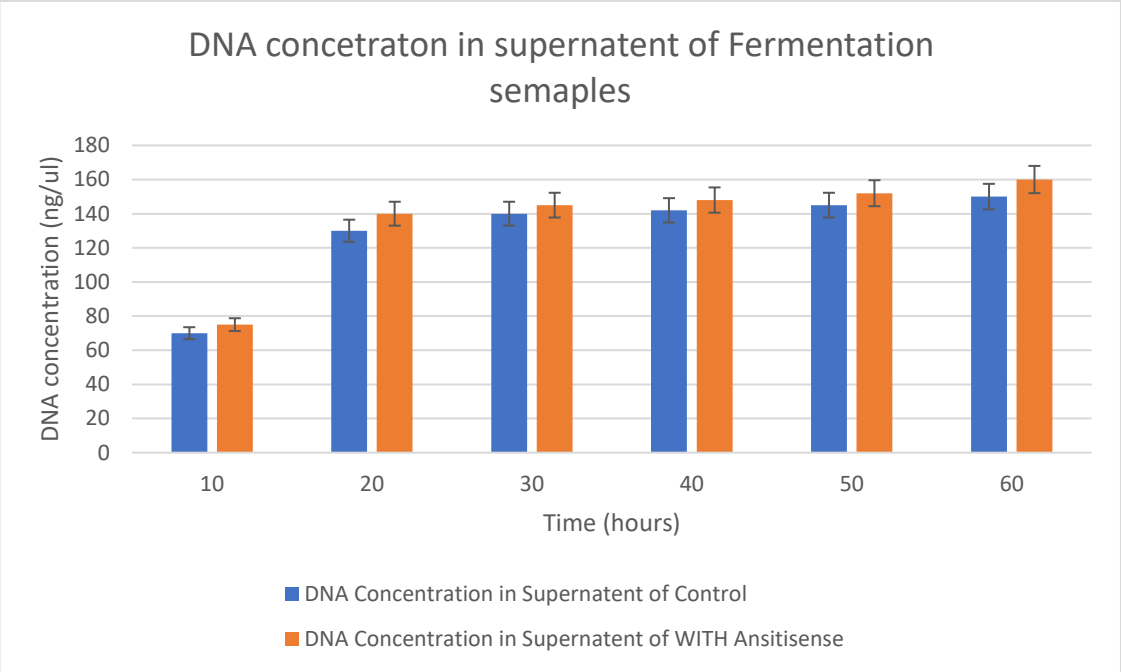


Figure 49 DNA Concentration measurements of the supernatant samples of Figure 45 fermentation (0.7L WV), W3110-wFab- fed batch ((a) Control, withOUT antisense) and ((b)-WITH antisense)

The DNA concentration of the supernatant samples from the experiment illustrated in Figure 45 were measured. This was to investigate the differences between the control and the antisense cells with respect to cell death that leads to host cell DNA release and therefore an increase in DNA quantity in the supernatant. As shown in the figure above there are no significant differences between the control and the antisense cells. Furthermore, it also shows that the viability does not decrease significantly as fermentation progresses post induction.

It may be deduced that the stability and slow increase in cell density as shown in both the control and WITH-antisense experiments post induction, are due to the change in temperature (from 32℃ to 27 ℃) that effects growth rate. Furthermore, as shown in the same figure, the production of the recombinant protein increases significantly post induction which suggests that most of the cellular activities are geared towards protein production.

5.2 Fab' expression in *Escherichia coli* BL21(DE3) fed-batch WITH and withOUT antisense

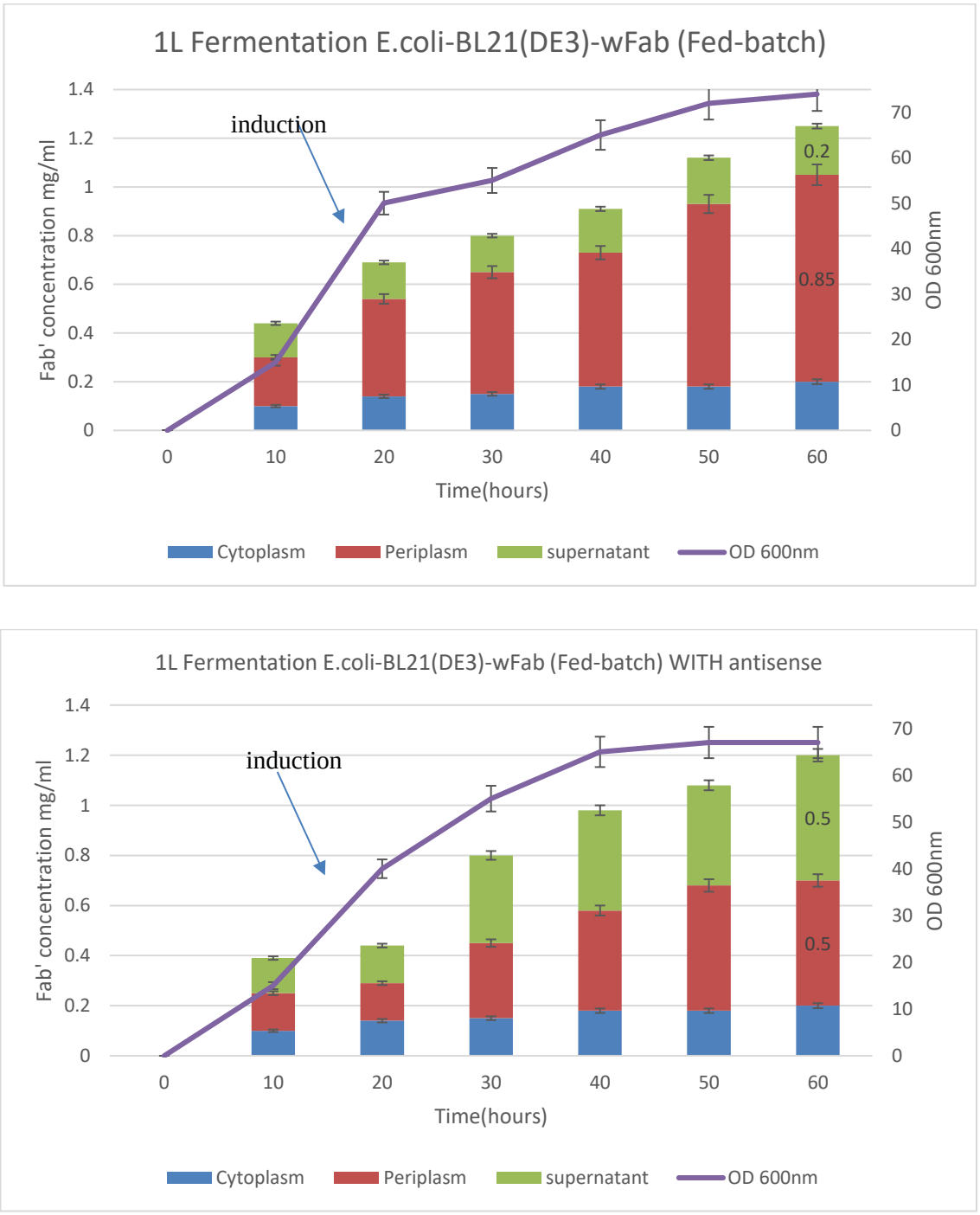


Figure 50 1L fermentation, BL21(DE3)-wFab- fed batch (top-withOUT antisense) and (Bottom-WITH antisense)

Control experiment above (top) indicates 1.25 mg/ml total, 70% in periplasm and 16% in supernatant. In the bottom (WITH antisense) a total titre of 1.2 mg/ml, 42% in periplasm and 42 % in supernatant is observed.

$$\boldsymbol{yield}(\%) = \frac{\boldsymbol{concentarion\ of\ product\ in\ supernatant\ mg/ml}}{\boldsymbol{sum\ of\ concentarions\ of\ product\ in\ all\ fradctions\ \ mg/ml}}\ \boldsymbol{x100}$$

Yields of >42% were achieved reproducibly, an increase from 16%. In other words, an approximately threefold increase in yields when adopting antisense technology was observed.

The result shows very similar release levels when compared with the W3110 strain in the same experimental procedure. Reproducibly and robustness are key observations

5.3 Optimisation, characterisation, and bioprocess improvements

5.3.1 Investigation of factors influencing extracellular release levels

The permeability of *E. coli*'s outer membrane during fermentation, specifically for the release of recombinant products that have accumulated in the periplasm, is a critical consideration in biotechnology and bioprocessing. The factors influencing this permeability can vary depending on the specific product and fermentation conditions. Factors such as temperature, pH, osmolarity, and nutrient availability in the fermentation medium can influence the outer membrane's properties. Optimizing these conditions may help enhance permeability while maintaining cell viability. It's important to note that while increasing outer membrane permeability can facilitate the release of a product from the periplasm, it should be done with careful consideration of the specific fermentation conditions and the potential impact on cell health and viability. Optimization and characterization of your chosen strategy are essential to achieve the desired results.

5.3.1.1 Temperature

Temperature can have a significant impact on both the permeability of the outer membrane and the productivity of recombinant proteins in *E. coli* during fermentation. The effects can be complex and depend on various factors, including the specific protein being expressed, the *E. coli* strain, and the fermentation conditions. Lowering the temperature of *E. coli* fermentation after induction can potentially improve the yields of recombinant protein under certain conditions. This strategy is known as "temperature induction" and is commonly used to enhance the production of recombinant proteins. However, the effectiveness of this approach depends on several factors, including the specific protein being expressed and the fermentation process design. Lowering the temperature after induction can promote the solubility of certain recombinant proteins. Many proteins tend to misfold or aggregate at higher temperatures, leading to lower yields. Reducing the temperature can mitigate these issues and improve protein solubility. Lowering the temperature can slow down cellular metabolism. After induction, the cell may redirect more resources toward recombinant protein production and secretion rather than growth and division. This can lead to increased protein yields. In some cases, high expression of recombinant proteins can lead to cell stress and lysis at elevated temperatures. Lowering the temperature can reduce this stress and prevent cell lysis,

thereby preserving the intracellular protein pool. Lowering the temperature typically slows down cell growth, which can allow for a longer period of recombinant protein expression. This extended expression period can lead to higher protein yields. lowering the temperature of *E. coli* fermentation after induction can be a valuable strategy to improve yields of recombinant protein, particularly for proteins that benefit from enhanced solubility, reduced proteolytic degradation, and improved folding at lower temperatures. However, it should be carefully evaluated and optimized for each specific protein and fermentation process to achieve the best results.

5.3.1.2 Magnesium sulphate

Lowering the magnesium sulphate (MgSO4) concentration in the *E. coli* fermentation media can potentially influence the release of recombinant products, but the effects can vary depending on the specific protein, strain, and fermentation conditions. MgSO4 is an essential component of growth media, and its concentration affects various aspects of bacterial physiology, including cell membrane stability, protein expression, and growth. Mg2+ ions play a role in stabilizing the structure of the outer membrane of Gram-negative bacteria, including *E. coli*. Lowering MgSO4 concentration may lead to increased outer membrane permeability, potentially facilitating the release of recombinant proteins into the surrounding media. This can be advantageous for downstream protein purification. *E. coli* has various protein secretion systems (e.g., Sec and Tat pathways) that transport proteins to the periplasm and beyond. Lowering MgSO4 concentration might affect the functionality of these pathways, potentially impacting the release of recombinant proteins. It can influence the periplasmic environment where many recombinant proteins are initially located. Lowering MgSO4 concentration should be considered as part of a broader process optimization strategy. It is essential to balance the potential advantages of increased protein release with the overall impact on cell viability, protein quality, and production yield. Reducing MgSO4 concentration in *E. coli* fermentation media can influence outer membrane permeability and potentially impact the release of recombinant products. However, the effects are complex and may vary depending on multiple factors. It is advisable to carefully evaluate the specific needs of your recombinant protein production process, conduct experimental trials, and optimize conditions to achieve the desired balance between protein release and overall fermentation performance.

5.3.1.3 Time of induction

The induction time in *E. coli* fermentation, specifically the timing of when recombinant protein expression is induced, can have a significant impact on the release of the recombinant product from the periplasm. The choice of induction time can affect both the quantity and quality of the recombinant protein that is ultimately released. The timing of induction can influence the level of recombinant protein expression. Inducing expression earlier in the fermentation process typically leads to higher intracellular protein levels. This can result in a larger pool of proteins that can potentially be released into the periplasm. The timing of induction often involves a trade-off between yield and protein quality. Early induction may maximize protein yield but could result in a higher proportion of misfolded or aggregated proteins. Late induction may yield higher-quality protein, but overall yields might be lower. The choice of induction time should align with the goals of the fermentation process. For example, if the priority is to maximize the total yield of recombinant protein, earlier induction might be preferred. If protein quality or proper folding is critical, later induction may be more suitable. The effect of induction time on the release of recombinant products from the periplasm of *E. coli* during fermentation is a critical parameter that must be carefully optimized. It involves a delicate balance between achieving high protein yields and ensuring proper protein folding and quality. The optimal induction time can vary depending on the specific recombinant protein, *E. coli* strain, and fermentation conditions, and it often requires empirical testing and process optimization to achieve the desired results.

In this project yield is defined as the amount of released product as percentage of total product produced.

$$yield(\%) = \frac{concentarion\ of\ product\ in\ supernatant\ (mg/ml)}{sum\ of\ concentarions\ of\ product\ in\ all\ fradctions\ (mg/ml)} \text{x100}$$

Equation 13

Media	Temperature	MgSO4	Induction time

• Base media
  • Optimization
• Feed media
  • optimization
	• Δ temperature after induction from 32°C to
	  • 25°C
	  • 22°C
		• Initial concentration
		  • Original(control)
		  • 10^{-1} of the control
			• Early (Before DOT spike)
			• Late (At DOT spike)

Table 13 investigated influential factors on Yield (%)

The factors mentioned above showed significant potential in improving yield % and are all industrially adaptable.

5.3.2 Optimised fab upstream bioprocessing

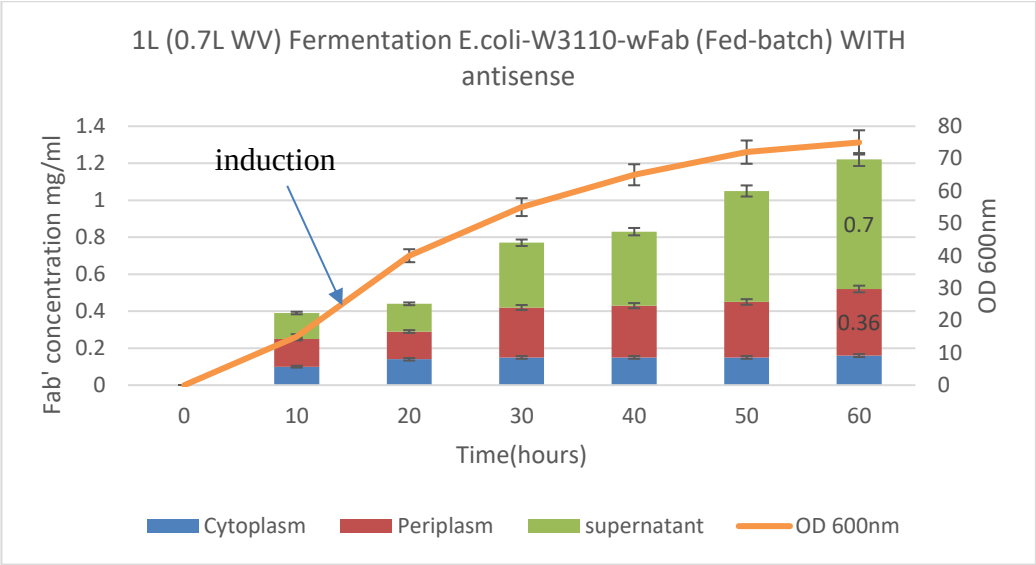


Figure 51 W3110 -wFab -with antisense - improved fermentation

In the figure above (WITH antisense) a total titre of 1.22 mg/ml, 29% in periplasm and 58% in supernatant is observed.

The above fermentation showed a prominsing improvement in terms of percentage of released product. This was achived by lowering the temperature further post induction compared with previous experiments, in this case being considered as control.

The ingredient of MgSO4 in the media takes part in the stability of the membrane therefore it was decided to alter its concentration and investigate its effect on the release levels, and it was found that lowering its concentration to the level stated in the base media will result in higher release levels.

1L fermentation – *Escherichia coli* w3110-Wfab' – **with antisense**			
Lane	Sample ID	Sample time	Fraction
1	0	0	n/a
2	3	30	supernatant
3	4	40	supernatant
4	5	50	supernatant
5	6	60	supernatant
6	All fractions of sample 6	60	Neat (un-fractioned) sample 6

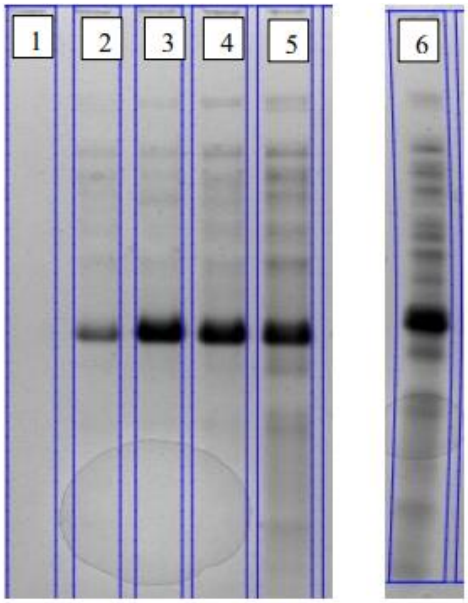


Figure 52 SDS-PAGE of improved 1L fermentation – *Escherichia coli* w3110-Wfab' – with antisense showing increase of amount of product in the supernatant as the fermentation goes on. The table above image of the gel describes the lanes in more detail.

The samples mentioned in the figure above were ran on an SDS-PAGE gel and qualitatively illustrate the release of the product of interest, in this case Fab' into the supernatant as the fermentation carries on. Lane 6 which is the whole of the last sample shows much background protein that is not present in the supernatant fraction off the

same sample, i.e. Lane 5. However, it can be deduced that some other impurities have escaped as well and looking at lane 5, slight background proteins are present. This observation can be used to determine optimal harvest time.

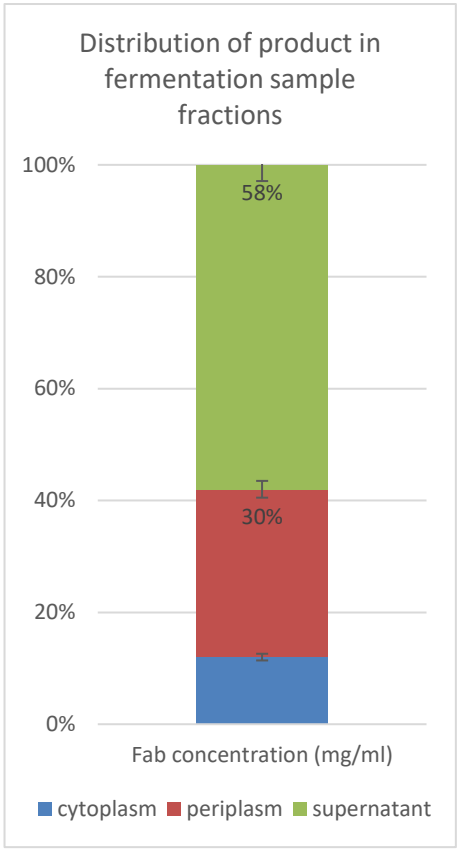


Figure 53 improved fab fermentation yield %. Final sample of fermentation (Figure 51) Total titre of 1.22 mg/ml, 30% periplasm 58 % supernatant. Reproducibility, n=3

Yield of 58% as shown above was achieved which was a significant improvement in the upstream bioprocess development and industrially appealing.

5.4 Optimised fab production – DoE

Factors influencing yield% during fermentation including temperature and MgSO4 concentration, were investigated for optimisation of yield as defined earlier.

Up to 60-64% release was achieved reproducibly.

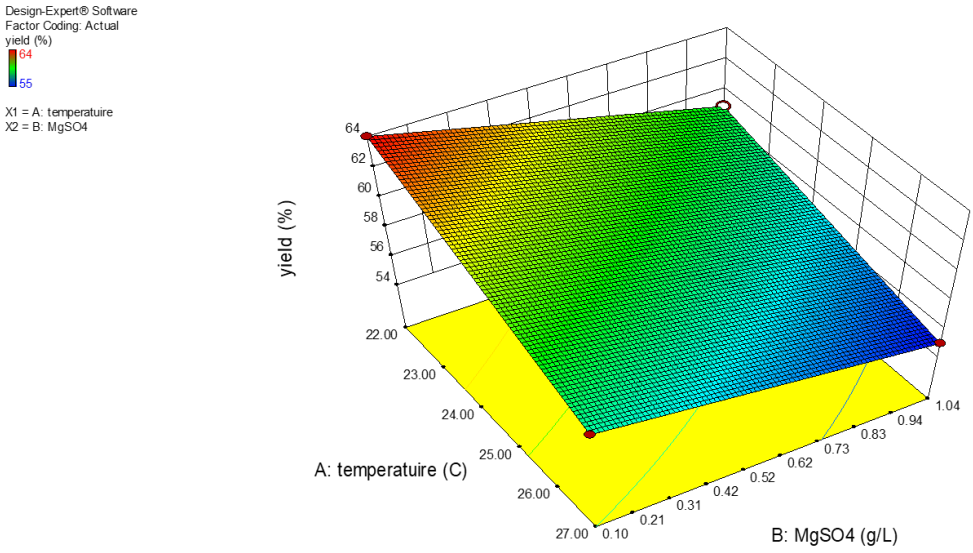


Figure 54 DoE results of Fab' fermentation with antisense

The two factors illustrated above influencing the release of product into the medium during fermentation proved to have significant impact on the yield. Further optimisation and investigation of multivariant factors studies can potentially improve the yield further.

5.5 Robustness studies

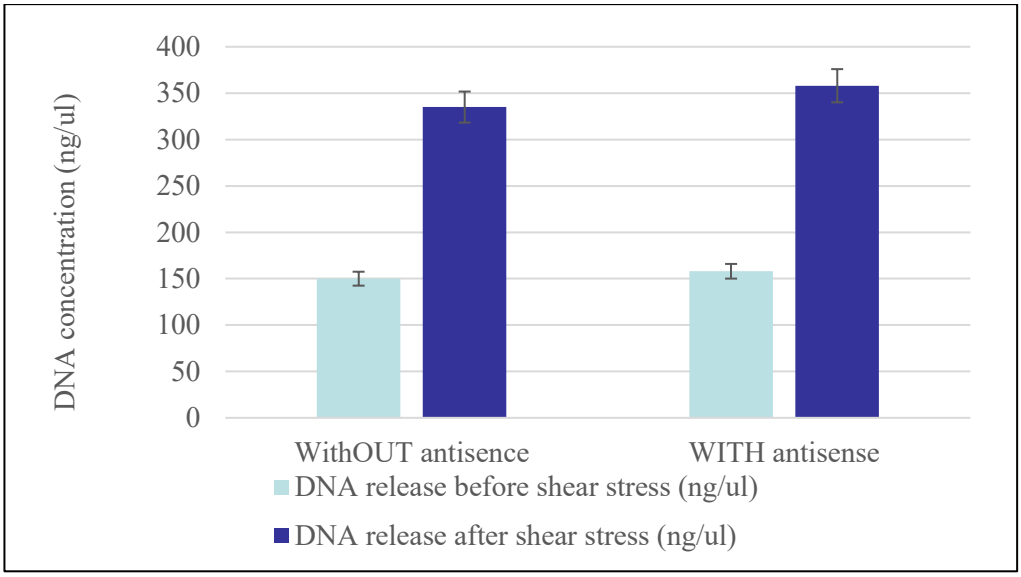


Figure 55 DNA release assays for final Fab' fermentation samples of with and without(control) antisense following utilisation of an ultra-scale-down (USD) shear device developed by UCL to mimic the shear stress that cells are exposed to in disk-stack centrifugation.

Robustness studies show No significant differences in cell integrity when exposed to shear stress equivalent to the shear stress that cells experience in early downstream processing. This is particularly important, as, had the cells' integrity been compromised with utilisation of antisense, the methodology would have been less applicable to industrial bioprocesses. However, it is evident that the cells' integrity is not compromised with antisense.

The DNA content will be released if the inner membrane is disrupted and comparing the Antisense cells with control, the amount of DNA released as result of the induced shear is not significantly different.

5.6 High cell density fermentations

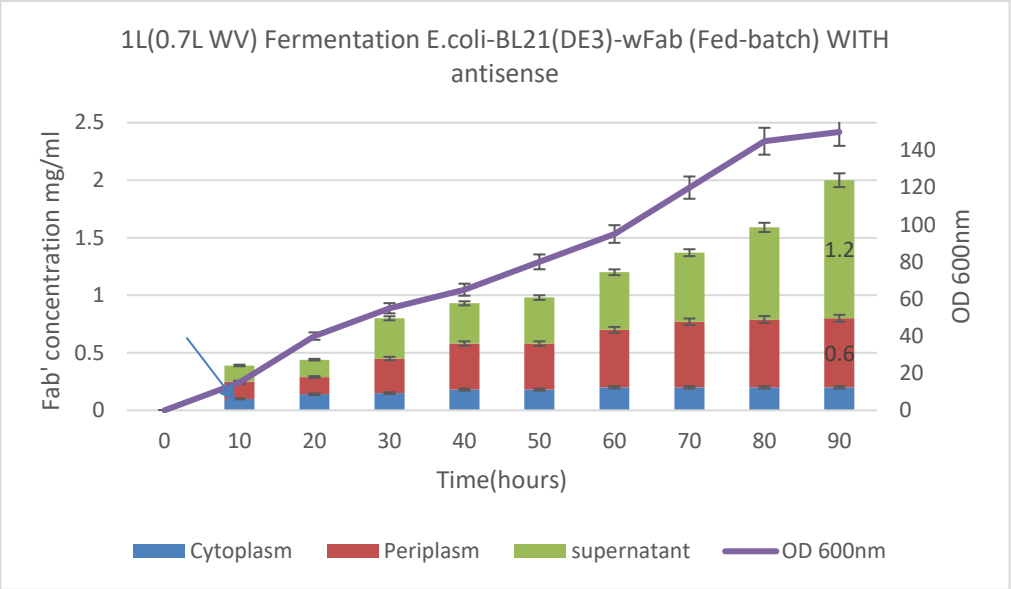


Figure 56 High cell density fed batch fermentation of BL21(DE3)-wFab with antisense, increased initial glycerol concentration source (x4). N=3, total titre 2mg/ml, OD 150 $_{600nm}$,

The figure above shows the utilisation of antisense technology for the mentioned application is valid for high cell density fermentations over 90 hours. The yield of 60% shows promising potential for industrial adaptation of the methodology.

1L fermentation – *Escherichia coli* w3110-Wfab' – **with antisense**			
Lane	Sample ID	Sample time	Fraction
1	Ladder	N/A	N/A
2	empty	n/a	supernatant
3	0	0	supernatant
4	6	60h	supernatant
5	7	70h	supernatant
6	8	80h	supernatant
7	9	90h	supernatant

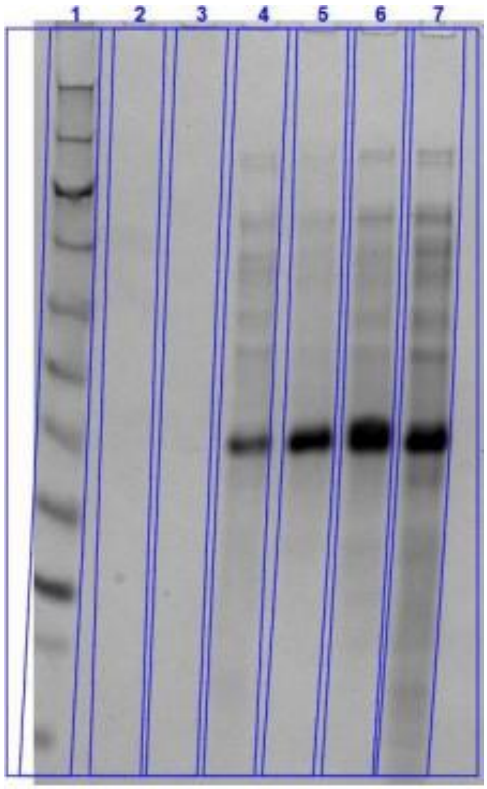


Figure 57 SDS-PAGE of High cell density 90hours 1L fermentation – *Escherichia coli* BL21(DE3)-Wfab' – with antisense showing increase of amount of product in the supernatant as the fermentation goes on.

The table above the image of the gel describes the lanes in more detail. The samples mentioned in the figure above were ran on an SDS-PAGE gel and qualitatively illustrate the release of the product of interest, in this case Fab' into the supernatant as the fermentation carries on. From Lane 7, it can be deduced that some other impurities have escaped as well and comparing this with lane 6 which is the supernatant at time 80 hours, there might be an interest in harvesting earlier in order to have an easier downstream processing at the cost of lower titre.

5.7 Conclusion

The fab fermentation study, including all the subsequent upstream process development trials, was shown to be reliable and reproducible, and as a result, it was suitable for use in an industrial setting. It was possible to reach yields of up to 60 percent, which is considerable and has the potential to result in comparable entire bioprocess yields as well as a reduction in the amount of time and money spent running the process.

Antisense technology may be successfully applied in *E. coli* strains in such a way that an RNA-antisense producing plasmid and a product of interest producing plasmid may be co-transformed in the cell. This may be beneficial to produce the desired product. According to the findings of this study, which utilised a variety of *E. coli* strains and recombinant proteins, the extracellular release of the recombinant protein into the fermentation broth is greatly increased after the induction of the process. In this work, fermentations at multiple scales, including 0.7L and 4L, were described, which proved that the process was scalable and reproducible. Even if it is interesting, the yield of up to 60 percent release of the recombinant product needs to be further tuned and improved so that it can become industrially relevant and attractive. In the event that the yields are greater, the industry may choose to adopt the technology in order to avoid the processes involving cell lysis. As a result, the overall number of recovery and purification steps involved in the production of recombinant proteins will be reduced. If this research is successful, there will be a huge increase in the industry's interest in it, and it may even result in a new bioprocessing strategy being implemented by the industry. It was possible to maintain growth, productivity, and cell integrity while simultaneously achieving extracellular release of sixty percent of the total amount of recombinant protein generated. This methodology may potentially result in comparable entire bioprocess yields while simultaneously resulting in a reduction in the amount of time that the process runs for and the cost that it incurs.

Chapter 6: Future work and industrial applications

This thesis described the use of a molecular biology-based strategy to solve a biochemical engineering problem, namely, to increase the levels of release of specific proteins expressed in the periplasm of *E. coli*, so that the proteins are present in the fermentation medium rather than being retained in the periplasm. Utilizing antisense RNA, the researchers suppressed the expression of murein lipoprotein, the most abundant protein in *E. coli* and a fundamental component of the cell membrane. Plasmids containing the antisense fragment were created and transformed into a variety of hosts. The release of overexpressed proteins, such as alpha-amylase and Fab fragments, as well as the influence of the alterations on the growth and productivity of the cells, were studied.

This project's research consisted of three major components: first, the baseline amounts of alpha-amylase release and *E. coli* growth were analysed so that subsequent alterations could be compared to the baseline studies. Second was the selection, design, and construction of the antisense strains themselves, including the final construct for antisense Lpp inhibition, which was inserted into the MCS of the plasmid pMMB66EH to allow it to be induced with IPTG; third, the construct was tested for release of alpha-amylase, which was also induced simultaneously with IPTG, and lastly in conjunction with Fab plasmids. This chapter discusses the overall study and describes potential future experiments that could be conducted to advance the research presented in this thesis.

After investigating the background production and release of the enzyme when it was induced with IPTG, the alpha-amylase assay was used to explore the release of overexpressed proteins for the first time. Comparisons were made between the levels of production achieved by strains that had the antisense plasmid present and those that did not have it, with the induction taking place during the exponential growth phase. Experiments with shaking flasks demonstrated that the periplasm kept a substantially lower level of amylase activity even before the cells were induced to create antisense Lpp RNA. This was the case both when the cells were activated and before they were induced. Gibbons, in his thesis (2012), made discoveries that were quite similar to these, noting that the antisense RNA necessary for the suppression of a separate gene was being created even in the absence of induction. The potential requirement for a method of antisense expression and regulation that is much more stringent.

It has been demonstrated that antisense inhibition can increase the amounts of release of a number of different target proteins; nevertheless, it is unable to provide for the release of the majority of target proteins that are present in an *E. coli* production host. This indicates that the system requires a large number of additional improvements, some of which could include altering the antisense system itself, while others could involve optimising the associated production systems and fermentation regimens.

Below, we will investigate some of the potential methods that can be used to raise the amounts of production and release of target proteins into the fermentation broth.

Because of its powerful tac promoter and the fact that it is not a proprietary plasmid, the non-proprietary plasmid pMMB66EH was chosen to serve as the vector for the insertion of the antisense fragments in this investigation. This is due to the RSF1010 replicon, which allows it to be used in conjunction with other production plasmids.

Because it is possible that artificially generated pieces of antisense RNA will not be expressed in vivo in significant amounts or remain stable over extended periods of time, a strong promoter is required to maximise the expression of antisense RNA. Other research (Aboul-Fadl, 2005) has found evidence of hairpin structures on the ends of antisense RNA molecules, and other research has attempted to enhance the stability of RNA within *E. coli* cells by incorporating hairpin structures into RNA. Both of these types of research are discussed more below (Crooke & Graham, 2013).

Therefore, it would be feasible to include stability components like these into the antisense system that is provided in this thesis, with the goal of determining whether or not doing so improves the stability of the antisense RNA molecules.

The findings of this thesis have demonstrated that antisense RNA can be utilised to begin the process of solving the problem of maximising the release of specified proteins. As a result of this, numerous antisense constructions were produced, of which one was investigated in greater detail. However, there are still many facets of the research that need additional examination, and in order to optimise the process, it is likely going to be necessary to apply the system to other cell membrane components, if not on an individual basis, then as part of a synergistic approach in which multiple components can be

inhibited together in a variety of different combinations. In addition, there are still many facets of the research that need additional examination. In addition, even though it has been demonstrated that the technology can improve the release of three different categories of proteins (one constitutive, one enzyme, and two Fabs), the degree to which the levels of release to the supernatant vary is not uniform from one protein to the next. For the technology to be useful in industrial practises, particularly if it is to be used as a broad expression system, it is important to define which proteins can be released, depending on characteristics such as size or pI. This is especially important if the technology is to be used as a broad expression system. In the event that additional research is conducted, the antisense technology may give a way that can significantly cut down on the amount of time and resources necessary for the recovery of industrially relevant proteins such as Fab fragments during the downstream processing step. Antisense technology could also be extended much further beyond the scope of this project. For example, it could be used to apply a level of control to many other aspects of the industry as a whole, ranging from biochemical engineering to metabolic engineering. This would be a significant step forward in the development of antisense technology.

If antisense RNA expression is used for the complete release of a therapeutic protein, such as a Fab fragment, then the protein will be present in the fermentation broth rather than the periplasmic region inside the cell. Because of this, the composition of the protein recovery and subsequent processing steps will be different.

To generate a therapeutic protein of value requires an effective technique that can be utilised with high-production systems such as HCDC fermentations. This is done to maximise the product yield as well as the purity of the final product and to eliminate any potential waste.

Processing that occurs farther downstream in the manufacturing chain can be a bottleneck in and of itself; hence, any method that would reduce the amount of time and money spent on this step would be an enormous boon to the production of protein at industrial levels.

The recovery of the protein from the supernatant, as opposed to the periplasm, is the result of a changed process that is brought about when the phase of cell disruption in bioprocessing is eliminated from a standard process stream. As was demonstrated, the

removal of the cell harvesting and subsequent disruption steps from the downstream processing sequence is made possible by the release of the Fab into the fermentation broth. This makes it possible for the fermentation broth to be directly recovered after the fermentation has been completed. Cell disruption leads to the release of the contents of the host cell into the medium, which dramatically increases the amount of contaminants present. Additionally, the purification of proteins such as a Fab fragment is made more effective when the media contains less host-cell contaminants. As a consequence of this, the subsequent processing may call for a reduced number of purification steps, including a step of capture chromatography followed by a step of polish chromatography, in order to achieve a more streamlined and effective level of purification. If the product was discharged directly into the supernatant, both the issue of inclusion bodies and the probable requirement for protein refolding would be eliminated. This results in a reduction not only in the total amount of money spent on items but also in the length of time necessary for the creation of the Fab fragments.

The standard fermentation is followed by a centrifugation step to harvest the cells, followed by a step to disrupt the cells, and an additional centrifugation step to harvest the broth at this point; the antisense fermentation requires a centrifugation step simply to remove the cell paste, which results in the broth being ready to go straight to filtration. This indicates that the antisense fermentations bypass two of the processes that take place before the two process streams converge into a single process stream. As a result, the downstream processing step is not receiving any new processes; rather, the purpose of the revalidation will be to demonstrate that the broth being processed yields the same output with the same quality. It is highly likely that the fermentation broth from the antisense stream will not contain the additional impurities that are the result of homogenization or other cell disruption steps. As a result, the quality of the broth (in terms of purity and yield of the target protein) should be improved in comparison to the initial process, which will allow for a very simple re-validation process. If the technique can be developed successfully in a manufacturing context, it will make it possible to conduct productive fermentations, which will result in the product being recovered in a manner that is easier and more effective, and it will provide more purity in the end product.

References

Aboul-Fadl, T. (2005). Antisense oligonucleotides: The state of the art. *Current Medicinal Chemistry*, *12*(19), 2193–2214. https://doi.org/10.2174/0929867054864859

Adams, R., Popplewell , G., Rapecki, S., & Tickle , S. (2009). Neutralising Antibody Molecules Having Specificity for Human IL-17.

Akopyan, K., Edgren, T., Wang-Edgren, H., Rosqvist, R., Fahlgren, A., Wolf-Watz, H., & Fallman, M. (2011). Translocation of surface-localized effectors in type III secretion. *Proceedings of the National Academy of Sciences*, *108*(4), 1639–1644. https://doi.org/10.1073/pnas.1013888108

Albiniak AM, Matos CF, Branston SD, Freedman RB, Keshavarz-Moore E, Robinson C.. (2013). High-level secretion of a recombinant protein to the culture medium with a Bacillus subtilis twin-arginine translocation system in *Escherichia coli*. *FEBS J.*. 280 (16), p3810-21.

Allman, T. (2020). *The Difference Between Batch, Fed-batch and Continuous Processes*. Blog. Retrieved 2021, from https://www.infors-ht.com/en/blog/the-difference-between-batch-fed-batch-and-continuous-processes/

Ammar, E. M., Wang, X., & Rao, C. V. (2018). Regulation of metabolism in *Escherichia coli* during growth on mixtures of the non-glucose sugars: arabinose, lactose, and xylose. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-017-18704-0

Andersen DC, Swartz J, Ryll T, Lin N, Snedecor B. (2001). Metabolic oscillations in an *Escherichia coli* fermentation.. *Biotechnol Bioeng.*. 75 (2), p212-8.

Andersen, D. C., Swartz, J., Ryll, T., Lin, N., & Snedecor, B. (2001). Metabolic oscillations in an *Escherichia coli* fermentation. *Biotechnol Bioeng*, *75*(2), 212-218.

Asenjo JA, Andrews BA. (1990). Enzymatic cell lysis for product release.. *Bioprocess Technol.* . 9 (1), p143-75.

Asenjo, J. A., & Andrews, B. A. (1990). Enzymatic cell lysis for product release. *Bioprocess Technol, 9*, 143-175.

Backert, S., & Meyer, T. F. (2006). Type IV secretion systems and their effectors in bacterial pathogenesis. *Current Opinion in Microbiology*, *9*(2), 207–217. https://doi.org/10.1016/j.mib.2006.02.008

Bahri, S. M., & Ward, J. M. (1990). Cloning and expression of an alpha-amylase gene from Streptomyces thermoviolaceus CUB74 in *Escherichia coli* JM107 and S. lividans TK24. *J Gen Microbiol, 136*(5), 811-818. doi:10.1099/00221287-136-5-811

Bahri, S. M., & Ward, J. M. (1990). Cloning and expression of an -amylase gene from streptomyces thermoviolaceus CUB74 in *Escherichia coli* JM107 and S. lividans TK24. *Journal of General Microbiology*, *136*(5), 811–818. https://doi.org/10.1099/00221287-136-5-811

Balasundaram, B., Nesbeth, D., Ward, J. M., Keshavarz-Moore, E., & Bracewell, D. G. (2009). Step change in the efficiency of centrifugation through cell engineering: co-expression of Staphylococcal nuclease to reduce the viscosity of the bioprocess feedstock. *Biotechnology and Bioengineering, 104*(1), 134-142. doi:10.1002/bit.22369

Bennett, C. F., & Cowsert, L. M. (1999a). Antisense oligonucleotides as a tool for gene functionalization and target validation. *Biochim Biophys Acta*, *1489*(1), 19-30.

Bennett, C. F., & Cowsert, L. M. (1999b). Application of antisense oligonucleotides for gene functionalization and target validation. *Curr Opin Mol Ther, 1*(3), 359-371.

Bentley, W. E., Mirjalili, N., Andersen, D. C., Davis, R. H., & Kompala, D. S. (1990). Plasmid-encoded protein: The principal factor in the “metabolic burden” associated with recombinant bacteria. *Biotechnology and Bioengineering*, *35*(7), 668–681. https://doi.org/10.1002/bit.260350704

Berkmen, M., Benedik, M. J., & Blasi, U. (1997). The Serratia marcescens NucE protein functions as a holin in *Escherichia coli*. *J Bacteriol, 179*(20), 6522-6524.

Blanchin-Roland, S., & Masson, J. M. (1989). Protein secretion controlled by a synthetic gene in *Escherichia coli*. *Protein engineering*, 2(6), 473–480. https://doi.org/10.1093/protein/2.6.473

Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA,Goeden MA, Rose DJ, Mau B, Shao Y. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science*. 277(5331):1453-62

Boulding, N., Yim, S. S. S., Keshavarz-Moore, E., Shamlou, P. A., & Berry, M. (2002). Ultra scaledown to predict filtering centrifugation of secreted antibody fragments from fungal broth. *Biotechnology and Bioengineering, 79*(4), 381-388. doi:10.1002/bit.10306

Bowering LC1, Bracewell DG, Kesharvarz-Moore E, Hoare M, Weir AN. (2002) Comparison of techniques for monitoring antibody fragment production in *Escherichia coli* fermentation cultures. *Biotechnol Prog*. 18(6):1431-8.

Branston SD, Matos CF, Freedman RB, Robinson C, Keshavarz-Moore E.. (2012). Investigation of the impact of Tat export pathway enhancement on *Escherichia coli* culture, protein production and early stage recovery.. *Biotechnol Bioeng*.. 109 (4), p983-91.

Braun, V. (1975). Covalent lipoprotein from the outer membrane of *Escherichia coli*. *Biochimica Et Biophysica Acta (BBA) - Reviews on Biomembranes*, *415*(3), 335–377. https://doi.org/10.1016/0304-4157(75)90013-1

Braun, V., & Sieglin, U. (1970). The covalent murein-lipoprotin structure of the *Escherichia coli* cell wall. The attachment site of the lipoprotein on the Murein. *European Journal of Biochemistry*, *13*(2), 336–346. https://doi.org/10.1111/j.1432-1033.1970.tb00936.x

Bruckner, C. G., Rehm, C., Grossart, H. P., & Kroth, P. G. (2011). Growth and release of extracellular organic compounds by benthic diatoms depend on interactions with bacteria. *Environmental Microbiology, 13*(4), 1052-1063. doi:10.1111/j.1462-2920.2010.02411.x

Brun, L. O., Stuart, J., Gaudichon, V., Aronstein, K., & French-Constant, R. H. (1995). Functional haplodiploidy: a mechanism for the spread of insecticide resistance in an important international insect pest. *Proc Natl Acad Sci U S A, 92*(21), 9861-9865.

Cascales, E., & Christie, P. J. (2003). The versatile bacterial type IV secretion systems. *Nature Reviews Microbiology*, *1*(2), 137–149. https://doi.org/10.1038/nrmicro753

Chatel, A., Kumpalume, P., & Hoare, M. (2013). Ultra scale-down characterization of the impact of conditioning methods for harvested cell broths on clarification by continuous centrifugation-recovery of domain antibodies from REC*E. coli*. *Biotechnology and Bioengineering*, *111*(5), 913–924. https://doi.org/10.1002/bit.25164

Chen, Z. Y., Cao, J., Xie, L., Li, X. F., Yu, Z. H., & Tong, W. Y. (2014). Construction of leaky strains and extracellular production of exogenous proteins in recombinant *Escherichia coli*. *Microb Biotechnol*, *7*(4), 360-370. doi:10.1111/1751-7915.12127

Choi, J. H., & Lee, S. Y. (2004). Secretory and extracellular production of recombinant proteins using *Escherichia coli*. *Applied Microbiology and Biotechnology*, *64*(5), 625–635. https://doi.org/10.1007/s00253-004-1559-9

Choi, J. H., Keum, K. C., & Lee, S. Y. (2006). Production of recombinant proteins by high cell density culture of *Escherichia coli*. *Chemical Engineering Science*, *61*(3), 876–885. https://doi.org/10.1016/j.ces.2005.03.031

Clapp, K. P., Castan, A., & Lindskog, E. K. (2018). Upstream Processing Equipment. *Biopharmaceutical Processing*, 457–476. https://doi.org/10.1016/b978-0-08-100623-8.00024-4

Crooke, R. M., & Graham, M. J. (2013). Modulation of lipoprotein metabolism by antisense technology: Preclinical Drug Discovery Methodology. *Methods in Molecular Biology*, 309–324. https://doi.org/10.1007/978-1-60327-369-5_14

Crooke, R. M., & Graham, M. J. (2013). Modulation of lipoprotein metabolism by antisense technology: Preclinical Drug Discovery Methodology. *Methods in Molecular Biology*, 309–324. https://doi.org/10.1007/978-1-60327-369-5_14

Crooke, S. T., Baker, B. F., Crooke, R. M., & Liang, X.-hai. (2021). Antisense technology: An overview and prospectus. *Nature Reviews Drug Discovery*, *20*(6), 427–453. https://doi.org/10.1038/s41573-021-00162-z

Dagleish, M. P., Brazil, T. J., & Scudamore, C. L. (2003). Potentiation of the extracellular release of equine neutrophil elastase and alpha-1-proteinase inhibitor by a combination of two bacterial cell wall components: fMLP and LPS. *Equine Veterinary Journal, 35*(1), 35-39. doi:10.2746/042516403775467496

Delepelaire, P. (2004). Type I secretion in gram-negative bacteria.*Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 1694 (1-3), 149-161.

DeLisa MP, Samuelson P, Palmer T, Georgiou G. (2002). Genetic analysis of the twin arginine translocator secretion pathway in bacteria.. *J Biol Chem*. 277 (33), p29825-31.

Devi, G.R. (2002) Prostate cancer: status of current treatments and emerging antisense therapies. *Curr. Opin. Mol. Ther.* 4,138–148.

Doran, P. M. (2013). *Bioprocess Engineering principles: Second edition*. Academic Press.

Dorceus, M. (2018). *Cell Culture Scale-Up in Stirred-Tank Single-Use Bioreactors*. Eppendorf.com. Retrieved 2021, from https://www.eppendorf.com/product-media/doc/en/633183/Fermentors-Bioreactors_Publication_BioBLU-c_Cell-Culture-Scale-Up-BioBLU-Single-Vessels.pdf

Dresler K, van den Heuvel J, Mu¨ller RJ, Deckwer WD (2006), Production of a recombinant polyester-cleaving hydrolase from Thermobifida fusca in *Escherichia coli.* Bioprocess Biosyst Eng 29:169–183

Duan, K., Liu, C. Q., Supple, S., & Dunn, N. W. (1998). Involvement of antisense RNA in replication control of the lactococcal plasmid pND324. *FEMS Microbiology Letters, 164*(2), 419-426. doi:10.1111/j.1574-6968.1998.tb13118.x

Edwards, E., Livanos, M., Krueger, A., Dell, A., Haslam, S. M., Mark Smales, C., & Bracewell, D. G. (2022). Strategies to control therapeutic antibody glycosylation during bioprocessing: Synthesis and separation. *Biotechnology and Bioengineering*. https://doi.org/10.1002/bit.28066

EKATO. (2021). *Conventional Impellers | EKATO*. EKATO. Retrieved 2021, from https://www.ekato.com/products/impellers/conventional-impellers/

Eppendorf. (2021). *The Eppendorf BioFlo® 320: The next generation universal bioprocess control station*. The Eppendorf BioFlo® 320 | The Next Generation Universal Bioprocess Control Station. Retrieved August 24, 2022, from https://web.eppendorf.com/BioFlo320/bioflo_configurator.html

Eppendorf. (2021). *The Eppendorf BioFlo® 320: The next generation universal bioprocess control station*. The Eppendorf BioFlo® 320 | The Next Generation Universal Bioprocess Control Station. Retrieved November 11, 2022, from https://web.eppendorf.com/BioFlo320/bioflo_features.html

Eppendorf.com. (2022). Retrieved 2022, from https://www.eppendorf.com/fileadmin/knowledgebase/asset/AT-de/73087.pdf

Eppendorf.com. (2022). Retrieved 2022, from https://www.eppendorf.com/product-media/doc/en/973619/Fermentors-Bioreactors_Application-Note_416_DASGIP-Parallel-Bioreactor-System_BioFlo-320_Scale-UpBiosimilar-Production-Process-CHO-Cells-Small-Bench-Scale.pdf

European Journal of Biochemistry. (2003) Comparison of different antisense strategies [diagram] in: European Journal of Biochemistry Volume 270, Issue 8, pages 1628-1644, 26 mar 2003

Fisher AC, Kim JY, Perez-Rodriguez R, Tullman-Ercek D, Fish WR, Henderson LA, DeLisa MP. (2008). Exploration of twin-arginine translocation for expression and purification of correctly folded proteins in *Escherichia coli*. *Microb Biotechnol.*. 1 (5), p403-15.

Forster, A. H., & Gescher, J. (2014). Metabolic Engineering of *Escherichia coli* for Production of Mixed-Acid Fermentation End Products. *Frontiers in Bioengineering and Biotechnology*, *2*. https://doi.org/10.3389/fbioe.2014.00016

French, C. and Ward, J.M. (1995). Improved production and stability of *Escherichia coli* recombinants expressing transketolase for large scale biotransformation. *Biotechnology Letters* 17:247-255

French, C. E., & Bruce, N. C. (1995). Bacterial morphinone reductase is related to Old Yellow Enzyme. *Biochem J, 312 (Pt 3)*, 671-678.

French, C. E., Hailes, A. M., Rathbone, D. A., Long, M. T., Willey, D. L., & Bruce, N. C. (1995). Biological production of semisynthetic opiates using genetically engineered bacteria. *Biotechnology (N Y)*, *13*(7), 674-676.

French, C. E., Hailes, A. M., Rathbone, D. A., Long, M. T., Willey, D. L., & Bruce, N. C. (1995). Biological production of semisynthetic opiates using genetically engineered bacteria. *Nature Biotechnology*, *13*(7), 674–676. https://doi.org/10.1038/nbt0795-674

French, C., & Ward, J. (1996). Production and modification of *E. coli* transketolase for large-scale biocatalysis. *Annals of the New York Academy of Sciences*, *799*(1 Enzyme Engine), 11–18. https://doi.org/10.1111/j.1749-6632.1996.tb33171.x

French, C., & Ward, J. M. (1995). Improved production and stability OF*E. coli* recombinants expressing transketolase for large scale biotransformation. *Biotechnology Letters*, *17*(3), 247–252. https://doi.org/10.1007/bf01190631

French, C., & Ward, J. M. (1996). Production and modification of *Escherichia coli* transketolase for large-scale biocatalysis. *Ann N Y Acad Sci, 799*, 11-18.

French, C., Keshavarz-Moore, E., & Ward, J. M. (1996). Development of a simple method for the recovery of recombinant proteins from the *Escherichia coli* periplasm. *Enzyme and Microbial Technology*, *19*(5), 332–338. https://doi.org/10.1016/s0141-0229(96)00003-8

French, C; Ward, JM; Keshavarz-Moore, E; (1996) Development of a simple method for the recovery of recombinant proteins from the *Escherichia coli*

Fu, X.-Y. (2010). Extracellular accumulation of recombinant protein by *Escherichia coli* in a defined medium. *Applied Microbiology and Biotechnology*, *88*(1), 75–86. https://doi.org/10.1007/s00253-010-2718-9

Fürste JP, Pansegrau W, Frank R, Blöcker H, Scholz P, Bagdasarian M, Lanka E. (1986) Molecular cloning of the plasmid RP4 primase region in a multi-host-range tacP expression vector. *Gene.* 48(1):119-31

Furste, J. P., Pansegrau, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M., & Lanka, E. (1986). Molecular cloning of the plasmid RP4 primase region in a multi-host-range tacP expression vector. *Gene, 48*(1), 119-131.

Fürste, J. P., Pansegrau, W., Frank, R., Blöcker, H., Scholz, P., Bagdasarian, M., & Lanka, E. (1986). Molecular cloning of the plasmid RP4 primase region in a multi-host-range TACP expression vector. *Gene*, *48*(1), 119–131. https://doi.org/10.1016/0378-1119(86)90358-6

Gene regulation by antisense RNA and DNA. (1991). *Journal of Cellular Biochemistry, 47*(S15D), 1-37. doi:10.1002/jcb.240470802

Gibbons, DJM. (2012). Towards Complete release from the *Escherichia coli* periplasm for improved manufacturing. Thesis submitted to UCL.

Gill, R. T., DeLisa, M. P., Valdes, J. J., & Bentley, W. E. (2001). Genomic analysis of high-cell-density recombinant *Escherichia coli* fermentation and “cell

conditioning" for improved recombinant protein yield. *Biotechnology and Bioengineering*, *72*(1), 85-95. doi:10.1002/1097-0290(20010105)72:1<85::AID-BIT12>3.0.CO;2-H

Gottschalk, U., & Spitali, M. (2017). DOWNSTREAM PROCESSING OF MONOCLONAL ANTIBODY FRAGMENTS. In *Process scale purification of antibodies* (2nd ed., pp. 537–558). essay, Wiley.

Green, E. R., & Mecsas, J. (2016). Bacterial secretion systems: An overview. *Microbiology Spectrum*, *4*(1). https://doi.org/10.1128/microbiolspec.vmbf-0012-2015

Green, E. R., & Mecsas, J. (2016). Bacterial secretion systems: An overview. *Microbiology Spectrum*, *4*(1). https://doi.org/10.1128/microbiolspec.vmbf-0012-2015

Haitjema CH, Boock JT, Natarajan A, Dominguez MA, Gardner JG, Keating DH, Withers ST, Delisa MP. (2013). Universal Genetic Assay for Engineering Extracellular Protein Expression.. *ACS Synth Biol*. 1 (1).

Hall, S. (2012). *Rules of thumb for chemical engineers* (5th ed.). Elsevier.

Han, K Lim, HC and Hong, J. (1992). Acetic acid formation in *Escherichia coli* fermentation. Biotechnology and Bioengineering. 39 (.), p663-671.

Harrison, J. S., Keshavarz-Moore, E., Dunnill, P., Berry, M. J., Fellinger, A., & Frenken, L. (1997). Factors affecting the fermentative production of a lysozyme-binding antibody fragment in *Escherichia coli*. *Biotechnology and Bioengineering*, *53*(6), 611-622. doi:10.1002/(SICI)1097-0290(19970320)53:6<611::AID-BIT9>3.0.CO;2-P

Harrison, S. T. L. (2011). Cell disruption. *Comprehensive Biotechnology*, 619–640. https://doi.org/10.1016/b978-0-08-088504-9.00127-6

Hayashi, K., Morooka, N., Yamamoto, Y., Fujita, K., Isono, K., Choi, S., Ohtsubo, E., Baba, T., Wanner, B. L., Mori, H., & Horiuchi, T. (2006). Highly accurate genome sequences of*Escherichia coli*K-12 strains MG1655 and W3110. *Molecular Systems Biology*, *2*(1). https://doi.org/10.1038/msb4100049

Henderson IR, Navarro-Garcia F, Desvaux M, Fernandez RC, Ala'Aldeen D. (2004). Type V protein secretion pathway: the autotransporter story. *Microbiol Mol Biol Rev.* 68 (4), 692-744.

Henderson, I. R., Navarro-Garcia, F., Desvaux Mickaël, Fernandez, R. C., & Ala'Aldeen, D. (2004). Type V protein secretion pathway: The autotransporter story. *Microbiology and Molecular Biology Reviews*, *68*(4), 692–744. https://doi.org/10.1128/mmbr.68.4.692-744.2004

Hsu, C. Y., Lin, M. H., Chen, C. C., Chien, S. C., Cheng, Y. H., Su, I. N., & Shu, J. C. (2011). Vancomycin promotes the bacterial autolysis, release of extracellular DNA, and biofilm formation in vancomycin-non-susceptible Staphylococcus aureus. *FEMS Immunology & Medical Microbiology*, *63*(2), 236-247. doi:10.1111/j.1574-695X.2011.00846.x

Infors HT. (2015). *Multifors 2 - parallel bioreactor system for small-scale bioprocesses*. Multifors 2 - Parallel bioreactor for bioprocess development | INFORS HT. Retrieved November 5, 2022, from https://www.infors-ht.com/en/bioreactors/bench-top-bioreactors/multifors2/

Inouye, S., Lee, N., Inouye, M., Wu, H. C., Suzuki, H., Nishimura, Y., Iketani, H., & Hirota, Y. (1977). Amino acid replacement in a mutant lipoprotein of the *Escherichia coli* outer membrane. *Journal of Bacteriology*, *132*(1), 308–313. https://doi.org/10.1128/jb.132.1.308-313.1977

Janke, F., Kober, L., & Glaser, R. (n.d.). *Scale-Up of a Biosimilar Production Process with CHO Cells from Small to Bench Scale*. Eppendorf.com. Retrieved 2022, from https://www.eppendorf.com/product-media/doc/en/973619/Fermentors-Bioreactors_Application-Note_416_DASGIP-Parallel-Bioreactor-System_BioFlo-320_Scale-UpBiosimilar-Production-Process-CHO-Cells-Small-Bench-Scale.pdf

Jazini, M., & Herwig, C. (2013). Substrate oscillations boost recombinant protein release from *Escherichia coli*. *Bioprocess and Biosystems Engineering*, *37*(5), 881–890. https://doi.org/10.1007/s00449-013-1059-3

Jeong H, Barbe V, Lee CH, Vallenet D, Yu DS, Choi SH, Couloux A, Lee SW, Yoon SH, Cattolico L, Hur CG, Park HS, Ségurens B, Kim SC, Oh TK, Lenski RE, Studier FW, Daegelen P, Kim JF. (2009). J Mol Biol. 394(4):644-52

Johnson TL, Abendroth J, Hol WG, Sandkvist M.. (2006). Type II secretion: from structure to function.. *FEMS Microbiol Lett*. 255 (2), 175-186.

Kager, J., Fricke, J., & Herwig, C. (2022). *A Generic Biomass Soft Sensor and Its Application in Bioprocess Development*. Eppendorf.com. Retrieved 2022, from https://www.eppendorf.com/product-media/doc/en/228755/Fermentors-Bioreactors_Application-Note_357_DASware_A-Generic-Biomass-Soft-Sensor-Its-Application-Bioprocess-Development.pdf

Kaiser, S. C., Werner, S., Jossen, V., Blaschczok, K., & Eibl, D. (2018). Power Input Measurements in Stirred Bioreactors at Laboratory Scale. *Journal of Visualized Experiments*, (135). https://doi.org/10.3791/56078

Kandra, K. (2020). *The Impeller: Why It Could Be Called The Heart Of The Bioreactor*. Blog. Retrieved 2021, from https://www.infors-ht.com/en/blog/the-impeller-why-you-can-call-it-the-heart-of-the-bioreactor/

Khushoo A, Pal Y, Mukherjee KJ (2005) Optimization of extracellular production of recombinant asparaginase in *Escherichia coli* in shaker-flask and bioreactor. *Appl Microbiol Biotechnol* 68:189–197

Kim JY, Doody AM, Chen DJ, Cremona GH, Shuler ML, Putnam D, DeLisa MP. (2008). Engineered bacterial outer membrane vesicles with enhanced functionality.. *J Mol Biol*. 380 (1), p51-66.

Kleiner-Grote, G. R., Risse, J. M., & Friehs, K. (2018). Secretion of recombinant proteins from*E. coli*. *Engineering in Life Sciences*, *18*(8), 532–550. https://doi.org/10.1002/elsc.201700200

Korotkov, K. V., Sandkvist, M., & Hol, W. G. (2012). The type II secretion system: Biogenesis, molecular architecture and mechanism. *Nature Reviews Microbiology*, *10*(5), 336–351. https://doi.org/10.1038/nrmicro2762

Korotkov, K. V., Sandkvist, M., & Hol, W. G. (2012). The type II secretion system: Biogenesis, molecular architecture and mechanism. *Nature Reviews Microbiology*, *10*(5), 336–351. https://doi.org/10.1038/nrmicro2762

Langer ES (2009) Capitalizing on novel expression systems. *Genet Eng Biotechnol News* 15:12

Lecher, J., Schwarz, C. K. W., Stoldt, M., Smits, S. H. J., Willbold, D., & Schmitt, L. (2012). An RTX transporter tethers its unfolded substrate during secretion via a unique N-terminal domain. *Structure*, *20*(10), 1778–1787. https://doi.org/10.1016/j.str.2012.08.005

Lee, N., & Inouye, M. (1974). Outer membrane proteins of*eschrichia coli*: Biosynthesis and assembly. *FEBS Letters*, *39*(2), 167–170. https://doi.org/10.1016/0014-5793(74)80043-8

Lee, S. Y. (1996). High cell-density culture of *Escherichia coli*. *Trends in Biotechnology*, *14*(3), 98–105. https://doi.org/10.1016/0167-7799(96)80930-9

Lewis, G., Taylor, I. W., Nienow, A. W., & Hewitt, C. J. (2004). The application of multi-parameter flow cytometry to the study of recombinant *Escherichia coli* batch fermentation processes. *Journal of Industrial Microbiology & Biotechnology*, *31*(7), 311–322. https://doi.org/10.1007/s10295-004-0151-8

Li Q, Xu X, Yang J, Nie Q, Xian M., (2008). Progress of antisense technology in metabolic regulation of bacteria. *Sheng Wu Gong Cheng Xue Bao*. 2008 Oct;24(10):1689-94

Li, B., & Sha, M. (2016). *Scale-Up of Escherichia coli Fermentation from Small Scale to Pilot Scale Using Eppendorf Fermentation Systems*. Eppendorf.com. Retrieved 2022, from https://www.eppendorf.com/product-media/doc/en/180447/Fermentors-Bioreactors_Application-Note_306_DASGIP-Parallel-Bioreactor-System_BioFlo-

320_BioFlo-610_Scale-Up-Escherichia-coli-Fermentation-Small-Scale-Pilot-Scale-Eppendorf-Fermentation-S.pdf

Liu, D. (2014). *Escherichia coli*☆. *Reference Module in Biomedical Sciences*. https://doi.org/10.1016/b978-0-12-801238-3.02291-1

Lusk, J. E., Williams, R. J. P., & Kennedy, E. P. (1968). Magnesium and the Growth of *Escherichia coli*. *Journal of Biological Chemistry*, *243*(10), 2618–2624. https://doi.org/10.1016/s0021-9258(18)93417-4

Martínez-Gómez, K., Flores, N., Castañeda, H. M., Martínez-Batallar, G., Hernández-Chávez, G., Ramírez, O. T., Gosset, G., Encarnación, S., & Bolivar, F. (2012). New insights into *Escherichia coli* metabolism: carbon scavenging, acetate metabolism and carbon recycling responses during growth on glycerol. *Microbial Cell Factories*, *11*(1). https://doi.org/10.1186/1475-2859-11-46

Matos CF, Branston SD, Albiniak A, Dhanoya A, Freedman RB, Keshavarz-Moore E, Robinson C. (2012). High-yield export of a native heterologous protein to the periplasm by the tat translocation pathway in *Escherichia coli*. *Biotechnol Bioeng.*. 109 (10), p2533-42.

Matos CF, Robinson C, Alanen HI, Prus P, Uchida Y, Ruddock LW, Freedman RB, Keshavarz-Moore E.. (2013). Efficient export of prefolded, disulfide-bonded recombinant proteins to the periplasm by the Tat pathway in *Escherichia coli*CyDisCo strains.. *Biotechnol Prog.*. 0 (0), p1-10.

McInerney, J., Aguiar, M., Thomas, M., & Sha, M. (2017). *Optimization of Computer Fluid Dynamics Modeling Using Experimentally Obtained Characteristics of Stirred-Tank Fermentors* (p. Poster). Eppendorf Manufacturing Corporation. Retrieved 2021, from https://www.eppendorf.com/fileadmin/General/Applications/Bioprocess_Landing_Page/Poster_CFD-modeling_approved_V1.pdf.

Merck (2019). *T7 Promoter System*. [online] Available at: https://www.sigmaaldrich.com/life-science/molecular-biology/cloning-and-expression/vector-systems/t7-promoter-system.html [Accessed 20 Dec. 2019].

Mergulhão, F. J. M., Summers, D. K., & Monteiro, G. A. (2005). Recombinant protein secretion in *Escherichia coli*. *Biotechnology Advances*, *23*(3), 177–202. https://doi.org/10.1016/j.biotechadv.2004.11.003

Millar, J. A., & Raghavan, R. (2021). Modulation of bacterial fitness and virulence through Antisense RNAS. *Frontiers in Cellular and Infection Microbiology*, *10*. https://doi.org/10.3389/fcimb.2020.596277

Mizuno, T., Chou, M. Y., & Inouye, M. (1984). A unique mechanism regulating gene expression: Translational inhibition by a complementary RNA transcript (micrna). *Proceedings of the National Academy of Sciences*, *81*(7), 1966–1970. https://doi.org/10.1073/pnas.81.7.1966

Morales VM, Bäckman A, Bagdasarian M.. (1991). A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants.. Gene. 97 (1), 39-47.

Morales, V. M., Bäckman, A., & Bagdasarian, M. (1991). A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. *Gene*, *97*(1), 39–47. https://doi.org/10.1016/0378-1119(91)90007-x

Müller S, Feldman MF, Cornelis GR. (2001). The Type III secretion system of Gram-negative bacteria: a potential therapeutic target?.*Expert Opin Ther Targets.*. 5 (3), 327-339.

Müller, M. (2005). Twin-arginine-specific protein export in *Escherichia coli*. *Research in Microbiology*, *156*(2), 131–136. https://doi.org/10.1016/j.resmic.2004.09.016

Nakashima, N., & Tamura, T. (2013). Gene silencing in *Escherichia coli* using antisense RNAs expressed from doxycycline-inducible vectors. *Letters in Applied Microbiology*, *56*(6), 436-442. doi:10.1111/lam.12066

Nandakumar MP, Cheung A, Marten MR (2006) Proteomic analysis of extracellular proteins from *Escherichia coli* W3110. *J Proteome Res* 5:1155–1161

Natale, P., Brüser, T., & Driessen, A. J. M. (2008). Sec- and tat-mediated protein secretion across the bacterial cytoplasmic membrane—distinct translocases and mechanisms. *Biochimica Et Biophysica Acta (BBA) - Biomembranes*, *1778*(9), 1735–1756. https://doi.org/10.1016/j.bbamem.2007.07.015

Neal, G., Christie, J., Keshavarz-Moore, E., & Shamlou, P. A. (2003). Ultra scale-down approach for the prediction of full-scale recovery of ovine polycolonal immunoglobulins used in the manufacture of snake venom-specific Fab fragment. *Biotechnology and Bioengineering, 81*(2), 149-157. doi:10.1002/bit.10454

NEB, . (2015). *Protein expression with T7 Express strains*. Retrieved November 23, 2022, from https://international.neb.com/-/media/nebus/files/application-notes/appnote_protein_expression_with_t7_expression_strains.pdf?rev=712238cab1174f80ae6e406ca62bfb6b

Nesbeth DN, Perez-Pardo MA, Ali S, Ward J, Keshavarz-Moore E.. (2012). Growth and productivity impacts of periplasmic nuclease expression in an *Escherichia coli*

Neutrons.ornl.gov. (2022). Retrieved 2022, from https://neutrons.ornl.gov/sites/default/files/lab/M1287-0054%20REV%20H%20BF%20310.pdf

Newton, J. M., Vlahopoulou, J., & Zhou, Y. (2017). Investigating and modelling the effects of cell lysis on the rheological properties of fermentation broths. *Biochemical Engineering Journal*, *121*, 38–48. https://doi.org/10.1016/j.bej.2017.01.009

Ni Y, Chen R.. (2009). Extracellular recombinant protein production from *Escherichia coli*. *Biotechnol Lett*. 31 (11), 1661-70.

Ni, Y., & Chen, R. (2009). Extracellular recombinant protein production from *Escherichia coli*. *Biotechnology Letters*, *31*(11), 1661–1670. https://doi.org/10.1007/s10529-009-0077-3

Ni, Y., Reye, J., & Chen, R. R. (2007). Lpp deletion as a permeabilization method. *Biotechnology and Bioengineering*, *97*(6), 1347–1356. https://doi.org/10.1002/bit.21375

Nienow, A. W. (1998). Hydrodynamics of Stirred Bioreactors. *Applied Mechanics Reviews*, *51*(1), 3–32. https://doi.org/10.1115/1.3098990

Nienow, A. W. (2006). Reactor Engineering in Large Scale Animal Cell Culture. *Cytotechnology*, *50*(1-3), 9–33. https://doi.org/10.1007/s10616-006-9005-8

Nino, L., Penuela, M., & Gelves, G. R. (2018). *Gas-Liquid Hydrodynamics Simulation using CFD in a Helical Ribbon Impeller Applied for Non-Newtonian Fluids*. Ripublication.com. Retrieved 2021, from https://www.ripublication.com/ijaer18/ijaerv13n11_84.pdf

Nisole A, Lussier F, Morley KL, Shareck F, Kazlauskas RJ, Dupont C, Pelletier JN (2006) Extracellular production of Streptomyces lividans acetyl xylan esterase A in *Escherichia coli* for rapid detection of activity. *Protein Expr Purif* 46:274–284

Noites, I. S., O'Kennedy, R. D., Levy, M. S., Abidi, N., & Keshavarz-Moore, E. (1999). Rapid quantitation and monitoring of plasmid DNA using an ultrasensitive DNA-binding dye. *Biotechnology and Bioengineering*, *66*(3), 195-201. doi:10.1002/(SICI)1097-0290(1999)66:3<195::AID-BIT8>3.0.CO;2-6

Packer, H. L., Keshavarz-Moore, E., Lilly, M. D., & Thomas, C. R. (1992). Estimation of cell volume and biomass of penicillium chrysogenum using image analysis. *Biotechnology and Bioengineering*, *39*(4), 384-391. doi:10.1002/bit.260390404

Paoli, T., Faulkner, J., O'Kennedy, R., & Keshavarz-Moore, E. (2010). A study of D-lactate and extracellular methylglyoxal production in lactate Re-Utilizing CHO cultures. *Biotechnology and Bioengineering*, *107*(1), 182-189. doi:10.1002/bit.22757

Papanikou, E., Karamanou, S., & Economou, A. (2007). Bacterial protein secretion through the translocase nanomachine. *Nature Reviews Microbiology*, *5*(11), 839–851. https://doi.org/10.1038/nrmicro1771

Papi RM, Shaitidou SA, Trikka FA, Kyriakidis DA ,(2005) ,Encapsulated *Escherichia coli* in alginate beads capable of secreting a heterologous pectin lyase. *Microb Cell Fact* 4:35

Park H, Yoon Y, Suk S, Lee JY, Lee Y. (2014). Effects of different target sites on antisense RNA-mediated regulation of gene expression. *BMB Rep.*. 1.

Patil SD, Sharma R, Srivastava S, Navani NK, Pathania R (2013) Downregulation of *yidC* in *Escherichia coli* by Antisense RNA Expression Results in Sensitization to Antibacterial Essential Oils Eugenol and Carvacrol. *PLoS ONE* 8(3): e57370. doi:10.1371/journal.pone.0057370

Pierce, JJ. Robinson, SC. Keshavarz-Moore, E. Dunnill, P.. (2002). A comparison of the process issues in expressing the same recombinant enzyme periplasmically in *Escherichia coli* and extracellularly in Streptomyces lividans. *Journal of Biotechnology*. 92 (1), 205-215.

Pitched-Blade Impeller - Accessories, Bioprocess | Eppendorf United Kingdom. Eppendorf.com. (2021). Retrieved 2021, from https://www.eppendorf.com/gb-en/eShop-Products/Bioprocess/Accessories/Impellers-p-78532236

Polissi A, Sperandeo P. (2014). The Lipopolysaccharide Export Pathway in *Escherichia coli* Structure, Organization and Regulated Assembly of the Lpt Machinery.. *Mar Drugs* . 12 (2), p1023-42.

Pope, B., & Kent, H. M. (1996). High efficiency 5 min transformation of *Escherichia coli*. *Nucleic Acids Research*, *24*(3), 536–537. https://doi.org/10.1093/nar/24.3.536

Qi, D. (2018). *Hydrometallurgy of Rare Earths*. Elsevier.

Qian, Z. G., Xia, X. X., Choi, J. H., & Lee, S. Y. (2008). Proteome-based identification of fusion partner for high-level extracellular production of recombinant proteins in *Escherichia coli*. *Biotechnol Bioeng*, *101*(3), 587-601. doi:10.1002/bit.21898

Ramalakshmi, S., Ramanan, R. N., Madhavan, S., Ooi, C. W., Chang, C. C. H., Harper, I. S., Lewis, D. M., Lee, A. K., He, L., & Seenichamy, A. (2021). Investigation of selective release of periplasmic proteins through pore size analysis and single-cell microscopy in *Escherichia coli*. *Biochemical Engineering Journal*, *171*, 108009. https://doi.org/10.1016/j.bej.2021.108009

Rayat, A. C. M. E., Chatel, A., Hoare, M., & Lye, G. J. (2016). Ultra scale-down approaches to enhance the creation of bioprocesses at scale: Impacts of process shear stress and early recovery stages. *Current Opinion in Chemical Engineering*, *14*, 150–157. https://doi.org/10.1016/j.coche.2016.09.012

Records AR.. (2011). The type VI secretion system: a multipurpose delivery system with a phage-like machinery.. *Mol Plant Microbe Interact.*. 24 (7), 751-7.

Rinas, U., & Hoffmann, F. (2004). Selective leakage of host-cell proteins during high-cell-density cultivation of recombinant and non-recombinant *Escherichia coli*. *Biotechnology Progress*, *20*(3), 679–687. https://doi.org/10.1021/bp034348k

Rodríguez-Carmona, E., Cano-Garrido, O., Dragosits, M., Maurer, A.M., Kunert, R., Mattanovich, D., Villaverde, A., Vázquez, F. (2012). Recombinant Fab expression and secretion in *Escherichia coli* continuous culture at medium cell densities: Influence of temperature. *Process biochemistry*, *47*(3), 446-452.

Rosano, G. Ã. L., & Ceccarelli, E. A. (2014). Recombinant protein expression in *Escherichia coli*: Advances and challenges. *Frontiers in Microbiology*, *5*. https://doi.org/10.3389/fmicb.2014.00172

Rushton-Type Impeller - Accessories, Bioprocess | Eppendorf United Kingdom. Eppendorf.com. (2021). Retrieved 2021, from https://www.eppendorf.com/gb-en/eShop-Products/Bioprocess/Accessories/Impellers-p-78100557

Russell, A. B., Hood, R. D., Bui, N. K., LeRoux, M., Vollmer, W., & Mougous, J. D. (2011). Type VI secretion delivers bacteriolytic effectors to target cells. *Nature*, *475*(7356), 343–347. https://doi.org/10.1038/nature10244

Russell, A. B., Peterson, S. B., & Mougous, J. D. (2014). Type VI secretion system effectors: Poisons with a purpose. *Nature Reviews Microbiology*, *12*(2), 137–148. https://doi.org/10.1038/nrmicro3185

Sankaran, K., & Wu, H. C. (1994). Lipid modification of bacterial prolipoprotein. transfer of diacylglyceryl moiety from phosphatidylglycerol. *Journal of Biological Chemistry*, *269*(31), 19701–19706. https://doi.org/10.1016/s0021-9258(17)32077-x

Santos, N. V. d., Santos-Ebinuma, V. d. C., Junior, A. P., & Pereira, J. F. B. (2018). Liquid–liquid extraction of biopharmaceuticals from fermented broth: trends and future prospects. *Journal of Chemical Technology & Biotechnology, 93*(7), 1845-1863. doi:10.1002/jctb.5476

Schimek, C., Egger, E., Tauer, C., Striedner, G., Brocard, C., Cserjan-Puschmann, M., & Hahn, R. (2020). Extraction of recombinant periplasmic proteins under industrially relevant process conditions: Selectivity and yield strongly depend on protein titer and methodology. *Biotechnology Progress*, *36*(5). https://doi.org/10.1002/btpr.2999

Schmidt, T. M., & Liu, D. (2019). *Escherichia coli*. In *Encyclopedia of microbiology* (fourth, pp. 171–182). essay, Elsevier.

Schottroff, F., Kastenhofer, J., Spadiut, O., Jaeger, H., & Wurm, D. J. (2021). Selective Release of Recombinant Periplasmic Protein From *E. coli* Using Continuous Pulsed Electric Field Treatment. *Frontiers in Bioengineering and Biotechnology*, *8*. https://doi.org/10.3389/fbioe.2020.586833

SelectScience. (2022). *Multifors 2 from INFORS HT*. SelectScience. Retrieved May 24, 2022, from https://www.selectscience.net/products/multifors-2/?prodID=223576

Seltmann, G., & Holst, O. (2002). Introduction. *The Bacterial Cell Wall*, 3–8. https://doi.org/10.1007/978-3-662-04878-8_1

Sesto, N., Wurtzel, O., Archambaud, C., Sorek, R., & Cossart, P. (2012). The excludon: A new concept in bacterial antisense RNA-mediated gene regulation. *Nature Reviews Microbiology*, *11*(2), 75–82. https://doi.org/10.1038/nrmicro2934

Sezonov, G., Joseleau-Petit Danièle, & D'Ari, R. (2007). *Escherichia coli* physiology in Luria-Bertani broth. *Journal of Bacteriology*, *189*(23), 8746–8749. https://doi.org/10.1128/jb.01368-07

Shibui, T and Nagahari, K. (1992). Secretion of a functional Fab fragment in *Escherichia coli* and the influence of culture conditions..*Appl Microbiol Biotechnol. 37(3):352-7*. 37 (3), p352-7.

Shiina, S., Ohshima, T., & Sato, M. (2004). Extracellular release of recombinant α-amylase from *Escherichia coli* using pulsed electric field. *Biotechnology Progress*, *20*(5), 1528–1533. https://doi.org/10.1021/bp049760u

Shiina, S., Ohshima, T., & Sato, M. (2004). Extracellular Release of Recombinant α-Amylase from *Escherichia coli* Using Pulsed Electric Field. *Biotechnology Progress*, *20*(5), 1528–1533. https://doi.org/10.1021/bp049760u

Shiloach, J., & Fass, R. (2005). Growing *E. coli* to high cell density—a historical perspective on method development. *Biotechnology Advances*, *23*(5), 345–357. https://doi.org/10.1016/j.biotechadv.2005.04.004

Shokri, A., Sandén, A., & Larsson, G. (2003). Cell and process design for targeting of recombinant protein into the culture medium of *Escherichia coli*. *Applied Microbiology and Biotechnology*, *60*(6), 654–664. https://doi.org/10.1007/s00253-002-1156-8

Sigma-Aldrich. (2014). LB Broth. Available: http://www.sigmaaldrich.com/catalog/product/sigma/l3022?lang=en®ion=GB. Last accessed 10th Oct 2014.

Simons, R. W., & Kleckner, N. (1983). Translational control of IS10 transposition. *Cell*, *34*(2), 683-691.

Simons, R. W., & Kleckner, N. (1983). Translational control of is10 transposition. *Cell*, *34*(2), 683–691. https://doi.org/10.1016/0092-8674(83)90401-4

Songer, J. G. (1997). Bacterial phospholipases and their role in virulence. *Trends in Microbiology*, *5*(4), 156–161. https://doi.org/10.1016/s0966-842x(97)01005-6

Su L, Chen S, Yi L, Woodard RW, Chen J, Wu J.. (2012). Extracellular overexpression of recombinant Thermobifida fusca cutinase by alpha-hemolysin secretion system in *Escherichia coli*

Su, L., Jiang, Q., Yu, L., & Wu, J. (2017). Enhanced extracellular production of recombinant proteins in *Escherichia coli* by co-expression with Bacillus cereus phospholipase C. *Microbial Cell Factories*, *16*(1). https://doi.org/10.1186/s12934-017-0639-3

Summers DK, Sherratt DJ.. (1984). Multimerization of high copy number plasmids causes instability: CoIE1 encodes a determinant essential for plasmid monomerization and stability.. Cell. 36 (4), p1097-1103.

Summers, D. K., & Sherratt, D. J. (1984). Multimerization of high copy number plasmids causes instability: Cole 1 encodes a determinant essential for plasmid monomerization and stability. *Cell*, *36*(4), 1097–1103. https://doi.org/10.1016/0092-8674(84)90060-6

Tabor, S. (1990). Expression using the T7<scp>rna</scp>polymerase/promoter system. *Current Protocols in Molecular Biology*, *11*(1). https://doi.org/10.1002/0471142727.mb1602s11

Tesfai, B. T., Wu, D., Chen, S., Chen, J., & Wu, J. (2012). Strategies for enhancing extracellular secretion of recombinant cyclodextrin glucanotransferase in *Escherichia coli*. *Appl Biochem Biotechnol, 167*(4), 897-908. doi:10.1007/s12010-012-9747-z

Thomas, S., Holland, I. B., & Schmitt, L. (2014). The type 1 secretion pathway — the hemolysin system and beyond. *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research*, *1843*(8), 1629–1641. https://doi.org/10.1016/j.bbamcr.2013.09.017

Tomizawa J, Itoh T, Selzer G, Som T., (1981), Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. *Proc Natl Acad Sci U S A.* 1981 Mar;78(3):1421-5

Tomizawa, J., & Itoh, T. (1981). Plasmid ColE1 incompatibility determined by interaction of RNA I with primer transcript. *Proc Natl Acad Sci U S A*, *78*(10), 6096-6100.

Tomizawa, J., & Itoh, T. (1981). Plasmid ColE1 incompatibility determined by interaction of RNA I with primer transcript. *Proceedings of the National Academy of Sciences*, *78*(10), 6096–6100. https://doi.org/10.1073/pnas.78.10.6096

Troisfontaines, P., & Cornelis, G. R. (2005). Type III secretion: More systems than you think. *Physiology*, *20*(5), 326–339. https://doi.org/10.1152/physiol.00011.2005

Typas T, Banzhaf Ml, Gross CA & Vollmer W. (2012). From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nature Reviews Microbiology*. 10 (1), 123-136.

van Ulsen, P., Rahman, S. ur, Jong, W. S. P., Daleke-Schermerhorn, M. H., & Luirink, J. (2014). Type V secretion: From Biogenesis to Biotechnology. *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research*, *1843*(8), 1592–1611. https://doi.org/10.1016/j.bbamcr.2013.11.006

Velur Selvamani, R. S., Friehs, K., & Flaschel, E. (2013). Extracellular recombinant protein production under continuous culture conditions with *Escherichia coli* using an alternative plasmid selection mechanism. *Bioprocess and Biosystems Engineering*, *37*(3), 401–413. https://doi.org/10.1007/s00449-013-1005-4

Voulgaris, I., Chatel, A., Hoare, M., Finka, G., & Uden, M. (2016). Evaluation of options for harvest of a recombinant*E. coli*fermentation producing a domain antibody using ultra scale-down techniques and pilot-scale verification. *Biotechnology Progress*, *32*(2), 382–392. https://doi.org/10.1002/btpr.2220

Voulgaris, I., Finka, G., Uden, M., & Hoare, M. (2015). Enhancing the selective extracellular location of a recombinant *E. coli* domain antibody by management of fermentation conditions. *Applied Microbiology and Biotechnology*, *99*(20), 8441–8453. https://doi.org/10.1007/s00253-015-6799-3

Wacker. (2012a). *the wacker secretion system- an Escherichia coli protein production system with unique properties.* Available: http://www.wacker.com/cms/media/documents/markets_brands/bio_secretionsystem.pdf. Last accessed 1st March 2014.

Wacker. (2012b). *Wacker Biotech and XL-protein Sign Long-Term Cooperation Agreement to Produce PASylated Biopharmaceuticals.*Available: http://www.wacker.com/cms/en/press_media/press-releases/archive2012/pressinformation-2012-detail~1_38465.jsp?from_all_summary=true. Last accessed 25th Feb 2014.

Waksman, G., & Hultgren, S. J. (2009). Structural biology of the chaperone–usher pathway of Pilus Biogenesis. *Nature Reviews Microbiology*, *7*(11), 765–774. https://doi.org/10.1038/nrmicro2220

Wang, S.-J., & Zhong, J.-J. (2007). Bioreactor Engineering. *Bioprocessing for Value-Added Products from Renewable Resources*, 131–161. https://doi.org/10.1016/b978-044452114-9/50007-4

Wellcome.ac.uk. (2008). *Secrets of secretion: characterising type IV secretion systems in gram-negative bacteria.* Available: http://www.wellcome.ac.uk/stellent/groups/corporatesite/@msh_publishing_group/documents/web_document/wtx053195.pdf. Last accessed 05th March 2014.

Wikipedia (2008) *File:gram negative cell wall.svg*, *Wikipedia*. Available at: http://en.wikipedia.org/wiki/File:Gram_negative_cell_wall.svg (Accessed: 06 August 2020).

Willey, J. M., Sandman, K., Wood, D. H., & Prescott, L. M. (2022). *Prescott's microbiology*. McGraw Hill.

Wurm, D. J., Slouka, C., Bosilj, T., Herwig, C., & Spadiut, O. (2016). How to trigger periplasmic release in recombinant*Escherichia coli*: A comparative analysis. *Engineering in Life Sciences*, *17*(2), 215–222. https://doi.org/10.1002/elsc.201600168

Www.biopharma.co.uk. (2014). *Cell disruption in the context of a bioprocess.* Available: http://www.biopharma.co.uk/2013/11/26/cell-disruption-in-the-context-of-a-bioprocess/. Last accessed 3rd March 2014.

Xu, B., Jahic, M., Blomsten, G., & Enfors, S.-O. (1999). Glucose overflow metabolism and mixed-acid fermentation in aerobic large-scale fed-batch processes with *Escherichia coli*. *Applied Microbiology and Biotechnology*, *51*(5), 564–571. https://doi.org/10.1007/s002530051433

Yang, H., Lu, X., Hu, J., Chen, Y., Shen, W., & Liu, L. (2018). Boosting Secretion of Extracellular Protein by *Escherichia coli* via Cell Wall Perturbation. *Applied and Environmental Microbiology*, *84*(20). https://doi.org/10.1128/aem.01382-18

Yau, S. Y., Keshavarz-Moore, E., & Ward, J. (2008). Host strain influences on supercoiled plasmid DNA production in *Escherichia coli.* Implications for efficient design of large-scale processes. *Biotechnology and Bioengineering*, *101*(3), 529-544. doi:10.1002/bit.21915

Zamecnik, P.C. & Stephenson, M.L. (1978) Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc. Natl Acad. Sci. USA* 75, 280–284.

Zhang G, Brokx S, Weiner JH.. (2006). Extracellular accumulation of recombinant proteins fused to the carrier protein YebF in *Escherichia coli*. *Nat Biotechnol.*. 24 (1), 100-104.

Zhang, G., Brokx, S., & Weiner, J. H. (2005). Extracellular accumulation of recombinant proteins fused to the carrier protein YebF in *Escherichia coli*. *Nature Biotechnology*, *24*(1), 100–104. https://doi.org/10.1038/nbt1174

Zhang, W.-Y., Dai, R.-M., & Wu, H. C. (1992). Deletion of internal twenty-one amino acid residues of *Escherichia coli* prolipoprotein does not affect the formation of the murein-bound lipoprotein. *FEBS Letters, 311*(3), 311-314. doi:10.1016/0014-5793(92)81127-8

Appendix

The sequence for the Lpp gene:

AGGGTATTAATAATGAAAGCTACTAAACTGGTACTGGGCGCGGTAATCC
TGGGTTCTACTCTGCTGGCAGGTTGCTCCAGCAACGCTAAAATCGATCA
GCTGTCTTCTGACGTTCAGACTCTGAACGCTAAAGTTGACCAGCTGAGC
AACGACGTGAACGCAATGCGTTCCGACGTTCAGGCTGCTAAAGATGAC
GCAGCTCGTGCTAACCAGCGTCTGGACAACATGGCTACTAAATACCGCA
AGTAA

The underlined section describes the RBS of the gene.

The sequence of sample pMMB66EH-Lpp:

GAATTCAGACGCTGGTTAGCACGAGCTGCGTCATCTTTAGCAGCCTGA
ACGTCGGAACGCATTGCGTTCACGTCGTTGCTCAGCTGGTCAACTTTAG
CGTTCAGAGTGTGAACGTCAGAAGACAGCTGATCGATTTTAGCGTTGCT
GGAGCAACCTGCCAGCAGAGTAGAACCCAGGATTACCGCGCCCAGTA
CCAGTTTAGTAGCTTTCATAAGCTT

The underlined parts represent the restriction enzymes EcoRI and HindIII. The bold section describes the antisense orientation of the Lpp gene.

Publication 1:

Utilizing Antisense Technology for Controlled Extracellular Release of Recombinant Proteins From *E. coli* During Fermentation (prepared for submission in 2023)

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Running Head: Utilizing Antisense Technology for Controlled Extracellular Release of Recombinant Proteins from *E. coli* during fermentation

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Abstract:

Escherichia coli is used to produce a wide variety of recombinant proteins. These expressed recombinant proteins were shown to mostly accumulate within the periplasmic region of the cell. After fermentation the proteins must be recovered by cell lysis operations, releasing the whole content of the cell including DNA and Host Cell Proteins which poses difficulties in Down Stream Processing. This study investigated the utilization of antisense technology to increase the released levels of product into the cell culture broth. This work includes the adoption of an appropriate plasmid vector to express as elected antisense RNA that would inhibit the translation of a cell membrane component, murein lipoprotein (Lpp), a major component of the outer cell membrane. The expression of the antisense RNA was controlled via an induction system. In strains containing antisense plasmids, the amount of extracellular release increased from 10 to 15 percent in unmodified strains to 50 percent. This was achieved without compromising the growth rates of the cells or productivity. In summary, this approach can be used to increase extracellular release levels of periplasmic proteins. Further research in optimization of the methodology can potentially increase the yield of released products as well as reduce the cost and time of bioprocessing.

Keywords: Antisense Technology, *E. coli*, Fermentation, Upstream Bioprocess Development, Extracellular release

Publication 2:

Fermentation Optimization: A Path to Elevated Extracellular Recombinant Protein Levels in High Cell Density *E.coli* Fermentation Via Utilisation of Antisense Technology (planned for submission in 2024)

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