A New Approach to Plasmid Upstream Processing for Vaccine and Gene Therapy Applications

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By

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Declaration

I, Olusegun O. Folarin, 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.

Signature

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Abstract

There has been a rise in the interest of plasmid DNA as therapeutics. The rise is evident in the number of ongoing clinical trials involving the use of plasmid DNA. To be useful as therapeutics, the DNA needs to be of high yield and high level of supercoiling. From the bioprocessing point of view, the level of supercoiling can potentially have an impact on the ease of downstream processing. We have approached meeting these requirements through plasmid engineering and developing an optimized fermentation strategy. Two different plasmids (small and large size) were developed. A 7.2kb plasmid was developed by insertion of Bacteriophage-Mu Strong gyrase-binding sequence (Mu-SGS) to 6.8kb pSVβ-Gal. Four *E. coli* strains were transformed with both the modified pSVβ-Gal398 plasmid and pSVβ-Gal. Small scale fermentation and analysis were carried out in triplicates cultures to screen for best-performing strains.

There was over 20% increase in the total plasmid yield with pSVβ-Gal398 in two of the strains. The supercoiled topoisomer content was increased by 5% in both strains leading to a 27% increase in the overall yield. The two strains were investigated further, and an increase in supercoiling and plasmid yield was also observed. The extent of supercoiling was examined by superhelical density (σ) quantification with pSVβ-Gal398 maintaining a superhelical density of -0.022 and pSVβ-Gal -0.019 in both strains. The compactness of the plasmid DNA was also quantified by hydrodynamic diameter (D_h) measurement using the Nanoparticle Tracking Analysis (NTA), and it was observed that pSVβ-Gal398 was more compact with a Dh of 40-59 nm compared to pSVβ-Gal with Dh of 70-90 nm for both strains examined. In order to investigate this approach can be scaled on plasmid, a 27 kb plasmid pSTFλ398 was constructed with its respective control plasmid, 26.6 kb pSTFλ. There was an almost 2-fold increase in the plasmid yield for the SGS

containing plasmid pSTFλ398 and a supercoiled content of 93%. In addition, SGS containing plasmid was maintained integrity when subjected to shear.

An approach was also developed to increase the plasmid yield by developing a batch fermentation for high cell density which involves supplementation with minimal media with amino acids. The media was supplemented with histidine and glycine which have been reported to be implicated in increasing plasmid yield, and methionine for suppressing acetate inhibition. The supplementation allowed the use of initial glycerol concentration as high as 100 g/L with a volumetric yield and plasmid yield of twice as much as using 100 g/L glycerol in the media without supplementation.

The report of this study has shown that plasmid modification with the Mu-phage SGS sequence and optimizing fermentation strategies have beneficial effects on improving not only the yield of total plasmid but also the supercoiled topoisomer content of therapeutic plasmid DNA during bioprocessing.

Impact Statement

Gene therapy offers hope to people suffering from genetic conditions that have impacted their quality of life. Developing a gene therapy requires the use of a vector for transporting the gene of interest into the host cell and naked plasmid DNA is one of the vehicles being used for this purpose. In addition to gene therapy, naked plasmid DNA is also being developed for vaccine application. The advantages of DNA vaccines over conventional vaccines include stability, ability to incorporate multiple antigens genes packed into the plasmid and ease of production. Especially in areas where cold chains are unavailable, developing vaccines that are relatively stable in the absence of these cold chains will make sure these vaccines are available for people living in these areas.

There have been a number of strategies aimed at developing a bioprocessing approach for the production of this molecule for therapeutic application. However, there are a number of challenges that are facing the manufacturing of these molecules. These challenges include quality of the DNA, loss during processing and the overall yield. Plasmid DNA are required to be in supercoiled form to be effective as a therapy. However, due to the manufacturing processing methods, plasmids get sheared and hence losses the supercoiling and become open-circular. The significance of supercoiling loss is that it leads to a lower process yield (as the quality is affected).

This research has tackled all of these challenges by developing approaches to solve the problems of quality (supercoiling) and hence the process yield. By developing a modified plasmid capable of maintaining high quality and developing approaches for the upstream processing, it is possible to improve the quality and the yield of the plasmid DNA.

The findings of this study can be put to use by incorporating the strategies to improve the yield and quality to industrial production of plasmid DNA by maintaining supercoiled level during the harsh conditions of downstream processing. The benefits will include a higher yield produced when a high cell density fermentation strategy can be implemented without the issue of overflow metabolism and contaminations associated with fed-batch cultures. By modification of the plasmid to improve the supercoiling (equivalent to its quality), there can be a higher overall yield achieved. In addition to improving the overall quality, the study also finds that these molecules are able to resist breakage to some extent under harsh conditions used in downstream processing methods.

This study also sheds light into research methodology for dealing with large DNA topoisomers by identifying the behaviours of topoisomers under different analytical conditions. It also provides opportunities for further research as it has opened up further questions about even improving the quality and yield of plasmid DNA.

Finally, the study's findings are being disseminated in peer-reviewed scholarly articles, with some of the findings published already. This will help inform the broader scientific community of the outcome of the study.

Table of Contents

List of Figures

List of Tables

CHAPTER 1. Introduction

1.1 INTRODUCTION

The past few decades have seen a geometric rise in the manufacture of biopharmaceutics for the treatment of diseases. This biopharmaceutics ranges from small molecules to proteins and DNA for gene therapy application. One biomolecule that has received much focus of recent is the plasmid DNA (pDNA) for the development of vaccines and gene therapy application.

Plasmid DNA has however been of such importance in the life science field for over half a century and has been employed in cloning, as an expression vector and for the production of recombinant proteins used to treat diseases that are due to the defective production or lack of essential proteins. However, it was of recent that it was observed that plasmid DNA in itself could be used as therapeutics. Since then, several studies have been dedicated to developing plasmid DNA for vaccines and gene therapy.

There has been breakthrough in the development of plasmid DNA as vaccine for veterinary purposes with approval of DNA vaccines for West Nile Virus in horses in 2005, infectious haematopoietic necrosis virus (IHNV) in salmon fish in 2005, growth hormone-releasing hormone in swine in 2007 and melanoma in dogs (conditional approval in 2007) (Kumaragurubaran and Kaliaperumal, 2013). Several plasmid DNA vaccines and gene therapy for human use are in clinical trials and are expected to be in the market in the next few years Table 1.1. Therefore, there is a need to develop economical bioprocessing methods for the large scale manufacturing of plasmid DNA.

Plasmid DNA vaccines have some advantages over the conventional vaccines of live attenuated, inactivated or killed, subunit vaccines and recombinant proteins. One

advantage of the plasmid DNA is that it elicits both humoral and cell-mediated immune response. There is also an elimination of the risk of virulence-associated with live attenuated virus vaccines. Unlike recombinant proteins that are very labile, plasmid DNA is highly stable; hence, transportation and storage are easier. This makes it easy to deliver vaccines across to the developing countries where cold chains are limited or non-existing. Plasmid DNA has a reduced risk of integration of foreign material it is carrying into the human chromosome (Nichols *et al.*, 1995), the production is also cheaper and faster when compared to the production of recombinant proteins, whole cells and viruses (Carvalho, Prazeres and Monteiro, 2009).

In gene therapy, delivery of the gene can be viral or non-viral. The viral method of gene delivery employs the use of viruses such as adenovirus and adeno-associated viruses, retroviruses and lentiviruses. However, there are some limitations associated with using viral vectors for gene delivery. These include the generation of toxic inflammatory responses against the virus thereby reducing their potency upon re-administration, random integration of the viral vector into the host chromosome and could lead to activation of proto-oncogenes, possible generation of replication-competent viruses by recombination with the host chromosome and insert-size limitation of the viral vector (Abraham, 2003).

Non-viral gene delivery involves the use of non-viral vector which the plasmid DNA belongs, alongside synthetic RNAs (siRNAs). The use of plasmid DNA for gene delivery in gene therapy eliminates or reduces the biosafety concern associated with viral vectors. Worldwide, there is about 452 pDNA-associated gene therapy in clinical trials. This amount to about 14.9% (Figure 1.1) of all the gene therapy clinical trials with only Adenovirus (18.5%) and retroviruses (16.9%) having more (Wiley, 2019). The list includes where plasmid DNA is used as the sole delivery agent and where it

is used as a starting material for viral delivery. Therefore, potentially high demand is expected of plasmid DNA for gene therapy in the near future.

According to data on ClinicalTrials.gov (2020), there are about 166 clinical trials of plasmid DNA vaccines on various infectious and non-infectious diseases as well as cancer. Table 1.1 contains selected list of diseases and the number of trials that are either recruiting, active or completed. A complete list of studies using the plasmid DNA for vaccine application can be found on clinicaltrials.gov

Table 1.1: Diseases for which plasmid DNA vaccines clinical trials are ongoing

Infectious diseases		**Non-infectious diseases**		**Cancer**	
Disease	No of Trials	Disease	No of Trials	Disease	No of Trials
HIV	58	**Hypersensitivity**	6	**Prostate cancer**	9
Influenza	19	**Alzheimer**	1	**Breast cancer**	7
Hepatitis virus	4			**Melanoma**	10
Ebola	11			**Ovarian cancer**	3
Coronavirus	3			**Pancreatic cancer**	1
Papillomavirus	2			**Lung cancer**	7
Herpes Simplex virus	2			**Intraocular melanoma**	2
Marburg	3			**B-cell lymphoma**	5
Dengue	3				
Zika Virus	3				
SERS	3				

Adapted from ClinicalTrials.gov (2020)

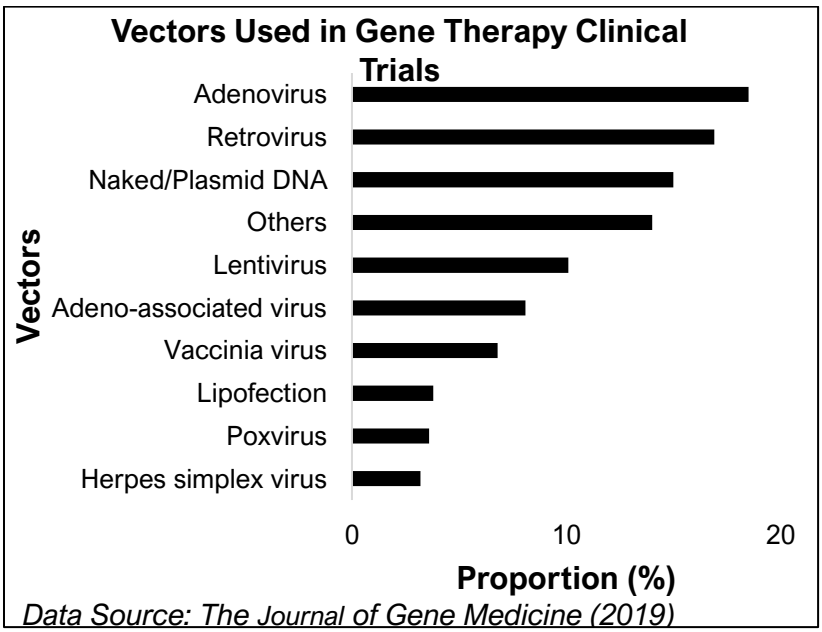


Figure 1.1: Chart showing the percentage of various vectors employed in gene therapy clinical trials as of 2019 (Wiley, 2019)

Attempt to use plasmid as a therapeutic agent is not new and dates back to late 1980s when it was being considered. However, there have been a lot of challenges over the years in developing successful therapeutics, one of which is the integration and adequate expression to elicit a response both as vaccine and gene therapy. When compared to viral vectors, this can be a bottleneck with plasmid DNAs. Viral vectors are easily integrated into the host chromosomes and hence expressed. It is of noteworthy that there have been success on that bottleneck by developing effective delivery methods to allow enough plasmid DNA get into the host cell (Suschak, Williams and Schmaljohn, 2017; Appelbe *et al.*, 2018; Beals, Kasibhatla and Basu, 2019).

However, the production of plasmid DNA on a large scale is still a challenge. Plasmid DNA is required in a large amount (mg) to elicit an immune response that is equivalent to the use of the conventional method (Leitner et al. 1999). This means that for the vaccination of a population for example during a pandemic, a large amount of the product will be required and a production scale of 100 m^3 (Hoare *et*

al., 2005) will be required to meet the demand. With the limitations of the current un-optimized production processes, and the bioprocess challenges of that scale of production, the demands for the product will not be met. Therefore, there is a need to develop a more robust processing method for the production of plasmid DNA.

The bioprocess operation stages involved in the production of plasmid DNA is shown in Figure 1.2. The optimization of the bioprocessing of plasmid DNA could involve the modification of host cell, modification of the plasmid vector, modification of the fermentation strategy as well as the modification of external factors during fermentation.

Tackling the challenges facing plasmid DNA bioprocessing will go a long way in the optimization of the process by improving the yield, productivity and level of supercoiling. This is essential as there as the demand for plasmid DNA for vaccines and gene therapy is expected to rise geometrically soon.

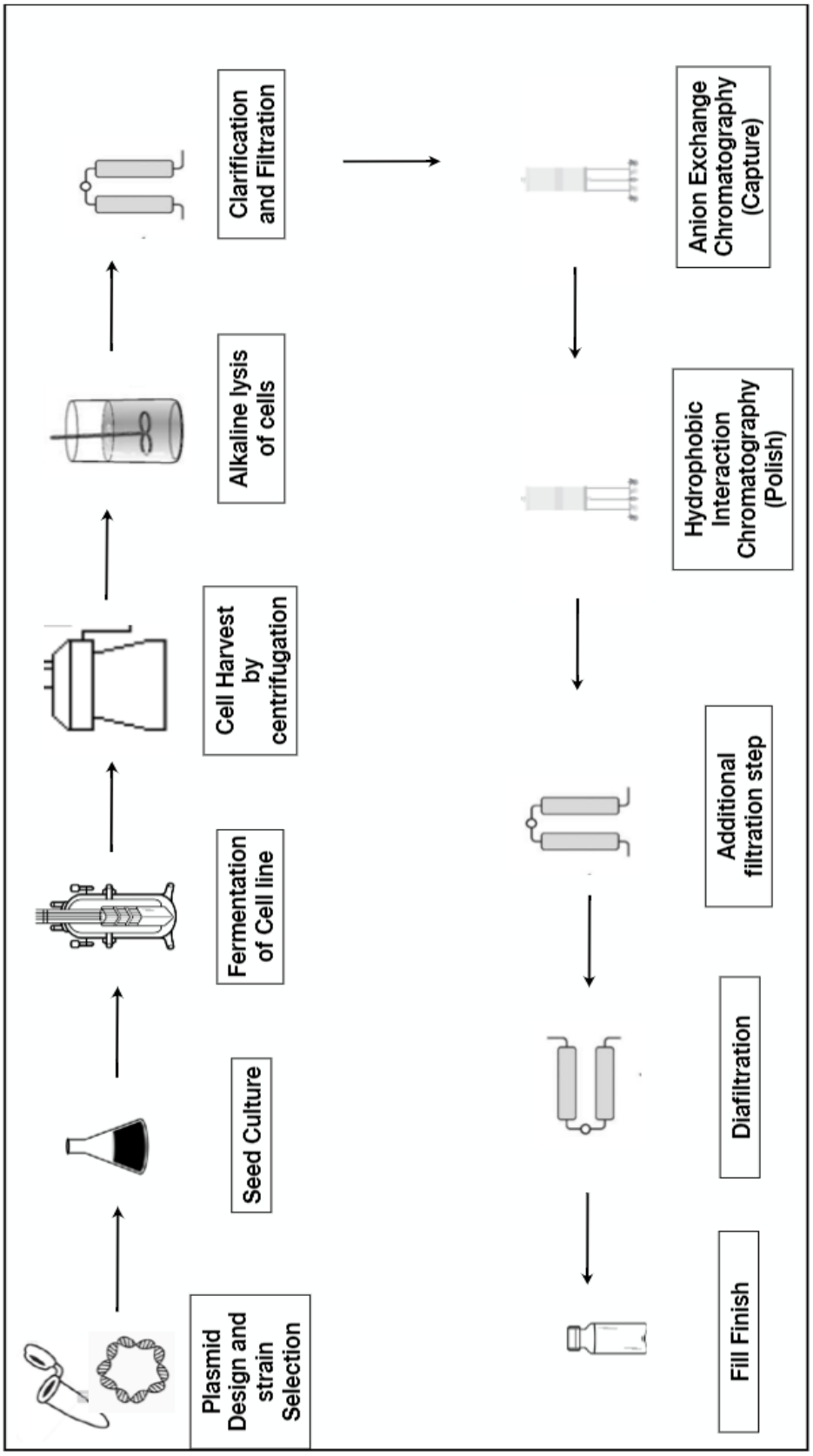


Figure 1.2: Bioprocess operations for the production of plasmid DNA including Master Cell Bank production, upstream processing, downstream processing and fill finish stages

REVIEW OF PLASMID DNA APPLICATION AND ITS BIOPROCESSING

1.2 Plasmids DNA for Therapy

The use of plasmid for gene therapy application stems from the knowledge that the ability to express a given protein within a specific locus in the chromosome could replace the protein if it were absent or non-functional. Plasmid DNA offers the opportunity to be able to provide the means to express the proteins and hence have been exploited. Plasmid biopharmaceuticals have also been advocated as a potential means to solve diseases that are not only caused by a single defect but also diseases caused by multiple factors such as cancer (Table 1.1) neurological diseases and cardiovascular diseases.

There is an additional demand of plasmid DNA as raw materials for viral vectors and as template for RNA vaccines. Plasmid DNA have been used as carriers in Chimeric Antigen Receptor (CAR) T cell where the contained plasmid as well as the cells are used. Plasmid DNA has also been used as starting materials for viral vectors (including lentiviral vectors and adeno-associated viruses) and RNA products (Hitchcock, 2016)

1.2.1 Genetic Disorders

Most genetic disorders are characterized by the absence or non-functional protein and at the genetic level, by a defect in a single gene (monogenic disorders). As a result of the defective genes, individuals suffering from these conditions have symptoms that could be from mild to life-threatening. While lifestyle changes easily manage some, others are challenging to manage. Gene therapy offers the promise of restoring a normal lifestyle by replacing the missing or defective protein at the

genetic level. maintain will involve introducing a correct version of the defective gene, and activate the expression of that gene to produce the corresponding protein.

There are certain conditions or genetic disorders that are the favourite targets for gene therapy due to their prevalence. Examples include haemophilia, which is an X-linked disorder characterized by excessive bleeding due to the defect in the clotting factors VIII and IX (Srivastava *et al.*, 2013), cystic fibrosis; a disease caused by the abnormal cystic fibrosis transmembrane regulator (CFTR) that affects multiple organs (Prickett and Jain, 2015), lysosomal storage disorders and severe combined immunodeficiencies, a disease characterized by profound reduction in the amount of T-lymphocytes produced (Naldini, 2015).

As of 2018, there are about 3000 gene therapy clinical trials ongoing, with 4.2% of these trials in phase III and 56.1% in phase I (Wiley, 2018) and 11.5% of all the trials being for monogenic diseases (diseases caused by a defect in a single gene). One of the drawbacks of developing gene therapies is that sometimes it does not get integrated into the chromosomes, hence leading to transient expression. The implication of transient expression is that multiple interventions are needed, which comes with the possibility of developing an autoimmune response, although no clinical evidence has been reported so far (Prazeres, 2011). However, plasmids are safer in this regard as they are less immunogenic compared to their viral counterparts (Schleef, 2013)

One of the goals of gene therapy is to develop single intervention therapy George et al. (2017) reported single intervention for the treatment of haemophilia B without the need for further treatment, using adeno-associated viral vector. They reported a near-elimination of bleeding in the subject. Although adeno-associated viral vector was used in the study, there are studies that have reported the use of

plasmid and naked DNA for cystic fibrosis (Rao and Zacks, 2014; Cooney *et al.*, 2016) and von Willebrand disease (Rao and Zacks, 2014).

For most of the studies in gene therapy that have been reported, polygenic and multifactorial diseases have been the major topics. These conditions are caused by numerous factors including genetic predisposition and environmental factors. Examples include cancer, cardiovascular diseases and neurological diseases.

1.2.2 Cancer

About two-thirds of all gene therapy clinical trials are devoted to treating cancer (Wiley, 2018). This is no surprise as one in three people are bound to have cancer in their lifetime. Also, there is a large number of studies devoted to the study and understanding of this group of diseases. The therapeutic strategies for developing gene therapy for treating cancer include prevention of the formation of blood vessels around the tumour (anti-angiogenesis), induction of apoptosis, stimulation of immune cells and activation of prodrugs capable of killing cancer cells (Cao, Cripps and Wei, 2010; Ortiz *et al.*, 2012).

Cancer cells grow rapidly and hence require nutrients and oxygen to support their proliferation. These nutrients are supplied by a network of blood vessels around the tumour mass. However, the tumour cells are able to stimulate the host cells to produce new vessels so that sufficient nutrients can reach them (Sherwood, Parris and Folkman, 1971). Extensive studies have shown that by interfering with the production of a new vessel which can be made possible by introducing antiangiogenic genes to the tumour mass, the calls can be starved and could potentially provide a means to treat cancer (Dimova, Popivanov and Djonov, 2014; Li *et al.*, 2018). Examples of studies that have targeted this strategy includes introducing the p53 genes to inhibit angiogenesis (Prabha, Sharma and

Labhasetwar, 2012), genes coding for a splice variant of the VEGF receptors (Kendall and Thomas, 1993) and some exploiting the antiangiogenic properties of cytokines like interleukin-12 (Fewell *et al.*, 2009).

Another therapeutic strategy for treating cancer is inducing apoptosis by introducing the missing tumour suppressor gene; an example is *p53* mentioned earlier. The p53 gene has been implicated in a lot of cancer, and a mutation in this gene leads to the malfunctioning of DNA repair and control. Re-establishing the wild-type p53 gene stimulates suicide in tumour cells. This approach has been attempted using both viral and non-viral vector such as plasmid (Tazawa, Kagawa and Fujiwara, 2013).

Suicide gene therapies have also been developed where a gene coding for a different enzyme is introduced into the tumour. Once the protein is expressed, a prodrug is introduced. The expressed foreign enzyme then converts the prodrug into an active cytotoxic drug that is capable of killing the tumour cells (Yazawa, Fisher and Brunicardi, 2002). Although shown on a proof of concept level, there is still a lot of research being carried out on this strategy (Zarogoulidis *et al.*, 2013).

All of the strategies being developed require a vector of one form or the other and plasmid DNA is one of the non-viral vector being developed as a carrier for these therapeutics.

1.2.3 Cardiovascular Diseases

Cardiovascular diseases are the leading cause of death worldwide. They are mostly caused by vascular dysfunction and can be as a result of multiple factors from genetic predisposition to environmental factors and lifestyle (Prazeres, 2011). Although some cardiovascular diseases are caused by a single gene defect,

however, these are very few as most cardiovascular diseases are caused by a combination of defects in multiple genes as well as environmental factors.

There are preventive and therapeutic actions that can be taken to reduce the burden of cardiovascular diseases. Most of these actions include lifestyle changes, medications, heart transplant and medical devices such as artificial pacemakers. However, gene therapies are being developed to reduce the impact of cardiovascular diseases, particularly coronary heart diseases, peripheral arterial diseases and cardiovascular diseases that involve blockage of the arteries (Katz *et al.*, 2015). Gene therapy strategies for cardiovascular disease treatment involve the induction of neovascularization by introducing angiogenic agent such as vascular endothelial growth factor (VEGF) and hepatocyte growth factors (HGF), so as to alter the balance of angiogenic and antiangiogenic agent for blood vessels development (Ylä-Herttuala *et al.*, 2017).

1.2.4 Neurological Disorders

Gene therapy approaches have been developed for the treatment of neurological disorders such as Parkinson's disease. Neurological disorders are characterized by a dysfunction in the nervous system, which can be mild to severe. Single and multiple genes could be implicated in neurological disorders; hence, a gene therapy approach strategy and suitable vectors are being studied. A detailed review of gene therapy targets for neurological disorders can be found in Deverman *et al*., (2018)

1.3 Plasmid DNA for Prophylaxis

Plasmid DNA has long emerged as a candidate for the prevention of infectious diseases. This reason is one of the most appealing application of plasmid DNA for therapeutics application. There is a long list of examples of infectious diseases to which plasmid DNA vaccines are being developed (both humans and animal). As a matter of fact, there are already approved plasmid DNA prophylaxis for veterinary use on the market.

Plasmid DNA vaccines can protect against infectious diseases agents by activating the innate immune system and production of antigen-specific antibodies. Plasmid DNA is also able to elicit both cell-mediated and humoral response. It has been reported that the immune response elicited by plasmid DNA depends on the route of administration (Prazeres, 2011). However, early studies showed that DNA vaccines are poorly immunogenic in humans (Laddy and Weiner, 2006), but recent advances in delivery and packaging have shown an improvement in immunogenicity in humans.

Usually, the antigen-coding genes find its way into the nucleus of the cell upon introduction, where it is transcribed into mRNA. This leads to the synthesis if the antigen with two possible routes if action: translocation to the endoplasmic reticulum, where it is degraded into smaller peptides to elicit immune response known as endogenous antigen processing (Leifert *et al.*, 2004; Liu, Wahren and Hedestam, 2006; Wahren and Liu, 2014) or secretion into the extracellular medium where it is taken up by antigen-presenting cells (APCs) known as exogenous antigen processing (Leifert *et al.*, 2004).

The uptake by APCs is then followed by presentation to T cells which can either involve the antigenic peptides binding to class I Major Histocompatibility Complex

(MHC) for recognition by $CD8^+$ T cells (Ramirez and Sigal, 2004; Basta and Alatery, 2007) or binding to class II MHC for recognition by $CD4^+$ T cells (Leifert *et al.*, 2004). $CD8^+$ T cells go on to proliferate into cytotoxic cells that are able to destroy foreign objects that present the same antigen while $CD4^+$ T cells proliferate into helper T cells and release of cytokines to aid B cells and $CD8^+$ cells.

1.4 Role of Plasmid-Borne Gene Product

The product of plasmid vaccines and gene therapies can act in different ways depending on what the end goal is; that is to act as vaccines and protect against infectious diseases or to act as gene therapy or for treating certain diseases. For vaccine application, the plasmid-borne product is used to either stimulate an immune response against specific antigenic substance or to boost an already existing immunity. Products for cancer treatments have roles mainly to block and kill and sometimes to stimulate an immune response. These roles are discussed below.

1.4.1 Stimulation of Immune Response

Plasmid-borne product for stimulation of immune response can act as prophylaxis or therapeutics. Prophylactic vaccines are administered to healthy people to prevent them from getting infected in the future. The vaccines carry genes coding for the antigens that are associated with the causative agents of the diseases. Once transfected, the antigen is expressed, and an immune response is elicited. A significant difference between DNA vaccines and traditional protein-based vaccines is that the antigens are synthesized upon transfection compared to introducing the antigens directly. The significance of employing DNA vaccines mode of immune response stimulation is that it mimics natural infection.

Therapeutic vaccines are used to treat an existing condition, for example, cancer. The endogenous expression of the antigen leads to stimulation of tumour immunogenicity and so the body's own immune system can be recruited for the elimination of cancer cells (Lowe, Shearer and Kennedy, 2006)

1.4.2 Boosting Physiological Responses

Plasmid-Borne products can also be used for physiological responses by increasing the expression of specific gene products. By adding more copies of the gene of interests, there is an acceleration and increase in the expression of the protein-product which are otherwise normal. Examples include an increase in the level of endogenous hormones, increase in the level of proteins like erythropoietin and increase in expression of vascular endothelial growth factor (VEGF) to promote vascularization in cases of cardiovascular diseases (Zhong *et al.*, 2003).

1.4.3 Cytotoxicity

The goal of cytotoxic therapy, particularly for cancer treatment, is to kill specific host cells, usually tumour cells. The product of gene therapy can be used to kill cancer cells indirectly. The product act as part of a more extensive, complex network of events that eventually lead to the death of the target cells. The product action could have roles including replacing missing protein, e.g. the tumour suppressor gene p53, stimulating the immune system or introducing new functions that ultimately leads to the death of cancer cells (Yazawa, Fisher and Brunicardi, 2002).

1.4.4 Blocking Expression of Certain Genes

Plasmid-borne product can also be used for blocking or inhibition of the expression of specific host gene, especially if the expression of this gene is implicated in a given pathology. Examples include the introduction of anti-sense RNA that is capable of hybridizing to a specific region of mRNA transcripts thereby inhibiting the expression of the gene product, the introduction of small interfering RNAs (siRNAs) that are capable of silencing the gene of interest. Usually, the product of these plasmid-based therapies is RNA transcripts (Kaykas and Moon, 2004).

1.4.5 Replacement of Defective Genes

Products of plasmid-based therapies can be used to replace defective or missing genes. This can be used to treat monogenic conditions such as cystic fibrosis (Rao and Zacks, 2014; Cooney *et al.*, 2016) and polygenic conditions where multiple genes are missing or defective. An example is the delivery of plasmid that is capable of increasing or replacing the gene for erythropoietin production in patients suffering from anaemia (Sebestyén *et al.*, 2007).

1.5 Considerations for the Design of Plasmid

For plasmid to serve as a vaccine, it must be able to elicit an immune response equivalent to that of the conventional methods. This means that the plasmid must be carrying an insert capable of leading to expression of the antigen to which the body can elicit a response to (Mor and Eliza, 2001). For gene therapy application, the plasmid insert is designed to express the missing protein or enzyme in the animal cell, hence restoring structure and function missing as a result of the absence of the gene (Friedmann, 1989).

The expression of the antigen in the cell is essential for the effectiveness of the plasmid as a vaccine because the expressed protein is the therapeutic aspect of the model. Therefore, there is a need to design the plasmid such that the insert will be expressed adequately in the cell. In addition to having the insert, there is also a need for the presence of replication machinery for the plasmid for the large scale production.

1.5.1 Concept of Plasmid Amplification

Plasmids are small, closed circular extra-chromosomal DNA molecules that are found in bacteria cells. They are usually negatively supercoiled and in a compact form in the bacteria cell except for extreme thermophiles (Valenti *et al.*, 2011). Plasmids confer some specific characteristics to their host such as antibiotics resistance, hydrocarbon catabolism and heavy metal resistance. They, in turn, use their host replication machinery to replicate in an autonomously controlled way (Summers, 1996).

Plasmids possess an origin of replication; a part of the circular DNA where the double strands are separated to signal to the start of the replication process. Initiation of plasmid replication requires a specific Rep protein; an initiator protein encoded by the plasmid. The Rep initiator protein interacts with specific DNA sequence at the origin of replication. The interaction of the Rep protein with the sequence signals the initiation of replication (Kornberg and Baker, 1992). For specific plasmid such as the ColE1, a replication initiator protein is not required. The plasmid makes use of DNA polymerase I (DNA Pol I) of the host to initiate plasmid replication (del Solar *et al.*, 1998; Brantl, 2014)

The origin of replication consists of a sequence that promotes the synthesis of RNAII and sequence that allows the hybridization of the RNAII to the DNA (Tomizawa,

Sakakibara and Kakefuda, 1975). Control of plasmid replication is initiated at the origin of replication. This ensures that there is a constant concentration of the plasmid in the host cell. A plasmid-encoded repressor initiates the regulation of plasmid replication.

A repressor protein (Cop), which is unstable, is constantly expressed by the plasmid and the synthesis and degradation of this repressor protein maintain the equilibrium concentration of the plasmid in the cell; a model known as the inhibition dilution model (Summers, 1996). Majority of plasmids developed for vaccines and gene therapy are derived from ColE1 plasmids. A detailed review of replication and regulation of plasmid is presented elsewhere (del Solar *et al.*, 1998).

1.5.2 Structural Characteristics of Plasmid

The topology of plasmids is vital for its therapeutic application. The topology of DNA is implicated in all of the chromosomal processes taking place in the cell. Hence, understanding the topology of plasmid DNA makes it easy to predict the stability and efficacy of plasmids as vaccines. Double stranded DNA including plasmids exist in different conformation, which may depend on the chemical environment, sequence and the organism in which the DNA exist.

The major conformation of double-stranded DNA are A, B and Z. The A conformation of DNA is a right-handed helical structure with a wide minor groove and a narrow major groove. The B-DNA, on the other hand, has a wider major groove and a narrow minor groove. The Z-DNA is a left-handed helical structure which results from the presence of methylated segment in the B-DNA (J. M. Berg, Tymoczko and Stryer, 2002). The most common conformations found in cells are the B- and Z- forms (Ghosh and Bansal, 2003).

Due to the nature of plasmids being circular, they also exist in structures such as knots and catenanes formation. In a higher level of topology, several plasmid molecules can exist as multimeric concatenations which form from recombination after replications (Higgins and Vologodskii, 2015).

Supercoil in plasmid DNA is created by the alteration of the twist around the axis of the double helix which places torsional stress on the circular DNA (Figure 1.3). The nicking of the closed DNA relieves the torsional strain on the DNA, and it becomes relaxed. There exists a mathematical relationship between the twist around the axis of plasmid DNA and its deformation, this is known as the linking number (Lk). Linking number is the number of intersection between one strand of DNA and the spanning surface of the other strand. Conventionally, linking number is positive if the DNA is of right-handed double helix structure. However, closed circular DNA is described using the linking number difference (ΔLk). The plasmid DNA is assumed to be supercoiled when $\Delta Lk \neq 0$.

The topology of plasmid is better expressed in terms of superhelical density (σ); which is a normalized form of ΔLk (Higgins and Vologodskii, 2015). Majority of plasmid DNA exists as negative supercoil, with σ value between -0.03 and -0.09 (Bauer, Crick and White, 1980).

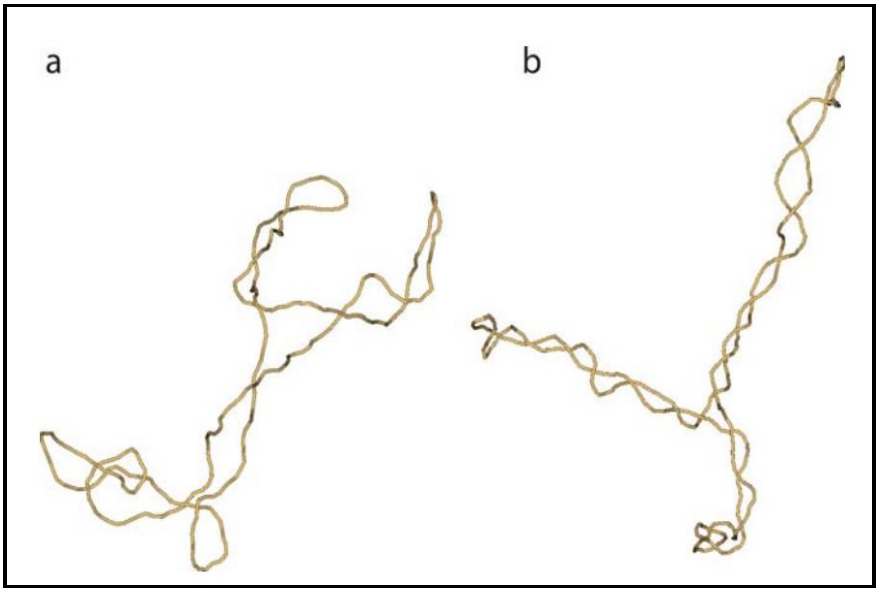


Figure 1.3: Supercoiled 4.4kb plasmid DNA with a superhelical density of (a) -0.03 and (b) -0.06 [Adapted from Higgins & Vologodskii, 2015]

1.6 Basic Components of Plasmid Construct

Plasmids need to be amplified before being administered into animals. Hence for the design of a plasmid, the major components to be considered are the insert (gene of interest), a eukaryotic promoter sequence, plasmid replication machinery and the selectable marker required for the selection of plasmid containing cells (Figure 1.4) (Montgomery and Prather, 2006).

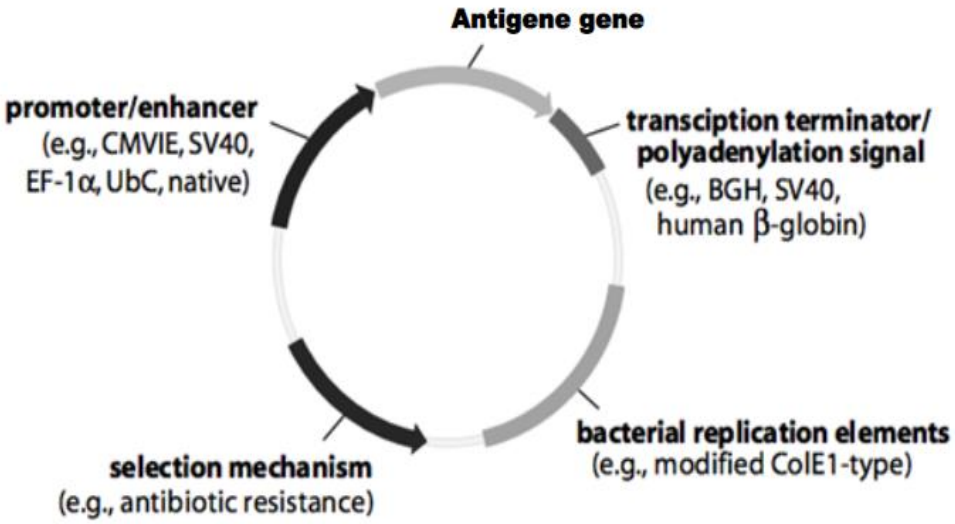


Figure 1.4: Basic components of a therapeutic plasmid [Adapted from Prather et al., 2003]

1.6.1 Origin of replication

The origin of replication is vital for the plasmid construct as it allows for amplification of the plasmid during production. There are a number of origins of replication for plasmids, and the selection of a suitable origin of replication for plasmid construct is dependent on the yield of the plasmid during bioprocessing. Majority of the gene therapy plasmids that have been constructed employs the theta replication origins (Williams *et al.*, 2009), which requires an RNA primer for DNA replication (del Solar *et al.*, 1998). The bulk of therapeutics plasmids constructed until date have used the origin of replications derived from pUC and pBR322 plasmids.

In the selection of an origin of replication for plasmid construct, the copy number of the plasmid is taken into consideration as this directly affects the yield of the plasmid during production. Majority of theta plasmid replication origins have been modified to yield a high copy number plasmid. pMB1 and ColE1 origins have been

modified by point mutation of *rop protein* gene involved in regulation to produce the derivatives pUC18 and pMM1 respectively. This has led to 30-40 fold increase in copy number (Lin-Chao, Chen and Wong, 1992; Camps, 2010). High copy number temperature-sensitive R1 derivatives have been designed and isolated with vaccines vector developed with the modified origin of replication (Hamann, Nielsen and Ingersle E, 2002).

One implication of engineering origin of replication of plasmid to yield high copy number plasmid is the loss of plasmid partitioning. In low copy number plasmids, partitioning occurs during replication to allow all daughter cells have at least a copy of the plasmid. This prevents the loss of plasmid and generation of plasmid-free cells (Guynet and de la Cruz, 2011). A dedicated system of partitioning ensures that all daughter cells have a copy of the plasmid (Nordstrom and Austin, 1989). In the absence of selection mechanism, segregational instability often occurs during the amplification of high copy number plasmid since segregation is random (Million-Weaver and Camps, 2014).

1.6.2 Selectable Marker

The plasmid is inserted into a bacterium for amplification; therefore there is a need to select the plasmid bearing population for propagation selectively. Conventional methods of selecting plasmid bearing population have involved antibiotics resistance gene coded on the plasmid and selecting against the antibiotics. β-lactamase is found in the majority of plasmid and confers resistance against β-lactam antibiotics such as ampicillin. However, the use of antibiotics is undesirable due to their use in the treatment of various infection in humans. If the

antibiotics are not removed entirely during the production of plasmid, there is a possibility of infectious bacteria developing resistance against them.

Plasmid bearing the kanamycin-resistance gene has also been developed for selection. The gene codes for an aminoglycoside enzyme that degrades kanamycin and thus allowing the use of kanamycin as it is not widely used in humans (Vandermeulen *et al.*, 2011). Generally, the antibiotic-free selection is preferred, especially for the production of therapeutics plasmid DNA. There have been some efforts made to develop antibiotic-free plasmid selection (discussed below). This will not only eliminate the use of antibiotics, it will also reduce the plasmid size and burden on the cell as there is no more expression of the product coded for by the gene (Cunningham, Koepsel, *et al.*, 2009).

1.6.3 Eukaryotic Promoter and Terminator Sequence for Gene Insert

The gene on the plasmid is expected to be expressed when it reaches the nucleus of the target cell, therefore it is important that the plasmid construct be designed to express the gene in a eukaryotic cell. One way of making this possible is to insert a eukaryotic promoter sequence close to the gene. This means the gene is only expressed in a eukaryotic cell. Typical eukaryotic promoter sequence that has been used in the construction of therapeutic plasmid include CMV, SV40 and RSV which are all viral promoters sequence (Manthorpe *et al.*, 2005). Other promoter sequences have also been demonstrated to be effective. For example, promoters from the human polyubiquitin C and elongation factor 1α resulted in gene expression up to four times more compared to the viral promoter sequences (Gill *et al.*, 2001).

The understanding of transcriptional termination control in eukaryotic cells is little; however, majority of therapeutic plasmid constructs have incorporated

polyadenylation sequence derived either from SV40 T-antigen, bovine growth hormone gene or the rabbit β-globin gene (Lara and Ramírez, 2012). Nuclear targeting sequence has also been included in the construct to increase the efficacy of the gene (Young, Benoit and Dean, 2003).

1.6.4 Plasmid Engineering for Therapeutics Application

Several strategies have been developed to increase the yield and efficacy of therapeutic plasmid for vaccine and gene therapy application. One of these strategies is engineering the plasmid of interest by modifying it while making sure it still maintains replicative capability. Some of these modifications are discussed below.

1.6.4.1 Reducing Plasmid Size

Reducing the size of the plasmid reduces the metabolic burden on the host cell, thereby increasing the productivity of the plasmid DNA (Rozkov *et al.*, 2004). Strategies have been employed to reduce plasmid size. There have been approaches to eliminate all prokaryotic sequence in a plasmid with exception to the sequence involved in replication. This has led to the design of mini-circle plasmids and Minimalistic Immunogenically Defined Gene Expression (MIDGE). The MIDGE is simply a small-sized linear double-stranded DNA which is covalently closed with stem-loop to prevent exonuclease attacks (Schakowski *et al.*, 2001). They are as small as 1.2 kb and have been reported to improve transgene expression (Schakowski *et al.*, 2007).

The mini-circles, on the other hand, are supercoiled plasmid DNA but without the origin of replication and antibiotics resistance gene. Usually, the whole plasmid is constructed with *attP* and *attB* sites. After amplification, the plasmid is excised at the

attP sites to yield two small DNAs; one of it containing the gene of interest and eukaryotic sequence and the other part containing the unwanted prokaryotic sequence (Darquet *et al.*, 1997). This mini-circles have been reported to be very effective (Dietz *et al.*, 2013). However, there is a lower yield from the bioprocessing point of view with as low 60% of the starting materials. In addition, dimeric plasmids and concatenates are purified alongside the plasmid of interest (Darquet *et al.*, 1997).

1.6.4.2 Development of Antibiotics-free Selectable Marker

In order to eliminate antibiotics in the selection of plasmid bearing cells, attempts have been made to modify both the plasmid and the host cell. Auxotrophic complementation has been developed whereby the cell is lacking an essential gene. An example is the development of glycine auxotrophy obtained by the mutation of *glyA* gene that codes for an essential enzyme in the glycine synthesis pathway. The modified cell is complemented with a plasmid bearing a copy of the gene and plasmid-bearing cells selected in a glycine free media (Vidal *et al.*, 2008).

Toxin-antitoxin based systems have also been developed. This mechanism is found in bacteria as a form of regulating metabolism but has been exploited for selection purposes (Yamaguchi, Park and Inouye, 2011). They are also known as post-segregational killing systems. The toxic protein (or RNA) and its inhibitor genes are inserted into the plasmid. The mechanism is based on the fact that the toxic protein has a more stable half-life than its inhibitor. When the plasmid is lost, there is an accumulation of the toxin with no inhibitor to bind; thus it induces cell death. This mechanism has been used in some studies (Pecota *et al.*, 1997; Van Melderen, 2002; Szpirer and Milinkovitch, 2005).

Other selection mechanisms that have been reported include operator-repressor titration (Cranenburgh *et al.*, 2001), antisense RNA regulator (Luke *et al.*, 2009), growth essential gene overexpression (Goh and Good, 2008) and mini-circles (Mayrhofer, Schleef and Jechlinger, 2009); all of which have been successful in eliminating antibiotics use in selection.

1.6.4.3 Increasing plasmid supercoiling and stability

Modification of the plasmid vector is essential for maintaining the supercoiled activity of the plasmid as well as its monomeric form. There have been a number of studies that have investigated external factors and their effect on plasmid supercoiling (O'Kennedy, Ward and Keshavarz-Moore, 2003; Yau, Keshavarz-Moore and Ward, 2008). However, there are very few reports that have investigated the effect of modification of plasmid DNA to increase supercoiling and superhelical density of plasmid DNA. DNA supercoil is introduced by the DNA gyrase, a Type II Topoisomerase which is tightly regulated alongside the DNA topoisomerase I; which relaxes the DNA. DNA gyrase overexpression in *E. coli* has only led to a slight increase in plasmid supercoiling (Snoep *et al.*, 2002).

The catalytic activity of the DNA gyrase involves binding to specific sequences on the DNA and using energy from ATP to introduce negative supercoil into DNA (Reece, Maxwell and Wang, 1991). Identifying the sequence and exploiting the extent of binding can increase the rate at which gyrase introduces supercoil on the DNA. A number of strong gyrase binding sites have been identified in plasmid pBR322, pSC101 and the bacteriophage Mu. The Mu phage gyrase binding sequence has been reported to have a strong affinity for the binding of gyrase, a property that

stimulates the replication and packaging of the Mu bacteriophage in its host (Pato and Banerjee, 1999).

This sequence is found in the middle of the 37 kb genome of the bacteriophage Mu (Oram *et al.*, 2003) and contain an 8bp where the gyrase binds and inserts supercoil to the Mu genome (Morgan *et al.*, 2002). Exploiting the efficiency of the Mu phage SGS for increasing plasmid supercoil and superhelical density will mean a potential increase in the supercoil nature of the plasmid further.

Further studies have shown that the length of the left and right of the key sequence on Mu phage SGS can also affect the efficiency of the sequence in inserting supercoil into plasmid DNA. In a separate study, Hassan et al. (2016) tested different lengths of the SGS and found out that the 398 bp sequence, which is the longest of the different SGS length tested, significantly increased the plasmid supercoiling and superhelical density of the 2.7 kb plasmid. It has also been reported that the sequence may be involved in the increased in segregational stability of the plasmid (Hassan, Keshavarz-Moore and Ward, 2016).

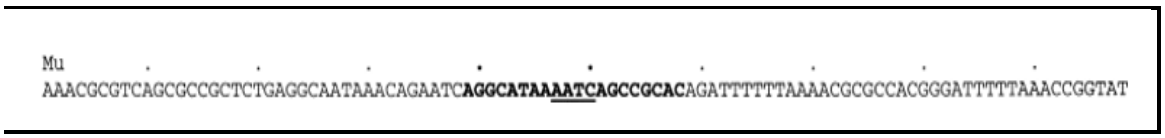


Figure 1.5: The Mu phage SGS sequence. The bold segment indicates the sequence implicated with nicking upon DNA gyrase binding.

1.7 Host Cell for Plasmid Amplification

The choice of the host cell is one of the deciding factors in the bioprocessing of biotherapeutics. Depending on the product, bacterial cells and fungal cells such

as *E. coli* and yeast cells have been the favourite strains for industrial applications. The properties of suitable host strain include the ability to be transformed or transfected by a plasmid which carries the gene of interest into the cell and the ability to culture them to high biomass.

1.7.1 Choice of Host Cell

E. coli has been employed as a suitable host for the production of recombinant proteins due to the vast repository of information available for the organism and its use in the production of plasmid DNA (Cai, Rodriguez and Hebel, 2009; Lara and Ramírez, 2012). Several strains of *E. coli* have been selected for the production of plasmid DNA based on laboratory use and commercial availability. Commercial *E. coli* strains include strains that have been adopted widely in industry and marketed for specific purposes, while research strains are those adopted for research purposes. These include *E. coli* K-12 strains like JM108 (Huber et al., 2005), DH5α (O'Kennedy, Baldwin and Keshavarz-Moore, 2000; Borja *et al.*, 2012; Gonçalves *et al.*, 2014), GALG20 (Gonçalves *et al.*, 2014), BL21(DE3) (Phue *et al.*, 2008), SCS1-L (Singer, Eiteman and Altman, 2009) and DH10B (Lahijani *et al.*, 1996).

Usually, host cells are selected based on experiments carried out on the various strains available. Yau *et al.,* (2008) demonstrated that the production of plasmid is strain/plasmid dependent, hence it is important to select suitable strain for the specific plasmid of interest.

1.7.2 Engineering of Host Cell for Plasmid DNA Production

Modifications targeted at improving the yield of plasmid DNA have been carried out on the host organisms. These modifications involve gene knockout and

gene overexpression in order to reduce the metabolic burden on the host organisms or direct the host machinery for plasmid DNA replication (Gonçalves *et al.*, 2012). Knabben *et al*. (2010) have also modified *E. coli* W3110 to yield strain VH33 where *pts* gene responsible for glucose transport has been knocked out and replaced with *galp*+ gene which codes for galactose permease, to overcome the issues of acetate accumulation when running batch culture using glucose as the carbon source. This has led to a reduction in acetate accumulation and increase in plasmid yield (doubling that of the parent cell) (Knabben *et al.*, 2010). Furthermore, they deleted several genes in the VH33 strain to yield a modified VH34; all of which has been targeted at increasing the plasmid yield.

Other study groups have modified central metabolic pathways by increasing flux into the pentose phosphate pathway (Flores *et al.*, 2004) and by knocking out the pyruvate kinase gene (Cunningham, Liu, *et al.*, 2009). However, the excessive modification of host cell may lead to over-modification and hence it becomes difficult to control and predict the physiological activities taking place in the host. Being able to control the host cell is very essential in large scale bioprocessing operations.

1.8 Cultivation Processes and Fermentation Strategy

Established bacterial cultivation techniques used for recombinant protein production have been adopted for the production of plasmid DNA. However, there is a need for increase in productivity for plasmid DNA production due to its large quantity required for therapy. Several approaches have been employed to increase productivity (volumetric plasmid yield and specific yield) of plasmid DNA. Some of these approaches are discussed in the following sections.

1.8.1 Culture Media

The culture media is the source of nutrient for growth and synthesis of materials in the cell: therefore, approaches to optimize media for plasmid DNA production have been attempted. Carbon to Nitrogen ratio (C: N) of the medium influences the plasmid yield. O'Kennedy *et al*., (2000) reported that at a specific ratio of carbon to nitrogen, the plasmid yield is increased. Supplementation of optimized concentration of glutamate and ammonium chloride have also been reported to improve plasmid DNA production (Voss *et al.*, 2004).

It has also been reported that semi-defined medium supplemented with casamino acid (SDCAS) supported high yield and plasmid stability compared to medium containing soy amino acids and LB medium (O'Kennedy, Baldwin and Keshavarz-Moore, 2000). In another study, Silva *et al.,* (2011) reported that tryptone as an amino acid source is enough to increase plasmid yield. Although amino acids are essential nutrients to cell growth, amino acid starvation and limitation has been reported to induce plasmid amplification (Hecker, Schroeter and Mach, 1985; Wróbel and Wegrzyn, 1997; Silva, 2011).

However, identification of the specific amino acid(s) involved in plasmid amplification and eliminating them from feed will make it possible for growth and plasmid amplification. (Dorward *et al.*, 2019) have identified that leucine starvation in the presence of histidine induces plasmid amplification while histidine and glycine were implicated in biomass increase.

1.8.2 Induction of Plasmid DNA Production

One of the approaches to increase plasmid yield involves the induction of plasmid DNA amplification. Both physical and chemical means have been

investigated with these methods increasing plasmid yield. Supplementation with chloramphenicol has long been used to induce plasmid amplification as it arrests protein synthesis. Addition of chloramphenicol to the culture has been reported to increases the plasmid copy number (PCN) by 50-fold (Summers, 1996). However, as the use of antibiotics are being avoided due to regulatory reasons and implementing this method is difficult.

Amino acid starvation has also been reported to induce plasmid amplification by increasing the plasmid yield 10-fold (Hecker, Schroeter and Mach, 1985; Wróbel and Wegrzyn, 1997; Silva, 2011). Another effective strategy that has been employed is the temperature increase, known as temperature shift, which is essential for plasmids with ColE1 origin derivatives. Temperature shift for plasmid amplification for is possible due to the point-mutation effected in the origin of replication of ColE1 plasmids; a mutation of guanine to adenine in sequence immediately before the RNAI sequence, and further deletion of the rom protein. This prevents RNAI from binding to RNAII, and the absence of Rom protein means that there is loss of control (Lin-Chao, Chen and Wong, 1992; Lahijani *et al.*, 1996; Singer, Eiteman and Altman, 2009). Temperature-shift induction can raise the plasmid copy number about 20-fold.

1.8.3 Fermentation Strategy

One way of improving plasmid yield is by increasing the cell biomass which is directly related to the plasmid yield as it is an intracellular product. High cell density culture is not only required for the production of plasmid DNA but also in the production of recombinant proteins. Several approaches have been tried in improving the biomass concentration for biopharmaceuticals productions such as

employing fed-batch fermentation mode, perfusion-culture fermentation strategy, electrodialysis, and host cell modification.

Batch cultures with high carbon source have been attempted in increasing the biomass yield during fermentation, however, when glucose is used as the carbon source at high concentration, it results in overflow metabolism causing the accumulation of acetate and other metabolites in the cell. The accumulated acetate further inhibits the cell growth (Luli and Strohl, 1990; Kleman and Strohl, 1994; Eiteman and Altman, 2006) hence limiting the biomass that can be achieved. Glucose as a carbon source has been replaced with glycerol in an attempt to reduce overflow metabolism. This leads to a decrease in growth rate which is beneficial for plasmid yield. However, most often, glycerol use as a carbon source has been used for fed-batch culture (Ongkudon *et al.*, 2011). Host cell modifications have been carried out to overcome the problem of overflow metabolism, and these strategies have yielded positive results (discussed in 1.7.2). However, this leads to an excessive modification to host cell with a potential challenge of controlling the cell.

Fed-batch culture has been investigated as a strategy to increase biomass concentration and hence, plasmid yield. Several studies have successfully implemented fed-batch culture for the production of plasmid DNA. The increase in biomass observed is due to the control of carbon source uptake over several hours, allowing time for plasmid replication and cell growth. In fed-batch culture for plasmid DNA production, the growth rate is kept low (~0.1 h^{-1}) and the dilution rate determined (Singer, Eiteman and Altman, 2009; Ongkudon *et al.*, 2011; Gonçalves *et al.*, 2014).

Although fed-batch culture is the strategy of choice, but achieving high cell density by running batch mode can further lead to a higher yield when fed-batch

strategy is combined and feeding is introduced. For these reasons different study groups have tried to eliminate overflow metabolism to allow for batch culture at high glucose concentration (Knabben *et al.*, 2010; Soto *et al.*, 2011; Borja *et al.*, 2012).

1.9 Recovery and Purification Process (Downstream Processes Consideration)

Although increasing plasmid yield during fermentation will positively affect overall productivity, the plasmid remains in the cell alongside impurities such as host cell proteins (about 55% of the cell content), RNAs, Chromosomal DNA and endotoxins. The plasmids have to be separated in the pure form without trading quality and the quantity. This is done by first lysing the cell open and separation from other impurities. Advances in the downstream processing of plasmid DNA are discussed below.

1.9.1 Cell Lysis

Mechanical disruption that has been employed in recombinant protein production has been introduced in nucleic acid separation. However, the high shear being employed in protein purification leads to degradation of nucleic acid (Lengsfeld and Anchordoquy, 2002). Although it has been shown that bead mill, which induces low shear, can release plasmid DNA with up to 90% of these molecules staying intact, this method was however carried out at small scale, and there is need to investigate the large scale feasibility and suitability (Prather, 2003). Thermal disintegration combined with lysozyme has also been investigated, but needs fine-tuning to be effective (Lee & Sagar 1996).

The most commonly employed method of cell lysis is alkaline lysis which uses alkaline solution to break the cell open by exploiting the cell membrane weakness and the pDNA remaining intact. However, scaling up this method is still a challenge as viscosity increases causing shear on the plasmid during mixing (Clemson and Kelly, 2003). Likewise, there could also be a pH gradient at a large scale leading to non-homogenous mixing and extraction. Although with the challenges listed above, the alkaline lysis is still the most promising extraction method that has been developed. RNase from bovine is sometimes added during alkaline lysis to degrade RNA, however as this is of animal origin, it is not acceptable.

An autolytic method has been developed using autolytic *E. coli* strains. Plasmid DNA is extracted by autolytic process under low concentration of ionic detergent and low salt buffer (Carnes *et al.*, 2009). The cell lysis stage however, still needs improvement as it is a vital aspect of the bioprocess.

1.9.2 Clarification

After cell lysis, solids are to be removed. This has proven to be a very crucial step due to the viscous nature of the lysate. Cell debris is removed by cross-flow filtration at a low flow rate. More recently, flocculation has been employed to reduce the clogging of the pore during filtration (Blom *et al.*, 2010). There have also been strategies to remove contaminants by precipitation with high salt buffer and chaotropic agent such as Polyethylene glycol (PEG) (Bo *et al.*, 2007). Impurity precipitation is sometimes followed by plasmid precipitation, and this is sometimes carried out using PEG or alcohol. The use of detergent for fractional purification of plasmid DNA has been reported (Lander *et al.*, 2002; Wahlund *et al.*, 2004).

1.9.3 Purification

Chromatography can be applied for the purification of plasmid DNA. Anion Exchange chromatography is employed in the capture stage while hydrophobic interaction chromatography (HIC) follows. This is to ensure that all contaminants (genomic DNA, RNA, open circular and linear pDNA) are removed from the purified plasmid.

One of the biggest challenges is the separation of the plasmid DNA isoforms in open circular and linear (Xenopoulos and Pattnaik, 2014). Anion Exchange chromatography that employs DEAE-Sepharose has been reported to yield plasmid DNA of sufficient purity (Li *et al.*, 2011). HIC is able to separate plasmid DNA because single stranded DNA is more hydrophobic. It has been used to separate plasmid DNA with yield and purity of 75 % and 98% respectively (Bo *et al.*, 2013). Other forms of chromatography have also been investigated and are showing positive results. These include size exclusion chromatography (Latulippe and Zydney, 2009), affinity chromatography (Wils *et al.*, 1997) and monolith use (Urthaler *et al.*, 2005).

1.10 Plasmid Formulation and Delivery

The success of plasmid DNA therapeutics cannot be overemphasized with respect to the number of clinical trials ongoing. Many studies are also being dedicated to the bioprocessing of plasmid DNA for biotherapeutics. However, one other area that is being investigated is the area of plasmid packaging and delivery of this therapeutics. Effective delivery is directly related to the effectiveness of the therapeutics. Upon introduction into the subject, the plasmid DNA is required to travel across capillaries into cells, and into the nucleus where expression takes place. This means that the plasmid is faced with lots of barriers which could restrict its

movement or break it down before it gets to the final destination. These barriers include harsh conditions such as pH, plasma and intracellular endonucleases, endosomes and lysosomes as well as nuclear pore complexes (Nishikawa and Huang, 2001).

The barriers faced by plasmid DNA on transfection will depend on the nature of the diseases, which in turn will depend on the route of administration of the plasmid. Plasmid DNA Vaccines, for example, are administered intramuscularly (Dean, Fuller and Osorio, 2003), therapeutics for the treatment of tumours are administered intratumorally (Mahvi *et al.*, 2007) while airway route is preferred for defects involving the respiratory tract or the lungs (Konstan *et al.*, 2004). Depending on the delivery route, packaging of plasmid DNA could range from naked plasmid to plasmid packaged in gold particles, polymeric microparticle, as well as cationic polymers (Prazeres, 2011).

Naked plasmid DNAs formulated in saline solution have been explored as a method of formulation of plasmid DNA. It is the simplest way of delivering Plasmid therapeutics to cells. However, studies have reported the low efficiency of this method of plasmid formulation when compared to other forms of formulation when delivered by injection (Wolff *et al.*, 1990). This has led to different methods of delivering naked plasmid DNA preparation with increased efficiency reported. One of the delivery methods that has been reported to be efficient is the liquid jet injector that delivers over 90% of the fluid using high speed (Mitragotri, 2006). Another method of delivery being investigated by several authors is the hydrodynamic delivery which involves intravascular delivery under hydrostatic pressure (Budker *et al.*, 1998). Other novel delivery methods being investigated include gene gun (Yang *et al.*, 1990) and electroporation (Titomirov, Sukharev and Kistanova, 1991).

To improve transfection of plasmid DNA as biotherapeutics, studies are being carried out about the possibilities of formulating plasmid in cationic lipids and polymers such as lipoplexes (Karmali and Chaudhuri, 2007) and polyplexes. This is then further packaged as compacted micro- and nanoparticles (Prazeres, 2011)

1.11 Analysis and Quantification of Plasmid

Like other nucleic acids, plasmid DNA can be quantified by spectrophotometric method which measures light absorbance at 260nm. Although there could be interference with RNAs and proteins. Further measuring the light absorbance at 280nm and finding the ratio of the absorbance at 260nm/280nm can be used to determine how pure the sample is and detect what contaminant is present. The Nanodrop spectrophotometer can be used to determine the purity of DNA samples using this method and only requires a tiny amount of the sample. This method, however, cannot be used to determine the topology of the plasmid. Other methods have been developed for this purpose and are discussed below.

1.11.1 Agarose Gel Electrophoresis

This method separates DNA based on charge and size on a gel subjected to electric field force. The DNA samples are placed at the cathode end and migrate through the gel microstructure towards the anode typically at a potential difference of 100V. Plasmids also migrate based on their topology, with supercoiled plasmid DNA migrating faster compared to open circular and linear DNA. The gel can be viewed by staining with a dye such as ethidium bromide and SYBR nucleic acid stain and viewed under UV light.

A DNA ladder of linear or supercoiled DNA can be run alongside and used to compare to check for the quality and size of the plasmid. By using a gel camera, the plasmid DNA samples can be quantified by densitometric analysis of the intensity of the bands. Hence agarose gel electrophoresis can be used to determine the level of supercoiling of the plasmid.

Although there are other methods for analysing the DNA content and supercoiled level (discussed below), agarose gel electrophoresis is most widely used method for topoisomer analysis. For research purposes, agarose gel electrophoresis can suffice as the assay method for quantification of DNA topoisomers.

1.11.2 Capillary Electrophoresis and HPLC

Capillary electrophoresis can also be used to quantify the different topoisomer content of plasmid preparations. It is sensitive and of high-throughput when compared to agarose gel electrophoresis. It separates topoisomers based on their electrophoretic mobility (Raucci, Maggi and Parente, 2000). To improve the sensitivity of capillary electrophoresis, it can be coupled with Laser-induced fluorescence (Holovics *et al.*, 2010; Mitchenall *et al.*, 2018).

Hydrophobic Interaction HPLC method have also been developed for quantification of plasmid DNA. This method coupled with capillary electrophoresis can be used for quantification of plasmid supercoiled topoisomer content. One benefit of using HPLC is that it can be used for process monitoring (Diogo, Queiroz and Prazeres, 2003)

1.11.3 Chloroquine-diphosphate Agarose Gel Electrophoresis

This method is used to determine the superhelical density which gives an indication of how supercoiled the plasmids are. It is based on the knowledge of chloroquine diphosphate unwinds the helical twist of DNA upon intercalation. The relaxation of the supercoiled DNA causes a reduction in the mobility on the gel during electrophoresis. This causes the topoisomers to separate into a group of bands which represents the number of superhelical turns present in the plasmid (Keller, 1975).

DNA standards with differing number of superhelical turns are prepared and run in parallel to the samples. This can be used to determine the absolute value of superhelical turns in the plasmid samples. Another modification of this method of topoisomer analysis employs the 2D electrophoresis, by running the gel twice, 90^{o} of one side to the other. This method can be used to determine the extent of the supercoiling in the plasmid. A detailed discussion of this method is found in Bowater *et al* (1992).

1.12 Research Objectives

The main objective of the study is to develop a new approach for the upstream processing of supercoiled plasmid DNA for vaccines and gene therapy applications. Specifically, this will involve the modification of plasmid DNA and the optimization of fermentation strategy and large scale production. Plasmid modification will be carried out on 6.8 kb plasmid with the insertion of the Mu SGS. In addition, a large plasmid (>20 kb) will be constructed and the effect of Mu SGS studied. The project will mainly investigate the impact of the insertion of Mu phage SGS into the plasmid on the host

strain ability to propagate the plasmid, as well as the impact on the supercoiled plasmid DNA production.

The modification of the plasmid DNA is likely to have a significant effect on the quality of the plasmid produced. However, it is necessary to select the host strain that will propagate the supercoiled plasmid DNA efficiently. The parameters that will be investigated for host strain selection will include growth profiles, supercoiled plasmid DNA yield, specific plasmid yield, plasmid copy number, superhelical density, plasmid segregational stability and the biomass yield. From the result, the best performing host strain will be selected for optimization studies.

A part of the study will also investigate batch fermentation by the optimization of the media by using glycerol as a carbon source and supplementing the feed with specific amino acids and elements that are aimed at increasing the biomass yield and achieving a high cell density culture. Consequently, the effect of increasing the biomass yield should translate to a high plasmid yield with better quality and so the project will involve using a design of experiment to determine the combination at which a high biomass and high plasmid DNA yield is achieved.

Lastly, the study will investigate the effect of the modification of the plasmid DNA on the integrity of the plasmid DNA during clarification and downstream processing, by measuring the compactness of the plasmid when exposed to shear conditions that a similar to what is observed during downstream processing of biomolecules. Exposure to shear will be done by using the ultra-scale down (USD) shear device that has been developed by the Department of Biochemical Engineering UCL

1.12.1 Significance of Study to the Industrial Production of Plasmid DNA

This is a new approach to supercoiled plasmid DNA production as a platform for large scale industrial production. Achieving high biomass is one of the strategies to increase plasmid DNA, and the study will investigate the optimization of media for high cell density culture. Furthermore, for plasmid DNA to be effective as therapeutics, it must maintain its supercoiled form during production. The study will also address the supercoiling of the plasmids to meet up with regulators requirement for plasmid DNA as a therapeutic product. The higher demand for plasmid DNA in vaccine development and gene therapy application will be on the rise in the near future; therefore, current processing techniques must be improved on to meet the proposed demand. Not only is the plasmid required in high yield, it must also retain its supercoiled and monomeric form. Engineering the plasmid vector will make this possible hence, the therapeutic activity can be retained.

CHAPTER 2. Materials and Methods

2.1 Materials

2.1.1 Plasmids Selected for the Study

The plasmid vector selected for this project is based on the background studies that have been carried out in the Department of Biochemical Engineering, UCL in the past years (O'Kennedy, Baldwin and Keshavarz-Moore, 2000; Yau, Keshavarz-Moore and Ward, 2008). This plasmid vector has specific characteristics that have influenced its selection for plasmid DNA production studies. Details about this plasmid vector are given below.

2.1.1.1 pSVβ-Gal

pSVβ-Galactosidase (referred to as pSVβ-Gal from here on) plasmid vector is a 6820bp plasmid vector which encodes the β-Galactosidase gene. The plasmid also contains the gene coding for ampicillin resistance which can be used for its selection upon transformation with a suitable host. The plasmid also contains the SV40 early promoter and enhancer sequence that drive the transcription of a specific lacZ gene on the plasmid. It was developed as a modification of the parent plasmid pRSV-β-Gal. The RSV sequence and the pBR322 Ori sequence were replaced with SV40 and pUC 18 sequences, respectively (Promega).

The SV40 promoter sequence contained in this plasmid makes it a suitable candidate for vaccine and gene therapy application as it can drive the transcription of genes inserted in it upon transfection into a mammalian cell. The Plasmid for this work was purchased from Promega Corporation (Southampton, UK). The plasmid contains multiple cloning site (MCS) and a pMB1 Origin of replication. The vector map is shown in Figure 2.1.

2.1.2 Mu Phage SGS sequence

The Mu-Phage SGS sequence used in this project was as reported by Hassan *et al.,* (2016). The 398bp sequence was selected among the three sizes that were reported in the study. The selection of the sequence as a strong binding site for DNA gyrase in *E. coli* was based on the report by Pato and Banerjee (1999). The Mu phage SGS sequence was drafted from the European Nucleotide Archive of the European Bioinformatics Institute (EBI). The sequence is found in position 17681bp-18078bp of the 37kb bacteriophage Mu genome. The sequence was prepared as a synthetic gene and cloned into the plasmid vector above by Genescript Corporation (Piscataway, NJ). The resulting plasmid vectors were labelled pSVβ-SGS and pUC57-SGS for pSVβ-Gal and pUC57 respectively.

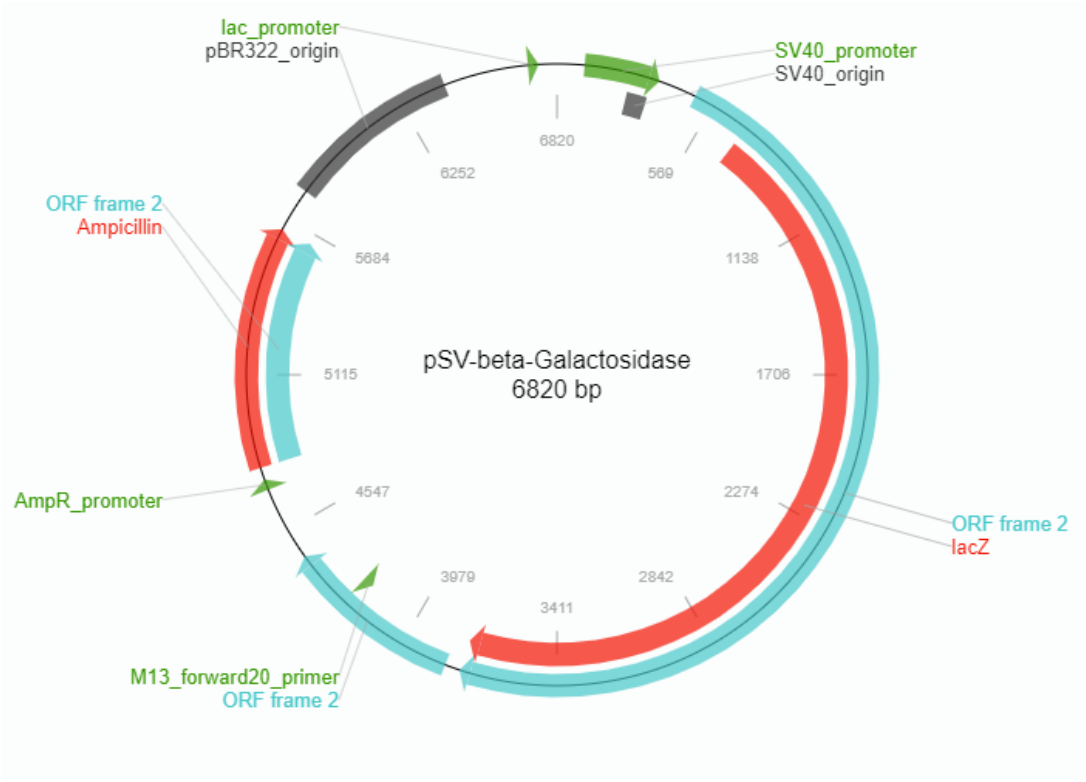


Figure 2.1: The plasmid map and features for the parent plasmid pSVβ-Gal used throughout the studies. Plasmid map illustration adapted from Addgene.org.

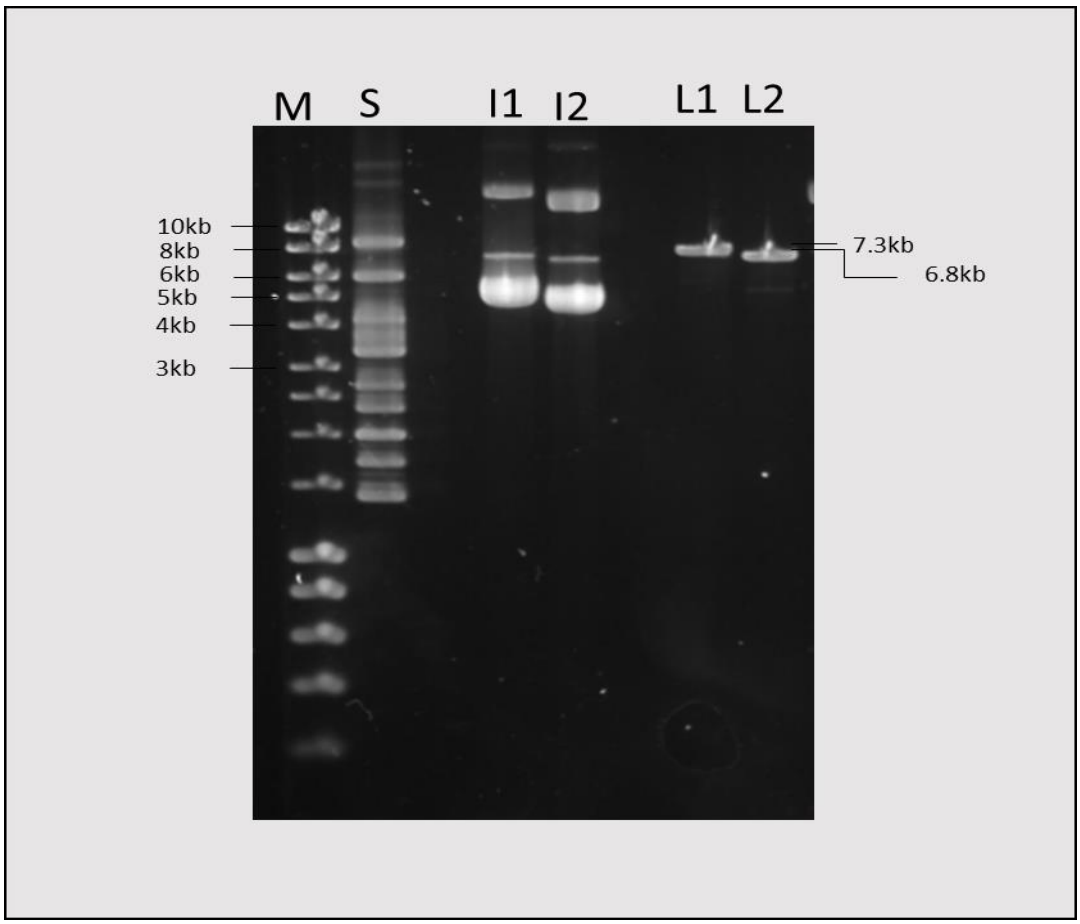


Figure 2.2: Gel analysis of the plasmid DNA used in the study. **[M]** represent the DNA Ladder used (HyperLadder™ 1kb), **[S]** represents the supercoil DNA ladder (NEB, UK), **[I1]** represent gel analysis of non-linearized plasmid pSVβ-Gal-SGS, **[I2]** represent gel analysis of non-linearized plasmid pSVβ-Gal, **[L1]** represent gel analysis of linearized Plasmid pSVβ-Gal-SGS and **[L2]** represent gel analysis of non-linearized plasmid pSVβ-Gal-SGS

2.1.3 *E. coli* Strains Selected for the Study

The *E. coli* strains selected for this study was based on the report of the studies that have been carried out in the Department of Biochemical Engineering, UCL in the past years (O'Kennedy, Baldwin and Keshavarz-Moore, 2000; Yau, Keshavarz-Moore and Ward, 2008). The strains were also selected based on their industrial relevance and commercial availability. A number of the strains are derivative of the parental *E. coli* strain K-12.

2.1.3.1 W3110

This strain was obtained from Professor Paul Dalby Laboratory at the Department of Biochemical Engineering, UCL. The strain was originally stored in 50% (w/v) glycerol as an electrocompetent strain, but the strain was made chemically competent, prepared in 15% glycerol; $CaCl_2$ solution and stored at -80^{o}C until transformation.

2.1.3.2 BL21(DE3)

The BL21(DE3) strain of *E. coli* was obtained from the laboratory of Professor Eli Kesharvarz-Moore at the Department of Biochemical Engineering, UCL. The strain was initially prepared in 50% (w/v) glycerol solution. A vial was expanded and then re-stock in 50% (w/v) glycerol. Part of the stock was made chemically competent before transformation with the plasmid vectors listed above.

2.1.3.3 HB101

Chemically competent *E. coli* strain HB101 was acquired from Promega Corporation (Southampton, UK) and stored at -80^{o}C until transformation

2.1.3.4 DH5α

Chemically competent DH5α cells were purchased from Life Technologies (Warrington, UK) and stored at -80^{o}C until transformation.

Table 2.1: Strains used in the study and their genotype

Strain	Genotype	Status
BL-21	$F^{-}\ dcm\ hsdS(r_B^{-}m_B^{-})gal\Delta\ (DE3)$	Commercial
DH5α	$F^{-}\ endA1\ glnV44\ thi$ $-1\ recA1\ relA1\ gyrA96\ deoR\ nupG\ \Phi 80dlacZ\Delta M15$ $-argF)U169,\quad hsdR17(r_K^{-}m_K^{+}),\lambda^{-}$	Commercial
HB101	$F-\ mcrB\ mrr\ hsdS20(rB$ $-\ mB-)\ recA13\ leuB6\ ara$ $-14\ proA2\ lacY1\ galK2\ xyl-5\ mtl$ $-1\ rpsL20(SmR)\ glnV44\ \lambda-$	Non-commercial
W3110	$F-\ \lambda-\ rph-1\ INV(rrnD,rrnE)$	Non-commercial

2.1.4 Culture Media

The culture media used in this study were prepared in RO water. For Luria Broth (LB), 2.0% (w/v) was prepared in RO water. The resulting solution was autoclaved at 120^{o}C for 20 minutes. The media pH was adjusted to 7.4$\pm$0.2 before autoclaving. Table 2.2 shows the composition of LB media.

Table 2.2: LB Powder Composition

Component	Amount (w/v)
Yeast Extract	5
Tryptone (Pancreatic Digest of Casein)	10
Sodium Chloride	5

Casamino-containing semi-defined media (SDCAS) was prepared by adding casamino acid to M9- minimal media. The composition of the minimal media and the trace elements prepared are shown in Table 2.3 and Table 2.4

Table 2.3: Minimal media composition used throughout this study with the exception of the glycerol which varies with the investigation. Modified from (García-Arrazola *et al.*, 2005)

Component	Amount (g/L)
Ammonium Sulphate	5
Sodium Phosphate monohydrate	2.8
Sodium Chloride	3.87
Citric Acid	4
Glycerol	30[1]
Trace Elements	10mL/L (prepared as in Table 2.4)
Magnesium Sulphate heptahydrate	1

Table 2.4: Trace elements composition for the preparation of minimal media. From (García-Arrazola *et al.*, 2005)

Component	Amount (g/L)
Citric Acid	100
Calcium Chloride hexahydrate	5
Zinc Sulphate heptahydrate	2.46
Manganese Sulphate tetrahydrate	2
Copper Sulphate pentahydrate	0.5
Cobalt Sulphate heptahydrate	0.427
Iron (III) chloride hexahydrate	9.67
Boric acid	0.03
Sodium Molybdate dihydrate	0.024

2.1.5 Agar Plates

For LB agar, 2% (w/v) of LB powder was dissolved in RO water with 1.5% (w/v) of agar. The mixture was mixed until dissolved. The resulting solution was autoclaved at 120^{o}C for 20 minutes. The media pH was adjusted to 7.4$\pm$0.2 before

[1] The glycerol concentrations used in the minimal media for this study varies for investigations with a maximum glycerol concentration of 100 g/L used

autoclaving. After autoclaving, plates were prepared by pouring agar into plates they solidify.

Table 2.5: LB agar composition

Component	Amount (g/L)
Yeast Extract	5
Tryptone (Pancreatic Digest of Casein)	10
Sodium Chloride	5
Agar	15

2.1.6 Buffers

The following buffers were used during the study:

1. Tris-Acetate-EDTA (TAE) Buffer 10× concentrated: This is composed of 0.4M Tris-Acetate; pH 8.3±0.2, 0.01M EDTA.Na2 (Sigma-Aldrich). 1× concentration was used throughout the electrophoresis experiment.
2. Tris-Borate-EDTA (TbE) Buffer 10× concentrated: This is composed of 0.45M Tris-Acetate; pH 8.3±0.2, 0.01M EDTA.Na2 (Sigma-Aldrich). 0.5× concentration was used throughout the electrophoresis experiment.
3. Gel loading dye, Purple 6× concentrated. This is composed of 2.5% Ficoll®-400, 10mM EDTA, 3.3mM Tris-HCl, 0.02% Dye 1, 0.001% Dye 2; pH 8.0 at 25^{o}C (New England Biolab, UK).

For plasmid extraction and purification, the buffers used were supplied along with the Qiagen Plasmid miniprep kit (manufacturer's details). The composition of the buffers is listed below.

1. Resuspension Buffer P1: 50mM Tris-HCl; pH 8.0, 10mM EDTA, 100µg/mL RNaseA. And LysBlue solution. The RNaseA and LysBlue were added to the Buffer after opening and stored at 4^{o}C. the LyseBlue solution contains

Thymophthalein which changes colour from colourless to blue at a pH shift to 9.3

2. Lysis Buffer P2: 200mM NaOH, 1% (w/v) Sodium Dodecyl Sulphate (SDS). Stored at room temperature.
3. Neutralization Buffer N3: 4.2M Guanidine HCl, 0.9M Potassium acetate. Stored at room temperature.
4. Wash Buffer PB: 5M Guanidine HCl, 30% isopropanol. It is stored at room temperature.
5. Wash Buffer PE: 10mM Tris-HCl; pH 7.5 and 80% ethanol. Ethanol was added after opening. The solution was stored at room temperature.
6. Elution Buffer EB: 10mM Tris-HCl; pH 8.5. Stored at room temperature.

2.1.7 Antibiotics Stock

Ampicillin stock was prepared by dissolving ampicillin in RO water to a stock concentration of 50mg/mL. This was then further diluted to give 100µg/mL, which is the ampicillin concentration for selection of plasmid transformed cells.

2.1.8 Restriction Enzymes

Restriction enzymes for nicking and cutting the plasmid into linear and open circular topoisomers were purchased from New England Biolabs (Hitchin, UK). These include *Hind III*, *NtBstNBI*, *EcoRI* and *BamHI*.

2.1.9 DNA Molecular Weight Marker

The DNA Molecular weight markers used in the analysis of results include λBstE II (New England Biolabs Hitchin, UK), λHind III (New England Biolabs Hitchin, UK),

Supercoiled DNA ladder (New England Biolabs Hitchin, UK) and Hyperladder-1 (Bioline London, UK)

2.2 Methods

2.2.1 Gene Synthesis

The Mu phage SGS sequence was drafted from the European Nucleotide Archive of the European Bioinformatics Institute (EBI). The sequence was sent to Genescript alongside the Plasmid pSVβ-Gal. The SGS sequence was synthesized and inserted in the multiple cloning site of the Plasmid.

2.2.2 Plasmid Fragmentation

Fragments were acquired using restriction endonucleases. The cut sites for the fragment of interest was identified on the target DNA. The DNA was incubated with enzyme at room temperature for 1 hour and buffer supplied. Once incubation was complete, enzymes were denatured by heating the reaction mixture at 55^{o}C. Fragments were purified to remove contaminants and loaded on gel to allow for separation. After electrophoresis was complete, the gel was stained using SyBR Gold and the fragment of interest identified and ready to be extracted.

2.2.3 Fragments Extraction

Once the fragment of interest was identified using agarose gel electrophoresis, the fragment was extracted using the GeneJet Gel Extraction Kit (Thermo Fisher Scientific, UK). An Eppendorf tube was weighed empty, the part of

the gel containing the fragment was excised and added on to the weighed Eppendorf tube. The DNA fragment was then extracted from the excised gel following the manufacturer's protocol. In some cases, the extracted fragment was further cleaned by using the DNA Clean & Concentrate Kit (Zymo Research, California USA). The pooled fragment was then stored at -20°C until it was required.

2.2.4 Fragments Ligation

Fragment ligations were carried out using ligase enzymes supplied by New England Biolab. Fragments to be ligated were pooled in a reaction tubes according to mole ratio required. T4 DNA ligase and buffer were added to the reaction tube to allow for successful ligation. The reaction was incubated at room temperature for 1 hour. The enzyme was heat-inactivated by heating the reaction tube at 65°C for 10 minutes. In the case where this was the final product, the reaction tube was chilled on ice and up to 5 µL was transformed into chemically competent cells.

Where the inactivated reaction tube was not the final product (in cases where multiple fragments were being ligated serially), the reaction tube was purified using the DNA Clean & Concentrate (Zymo Research) and used for subsequent ligation steps.

2.2.5 Transformation

E. coli strain W3110 and BL21(DE3) were non-competent cells when they were obtained. These were made chemically competent and grown in 85mM $CaCl_2$, 20%(v/v) glycerol and stored in vials. Other strains were already chemically competent when purchased. All of the strains, both in-house acquired and commercially purchased, were then transformed with plasmids pSVβ-Gal and pSVβ-

Gal-SGS using standard heat-shock, calcium chloride technique. The cells were transferred into sterile cryovials and stored at -80°C until it was required.

2.2.6 Plate Culture

The transformed cell stored in glycerol were thawed and used to streak LB agar plates which contain ampicillin antibiotics for selection and growth of plasmid containing cells. Ampicillin concentration used was (100µg/mL). This is enough to select against the host. The plates were then incubated at 37°C for 24 hours. They were wrapped in film and stored at 4°C until they are required.

2.2.7 5mL and 10mL Cultures

5 mL and 10 mL cultures containing LB media and the appropriate antibiotics (ampicillin) were grown in 50 mL falcon tubes for 16.5 hours at 37°C in an incubator with an agitation speed of 250 rpm. The media were inoculated with single picked colonies from freshly prepared agar plates.

2.2.8 200mL Shake Flask Cultures

The inoculum for the 200 mL shake flask was prepared as in Section 2.2.7. 200 mL of media was added to flasks and sterilized. The flasks were inoculated with the inoculum to the desired initial OD 600nm. The shake flasks were placed in an incubator at 37°C and shaking speed of 200 rpm. OD readings were taken every hour until the end of the fermentation.

2.2.9 1 L Batch Fermentation

The reactors were prepared according to the standard operating procedure in Appendix C. Four reactors were run in parallel. The reactors were filled with 800 mL of media and sterilized in the Getinge Autoclave alongside acid and alkali bottles (sterilized empty) and antifoam bottles. After sterilization is complete, the reactors were set up for inoculation. All necessary components were attached and connected to the control system. Airflow was turned on, and the stirrer started. The gas analyzer and water chiller were turned on while the antifoam probe was pulled up and connected. Acid and alkali bottles were filled and primed for all reactors. Data logging software was started, and fermentation logging confirmed. Acid and alkali were added to maintain a pH of 6.98, while the DO was set at 30%. The initial stirrer speed was set at 400 rpm, but was increased during the course of fermentation to maintain a DO not less than 30%

When the temperature, pH reached the setpoint, the reactors were inoculated with cultures from 200 mL shake flask culture that has been incubated till the OD reached the required level. 900 µL of ampicillin was added to the reactor. The reactors were inoculated to a starting OD of 0.5. The OD was monitored throughout the fermentation, and dry cell weight and plasmid content were determined every four hours.

A stable or dropped optical density signalled the end of fermentation. The cells were harvested of cooled to a temperature of 4^{o}C until required. In situations where the cells were not required, the cells were killed by autoclaving, and the reactors cleaned according to protocol.

2.2.10 Optical Density Measurements

At the end of each fermentation, samples were taken from the culture, and the optical density was measured with a spectrophotometer at a wavelength of 600nm. The OD was used to predict the biomass yield per mL of culture which indicates how well the strains have grown.

2.2.11 Dry Cell Weight Determination

In order to determine the dry cell weight. 1 mL Eppendorf tubes were labelled and dried in an oven at 100oC until a constant weight was reached. The weight was recorded as initial weight, and 1 mL of cell culture added to the Eppendorf tube. The tubes were spun down and the supernatant discarded. The tubes were placed in the oven and dried until the weight was constant. The difference between the initial weight and the final weight of the tube was determined and taken as the dry cell weight (in g/L).

2.2.12 Plasmid DNA Extraction and Purification

Plasmid DNA samples from the fermentation were extracted and purified using the Qiagen Spin Mini Prep kits (Qiagen Ltd, Sussex UK). The procedure was followed according to the protocols provided along with the kits. Buffers and solutions provided along with the kits and labelled. The compositions of these solutions and buffers are explained in section 2.1.6. 5mL of overnight bacterial culture was pelleted by centrifugation at 4000rpm for 10 minutes at 15°C. The pellet was completely re-suspended in 250 µL buffer P1 and then was transferred to a micro-centrifuge tube. 250 µL of buffer P2 was then added and mixed by inverting multiple times until the

solution turned blue (due to the presence of LyseBlu). 350 µL of Buffer N3 was then added and mixed immediately and thoroughly until the solution turned colourless.

The solution was then centrifuged at 10000 g for 10 minutes in a benchtop centrifuge (Eppendorf 5424R Microcentrifuges). The resulting supernatant was then transferred to the QIAprep spin column (provided along with the kit) by pipetting. This was then centrifuged at 10000 g for 60 seconds using the benchtop centrifuge mentioned above. The flow-through was discarded, and the QIAprep spin column was washed by adding 500 µL of buffer PB. This was again centrifuged for 60 seconds. Further wash was carried out by adding 750 µL of Buffer PE, then centrifuged for 60 seconds and the flow-through discarded. The column was further centrifuged for one minute to remove residual buffer.

The QIAprep spin column was placed in a clean 1mL micro-centrifuge tube. The DNA was eluted from the column into the micro-centrifuge tube by adding 50 µL of buffer EB (10mM Tris.Cl, pH 8.5) to the centre of the column. It was allowed to stand for one minute and centrifuged for one minute. The microcentrifuge tubes were then stored at 4^{o}C.

2.2.13 Plasmid DNA Purity Determination

The plasmid DNA purity was determined by measuring the absorbance ratio of A260nm/A280nm, using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Willington, DE USA). The ratio of the absorbance at these wavelength indicates the purity of the sample and if it is contaminated with protein, DNA or RNA. A ratio of ~ 1.8 is said to be pure. If the ratio is less than 1.8, then the sample is contaminated with protein, or if it is greater than 1.8, it is contaminated with RNA. The plasmid DNA purity was also determined partially with agarose gel electrophoresis.

2.2.14 Plasmid DNA Yield Determination

The plasmid yield was determined by measuring the concentration of the plasmid DNA from purified samples. The concentration was determined spectrophotometrically using the Nanodrop ND-1000 Spectrophotometer. The DNA concentration was determined by the absorbance of the sample at a wavelength of 260nm. The concentration was recorded in ng/µL. The total plasmid yield in mg/L was determined by multiplying the concentration by the eluent volume per 1mL of the sample. The plasmid yield per biomass yield was determined by multiplying the total plasmid yield by the optical density recorded.

2.2.15 Plasmid DNA Nicking and Linearization

To determine the different topoisomers (linearized, open-circular and supercoiled) of plasmid DNA present in the samples, linearized and open circular plasmid samples were prepared and run alongside the samples during agarose gel electrophoresis. To prepare open-circular plasmid, 2 µL of the enzyme Nt.Bst.NBI was added to the preparation of 2 µL plasmid DNA, 5 µL of 10X buffer and 41 µL of water. The mixture was incubated at 55^{o}C for 1 hour followed by inactivation of the enzyme at 80^{o}C for 20 minutes. To prepare the linear plasmid, 2 µL of the enzyme BamHI was added to the preparation of 2 µL plasmid DNA, 5 µL of 10X Cutsmart buffer and 41 µL of water. The mixture was incubated at 37^{o}C for 15 minutes.

2.2.16 Plasmid Topology Determination

The plasmid DNA topology compositions and percentage intensities were determined using agarose gel electrophoresis. 1% (w/v) agarose gel was prepared in 1X TAE (Tris-acetate-EDTA) buffer (Sigma-Aldrich). The gel was pre-stained ethidium bromide (500µg/mL) and was run in 1X TAE (Tris-acetate-EDTA) buffer at

90V for 2 hours. The gel was viewed under UV, and the image acquired using the AlphaImager Mini System (ProteinSimple, California, USA). The AlphaView CA Software (ProteinSimple) was used to identify the bands and as a densitometry tool to quantify the intensity of the bands on the acquired image of the gel. The lanes represent each sample and the bands on each lane represent the different topoisomers of the plasmid present in the sample.

Agarose gel electrophoresis was used for quantifying plasmid topology as this was the set up that was available in the laboratory. Other methods that can be used to determine the plasmid topology contents include capillary electrophoresis and HPLC methods.

2.2.17 Plasmid DNA Super-Helical Density Determination

The plasmid super-helical density was determined using chloroquine Agarose Gel electrophoresis, according to Bowater (1992). 1% (w/v) agarose was prepared in 0.5X TBE (Tris-Borate-EDTA) buffer (Sigma Aldrich). 0.6 mg/L of chloroquine diphosphate was used to pre-stain the gel before running. Two samples were loaded 10 cm apart, and electrophoresis carried out in the first dimension at 3V/cm for 17 hours with 0.5X TBE as the running buffer. The electrophoresis was stopped, and the gel was placed in 0.5X TBE buffer with 3mg/L chloroquine diphosphate and allowed to soak for 3 hours. Electrophoresis was carried out in the second dimension; 90^{o} to the first at 3.3V/cm for 21 hours. The gel was then rinsed three times in 1X TBE for an hour each. The gel was stained with SYBR gold nucleic acid stain for 2 hours before visualization under UV light. The superhelical density was calculated.

2.2.18 Determination of Plasmid Copy Number

To determine the plasmid copy number (PCN), the method of Smith and Bidchoka (1998) was adapted. 5mL cultures were grown overnight for 16.5 hours in LB media. Samples were taken to determine the optical density. Plasmid DNA from the samples was extracted and the plasmid yield determined according to the method discussed above. One mL of the culture was taken and serially diluted in sterile RO water. 50µL of the dilution was aliquoted onto LB agar containing ampicillin to select against plasmid-free cells. The cells were spread on the agar using sterile cell spreader. The plates were incubated at 37^{o}C for 24 hours. Colonies were counted after 24 hours and the number of bacterial cells per mL was calculated using the equation below.

$$Cells\ per\ mL = \frac{Number\ of\ colonies}{dilution\ factor\ \times volume\ of\ sample\ plated\ (mL)} \qquad [1]$$

The size of the plasmid in kb was used to calculate the mass of the plasmid in ng using Avogadro's number. The number of plasmid molecules per bacteria cells was calculated using the equation below.

$$\frac{Plasmid}{cell} = \frac{6.02 \times 10^{23}}{1\ mole} \times \frac{1\ mole}{single\ plasmid\ mass\ (ng)} \times \frac{amount\ of\ plasmid\ (ng)}{1\ mL\ of\ solution} \times \frac{1\ mL\ of\ culture}{Number\ of\ cells} \qquad [2]$$

CHAPTER 3. Assembly of 27kb Plasmid: Challenges and Approach

3.1 Introduction

One of the potentials of plasmid DNA for biotherapeutics application is the possibility of packaging multiple genes into a plasmid. The technical consequence of this possibility of multiple genes packaging is the increase in the size of the plasmid being used. It is therefore pertinent to investigate the feasibility of large plasmid (>20 kb) bioprocessing.

Like a small plasmid, the large plasmid must be a high copy number plasmid for the sake of yield, contain selection marker and multiple cloning site for the insertion of genes of interest. Issues with large plasmid bioprocessing have included the shear sensitivity of the plasmid during downstream processing which could potentially lower the overall plasmid yield.

The challenge, however, is to construct a large plasmid in order to be able to investigate and subsequently tackle the bioprocess challenges. In addition to the regular parts of the plasmid, the large plasmid would also be investigated by insertion of the Mu-phage Strong Gyrase-Binding Site (Mu-SGS).

There are several approaches that could be employed in plasmid construction. However, most of these approaches tend to focus on the assembly of small sizes. Also, the choice of approach was based on the available fragment DNA.

3.2 Fragment generation for plasmid construct

Being the workhorse of molecular biology, DNA assembly is one of the most important protocols for all molecular biology research. The traditional restriction

digestion and ligation methods have been a very effective way of assembling DNA fragment. However, the limitations of this method have prompted the development of new and end efficient way of assembling DNA fragment for molecular biology purposes. All of the approaches of DNA assembly either follow the serial strategy, where a single fragment is added at a time or parallel strategy where all fragments are added in a single step (Gao *et al.*, 2013).

The size of the large plasmid to be constructed is limited by the available fragments to be ligated. Plasmid pSVβ and the SGS containing counterpart was decided to be used as the base plasmid. Since these plasmids are larger than 6kb, the challenge is to generate fragments or a fragment larger than 20kb to achieve a plasmid greater than 20kb in size.

3.2.1 Choice of TOL Sequence for Large (30+kb) plasmid

In order to achieve plasmid of 30+ kb size, fragments from plasmid pMT103 have been acquired. This plasmid is composed of 24kb *HindIII* fragment of the TOL catabolic plasmid pWW0 of pseudomonas responsible for the breakdown of toluene (Greated *et al.*, 2002) subcloned into pBR322 plasmid, a 4.3kb plasmid, at the *HindIII* site (Figure 3.1).

The decision to construct a new plasmid and not to use the pMT103 was due to the fact that the base plasmid of pMT103 (pBR322) is a medium copy number plasmid with copies of about 15-20 copies per cell (Bolivar *et al.*, 1977). This is due to the origin of replication present in this plasmid. Since the use of plasmid as therapeutics requires a higher yield, a high copy number plasmid is best suited for the investigation, hence the use of the pSVβgal as the base plasmid for the plasmid construction.

The TOL sequence of pMT103 plasmid was excised to be used for the construction of the large plasmid. It is understood that there is no ori sequence present in the TOL sequence hence the only origin of replication will be from the other fragment

pSVβ-gal is a high copy number plasmid (~500 – 700 copies per cell) due to its origin of replication being derived from pUC18 sequences (Vieira and Messing, 1982). By linearizing pSVβ-gal and the SGS containing counterpart and subcloning the 24kb *HindIII* fragment of the pMT103, we can generate a 31kb plasmid.

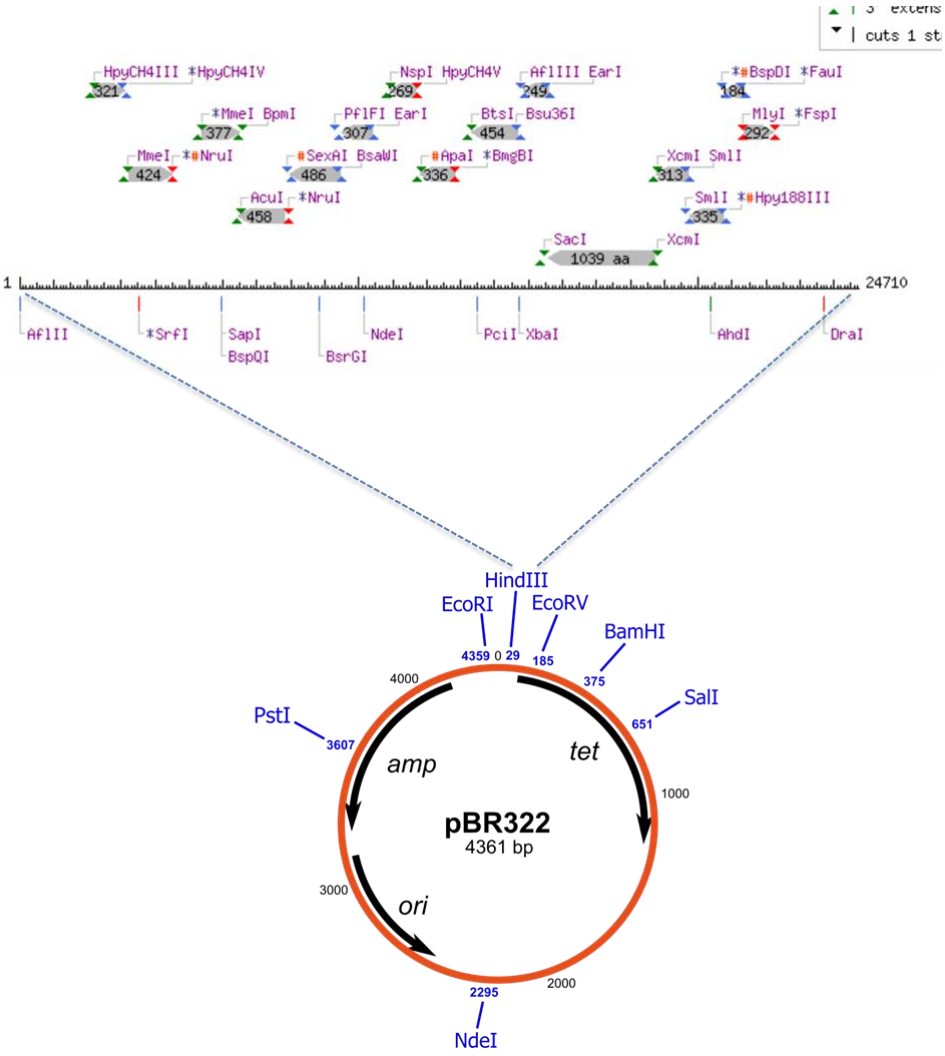


Figure 3.1: The plasmid map of the base plasmid pMT103 used for the construction. Fragments were extracted from this plasmid for construction of the plasmid of interest. The plasmid itself was constructed with pBR322 backbone and *HindIII* TOL fragment of plasmidpWW0.

3.3 Choice of assembly method and limitation

There are different methods of DNA assembly that we could use to construct the large plasmid. As much as these methods are very efficient and quick, there are limitations which would hinder us from being able to use them for the assembly of the plasmid of interest.

3.3.1 Golden gate DNA Assembly

The Golden gate DNA assembly method allows for multi-fragment parallel assembly, with up to 10 pieces of DNA assembly in a single pot. It uses enzymes cutting outside restriction site and leaving behind ssDNA of typically four nucleotides. An advantage of using golden gate assembly method is that scar sites can be avoided (Engler *et al.*, 2009)

3.3.2 Modular Cloning (MoClo)

The modular cloning method establishes standard overlaps, defined and validated modules. It uses type IIS restriction enzymes to assemble DNA fragments in a linear order (Weber *et al.*, 2011). Polymerase Chain Reaction (PCR) method is used to create the overlaps in the fragments. As in the case of our constructs, the fragments are already available and will not be generated by PCR which would have made it easier to add the overlaps, hence this method is not suitable for the assembly of the construct. Although it is possible to add an overlap to already available fragments, it would be practically impossible to add the overlap to a fragment as large as 24kb due to the limitation of PCR sizes.

3.3.3 Methylated-Assisted Tailorable Ends Rationale

Sometimes, the choice of restriction site may be present multiple times in the fragment. To avoid off-target restriction digest, this method uses type IIS enzymes digesting at only methylated sites. Primers containing methylated cytosine can be used to introduce methylated cytosine and the PCR product digested with the methyl-recognising enzyme (Chen *et al.*, 2013).

Since this method also involves the use of PCR, the limitation of PCR sizes rules the method out for use in the plasmid construct assembly.

3.3.4 Iterative Capped Assembly

The iterative capped assembly joins DNA fragments with 4-nt overhangs that are added by a type IIS restriction enzyme as a growing chain attached to a support bed. The ICA strategy allows for serial ligation but in a quick manner (Briggs *et al.*, 2012). However, it is limited as only designed sequence can be created. This makes it unsuitable for use in the assembly of the construct.

3.3.5 PCR methods

There are PCR methods that can be used for DNA assembly. These methods do not require restriction enzyme digestions and no ligation steps are required. They use a polymerase extension. Like PCR limitations with regards to sizes, these methods are also limited by sequence length which has been enforced by the polymerase (Borg *et al.*, 2016). Examples include TA and TOPO cloning that joins a PCR product insert with a vector using the terminal transferase activity of *Taq* DNA polymerase (Brownstein, Carpten and Smith, 1996), sticky end polymerase chain

reaction that mimics digestion-ligation but without the restriction digestion (Zeng, 1998) , overlap extension PCR (Shuldiner, Scott and Roth, 1990), chain reaction cloning and ligase cycling reaction (Pachuk *et al.*, 2000).

3.4 An attempt at using Conventional Restriction Digestion-Ligation Method

With the limitations of all the methods highlighted above, an attempt was made to use the conventional restriction digestion ligation method, where the fragments are generated having matching restriction overhangs and ligated to yield the plasmid of interest. The available fragment (*HindIII* TOL fragment) that is to be cloned into pSVβ-gal398 was excised from its parent plasmid at the *HindIII* site. This resulted in both terminals of the fragment bearing the *HindIII* restriction site overhangs. The backbones were also linearized at this site thereby leading to non-directional ligation option as the only option (Figure 3.2).

The ligation was carried out at different fragment-backbone mole ratio to increase the chances of success (Table 3.1). In the first attempt, the number of moles of the fragment was kept constant and that of the backbone varied. This was decided as the fragment is three times larger than the backbone. The ligation was carried out using Quick ligase reaction mix from NEB and 100uL of transformation reaction plated out.

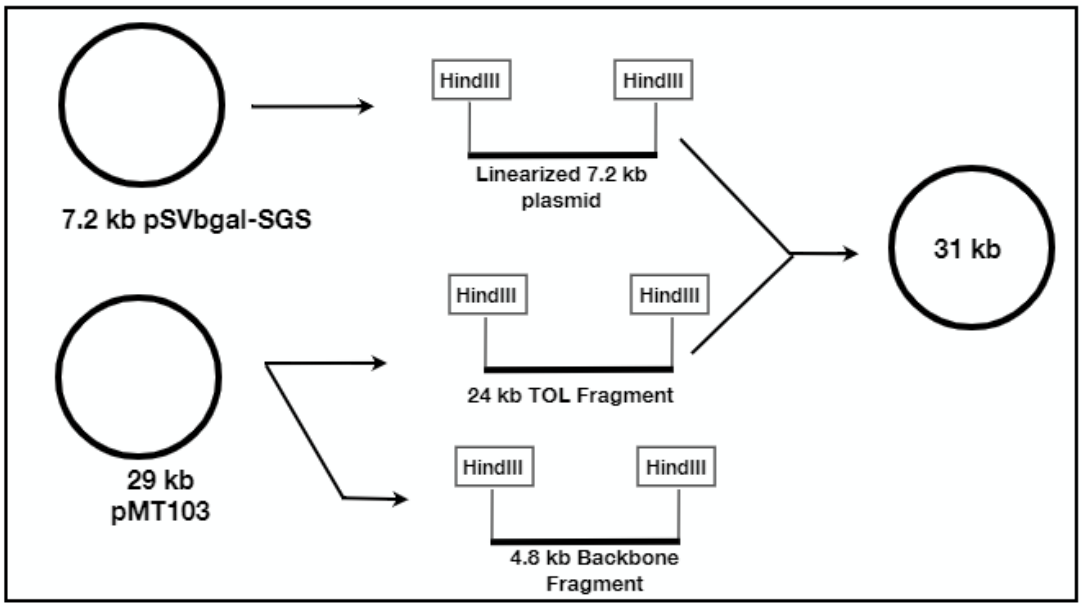


Figure 3.2: Flowchart of the plasmid assembly plan from sources of fragments to the final construct

On transformation and spread on LB agar plates, there were no colonies observed for all transformant and mole ratio. In order to troubleshoot this problem, controls were made. One control reaction had a linearized backbone and the quick ligase reaction mixture, and another control had the complete plasmid for transformation. Two different strains of chemically competent *E. coli* were used as these have been the strains for the project. The outcome of transformation confirmed that the cells were indeed chemically competent as there were colonies formed on plates with transformant of the complete parent plasmid.

Table 3.1: Ligation reaction table for the ligation of the HindII digest TOL fragment and the backbone, in this case, pSVβ-gal and pSVβ-gal398. 5 µL of the ligation reaction was used to transform 50 µL of cells. In the first attempt, 100 µL was plated but no colonies found. In a second attempt, 150 µL was plated. The colonies are shown in the last row of the table.

Reaction Components	**Control 1**	**Control 2**	**Mole Ratio 1:1**	**Mole Ratio 1:2**	**Mole Ratio 1:3**	**Mole Ratio 1:5**
HindIII Fragment	8 µL (50 ng)	-	8 µL (50 ng)	8 µL (50 ng)	8 µL (50 ng)	8 µL (50 ng)
Backbone (ng)	-	3 µL	1 µL	2 µL	3 µL	5 µL
Nuclease-Free Water	7 µL	12 µL	-	-	-	-
Quick Ligase buffer Mix	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL
Total	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL
Colonies	-	50	-	4	6	3

There were no colonies on plates with transformant of the ligase reaction containing the linearized plasmid either. This observation influenced a preliminary suggestion that the problem was likely to be the quick ligase reaction used for the ligation. However, with another round of transformation of cells and plating of 150uL yielded colonies but few in the Control with only the linearized backbone ligation reaction and in few plates; one for each backbone-fragment ligation reaction Table 3.1.

The colonies were screened using the traditional diagnostic method. The rapid PCR colony screening could not be used due to the size of the construct expected from the screening (Walser *et al.*, 2009). All colonies were selected from both plates and screened for the presence of the construct of interest. None of the colonies selected was the transformant of interest, instead the DNA size observed was that of the backbone pSVβ-gal and pSVβ-gal398. This suggests that there was

recircularization of the backbone which could have been a spontaneous reaction, or uncut plasmid remaining from linearization as there was a possibility of incomplete linearizing. It was impossible to tell if there was circularization of the backbone as there was no selection marker on the fragment. Figure 3.3: Schematic flow chart of the steps taken in the initial approach to construct the large plasmid Figure 3.3 shows a schematic flow chart of the steps taken for this approach.

This is no surprise as there was bound to be spatial constraint with the size of the fragment used (24kb) (Zeng *et al.*, 2017). Recircularization of plasmid backbone is one of the limitations of non-directional cloning where the two terminals have the overhangs of the same restriction sites. An adjustment was made to prevent the recircularization of the plasmid backbone.

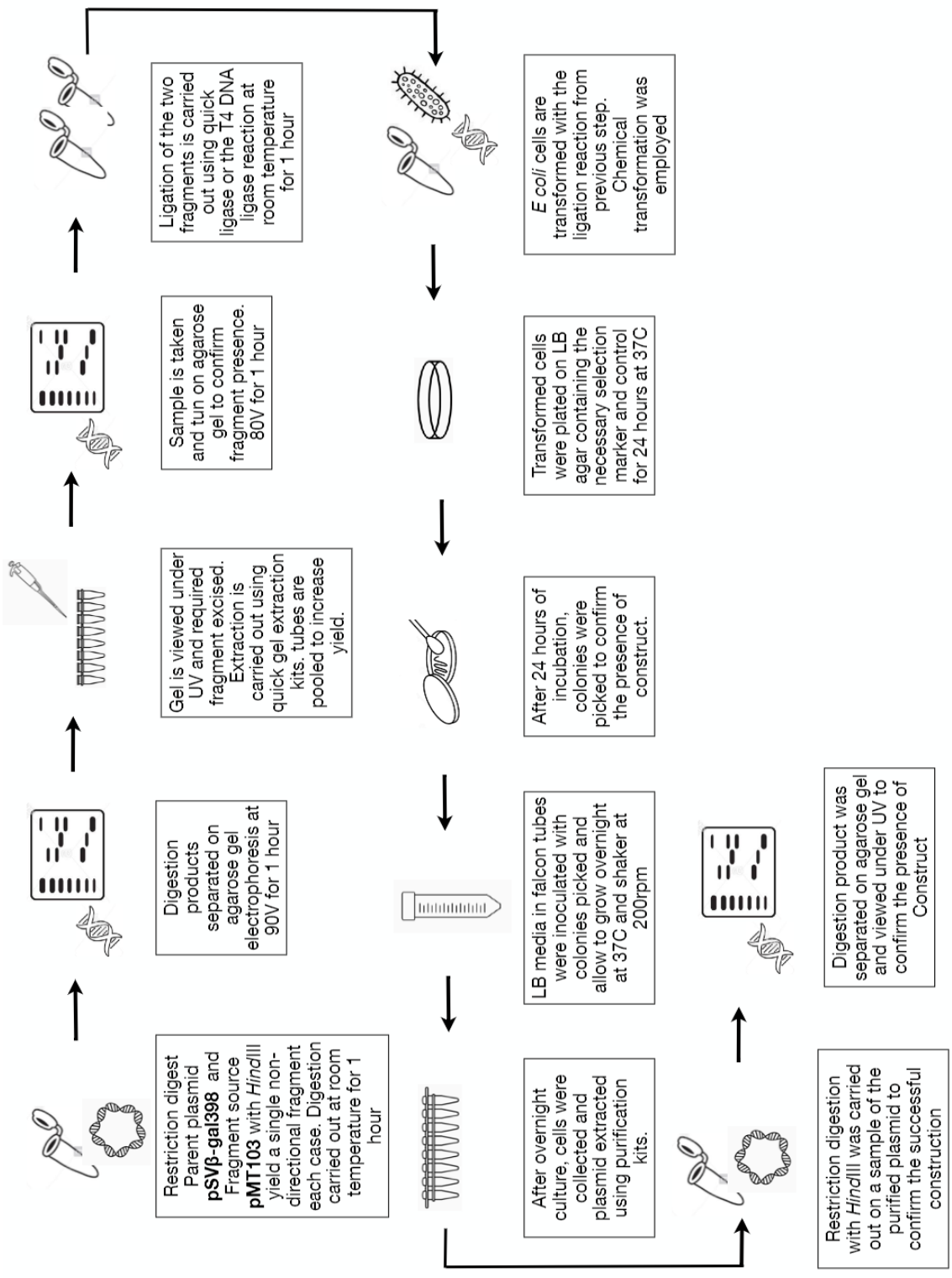


Figure 3.3: Schematic flow chart of the steps taken in the initial approach to construct the large plasmid

3.4.1 Dephosphorylation of plasmid Backbone

To solve the problem of recircularization of plasmid, the backbone was dephosphorylated by using the calf intestinal alkaline phosphatase (CIP). The enzyme CIP dephosphorylates the DNA by catalysing the removal of the 5'-phosphates group from nucleic acid, thereby preventing recircularization of linearized plasmid. A new ligation reaction was set up including control samples with tubes containing dephosphorylated samples only (without the *Hind*III TOL fragment inserts). Table 3.2 shows the results of the outcome of transformation in the form of whether colonies are expected and observed. It was expected that there would be no colonies present on plates streaked from the tube containing dephosphorylated backbone only. However, there were some colonies present which suggested that we may have some incomplete dephosphorylation or contamination.

Table 3.2: Ligation reaction table for the ligation of the HindII digest TOL fragment and the CIP-dephosphorylated backbone, in this case, dpSVβ-gal and dpSVβ-gal398. Ligation of non-dephosphorylated backbone and fragment was also carried out as a form of control. The presence or absence of colonies is shown in the last row of the table.

Reaction Components	**Control 1**	**Control 2**	**pSVβ-gal**	**pSVβ-gal (dephos.) / Fragment**	**pSVβ-gal (dephos.)**	**pSVβ-gal (dephos.) / Fragment**	**pSVβ-gal (dephos.) / Fragment**	**pSVβ-gal398**	**pSVβ-gal398/ Fragment**	**pSVβ-gal (dephos.)**	**pSVβ-gal398 (dephos.) / Fragment**	**pSVβ-gal398 (dephos.) / Fragment**
HindIII Fragment	5 µL (50 ng)	-	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL
Backbone (ng)	-	3 µL	2 µL (75 ng)	2 µL (75 ng)	2 µL	1 µL	2 µL	2 µL	2 µL	2 µL	1 µL	2 µL
Nuclease-Free Water	12 µL	14 µL	10 µL	10 µL	10 µL	11 µL	10 µL	10 µL	10 µL	10 µL	11 µL	10 µL
Buffer	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL
T4 DNA Ligase	1 µL (10 U)	1 µL (10 U)	1 µL (10 U)	1 µL (10 U)	1 µL (10 U)	1 µL (10 U)	1 µL (10 U)	1 µL (10 U)	1 µL (10 U)	1 µL (10 U)	1 µL (10 U)	1 µL (10 U)
Total Reaction	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL
Colonies Expected			**Y**	**Y**	**N**	**Y**	**Y**	**Y**	**Y**	**N**	**Y**	**Y**
Colonies Present			**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**

It was suspected that the colonies observed from plates streaked with ligation reactions containing dephosphorylated backbone and inserts might not have the construct of interest. However, to confirm the suspicion, colonies from these plates were screened. Based on the number of colonies observed, ten colonies were screened from each plate. The outcome was negative as re-circularized backbone was observed on gel in all diagnosed colonies. One possible reason could be a problem posed by the subsequent steps after the reaction is complete as calf intestinal phosphatase enzyme is challenging to heat-inactivate after use (Lu *et al.*, 2010). Usually, the reaction is cleaned up after use to remove any CIP present, but if the clean-up is incomplete, there is a possibility of the insert getting dephosphorylated during ligation step. There is also a chance that dephosphorylated plasmid is lost during the clean-up step.

A different dephosphorylating enzyme was used to overcome the challenges posed using CIP. The Shrimp Alkaline Phosphatase (rSAP) has been reported to have a specific higher activity than CIP, and it can be heat-inactivated; the two major challenges of CIP. The dephosphorylation and inactivation were carried out according to manufacturer's protocol supplied alongside the enzyme. The outcome was no different than observed with CIP with multiple runs, which suggested that the problem with ligation might be the stearic hinderances of the 24 kb fragment with the 7.2 kb backbone plasmid. The challenges meant that a different approach was needed to construct the large plasmid. The steps taken using this approach is shown in Figure 3.4

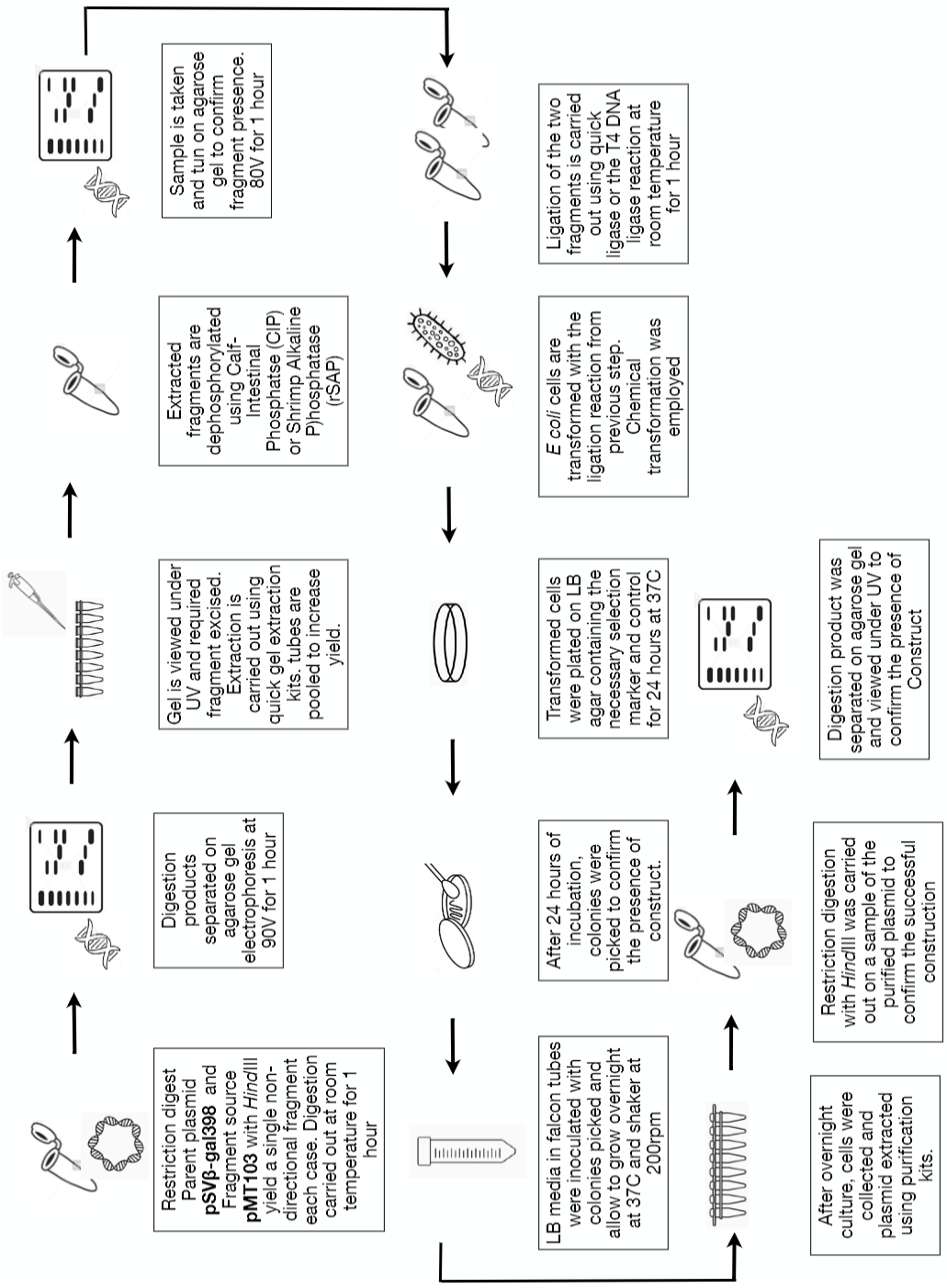


Figure 3.4: Schematic flow chart of the steps taken in the modification of initial approach to include a dephosphorylation step in order to construct the large plasmid

3.4.2 Re-design of the Construct using multiple short and medium-sized fragments.

To overcome the challenges of steric hindrances that result from using the large fragment, it was decided to use reduced-size fragments generated in such a way that directional ligation is possible. It was impossible to split the 24 kb fragment into multiple fragments for the construction as there were no unique restriction sites on the fragment that are also present on the plasmid backbone. However, this fragment was still useful as a 7.85 kb (named the α fragment) fragment was generated from the 24 kb fragment by digesting with two restriction enzymes (*EcoRI* and *HindIII*) hence generating a fragment with the overhangs for these enzymes (Figure 3.5).

The backbone fragment was generated by digesting 6.8 kb pSVβ-gal and the 7.2 kb pSVβ-gal398 with the enzymes *BamHI* and *HindIII* (Figure 3.6), generating two fragments in each case. For the 7.2 kb pSVβ-gal398, the digestion resulted in a 3.47kb (named the S fragment) fragment containing the origin of replication, selection marker and the SGS sequence, and a 3.74 kb fragment. The very similar size of these fragments meant that there was difficulty in extracting the fragment of interest. To overcome this challenge, the fragments were separated on gel with high gel concentration and long electrophoresis run time.

For 6.8 kb pSVβ-gal, the resulting fragments are the 3.08 kb (named the V fragment) fragment containing the origin of replication and selection marker; and a 3.74 kb fragment. Extracting the fragment of interest did not pose too much a challenge as the sizes were well resolved on the gel (Figure 3.7).

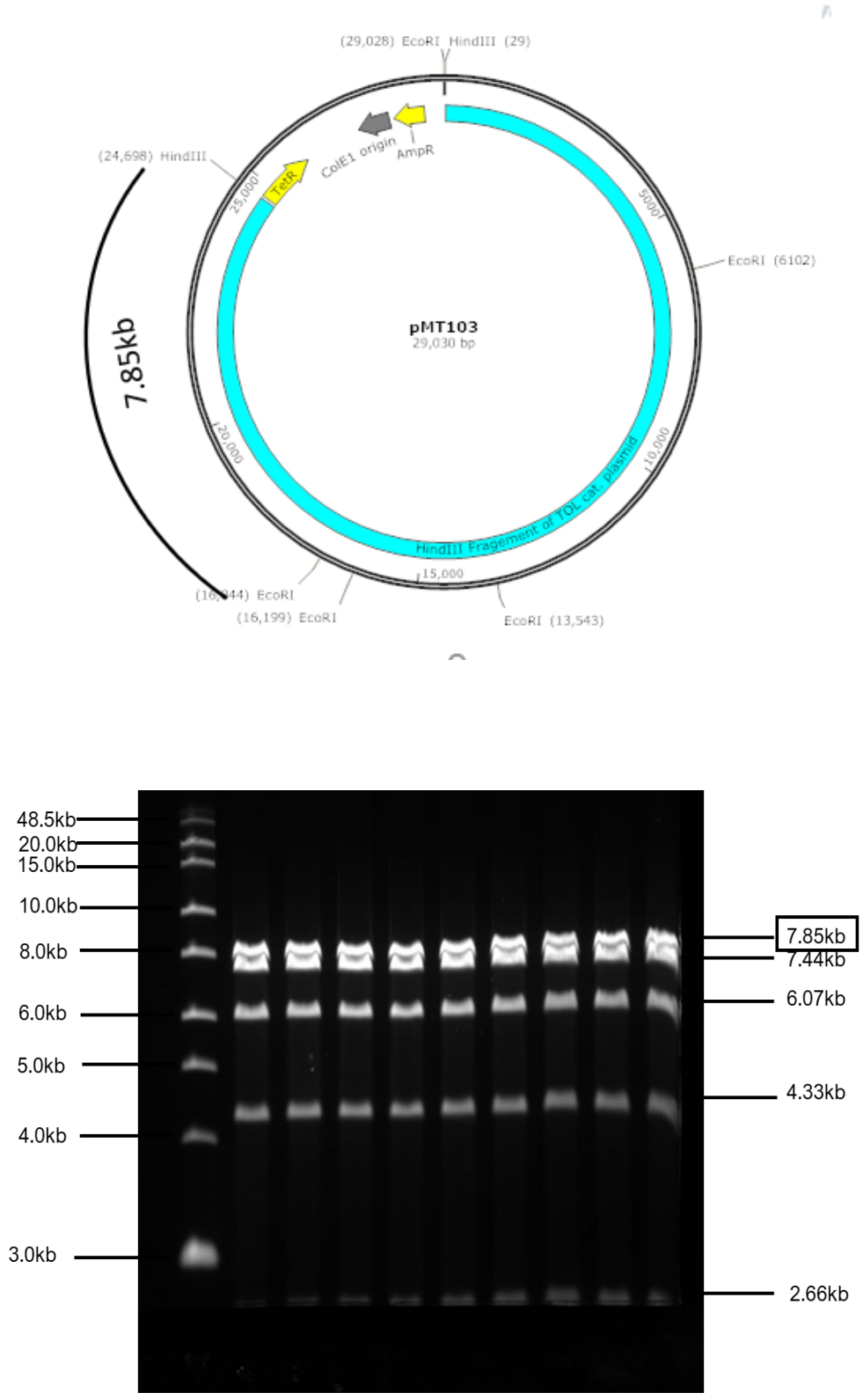


Figure 3.5: Digestion of the 24kb plamid pMT103 with *HindIII* and *EcoRI* produced 7.85 kb (α), 7.44 kb, 6.07 kb 4.33 kb and 2.66 kb fragments. The 7.85kb fragment was extracted to be used in the construction of the plasmid of interest. TOP: Plasmid map, BOTTOM: Restriction digestion gel electrophoresis image. All lanes were loaded with the same restriction digest preparation to increase yield during extraction

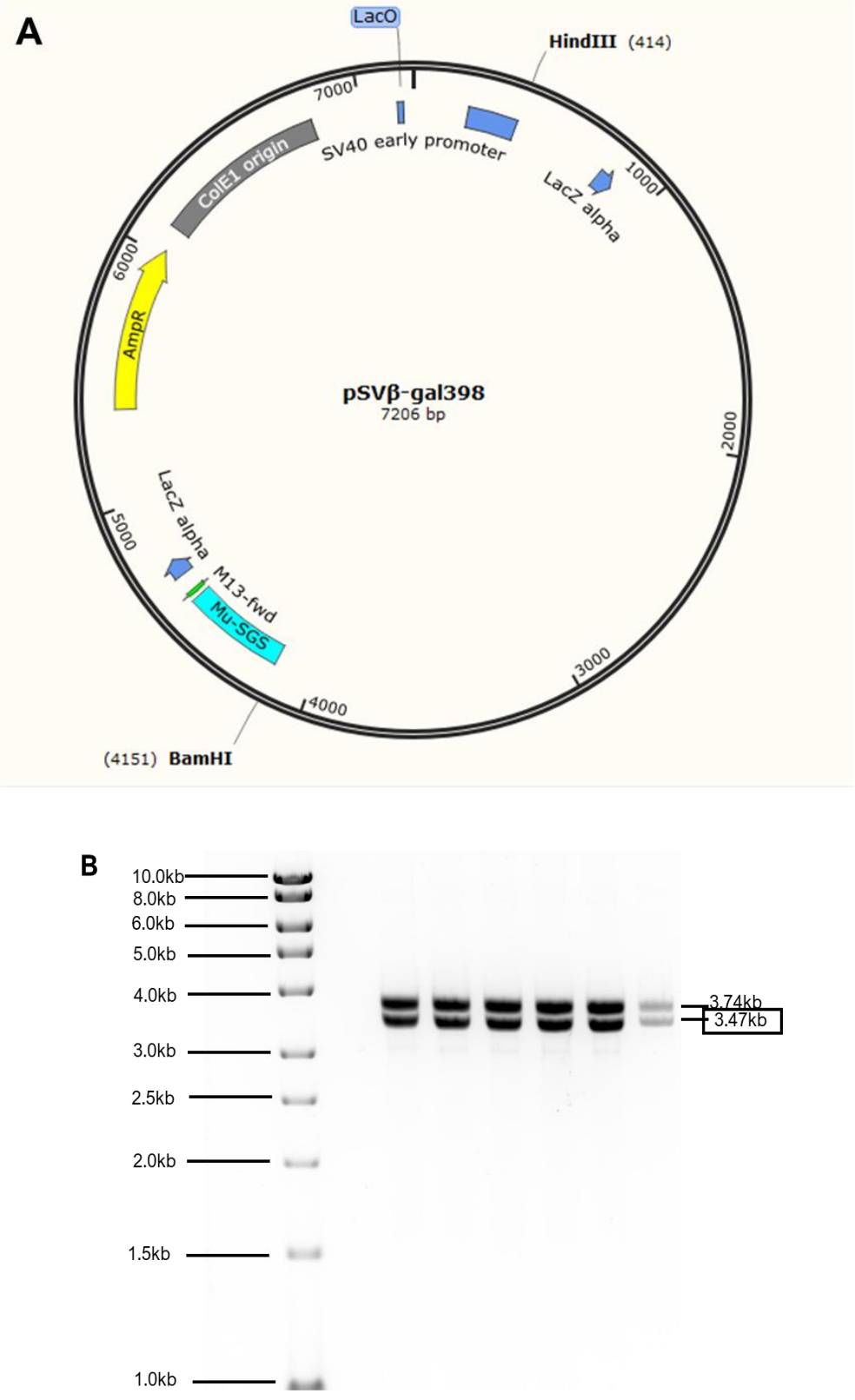


Figure 3.6: pSVβ-gal398 plasmid map showing the restriction site for the generation of fragment S. The restriction-digest gel electrophoresis image is shown at the below the plasmid map. All lanes were loaded with the same restriction digest preparation to increase yield during extraction

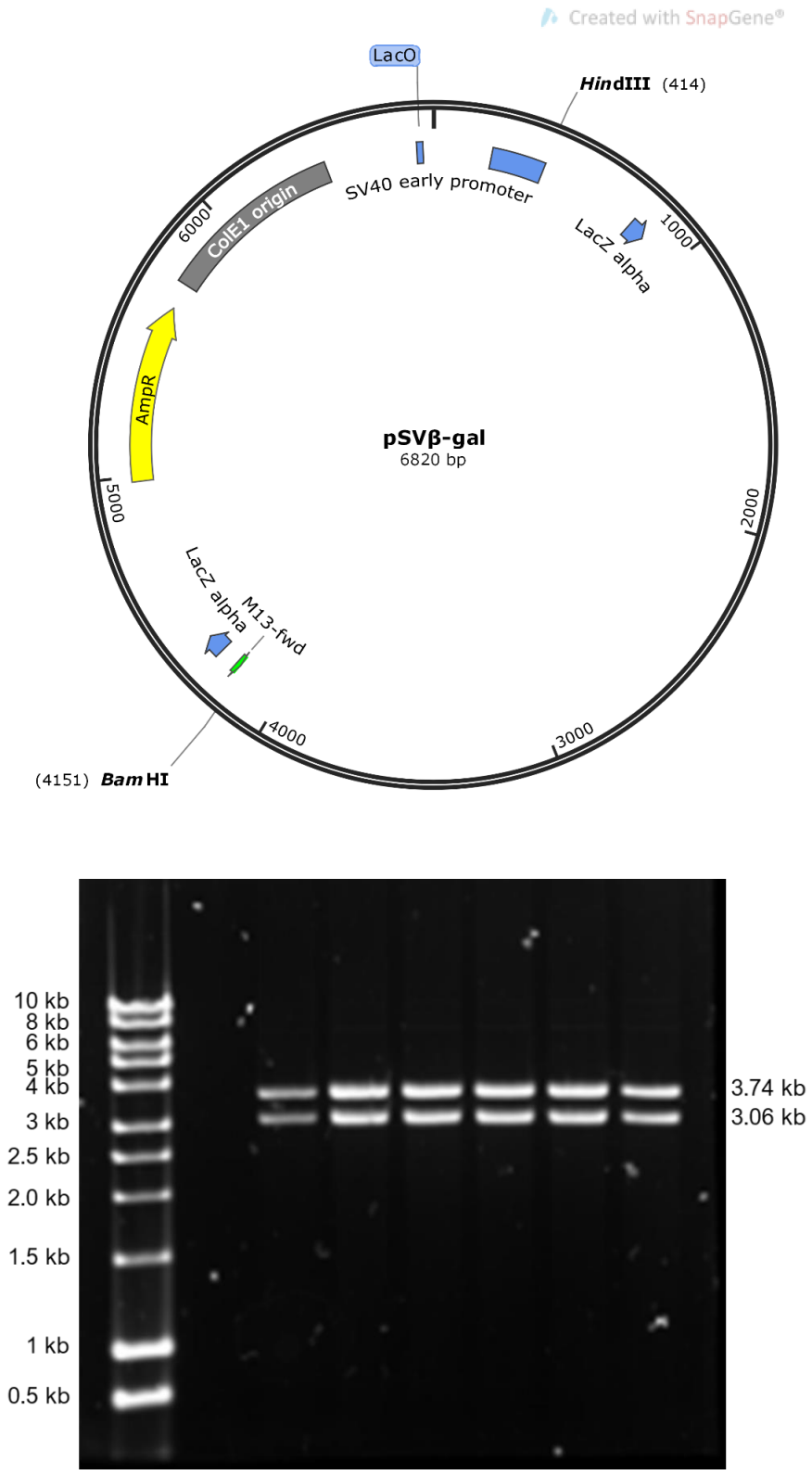


Figure 3.7: pSVβ-gal plasmid map showing the restriction site for the generation of fragment S. The restriction-digest gel electrophoresis image is shown at the below the plasmid map. All lanes were loaded with the same restriction digest preparation to increase yield during extraction

The implication of this is that there is a possibility of directional ligation with the *HindIII* ends of these fragments and the α fragment from pMT103 digestion (Figure 3.1). However, this would only result in an 11 kb linear fragment. There is a need for a 10 kb fragment at the least to complete the construct.

The rest of the fragment was acquired from the bacteriophage lambda's DNA. The bacteriophage lambda-DNA is a linear 48.5 kb DNA which is available commercially. This DNA has been widely used in the construction of Double-stranded (ds) DNA ladder by digesting with the *BstEII* enzyme (Casjens and Hendrix, 2015). Careful analysis of the restriction enzyme sites on the Lambda DNA revealed the possibility of acquiring fragments from this DNA in the construction of the >20 kb plasmid. The DNA was restriction-digested with the *EcoRI* and the *BamHI* restriction enzymes. The resulting fragments yielded a 15.7 kb (termed λ fragment) with *EcoRI* and *BamHI* overhangs. The flowchart for the assembly plan I shown in Figure 3.8

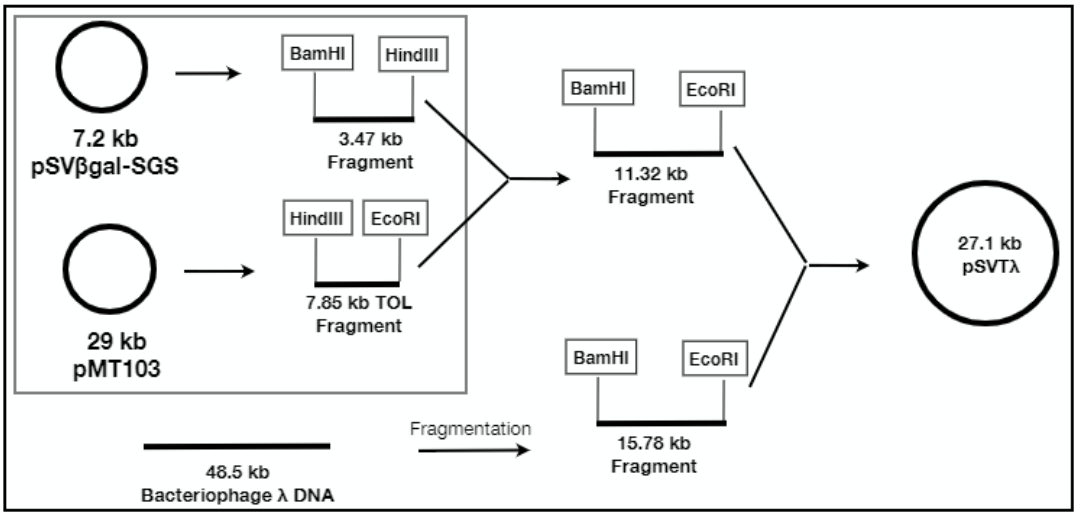


Figure 3.8: Flowchart showing plan for the assembly of a 27 kb plasmid involving ligation of fragments from three sources employing the serial ligation strategy.

This meant that a complete circularized plasmid with overlapping overhangs from the three fragments is possible (Table 3.3). A serial ligation strategy was adopted for the ligation of the fragments. The S fragment was ligated with the α fragment in the first step of the serial ligation in the ratio 3:1 and 1:1. The resulting ligation reaction was cleaned, and a second step ligation was carried out with the cleaned products of the first step and the λ fragment. The final ligation reaction was used to transform *E. coli* TOP10 chemically competent cells and electrocompetent cells by heat shock and electroporation, respectively.

The two protocols were used as it has been reported that large plasmids are better transformed by electroporation than heat shock method (Tu *et al.*, 2016). However, there was a successful transformation with the heat-shock method than the electroporation method as colonies were detected with the heat shock reaction and no colonies in the electroporation reaction. This was likely due to the pulser used or errors with preparation because the colonies observed with the heat shock method only had the re-circularized 7.2 kb pSVβ-gal398. The small size of the plasmid observed in the colonies formed from the heat shock method meant that we should have some colonies from plates with electroporation transformed cells.

Table 3.3: Sizes (in kb) of fragments used in the final construct and the overhangs.

Fragment	**Overhang A**	**Overhang B**	**Size (kb)**
S	*BamHI*	*HindIII*	3.47
V	*BamHI*	*HindIII*	3.06
α	*HindIII*	*EcoRI*	7.85
λ	*BamHI*	*EcoRI*	15.78

The explanation for the observed re-circularized plasmid is that there is cross-contamination between the two fragments resulted from the digestion of pSVβ-gal398. This was possible as the sizes of the fragments meant the bands were very close on the gel, and the extraction process might have involved cutting the unwanted band along with the band of interest. As explained earlier, a second digestion and extraction were carried out on pSVβ-gal398, and a high gel concentration coupled with a long run time was used in the separation of the fragments as bands on gel. The serial ligation strategy was employed again for the ligation process. The heat-shock method was used for transformation and there were colonies formed on the plate. Ten (10) colonies were selected, and only two of the colonies were transformed the complete plasmid construct.

The same strategy was used for the control plasmid, i.e. plasmid without SGS. The V fragment was ligated serially with the α fragment, and the resulting ligation mixture was ligated (after clean-up) with the λ fragment. There were no challenges in getting the plasmid construct as the two fragments from pSVβ-gal digestion were separated distinctly as bands on gel. There was a little chance of contamination of the fragment of interest (V) with the unwanted fragments. The plasmid maps of the two successful constructs are shown in Figure 3.9 and Figure 3.10.

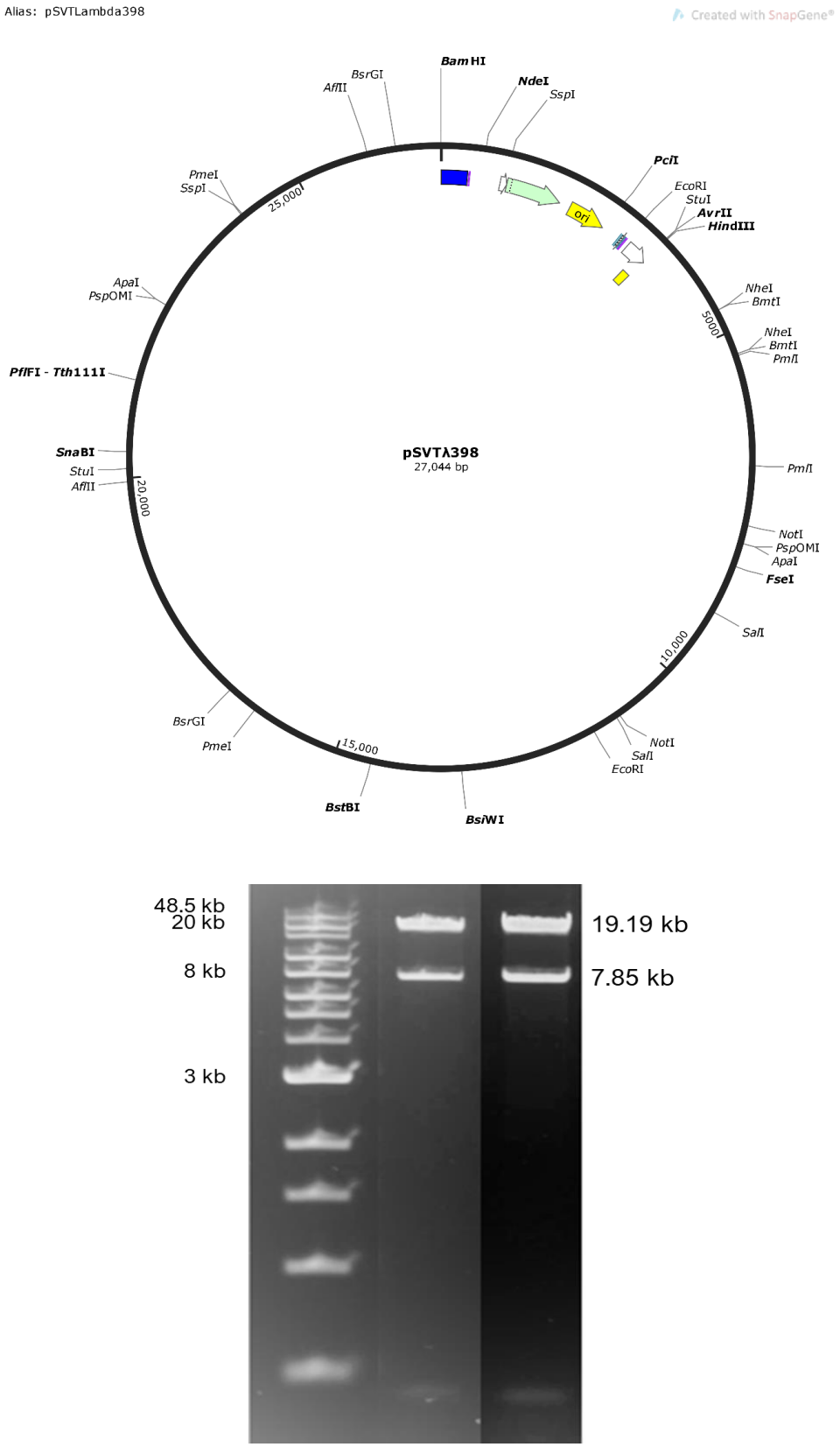


Figure 3.9: The plasmid construct pSVTλ398 containing the SGS sequence and the gel image of the diagnostic restriction digest with *HindIII* and *EcoRI* for confirmation of plasmid size

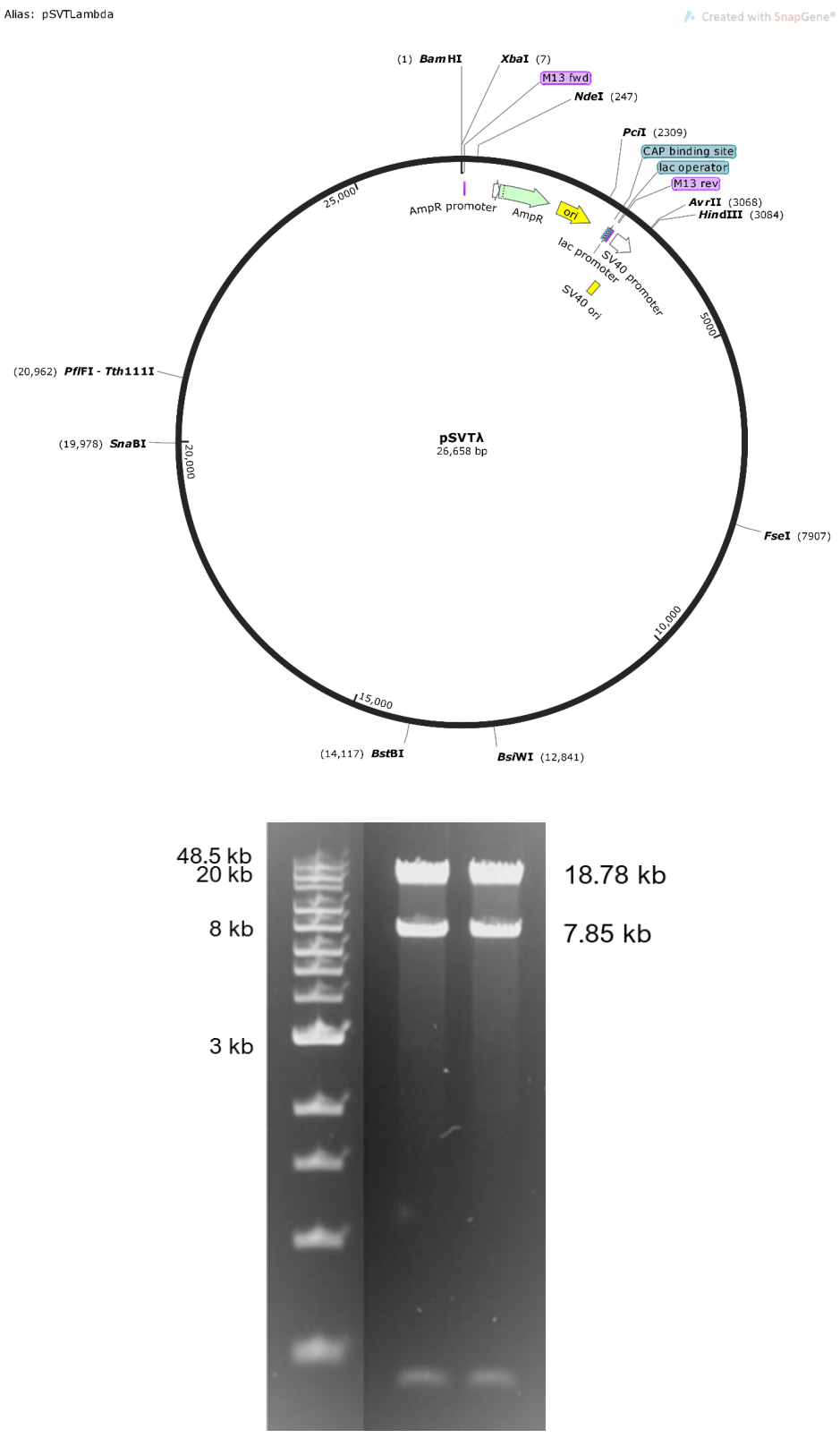


Figure 3.10: The plasmid construct pSVTλ containing the SGS sequence and the gel image of the diagnostic restriction digest with *HindIII* and *EcoRI* for confirmation of plasmid size.

3.4.3 Transformation of *E. coli* strains DH5α and HB101 with plasmid constructs

E. coli TOP10 strain was used for transformation during the plasmid construction, but the strain is not the strain of choice for plasmid bioprocessing. The plasmid constructs were used to transform *E. coli* strains DH5α and HB101, which have been reported to be efficient strains for plasmid amplification (Yau, Keshavarz-Moore and Ward, 2008). These plasmid-strains combinations were used for further studies.

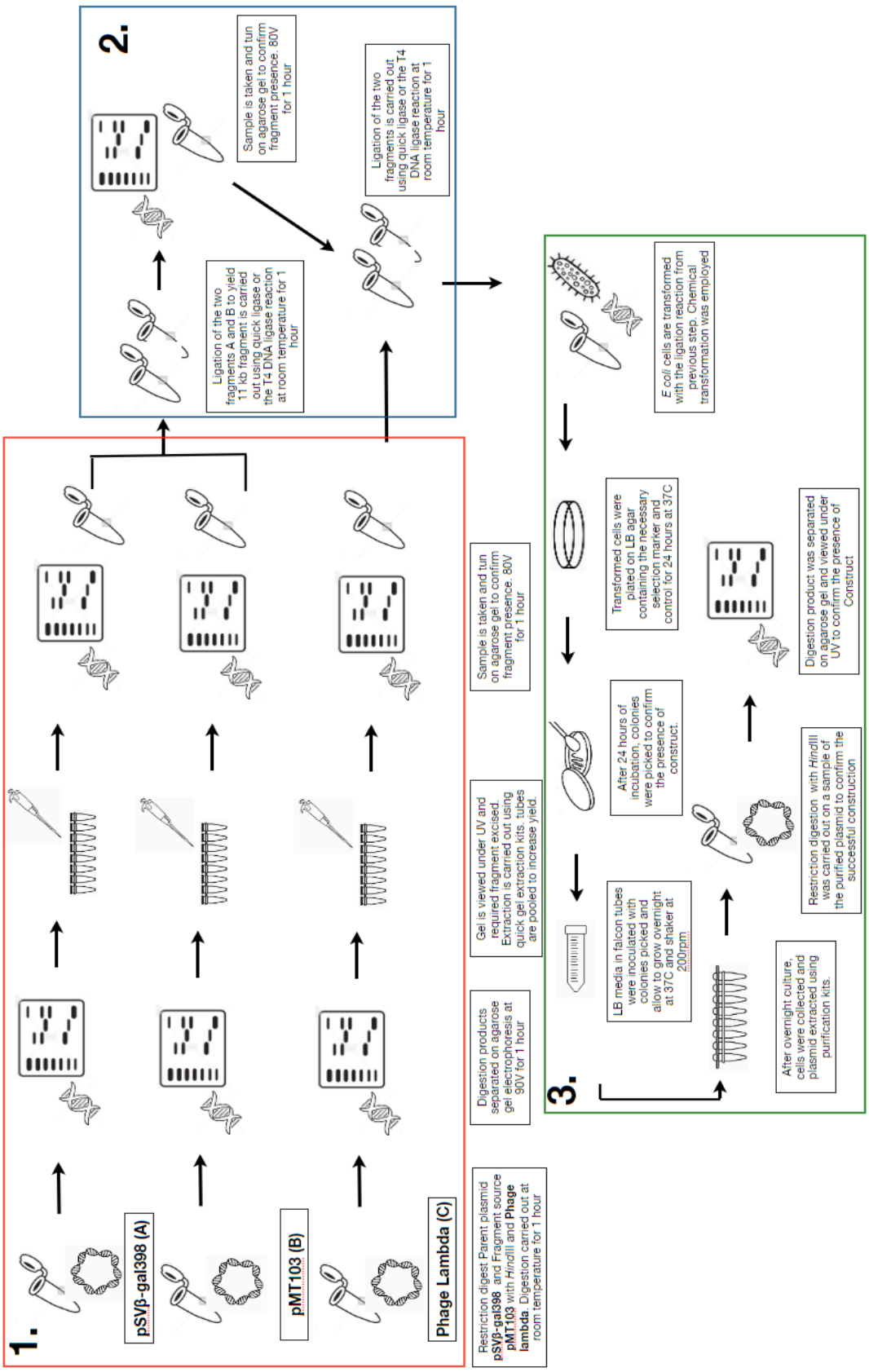


Figure 3.11 Schematic flow chart of the steps taken in the re-design approach to construct the large plasmid. Section 1 details the processes of getting the fragments from their sources, section 2 is the serial ligation step and 3 transformation

3.5 Conclusion

As one of the aims of the studies, the construction of a large plasmid was essential. Initial attempts to construct the plasmid greater than 20 kb was met with challenges due to the non-directional nature of the cloning method as well as stearic hindrances associated the sizes of the fragment.

To overcome these challenges, a directional approach was employed. Fragments from the small plasmid pSVβ-gal398 and plasmid pMT103 were extracted by digestion with multiple restriction enzymes. A larger fragment was obtained from bacteriophage lambda DNA to achieve a construct size of 27 kb for SGS containing plasmid and 26.6 kb for the non-SGS-containing plasmid.

CHAPTER 4. Effect of Mu Strong Gyrase Binding Sequence on Plasmid Yield and Quality for Biotherapeutics Application

4.1 Introduction

Supercoiling is an essential feature for quality measurement during plasmid bioprocessing. An ample amount of plasmid DNA (pDNA) is required (in mg) because not all of it gets integrated during transfection and must be mostly in the supercoiled form (in comparison to linear and nicked form) to be effective for biotherapeutics application (Cupillard *et al.*, 2005). Supercoiling is required for various physiological process of DNA including replication and transcription and also promotes recombination processes (Baranello *et al.*, 2012). As a therapeutic agent, plasmid DNA will be required to get integrated into the host chromosome, transcribed into mRNA and eventually translated and express the required protein. Therefore, two bioprocess challenges that need to be addressed are the yield and the quality (i.e. maintaining a high supercoiled fraction) of the plasmid DNA.

Increasing the plasmid yield has been well studied, but there is not much information about increasing the supercoiling of the plasmid. A study carried out by Adamcik et al., highlight how temperature affects the distribution of topoisomers in plasmid preparation and the impact on supercoiling of the preparation (Adamčík *et al.*, 2002). For instance, it has been reported that by achieving a high cell density culture, the plasmid yield can be improved as plasmid production is directly linked to the biomass yield of culture (Lara and Ramírez, 2012).

Some of these reports have achieved high biomass yield by engineering the cell using gene knockout or gene overexpression. Examples include the knockout of *pts* gene responsible for glucose transport and replacement with *galp*+ which codes for

permease (Knabben *et al.*, 2010; Gonçalves *et al.*, 2014), modification of pentose phosphate pathway (Flores *et al.*, 2004) and knocking out the pyruvate kinase gene (Cunningham, Liu, *et al.*, 2009).

In addition to a high supercoiled amount being required for the effectiveness of plasmid use as vaccine and gene therapy applications, it makes plasmid more compact as the plasmid becomes coiled up due to the twisting and writhing associated with supercoiling. This is potentially essential for downstream processing of plasmid as these molecules are sensitive to shear during downstream processes such as centrifugation, flow through pumps (M. S. Levy *et al.*, 1999; Zhang *et al.*, 2007) and filtration. The sensitivity of plasmid DNA to shear is also dependent on the size of the plasmid, hence by making plasmid more compact it is potentially possible the sensitivity of exposure to shear.

Several studies have investigated external factors and their effects on plasmid supercoiling (Mizushima *et al.*, 1997; O'Kennedy, Ward and Keshavarz-Moore, 2003; Yau, Keshavarz-Moore and Ward, 2008). However, there are very few reports that have investigated the effect of modification of plasmid DNA with aim of increasing supercoiling and superhelical density (a measurement of the compactness of the DNA) of plasmid DNA (Hassan, Keshavarz-Moore and Ward, 2016). DNA negative supercoiling is introduced by the DNA gyrase—a Type II Topoisomerase which is tightly regulated alongside the DNA topoisomerase I—which relaxes the DNA (Champoux, 2001). DNA gyrase overexpression in *E. coli* has only led to a slight increase in plasmid supercoiling (Snoep *et al.*, 2002).

Exploiting the Mu-phage SGS ability to enhance DNA supercoil and superhelical density will mean increasing the supercoil nature of the plasmid further. Hassan et al. (2016), have published studies that have shown that the length of the

left and right of the key sequence on Mu-phage SGS can also affect the efficiency of the sequence in inserting supercoil into plasmid DNA. In this study, they tested different length of the SGS and found out that the 398bp sequence, which is the longest of the different SGS length tested, significantly increased the plasmid supercoiling and superhelical density of a 2.7 kb plasmid. This present study builds on that report to look at the impact of this sequence on larger plasmids and investigate the dependency of the effect on the plasmid DNA size.

Strain selection is also important for plasmid production. Several strains of *E. coli* have been selected for the production of plasmid DNA based on laboratory use and commercial availability. These include *E. coli* K-12 strains like JM108(Huber H, Pacher C, Necina R, Kollmann F, 2005), DH5α (O'Kennedy, Baldwin and Keshavarz-Moore, 2000; Borja *et al.*, 2012; Gonçalves *et al.*, 2014), GALG20 (Gonçalves *et al.*, 2014), BL21(DE3) (Phue *et al.*, 2008), SCS1-L (Singer, Eiteman and Altman, 2009) and DH10B (Lahijani *et al.*, 1996). Usually, host cells are selected based on experiments carried out on the various strains available. Yau *et al.,* (2008) demonstrated that there is no direct correlation between the strain and the plasmid, therefore testing on different strains should be carried out when selecting a strain for specific plasmids.

Chapter aims and objectives are as follows:

- To investigate the effect of Mu-SGS presence on the yield and supercoiling of a medium-sized 7.2kb plasmid
- To investigate the effect of Mu-SGS presence on the yield and supercoiling of a large-sized 27kb plasmid
- To determine the superhelical density of plasmid with SGS

- To investigate the impact of SGS presence on the compactness of the plasmid by using the Nanoparticle Tracking Analysis
- To investigate the impact of the SGS on the integrity of plasmid during downstream processing.
- To investigate the effect of SGS presence on the segregational stability of the plasmid.

4.2 Results and Discussion

4.2.1 Plasmid Yield, Quality and Strain Selection

Supercoiling is essential for the application of DNA as therapeutics. DNA supercoiling is essential for processes such as replication and transcription (Baranello *et al.*, 2012), as well as unknotting and decatenation of double-stranded DNA—both of which are required for efficient DNA metabolism (Witz and Stasiak, 2009). Studies have been carried out to investigate the impact of supercoiling on the efficacy of vaccine with one of those reports suggesting that a plasmid preparation containing above 70% supercoiled topoisomer is capable of inducing immunity compared to the open circular topoisomer and linear topoisomer (Cupillard *et al.*, 2005). However, according to the FDA guidelines, it is specified that the minimum supercoiled conformation of plasmid preparation must be 80% (FDA, 2015). Hence, it is crucial to focus on increasing the supercoiling topoisomer of plasmid preparation for efficacious therapeutics.

In this study, the 398bp Mu-phage SGS sequence was introduced into a 7.2kb plasmid pSVβ-gal. Initial studies were carried out in small-scale (5 mL cultures) with different strains of *E. coli* transformed with the SGS and non-SGS-containing plasmids. The aim of investigating different strains is to observe the dependence of Mu-SGS on the host strain and to select strains which are prominently affected by

the SGS presence on the plasmid. The selected strains were based on the previous report by Yau *et al.,* (2008) who reported that plasmid amplification and optimization is host-strain dependent.

Figure 4.1 shows the results observed for the four different strains selected with respect to the plasmid yield and supercoil topoisomer content of the extracted plasmid. A higher plasmid yield was observed for SGS-containing plasmid pSVβ-gal398 relative to the parent plasmid pSVβ-gal. The increase plasmid yield was observed in the strains DH5α and HB101, with a statistically significant increase of 36% and 27% respectively (p-values < 0.05), but this was not the case for the other two strains W3110 and BL21(DE3). It is not surprising as the strains W3110 and BL21(DE3) are designed for recombinant protein expression and not for plasmid production (Rosano and Ceccarelli, 2014).

The high plasmid yield observed in DH5α agrees with the review of Lara and Ramirez (2012), highlighting DH5α as being a high plasmid-yield producing strain which was specifically designed for plasmid production. *E coli* strain HB101, on the other hand, has not been extensively employed for plasmid production; however, there are a few studies that have reported high plasmid yield using this strain of *E. coli* (Duttweiler and Gross, 1998; Yau *et al*., 2008). The significance of the increase in plasmid yield is implicated in the overall plasmid yield with a typical bioprocess yield reported to be around 65% for a plant designed to produce 141g per batch (Freitas *et al.*, 2006). It is also important to note that this value could be lower depending on the plasmid size, as increase in size of plasmid leads to a higher metabolic stress and nutrient requirement for the cells.

The supercoiled topoisomer content was also considered for strain-dependence amplification and the effect of SGS presence on the plasmid, as this is one of the

bioprocess requirement for plasmid DNA production for therapeutics application (Williams *et al.*, 2009). Like plasmid yield discussed above, a high supercoiled content was observed in the *E. coli* strains DH5α and HB101 with supercoiled content for both SGS-containing plasmid and non-SGS -containing plasmid exceeding 90% as shown in Figure 4.1.

Despite the two plasmids peaking above 90% supercoiled content, there was a statistically significant increase (5% with a p-value< 0.05) in the supercoiled content for pSVβ-gal398 compared to the parent plasmid pSVβ-gal. This shows the impact of the presence of the Mu-Phage SGS sequence which has been reported to increase the supercoiling of DNA due to strong binding of the Gyrase (Oram *et al.*, 2003; Hassan *et al.*, 2016). The binding of the gyrase to the SGS site induces a nick on the plasmid and enhances the supercoiling to the plasmid (Pato and Banerjee, 2000; Snoep *et al.*, 2002).

The insertion of supercoiling is implicated in the superhelical density of the plasmid— discussed in detail in sections to follow. In the case of the *E. coli* strain BL21(DE3) that was also investigated, a large proportion of the preparation was nicked (Figure 4.1). This shows that the effect of SGS presence on the supercoiling of the plasmid DNA is strain-dependent. A similar result was observed for W3110. The increase in the amount of supercoiled plasmid in the preparation coupled with the increase in plasmid yield in both HB101 and DH5α further leads to an increase in the overall productivity with 27% for pSVβ-gal398 compared to the unmodified pSVβ-gal.

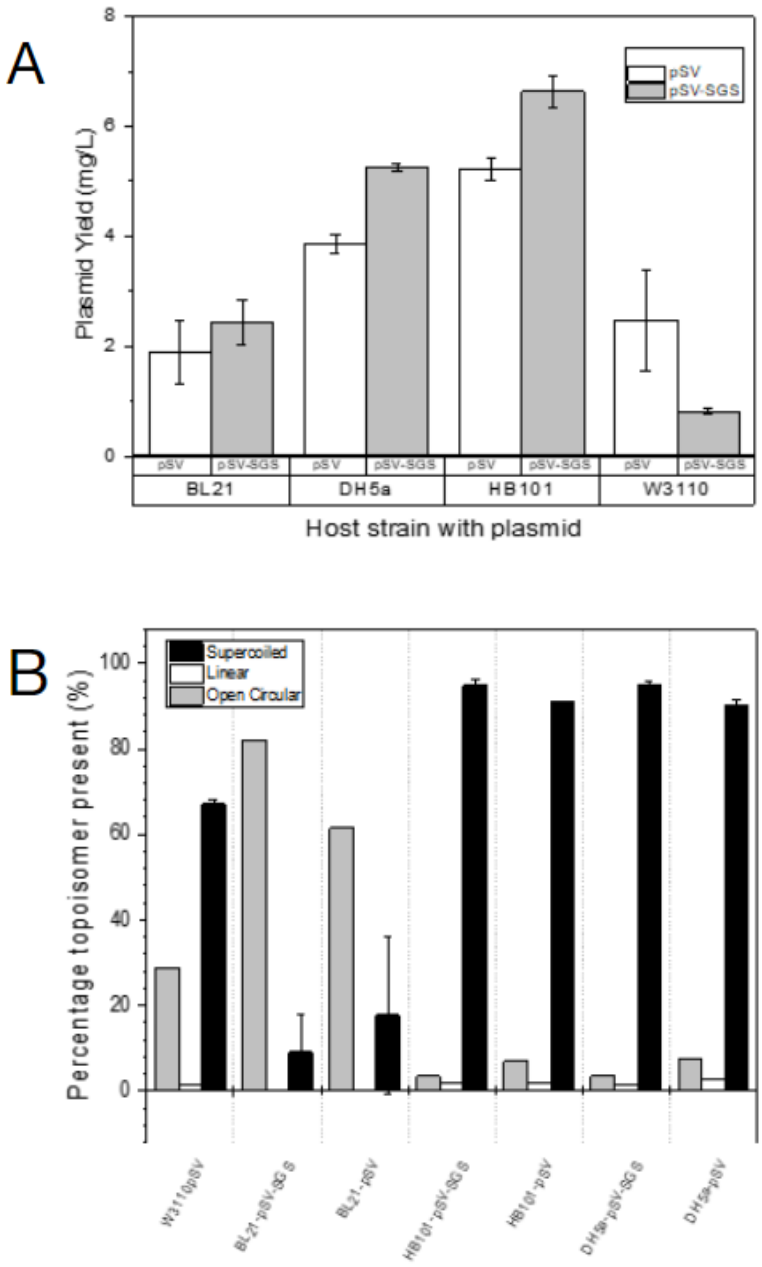


Figure 4.1: Plasmid yield and supercoiling of pSVβ-gal398 (pSV-SGS) and pSVβ-gal (pSV) amplified in the four strains. All analyses were done in triplicates. (A) The total plasmid yield (mg/L) of pSVβ-gal398 and pSVβ-gal amplified in the four strains. (B) Percentage supercoiled topoisomer content of plasmid extracted from the four strains used. The differences observed were tested statistically with $p<0.05$.

Based on the result of the initial screening studies on the strains, the two efficient strains were selected for further studies. Figure 4.2 shows the results of the fermentation growth curve for *E. coli* strains DH5α and HB101 harbouring pSVβ-

gal398 and pSVβ-gal. The final biomass yield observed agreed with initial studies with no significant differences between accumulated biomass in host strain harbouring pSVβ-gal398 and strain harbouring pSVβ-gal. We do not expect that there would be a significant difference, although it can be argued that the presence of the extra SGS sequence in the pSVβ-gal398 could have added to the metabolic stress on the strain (Diaz Ricci and Hernández, 2000), but since it is not expressing any additional recombinant product except for the selectable marker, the effect would be expected to be minimal.

However, there was a lower growth rate observed in the strain HB101 but not in DH5α. The presence of the extra sequence on the plasmid could have led to the reduced growth rate observed particularly in HB101 (Bolivar and Backman, 1979). In addition, the differences in the genotypes of the two strains (Table 4.1) could explain the observation. The fact that the decrease in growth rate was observed only in HB101 reiterates the reports that bioprocessing of plasmid DNA is strain-dependent (Yau *et al*., 2008)

Table 4.1: Strains selected for the study and their genotype

Strain	Genotype	Source	Status
BL-21	$F^- \; dcm \; hsdS(r_B^- m_B^-) gal\Delta \; (DE3)$	(Studier and Moffatt, 1986)	Commercial
DH5α	$F^- \; endA1 \; glnV44 \; thi - 1 \; recA1 \; relA1 \; gyrA96 \; deoR \; nupG \; \Phi 80dlacZ\Delta M15 \; \Delta(lacZYA - argF)U169, \; hsdR17(r_K^- m_K^+), \lambda^-$	(Hanahan, 1985; Grant *et al.*, 1990)	Commercial
HB101	$F - \; mcrB \; mrr \; hsdS20(rB - \; mB-) \; recA13 \; leuB6 \; ara - 14 \; proA2 \; lacY1 \; galK2 \; xyl - 5 \; mtl - 1 \; rpsL20(SmR) \; glnV44 \; \lambda -$	(Boyer and Roulland-dussoix, 1969)	Non-commercial
W3110	$F - \; \lambda - \; rph - 1 \; INV(rrnD, rrnE)$	(Hayashi *et al.*, 2006)	Non-commercial

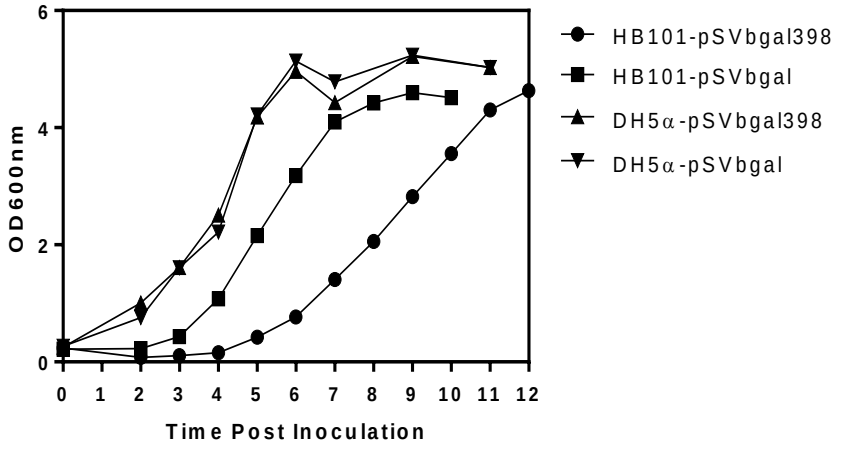


Figure 4.2: Characteristics of shake flask fermentation of the two strains HB101 and DH5α. Charts showing the growth curve of the strains bearing both the non-SGS plasmid and SGS containing the plasmid. Results are mean of triplicate cultures. Error bar invisible due to the minimal standard deviation from the mean.

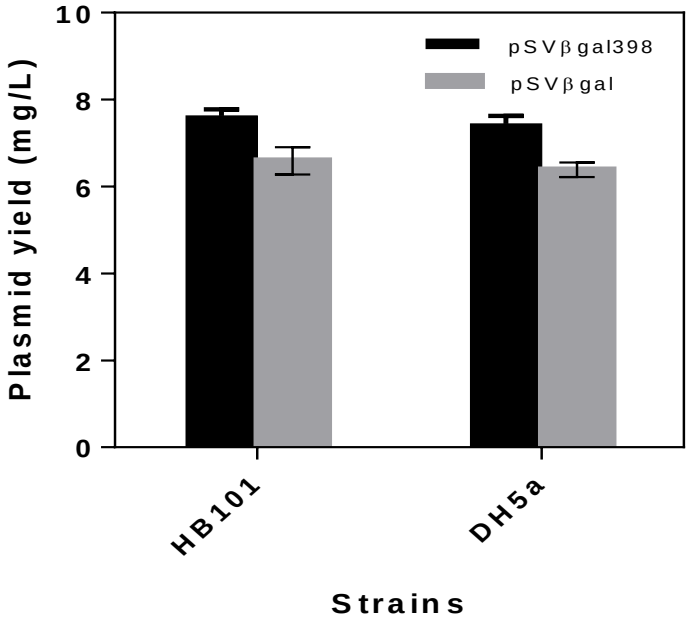


Figure 4.3: Characteristics of shake flask fermentation of the two strains HB101 and DH5α. Charts showing Plasmid yield of pSVβ-gal398 and pSVβ-gal amplified in the two strains. Results are mean of triplicate samples (n=3).

The plasmid yield was observed to be higher for pSVβ-gal398 in both strains—7.63mg/L and 7.44mg/L in HB101 and DH5α respectively— relative to the

parent plasmid pSVβ-gal with a yield of 6.59mg/L and 6.38mg/L in HB101 and DH5α respectively (Figure 4.3). The increase in plasmid yield is believed to be as a result of an increase in plasmid copy number which is shown in Figure 4.4. The plasmid copy number was investigated as it was clear that the increase in plasmid yield observed is not as a result of an increase in biomass yield since the final biomass was similar for the two strain-plasmid combinations for the two strains.

The increase in plasmid copy number could have been influenced by the presence of SGS on the plasmid. However, this needs further investigation. Another possible reason for the increase in plasmid yield specifically in HB101 could be as a result of the low growth rate because a low growth rate has been reported to support plasmid amplification (Williams *et al.*, 2009).

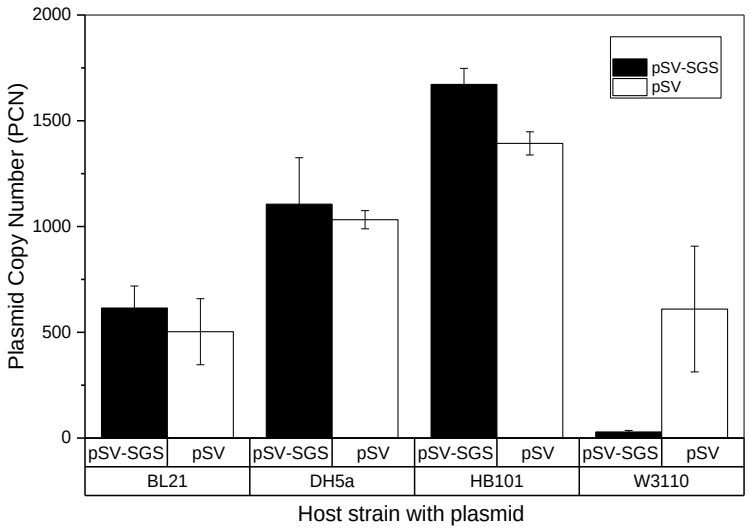


Figure 4.4: Plot of the effect of Mu-SGS on plasmid copy number on the four strains used. Plasmid copy number was determined according to the method described in Section 2.2.18. Plasmid copy number (PCN) is shown as mean± S.E.M of triplicates analysis.

The result of the supercoil topoisomer content is shown in Figure 4.5. While there was a maintenance of over 90% supercoiled content in DH5α, the supercoiled content of plasmid amplified in HB101 peaked at 80% in contrast to results observed

in the initial screening studies. One explanation for the low supercoiled topoisomer content observed in HB101 compared to DH5α could be because the latter strain was specifically designed for plasmid production. Overall, there was an increase in the supercoil topoisomer content for pSVβ-gal398 amplified in both strains compared to pSVβ-gal.

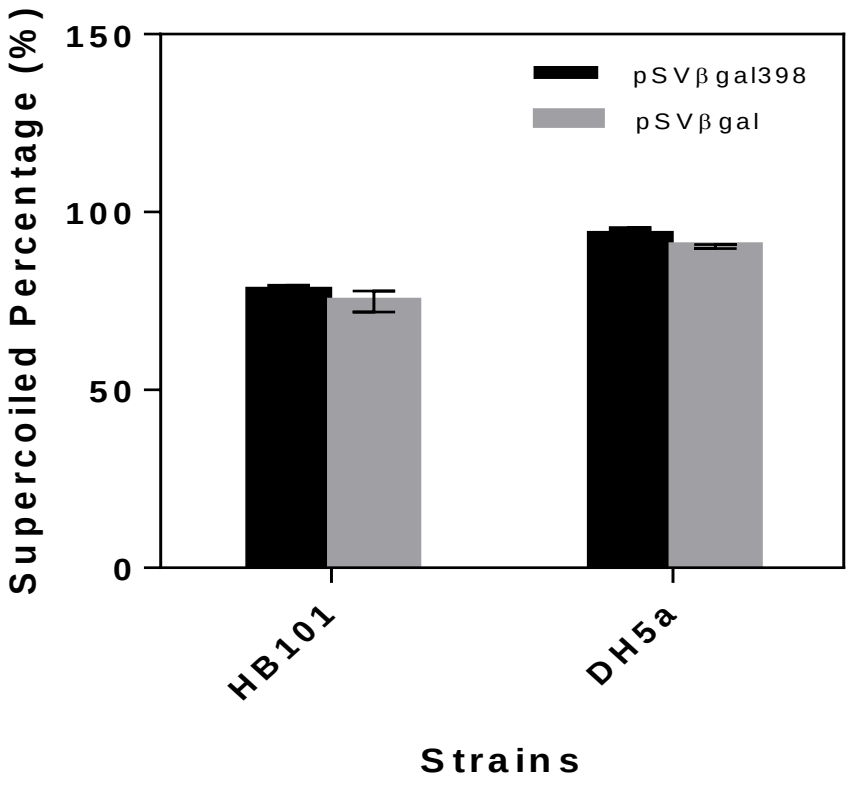


Figure 4.5: Supercoiled topoisomer content of plasmid preparations from shake flask fermentation experiments for *E. coli* strains HB101 and DH5α, n=3. Percentage supercoiling was determined by measuring the intensity of the supercoiled band on gel relative to the amount of DNA loaded on gel. Supercoiled content shown as a mean percentage ± S.E.M.

4.2.2 Plasmid Superhelical Density

The superhelical density of plasmid is a measure of how supercoiled a plasmid is, in terms of the twist (that is the winding of the DNA helix around its axis)

and writhe (the number of times the DNA helix crosses itself) of the two strands on one another (Higgins and Vologodskii, 2015). The superhelical density of DNA also measures if DNA is positively supercoiled or negatively supercoiled. This is determined using the linking number, which describes—quantitatively—the topology of the plasmid in terms of twists and writhes.

Apart from extreme thermophiles, negatively supercoiled DNA is the most preferred topological form of DNA as it facilitates the interaction between replication/transcriptional proteins and DNA (Koster *et al.*, 2010). Mu-phage SGS influences the supercoiling and compactness of DNA. One way to quantify the extent of supercoiling is by measuring the superhelicity of the DNA; in this case, the plasmid.

Figure 4.6 shows a 2-dimensional chloroquine agarose gel which is run to determine the linking difference of the plasmid. Each band represent the different level of supercoil that is found on the plasmid. By running a 2-Dimension chloroquine agarose gel, it was observed that the plasmids (both pSVβ-gal398 and pSVβ-gal) are negatively supercoiled. This is shown by the pattern of bands migration on the second dimension of the gel. The plasmids are expected to be negatively supercoiled because this is the form that supports cellular processes in *E. coli* (Higgins and Vologodskii, 2015).

Figure 4.6 shows the result of the comparison of the superhelical density of the two plasmids (pSVβ-gal398 and pSVβ-gal) amplified in the two strains HB101 and DH5α. As shown in the graph, the superhelical density of pSVβ-gal398 was approximately -0.022 in both strains compared to -0.019 for pSVβ-gal. This agrees with an earlier report by Hassan *et al.* (2016) who observed a similar result in a 2.8kb plasmid.

The increase in superhelicity observed in plasmid bearing the SGS sequence translates to high negative supercoiling. The implication of the high superhelicity to bioprocessing challenges is the possibility of maintaining the integrity of the plasmid upon shear exposure during downstream unit operations such as centrifugation and filtration. In addition to shear, there are other factors that could affect the supercoiling of plasmid during bioprocessing including pH and salt concentration of media used, particularly during alkaline lysis and chromatography. An increase in media pH can lead to loss of supercoiling (Meacle *et al.*, 2004).

More so, it has been reported that supercoiling is essential for efficient transfection. Therefore, the increase in superhelicity of the plasmid could potentially increase the transfection efficiency of the plasmid (Weintraub, Cheng and Conrad, 1986). The increase in superhelical density is equivalent to an average of two extra linking number difference (*ΔLk*) in pSVβ-gal398 compared to pSVβ-gal.

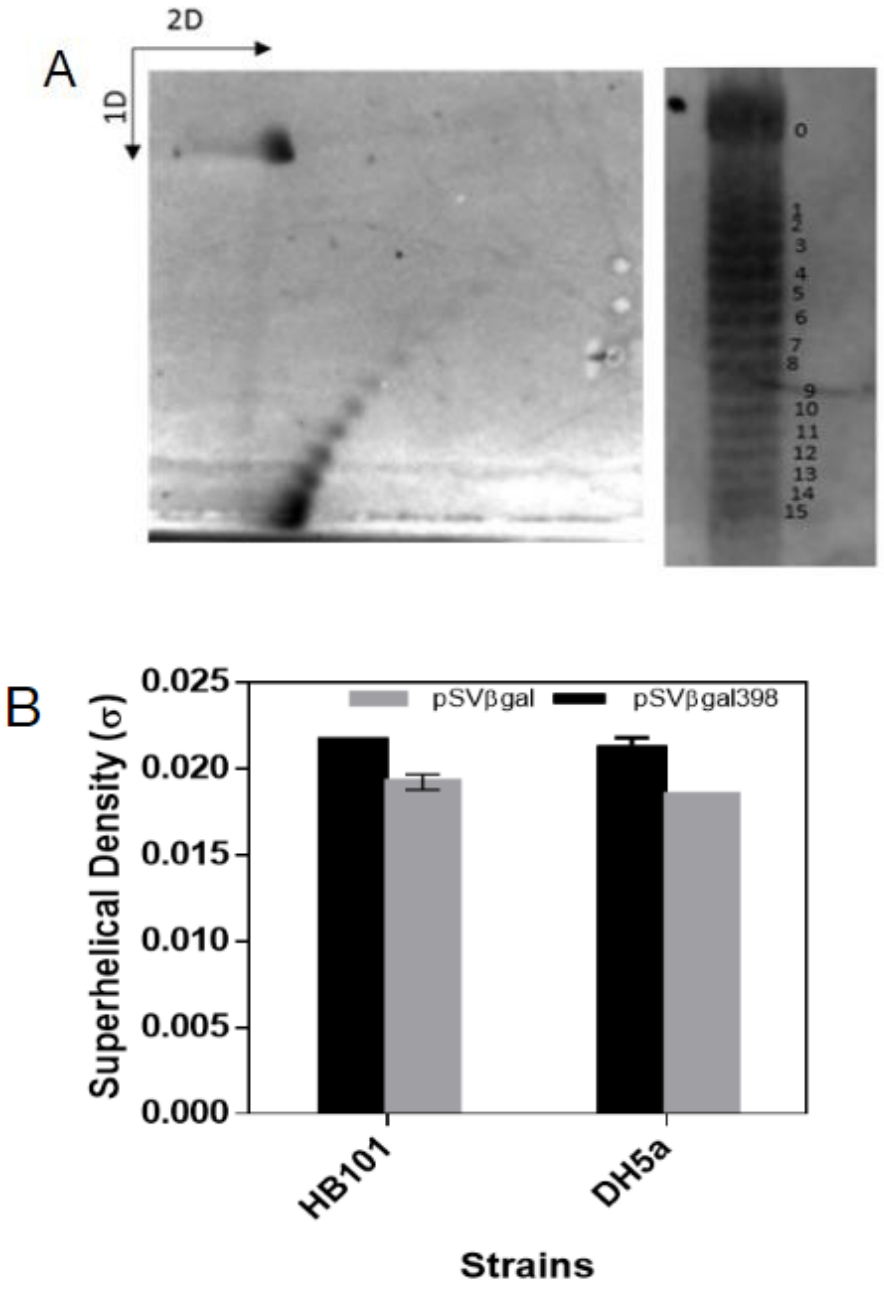


Figure 4.6: Superhelical density determination of plasmid amplified in DH5α and HB101. (A) 2D- and 1D- chloroquine-agarose gel electrophoresis showing the distribution of topoisomers of the plasmid. The bands depict the linking difference which is then used to calculate the superhelical density. (B) Chart showing the superhelical density of pSVβ-gal398 and pSVβ-gal amplified in the two strains. N=4. The difference observed is statistically significant in the two strains ($p<0.05$)

4.2.3 Plasmid hydrodynamic diameter.

To further quantify how supercoiled the plasmid is, the plasmid hydrodynamic diameter was measured. Size changes induced by SGS presence can be determined by measuring the hydrodynamic diameter of the plasmid using Nanoparticle Tracking Analysis (NTA). The NTA measures the hydrodynamic diameter based on the diffusion coefficient of the particle in Brownian motion and then tracks the particle to establish the diameter of the particle. The plasmid hydrodynamic diameter (D_h) was determined using the Nanosight NS300 (Malvern, UK) and both the plasmids pSVβ-gal and pSVβ-gal398 amplified in the strains HB101 and DH5α were examined.

To estimate the size of the supercoiled plasmid, the hydrodynamic diameter D_h of the linearized and Nicked plasmids were measured. The linear and nicked plasmids were prepared, as explained in the Materials and Method. Figure 4.7 shows the average hydrodynamic diameter of the linear and nicked plasmid pSVβ-gal398.

It is expected that the nicked plasmid will be larger than the linear plasmid as it has been observed during gel electrophoresis Figure 4.8 that supercoiled plasmid will migrate the fastest due to its tight packing, followed by a linear plasmid, with the nicked plasmid migrating the slowest. The supercoiled plasmid is therefore expected to have a smaller size relative to the linear and nicked plasmids.

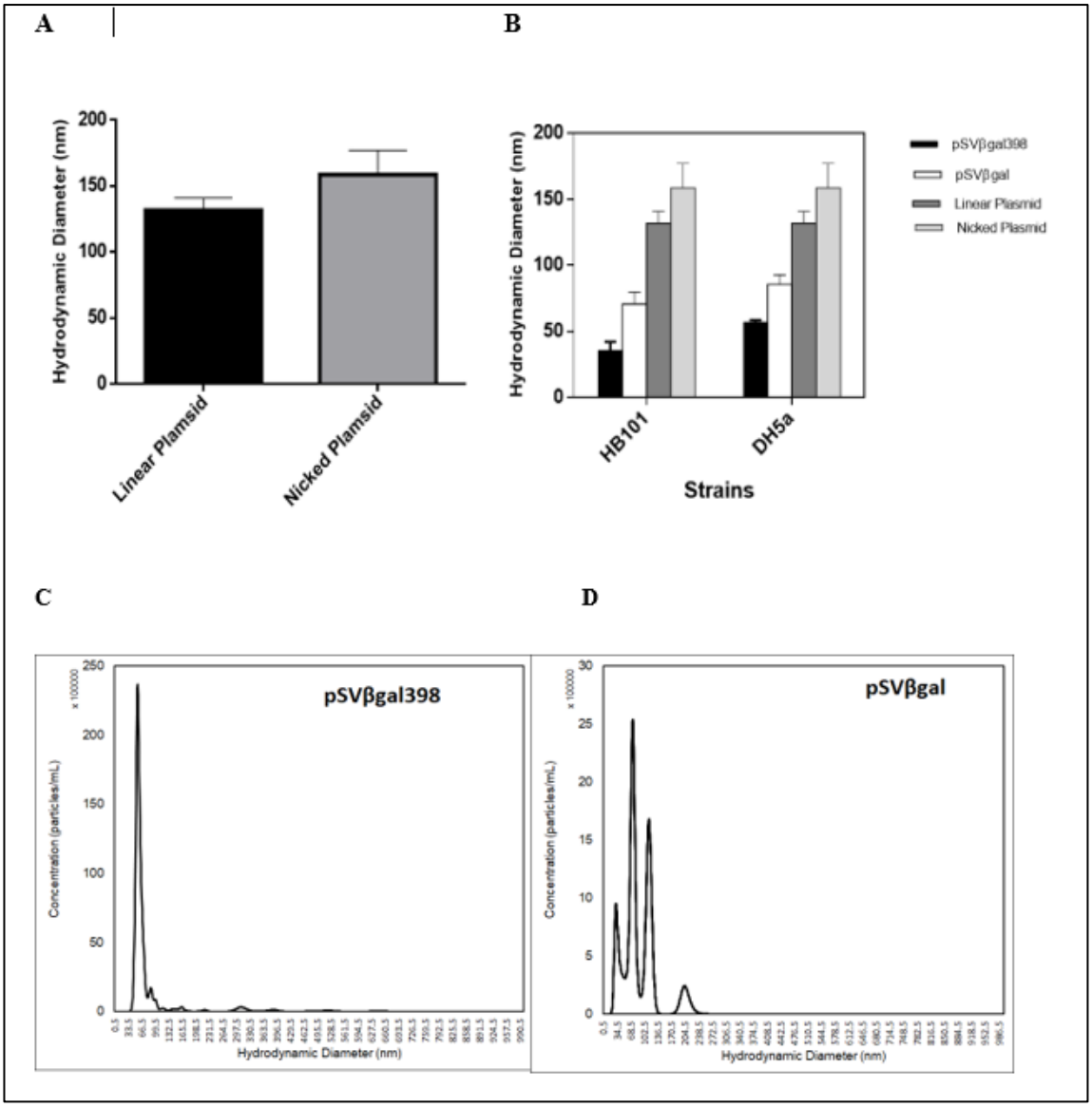


Figure 4.7: Particle size determination using the Nanoparticle Tracking Analysis which tracks particle based on Brownian motion. Charts showing (A)The hydrodynamic diameter (D_h) of linearized and nicked plasmid (B) the hydrodynamic diameter of supercoiled plasmid pSVβ-gal398 and pSVβ-gal in the two strains. The sizes are compared to the nicked and linearized plasmid which are larger than the supercoiled form of the plasmid. (C) and (D) Particle size distribution plasmid pSVβ-gal398 and pSVβ-gal from the strain DH5α. It shows a plot of diameter against concentration in particles/mL. Analyses were done in triplicates for all samples

The NTA can determine the number-weighted particle size compared to dynamic light scattering techniques (DLS) which determine an intensity-weighted average. The number-weighted assessment is particularly useful in preparations containing polydisperse particles. The plasmid preparation is expected to be

polydispersed as the sample contains the three different topoisomers: supercoiled, nicked and linear plasmid with the latter two albeit in minimal amounts. In addition, the supercoiled plasmid particles are expected to be in different random coiled form with different hydrodynamic diameter, but with sizes less than the linear and nicked plasmids. Due to the nature of the polydispersity of the samples, NTA-mode measurement was used for analysis. The NTA mode value represents the peak of the particle size on the histogram of a number-weighted distribution.

The average size of the supercoiled plasmid was observed to be 88.3nm. However, this is not a good representation of the hydrodynamic diameter of supercoiled plasmid DNA because it is the average of all available plasmid irrespective of the forms. One statistical term that gives the true hydrodynamic diameter of the plasmid is the modal value. This shows the hydrodynamic diameter of the bulk of the plasmid present as the measurement is number-weighted. When compared to the linearized plasmid and nicked plasmid, the plasmid size is smaller with an observed modal size of 40-100nm for all supercoiled plasmid preparation.

This observation is expected as agarose gel electrophoresis analysis showed that supercoiled plasmid DNA is more compact and migrates faster on gel followed by linear then nicked plasmid. More so, semi densitometry analysis of topoisomer content has shown (Figure 4.7) that the bulk of the plasmid preparation is made up of supercoiled plasmid topoisomers (although it could be the other way round, especially for large plasmid DNA and the gel concentration as shown in Appendix A). Therefore, the modal hydrodynamic diameter observed is suspected to be for the supercoiled topoisomers.

The hydrodynamic diameter observed also highlighted the compactness of the pSVβ-gal398 compared to pSVβ-gal. For plasmids amplified in the *E. coli* strain

HB101, the Hydrodynamic modal diameter for the pSVβ-gal398 is 40nm while the modal hydrodynamic diameter of the pSVβ-gal is 70nm (Figure 4.7). This supports the observation from the 2D chloroquine agarose gel electrophoresis, where the superhelical density of pSVβ-gal-SGS was found to be higher than pSVβ-gal. The same was observed for plasmids amplified in the *E. coli* strain DH5α where the modal hydrodynamic diameter of the pSVβ-gal398 and pSVβ-gal were observed to be 57nm and 86nm respectively (Figure 4.7). The compactness observed can be attributed to the presence of the Mu-SGS on the plasmid as reported earlier.

Although, earlier report by Hassan et al., (2016) and section 4.2.2 have analyzed the influence of Mu-SGS on the supercoiling of plasmid by determination of the superhelical density, this study highlights the possibility of investigating plasmid DNA hydrodynamic size using the Nanoparticle Tracking Analysis (NTA) and to further prove the influence of Mu-SGS presence on the compactness of the plasmid DNA; a potential requirement for DNA uses for therapeutic applications.

Surprisingly, the effect of Mu-SGS on plasmid supercoiling was observed to be more prominent in plasmids amplified in HB101 in comparison to DH5α—a common *E. coli* strain for plasmid production. Although there was an impact of Mu-SGS on the plasmid in both strains, the superhelical density and plasmid hydrodynamic diameter (D_h) shows that the plasmids amplified in HB101 are more compact and supercoiled than plasmids amplified in DH5α (Table 4.2). Yau *et al.* (2008), reported a strain dependency on plasmid production. This study further emphasizes the selection of the right strain for specific plasmid during bioprocessing.

Table 4.2: Superhelical density and hydrodynamic diameter of the plasmids amplified in different strains. The hydrodynamic diameter is expressed as mean + SEM and n = 3.

	HB101		DH5α	
	pSVβ-gal398	**pSVβ-gal**	**pSVβ-gal398**	**pSVβ-gal**
Superhelical density	−0.0218	−0.0192	−0.0204	−0.0185
Hydrodynamic diameter	39.53 ± 6.52 nm	70.77 ± 8.84 nm	56.97 ± 1.55 nm	85.50 ± 7.19 nm
Compactness	44% more compact		34% more compact	

4.2.4 Impact of Increase Supercoiling on Shear Resistance

One of the hypotheses of the significance of a highly supercoiled plasmid is the maintenance of plasmid integrity during downstream processing. Plasmids DNA molecules get exposed to elongational shear stress during centrifugation process as well as in pumps (Zhang *et al.*, 2007; Kong *et al.*, 2008; Xenopoulos and Pattnaik, 2014). After fermentation and alkaline lysis processes, plasmid get exposed to shear during the clarification processes, which can either be centrifugation or filtration. During centrifugation, plasmid get exposed to shear as high as 10^4 s^{-1}. Shear stress causes nicking of plasmid hence reducing the supercoiled plasmid topoisomer content of plasmid preparation. Figure 4.8 and Figure 4.9 show the supercoil percentage topoisomer of plasmid preparations exposed to shear using the Ultra-scale down (USD) shear device— which mimics shear during centrifugation (discussed in Chapter 2).

The results showed that there was no significant change in topology observed for plasmids exposed to shear. The supercoiled plasmids remained intact despite exposing them to high shear. This observation is not surprising as it has been reported that plasmid with size <13kb are affected minimally by shearing during downstream processing with significant shear observed for plasmid larger than 13kb (Levy *et al.*, 1999; Zhang *et al.*, 2007). Therefore, the effect of the SGS sequence—

increasing supercoiling— on the plasmid integrity could not be determined from this experiment.

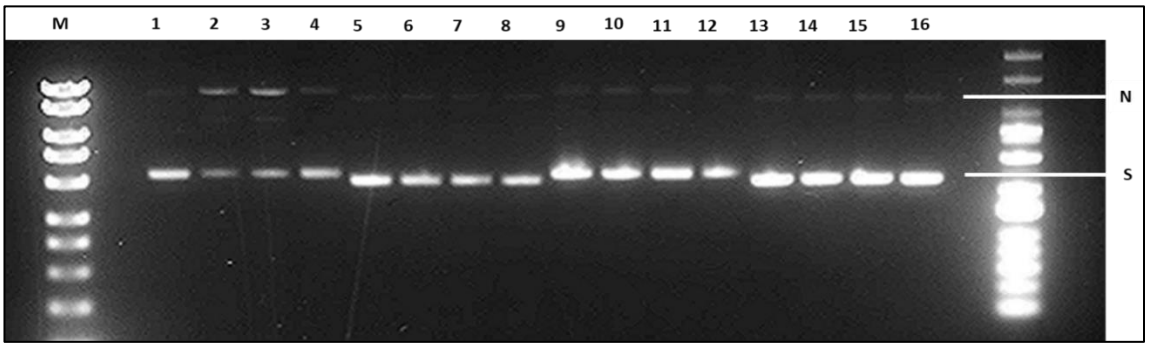


Figure 4.8: Gel image of the samples after shearing; M= marker, Lane 1 = DH5α/ pSVβ-gal398 control sample, Lane 2-4 = DH5α/ pSVβ-gal398 sheared samples, Lane 5= DH5α/ pSVβ-gal control sample, Lane 6-8 = DH5α/ pSVβ-gal sheared samples, Lane 9 = HB101/ pSVβ-gal398 control sample, Lane10-12 = HB101/ pSVβ-gal398 sheared samples, Lane 13= HB101/ pSVβ-gal control sample, Lane 14-16 = HB101/ pSVβ-gal sheared samples, N= Nicked plasmid and S= supercoiled plasmid. Supercoiled topoisomer content after exposure to shear using the USD shear device, expressed as a fraction of the control (supercoiled content of non-sheared sample), n=3

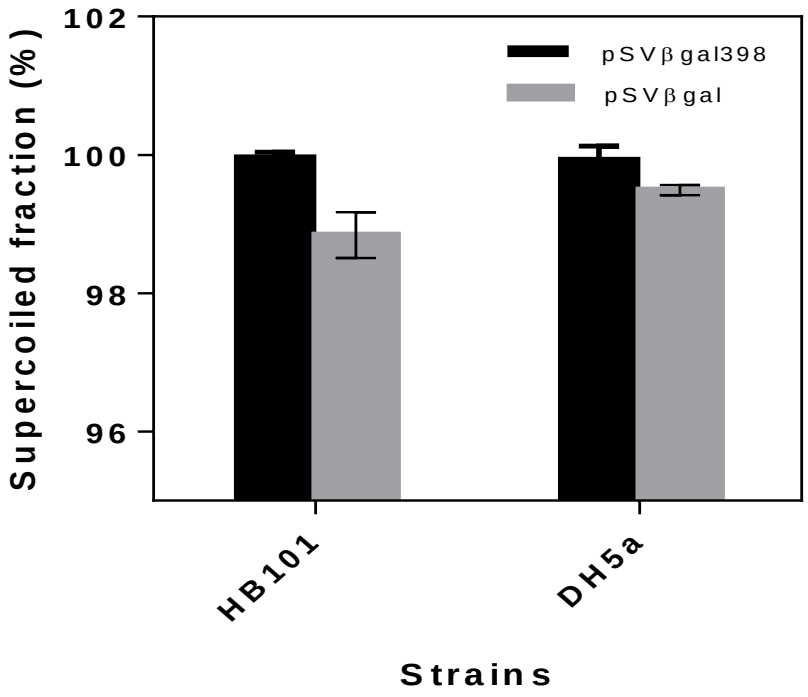


Figure 4.9: Supercoiled topoisomer content after exposure to shear using USD shear device, expressed as a fraction of the control (supercoiled content of non-sheared sample), n=3

4.2.5 Effect of SGS on the biomass yield and growth curve of a 27kb plasmid

Many indications are pointing towards the potential of plasmid size used in gene therapy application reaching over 50kb in size (Levy, O'Kennedy and Ayazi-Shamlou, 2000). The potentials of large plasmid DNA for therapeutic application means that it is necessary to develop a bioprocessing approach for large plasmid size.

The 27kb plasmid (pSTFλ398) was designed to bear the Mu SGS sequence as discussed in the earlier chapter and transformed to the two strains that have been investigated in the study (HB101 and DH5α). Figure 4.10 shows the growth curve and biomass yield observed when these strains were cultured in complex media.

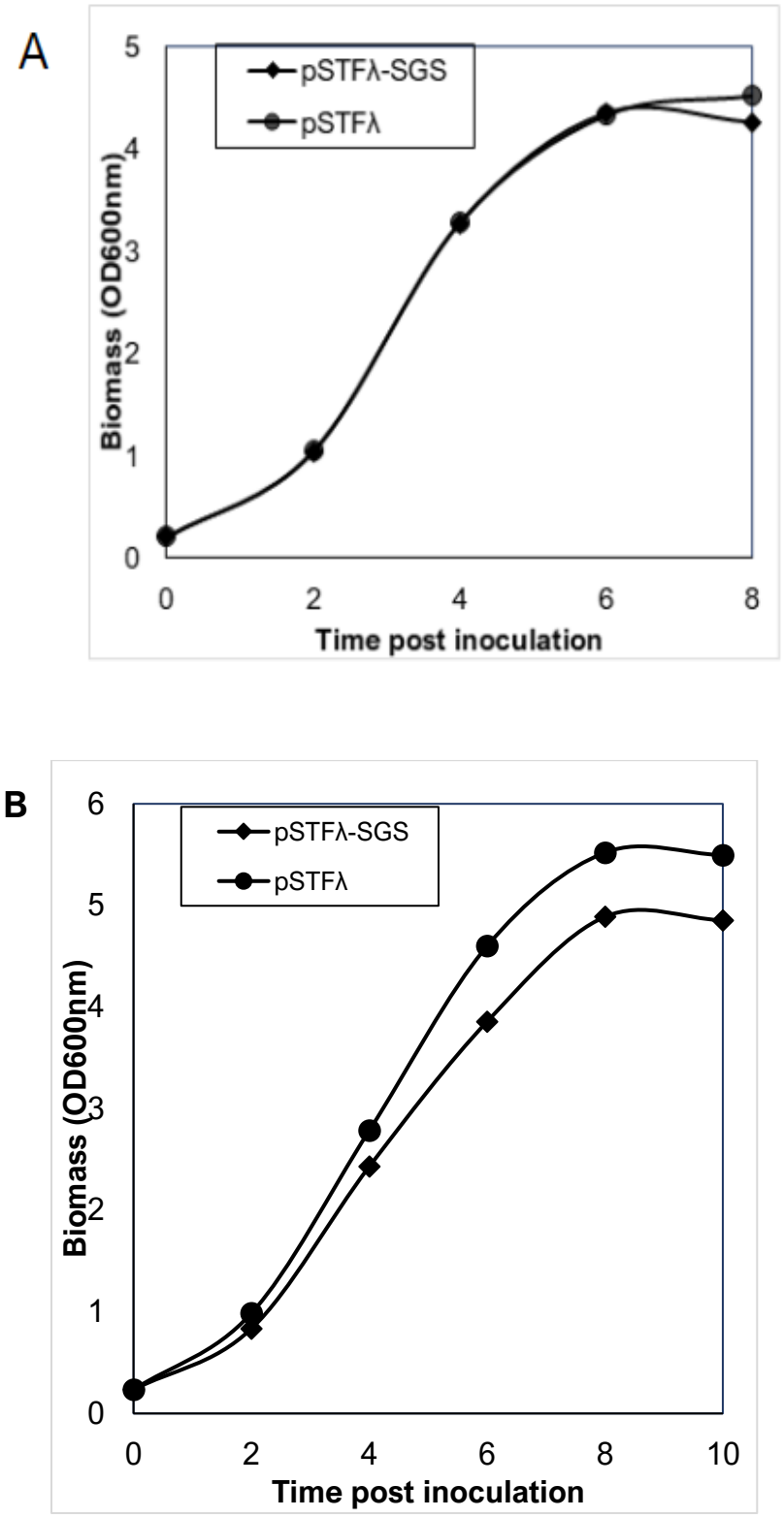


Figure 4.10: Growth curve for (A) DH5α and (B) HB101 bearing the 26.6 kb pSTFλ and 27 kb pSTFλ398 plasmids. The OD readings are the average of independent fermentation cultures.

In the strain DH5α, the growth curve of the control plasmid pSTFλ and the SGS plasmid containing pSTFλ398 cells are similar, having same growth rate and achieving the same biomass yield until the stationary phase when the strain with plasmid bearing SGS dropped in biomass. The observed similarity agrees with previous observation for the 7.2kb pSVβgal-398 and 6.8kb pSVβgal in DH5α, where the growth rate and biomass yield was similar for both plasmid-strain combinations (4.2.1).

In HB101, just like it was observed for the 6.8 kb pSVβgal and 7.2 kb pSVβgal-398, the growth rate differs with the HB101-27 kb pSTFλ398 plasmid having a lower growth rate than its non-SGS containing counterpart. This could be due to the fact that extra 400bp in the plasmid confers some extra stress on the cell. However, the final biomass yield was significantly lower in this strain.

This is in contrast to what was observed for the 7.2kb pSVβgal-398 where the final biomass achieved was similar to the strain transformed with the non-SGS-containing plasmid. One possible explanation could be that the size of the plasmid confers high metabolic stress on *E.coli* due to the requirement for replication and maintenance of the plasmid (Silva, Queiroz and Domingues, 2012). An extra 400bp might have a significant impact on an already stressed cell.

4.2.6 Total Plasmid Yield and Supercoiled Plasmid Yield

The effect of SGS on the total and supercoiled plasmid yield is shown in Figure 4.11. The total plasmid yield was significantly higher for the SGS-containing pSTFλ398 in both strains. In DH5α, the total plasmid yield for pSTFλ398 was 8.44 mg/L and 5.29 mg/L for pSTFλ. This is about a 60% increase in the plasmid yield for SGS-containing plasmid. In HB101, the total plasmid yield for pSTFλ398 plasmid was

13.45 mg/L and 7.75 mg/L for pSTFλ. The total plasmid yield for SGS-containing plasmid was almost double the yield in the non-SGS containing plasmid. This further reiterates that the effect of SGS on plasmid yield is universal, irrespective of the plasmid size and construct. It also echoes the possibility of increasing plasmid through rational engineering (Gonçalves *et al.*, 2012).

The effect of SGS presence was also echoed on the supercoiled plasmid yield, particularly in *E. coli* strain HB101. The percentage supercoiling determined by semi-densitometric method revealed that there was a higher supercoiled plasmid content in plasmid preparation of SGS containing plasmid (pSTFλ398). The supercoiled content in 27kb plasmid peaked at 93% and pSTFλ peaked at 91% (significant difference with p-value < 0.05). Even though the difference looks like a slight difference, the effect can be observed in the total supercoiled plasmid yield (Figure 4.11) with a relative yield of 76%.

In DH5α, the total supercoiled content was lower than expected. The strain is widely used for plasmid production due to its *endA* and *recA* mutations that confer stability on the plasmid (Taylor, Walker and McInnes, 1993). In addition to these mutations, there is also a *gyrA* mutation that confers resistance against nalidixic acid. Since the *gyrA* gene is implicated in the introduction of supercoiling in DNA, it is possible that the presence of this mutation affects the supercoiling ability of *E. coli* gyrase on a large plasmid. No such effect was observed in a small-sized plasmid (7.2 kb). Due to the very low supercoil content, there was no significant effect of Mu- SGS presence on the supercoiled content of the plasmid amplified in DH5α.

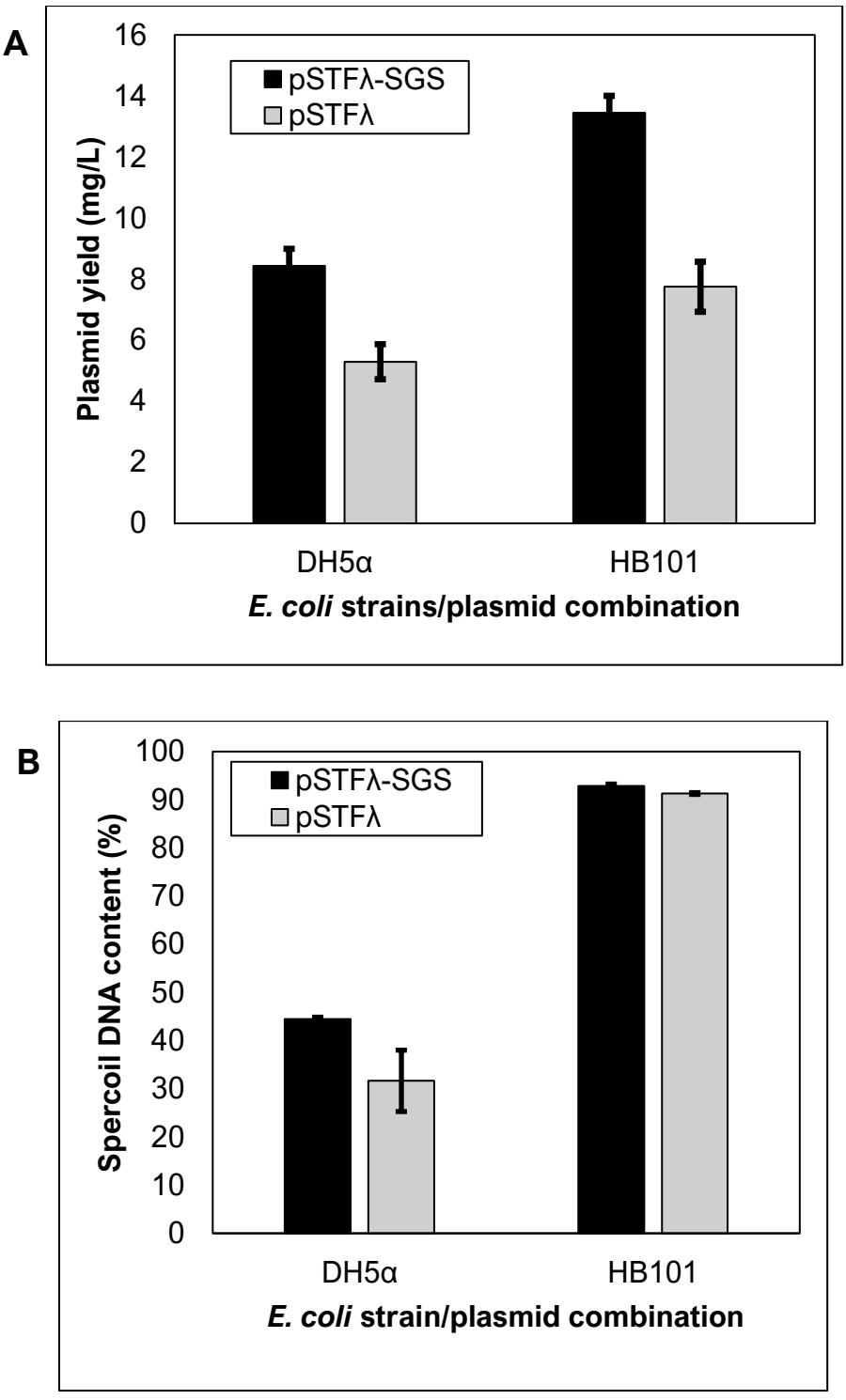


Figure 4.11: Plot showing the total volumetric plasmid yield and supercoiled content of plasmids. (A) The plasmid yield (in mg/L) for the two plasmids in the two strains. A higher plasmid yield is observed in pSTFλ398 in both strains compared to pSTFλ. (B) Supercoiled content is shown for both strains with HB101 having a higher supercoiled content for both plasmids. All values are mean of triplicate analyses

4.2.7 Effect of shear stress on the integrity of the plasmid

Consideration of the bioprocessing of large plasmid is essential as large plasmids are susceptible to shear during downstream processing (Kong *et al.*, 2008; Xenopoulos and Pattnaik, 2014). Levy *et al.* (1999) have reported the increase in shear sensitivity of plasmid with an increase in size and ionic strength of the environment when these plasmids are exposed to shear conditions similar to exposure during bioprocessing with the highest damage occurring when there is an interaction between the plasmid and air-liquid interface. The resulting shear induction occurs both post-alkaline lysis and post-purification. Plasmid DNA is also exposed to shear during the preparation for application such as spray-drying, nebulization and vial-filling (Lengsfeld and Anchordoquy, 2002).

During homogenization and centrifugation, plasmid DNA is exposed to shear rate as high as $10^5 – 10^6$ s^{-1} (M.S. Levy *et al.*, 1999; Zhang *et al.*, 2007). It is, therefore, essential to tackle this problem. The plasmid extracted from the two strains were subjected to shear and the integrity was determined afterwards. Like the 7.2kb pSVβ-gal398 and 6.8kb pSVβ-gal, the 27kb pSTFλ398 and 26.6kb pSTFλ were subjected to shear at an energy dissipation rate of 10^6 W/kg. Unlike the small plasmids that maintained their integrity at this shear rate, the large plasmids were sheared completely when observed on gel (Figure 4.12). This is not surprising as it has been reported that large plasmids are susceptible to shear (Levy *et al.*, 1999). The energy dissipation rate was then lowered, and the plasmids were exposed to varying energy dissipation rate.

When these plasmids were exposed to energy dissipation rate of 10^4 W/kg, which is equivalent to 6000rpm on the USD shear device, there was no effect observed compared to the control for both plasmids. The integrity of the plasmids

was maintained at this shear rate. However, with increasing the energy dissipation rate to 10^5 W/kg which is equivalent to 12000rpm, the supercoiled contents in both plasmids dropped, with a supercoiled fraction of 76% for 27kb pSTFλ398 and 70% for the 26.6kb pSTFλ relative to the control plasmid in both cases (Figure 4.13). Despite both plasmids having a reduced supercoiled plasmid content on exposure to shear, the SGS-containing plasmid was able to maintain a higher fraction. This can be attributed to the increase in the superhelical density of the plasmid.

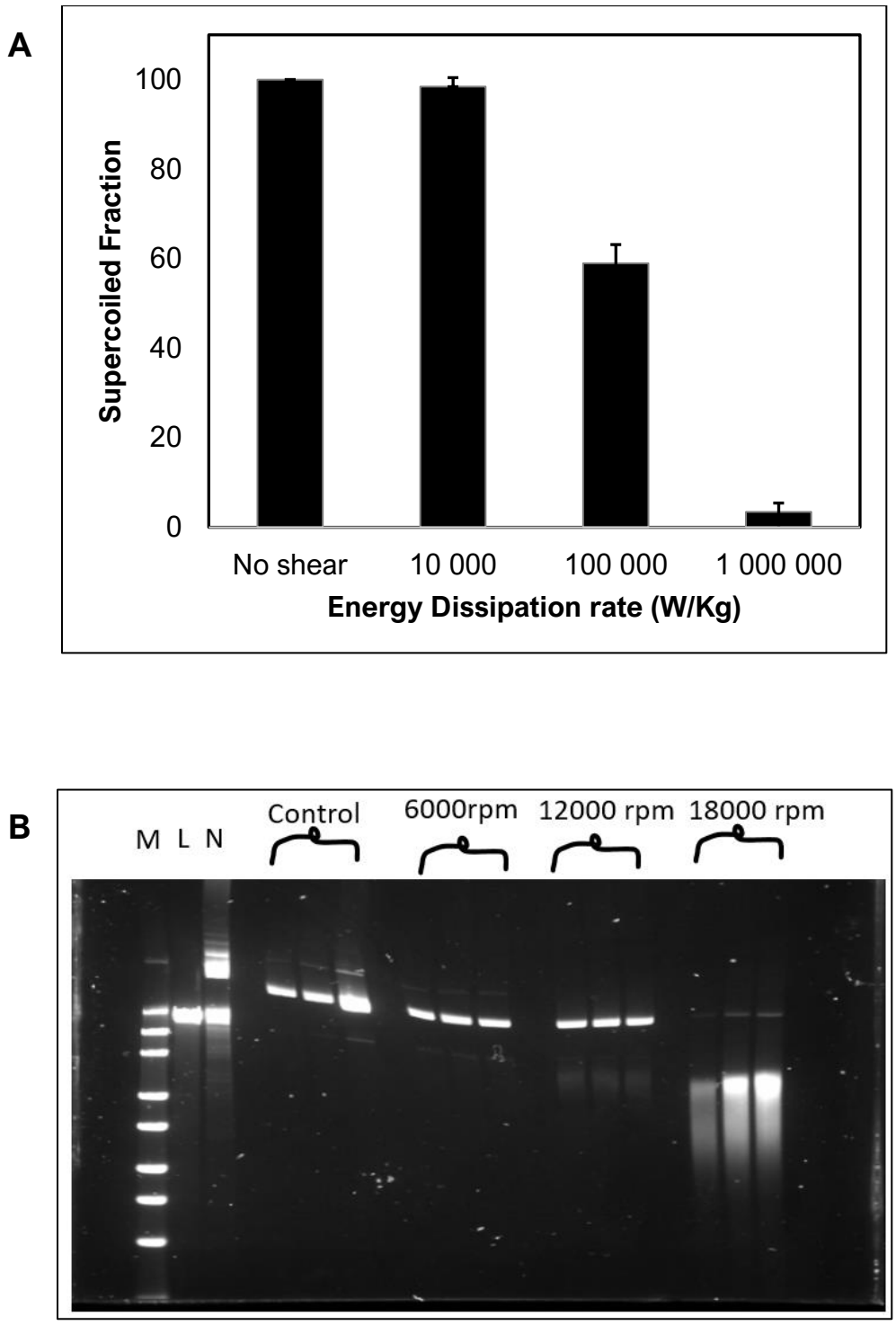


Figure 4.12 Exposure of large plasmid to different shear rates. (A) shows the plot of the supercoiled fractions of the pSTFλ398 over a selected range of energy dissipation rate Results are shown as mean+ S.E.M (n=3). (B) Gel image of the sheared plasmid run at 80V for 9 hours over a range of rotational speed (rpm) equivalent to energy dissipation rate. The bendy bands are due to the potential difference and the long hours of run.

Again, the importance of plasmid engineering for improved bioprocessing of plasmid for therapeutics application is being highlighted here, where the integrity of the plasmid is maintained even when exposed to shear conditions. The reduced sensitivity to shear could be translated to higher yield as less plasmid will be lost to nicking due to downstream processing conditions which are necessary to get the plasmid to the pure form (Xenopoulos and Pattnaik, 2014).

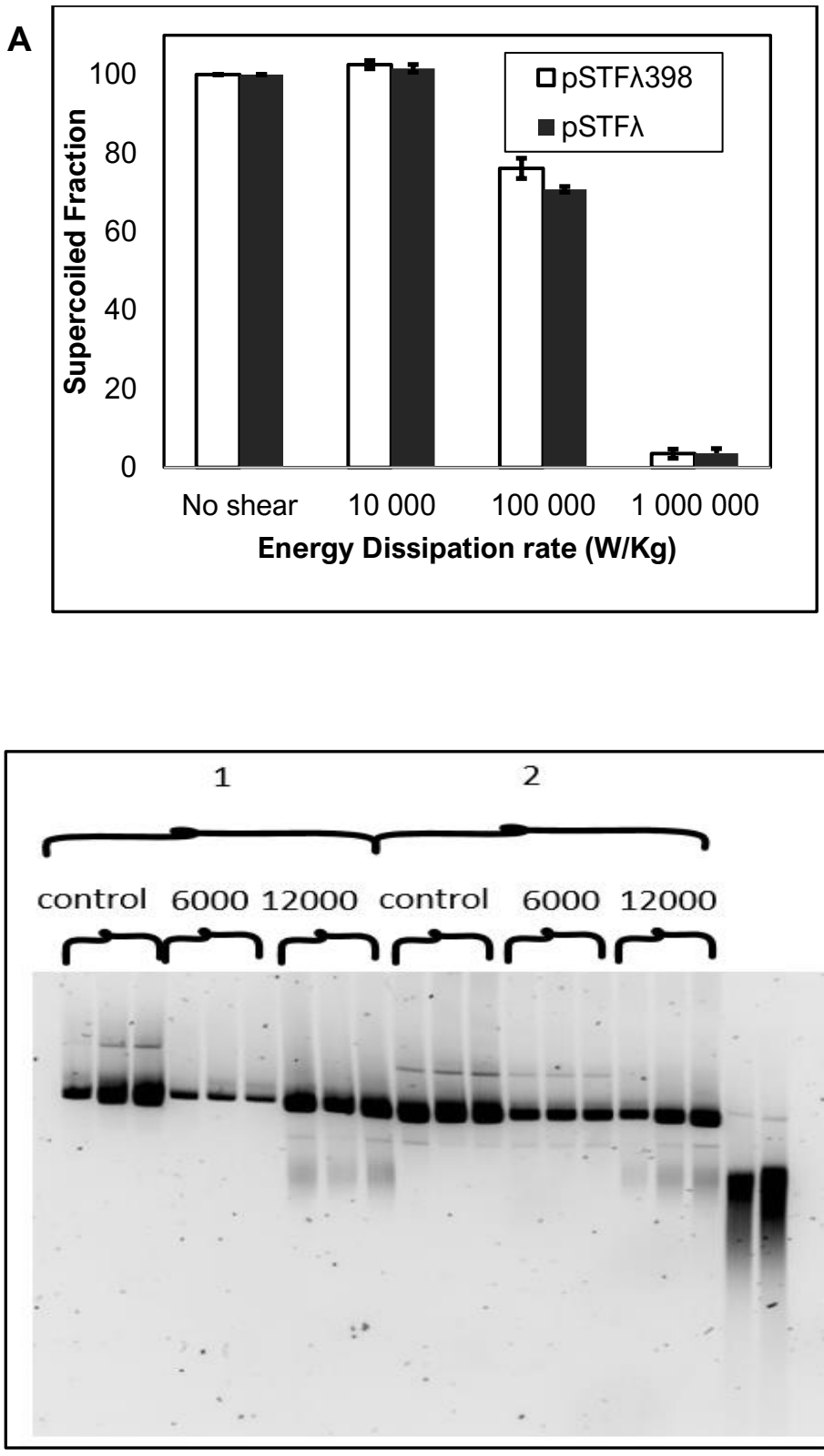


Figure 4.13: Effect of shear on pSTFλ398 and pSTFλ. (A) shows the plot of the supercoiled fraction of the two plasmids over a selected range of energy dissipation rate. Results are shown as mean+ S.E.M (n=3). (B) Gel image of the sheared plasmid run at 80V for 9 hours over a range of rotational speed (rpm) equivalent to energy dissipation rate in A. 1 is pSTFλ398 and 2 is pSTFλ

4.2.8 Effects of SGS presence on Plasmid Segregational Stability of the plasmid

High copy number plasmids tend to be segregationally unstable due to the incomplete partitioning that is associated with them (Field and Summers, 2011). Unlike low copy number plasmid, high copy number plasmids tend to be lost during fermentation, giving rise to a population of plasmid-free cells hence impacting on the plasmid yield (Grabherr *et al.*, 2002). To combat these challenges, selection pressure is introduced, mostly with the use of antibiotics to maintain plasmid-containing cells. This method, however, is unsuitable for industrial application as the regulator's requirements involve elimination of the use of antibiotics in industrial production of biotherapeutics.

It has been shown previously that the Mu-SGS-carrying plasmids are more stable segregationally than their counterparts not containing the SGS (Hassan *et al.*, 2016). This study was carried out in 2.7 kb pUC57 plasmid. It was decided also to investigate if this effect will be observed in the segregational stability of the 27kb plasmid pSTFλ398. This is important, as one of the root causes of segregational instability is the size of the plasmid used as it confers a metabolic burden on the cell (Silva, Queiroz and Domingues, 2012). The investigation was carried out with both strains DH5α and HB101. The method is described in the materials and method chapter.

The plasmid loss was monitored over 50 generations, with the assumption of 20 minutes doubling time for *E. coli*. The significance of monitoring over 50 generations is that it allows the fermentation time of about 17 hours, which is about the fermentation run time for a batch culture in the industry. Hence by being able to

maintain the plasmid segregational stability over the course of the fermentation, product yield is not affected as plasmid-containing cells are not lost.

Plasmid loss was observed, which is not surprising as this plasmid is a high copy number plasmid due to the origin of replication it is carrying. High copy number plasmids tend to be lost as plasmid over generations. This is due to the partitioning issues with high copy number plasmid (Million-Weaver and Camps, 2014). Coupled with the size of the plasmid, and its associated metabolic burden, it is expected that there is going to be plasmid loss. This was the case with the 26.6 kb bearing no SGS. The plasmid loss was observed in both strains used (Figure 4.14).

By the 30th generation, about 50% of the plasmid containing cells are lost, confirmed by plaiting cells cultured without selective pressure. This plasmid loss increased to 60% by the 50th generation. In DH5α, there was a plasmid loss of about 78% by the 40th generation which is higher than observed in HB101. Also, there was a loss of viability over time in DH5α (Figure 4.15).

Again, this could be due to high metabolic stress conferred on the cell by the plasmid. Although this can be combated by introducing selection pressure such as antibiotics addition, however, this is not suitable industrially as explained earlier. This would eventually lead to a massive reduction in product yield especially as our product is the plasmid. More so, if the product is a recombinant protein, as the product is directly proportional to plasmid content, there is also a reduction in product yield.

The problem of plasmid loss seems to be resolved in strains bearing the 27 kb plasmid (Figure 4.14 and Figure 4.15). There was no significant plasmid loss observed over 50 generations. As this was not the case in the plasmid without SGS, it can be concluded that the SGS confers some plasmid stability. This agrees with

the investigation carried out on 2.7kb plasmid (Hassan, Keshavarz-Moore and Ward, 2016), which is one-tenth the size of the plasmid used in that study, and the effect is still pronounced.

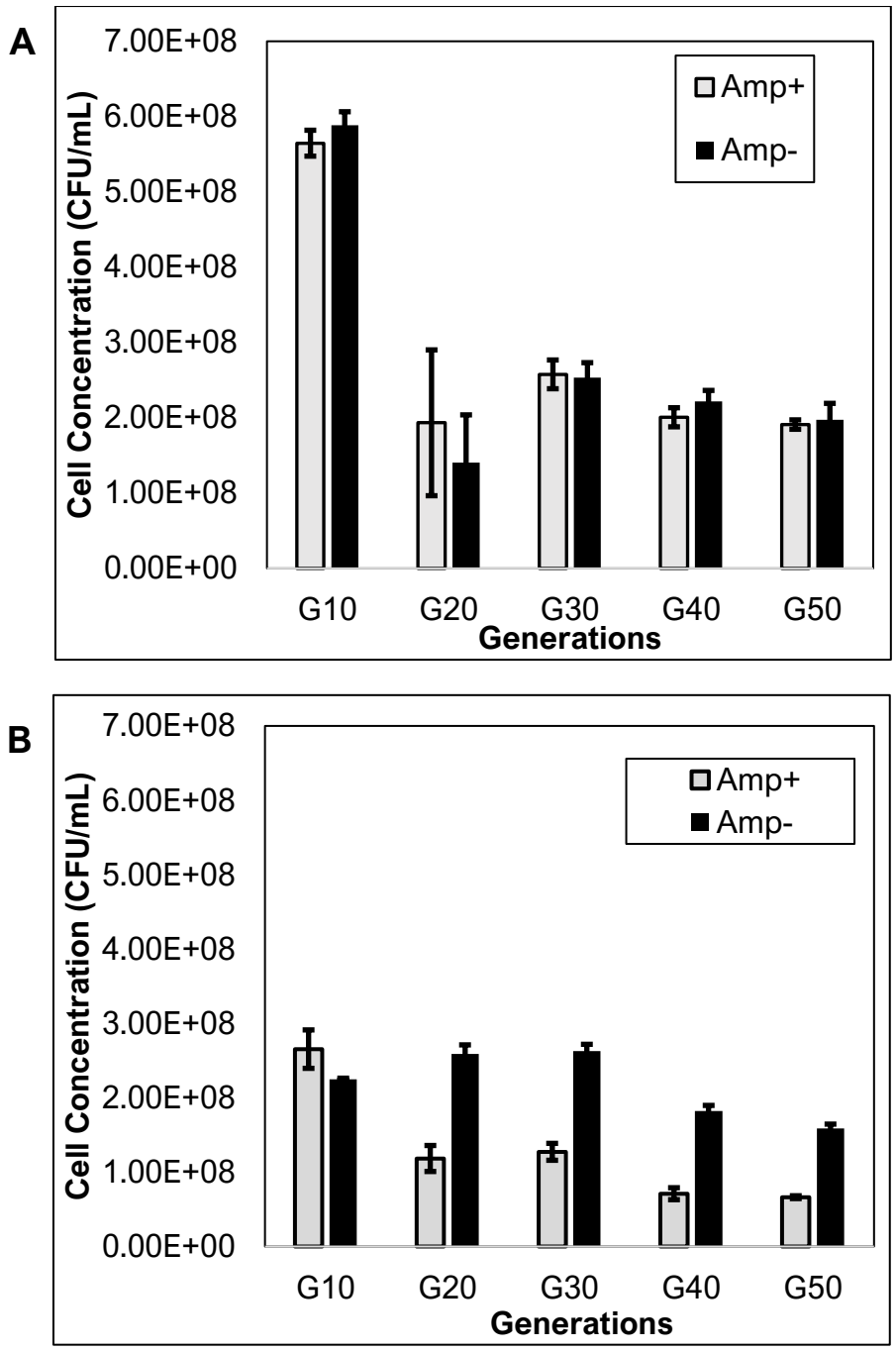


Figure 4.14: Stability studies on *E. coli* strain HB101, over 50 generations, with the measurements taken after every ten generations, cultured in 5 mL tubes. The bar represents colony-forming units per mL of cell culture. Plates were streaked and left in the incubator for 24 hours (A) 27kb plasmid with SGS, (B) 26.6kb without SGS

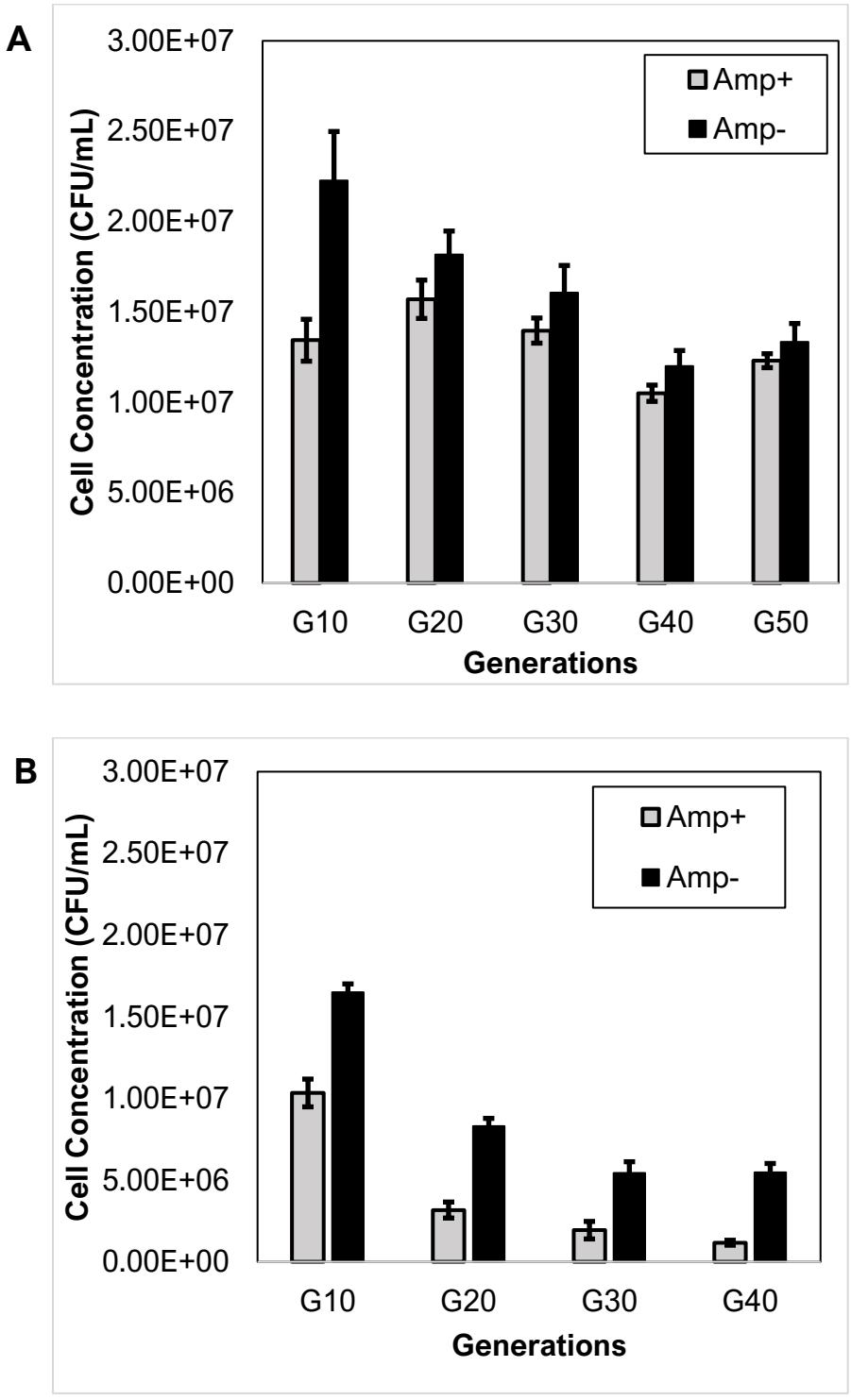


Figure 4.15: Stability studies on *E. coli* strain DH5α over 40 generations with the measurement taken after every 10 generations, cultured in 5 mL tubes. The bar represents colony-forming units per mL of cell culture. Plates were streaked and left in the incubator for 24 hours. (A) 27kb plasmid with SGS, (B) 26.6kb without SGS

The implication of this observation is that there is no need for selection pressure for industrial-scale production of biotherapeutics (plasmids and recombinant proteins) as the size can stay stable over generations without loss of plasmid containing cells, as long as the plasmid bears the Strong gyrase Binding sequence (SGS). Although it cannot be concluded that this effect is universal irrespective of the bacterial strain used as only two *E. coli* strains were used in the study, there is a chance that the effect can be replicated irrespective of strain and plasmid size.

4.3 Conclusion

The results presented in this chapter has shown that supercoiled plasmid yield can be improved as this is required for the application of plasmid as biotherapeutics. The benefit of Mu-SGS in improving the supercoiling of the plasmid DNA was exploited and it was found that this is strain-dependent as the effect was more pronounced in strain HB101 compared to DH5α. The effect on plasmid yield and supercoiled content is not specific to plasmid size as the improvement was observed both in a small size 7.2 kb plasmid and large size 27 kb plasmid.

One of the benefits of increase in supercoiling is the increase in compactness which potentially means maintenance of integrity by being less sensitive to shear during downstream processing, hence the possibility of integrity maintenance during downstream processing was investigated by subjecting purified plasmid to shear using the ultra-scale down (USD) shear device which is able to mimic conditions in an industrial scale centrifuge. In 27kb SGS containing plasmid, there was about 8% reduction in the plasmid breakage compared to the non SGS plasmid. In the 7.2kb plasmid, the reduction is not very pronounced as both the SGS containing plasmid and non-SGS plasmid are able to maintain about 90% supercoiled fraction.

The extent of supercoiling was quantified by measuring the superhelical density of the DNA; a term which employs the linking difference of the plasmid. To investigate the effect of the Mu-SGS presence on the overall compactness, the hydrodynamic diameter of the plasmid was measured using Nanoparticle tracking analysis (NTA). This method was used over the Dynamic Light Scattering (DLS) method because it employs number-weighted measurement in contrast with the intensity-weighted average of the DLS. Number-weighted measurement is more reliable as the plasmid preparation is poly-dispersed. The supercoiled plasmid hydrodynamic diameter was compared to the linearized and nicked plasmids. The results from NTA also

confirmed that the Mu-SGS containing plasmid was more supercoiled compared to the parent plasmid. This also highlights the possibility of quantifying the extent of plasmid supercoiling using the Nanoparticle Tracking Analysis (NTA).

Finally, the possibility of maintenance of plasmid segregational stability in 27kb plasmid was also investigated. Plasmid loss is particularly common in high-copy number plasmid and prominent in large plasmid as well. It was observed that the presence of Mu-SGS leads to maintenance and retention of plasmid up to the 50^{th} generation with the non-SGS plasmid getting lost over generations. The implication of this loss is that plasmid-free cells are generated which can have an impact on product yield and overall productivity. Hence by introducing the SGS into the plasmid, it is possible to eliminate the use of selection pressure, particularly antibiotics, and still maintain productivity equivalent to the use of selection pressure. The effect of SGS on plasmid stability was not limited to a single strain. This stability effect was observed in the two strains used in the study (HB101 and DH5α).

Overall, the results reported in this chapter is a step towards improving the productivity of supercoiled plasmid DNA bioprocessing for biotherapeutics application.

CHAPTER 5. Amino Acid Supplementation-Based High Cell Density fermentation for Supercoiled Plasmid

5.1 Introduction

The overall goal of plasmid bioprocessing is the production of high supercoiled plasmid yield. Since plasmid is intracellularly linked to the cell, the goal can be achieved by generating a large quantity of plasmid-containing cells during fermentation. The challenge, however, is achieving high cell density culture for high plasmid yield because by having a high initial carbon source, there is a possibility of accumulating toxic substances which eventually inhibits the growth of the cells (Luli and Strohl, 1990; Basan *et al.*, 2015).

Generally, achieving a high cell density culture is made possible by fed-batch fermentation where the carbon source is fed to avoid overflow metabolism associated with the use of high initial carbon source (Kesson, Hagander and Axelsson, 2001). The initial aim of the chapter was to develop a high cell density batch culture and to incorporate the strategy with fed-batch strategy to achieve even a higher cell density. However, due to time constraints, only the batch strategy was completed. Glucose has long been used as the carbon source because it is the choice of carbon for *E. coli* metabolism, however, running a batch fermentation with high initial glucose concentration as high as 100 g/L is almost impossible.

The composition of media used for culturing plasmid-producing *E. coli* cells are critical to obtaining high plasmid yield and the desired supercoiled quality. Nutrients are chosen based on their lot consistency. Usually complex media tend to produce high cell density culture but there is a poor lot consistency with their use and sometimes, the compositions are of animal origin. Chemically defined media, and to

some extent semi-defined media are the appropriate choices. This means they need to be designed such that consistency can be guaranteed and analytical characterization is possible.

The source of Nitrogen is as important as the source of carbon. The majority of the studies that have used complex media which derived their nitrogen from animal extracts such as tryptone (Carnes, Hodgson and Williams, 2006; Williams *et al.*, 2009) and yeast extract (Silva, 2011). For studies that have been conducted using defined media, an alternative source of Nitrogen is required. While ammonium salt has been the source of nitrogen (with defined media) for recombinant protein production, this has not been the case for plasmid DNA production. A yield of about 50mg/L plasmid DNA has been reported when ammonium chloride was used as the source of nitrogen (Voss *et al.*, 2004), this is low in comparison to yield acquired using complex nitrogen source (Lahijani *et al.*, 1996).

Some examples of media designed for high plasmid production include supplementing the media with ammonium salt as a source of nitrogen (Wang *et al.*, 2001), supplementing with specific amino acids , supplementing media with casamino acid to yield a semi-defined media (SDCAS) (O'Kennedy, Baldwin and Keshavarz-Moore, 2000; O'Kennedy, Ward and Keshavarz-Moore, 2003).

The aims and objectives of this chapter include:

- Characterize the batch fermentation using glycerol and semi defined casamino acid.
- Investigate the effect of high glycerol concentration on biomass and supercoiled plasmid yield.
- Investigate the effect of supplementing defined media with selected amino acids on the biomass yield and supercoiled plasmid yield.

5.2 Results and Discussion

5.2.1 Batch Fermentation Using Semi-Defined Casamino Acid and Glycerol

Batch fermentation was set up using the 1L Applikon Biotechnology reactors. Glycerol was selected as the carbon source, and the media was supplemented with casamino acid as it has been reported that supplementation of fermentation media with casamino acids improve the supercoiled form of the plasmid (O'Kennedy, Ward and Keshavarz-Moore, 2003). Casamino acids have been used in combination with glucose as the carbon source. For this study, glycerol was used as the sole carbon source due to the benefits over the use of glucose, including absence or reduction of overflow metabolism and cheaper source of carbon (Yazdani and Gonzalez, 2007). 50 g/L initial glucose concentration and 10 g/L casamino acid were used in the medium.

5.2.1.1 Biomass Yield on Semi-Defined Casamino Acid and Glycerol

Figure 5.1 shows the typical growth curve observed when the *E. coli* strain DH5α containing the pSVβ-gal398 plasmid was grown in the defined media (DM) supplemented with 50 g/L glycerol and 10 g/L casamino acid. The fermentation had a lag phase of about 4 hours before the exponential phase was observed for about 10 hours. The stationary stage was reached at about 16 hours post-inoculation. The mean OD600nm reached was 63 and biomass yield of 18.2 g/L DCW. This is not uncommon for fermentation involving glycerol. The maximum specific growth rate (μ_{max}) was similar to values reported for glycerol-containing media (Islas-Lugo *et al.*, 2016). In order to investigate the influence of casamino acid presence in the media, a parallel duplicate fermentation was run with only glycerol as the carbon source and

no casamino acid added. Figure 5.1 shows the growth curve of this run. Unlike the casamino acid-containing media, the fermentation run involving only glucose peaked at an OD600nm of 46 and biomass yield of 17.95 g/L DCW.

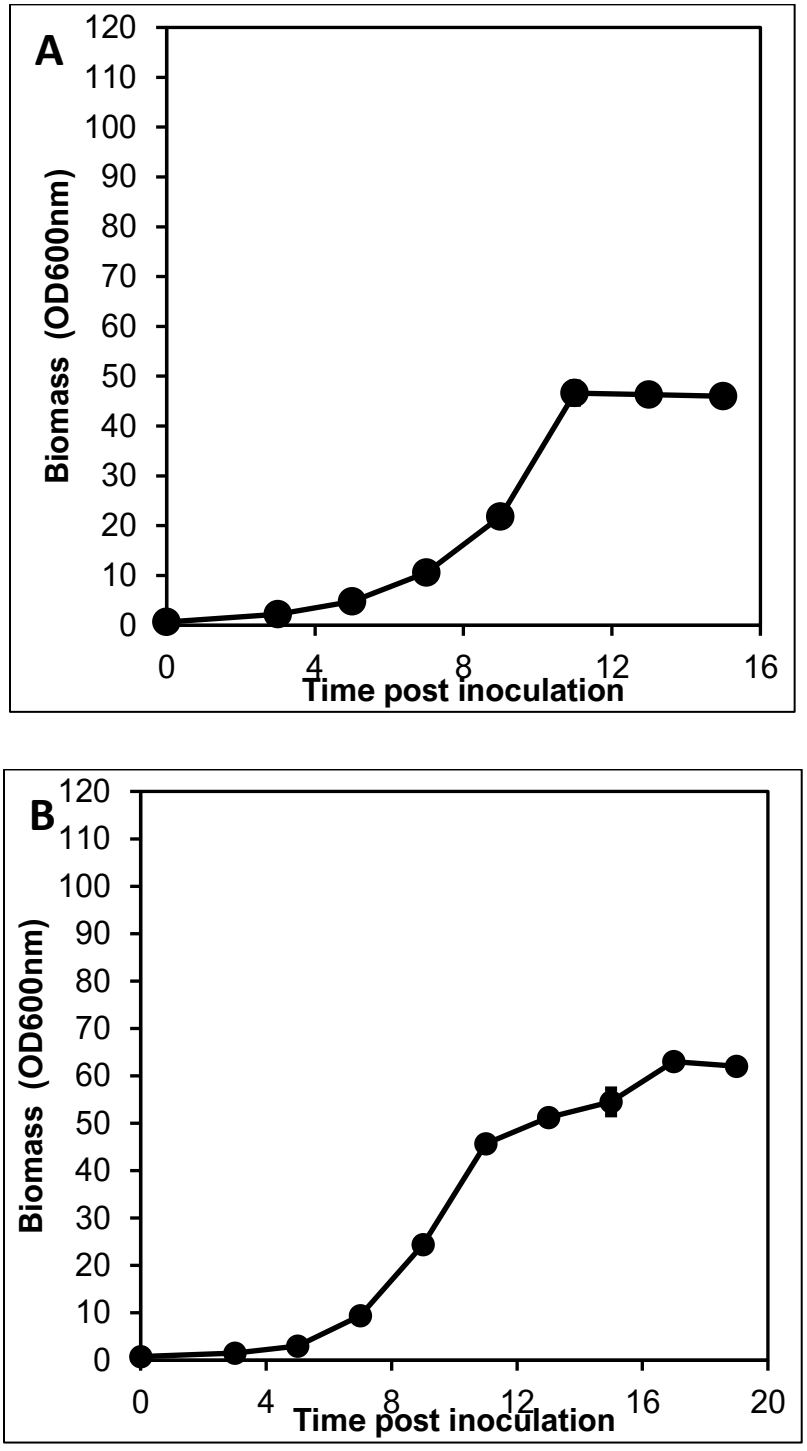
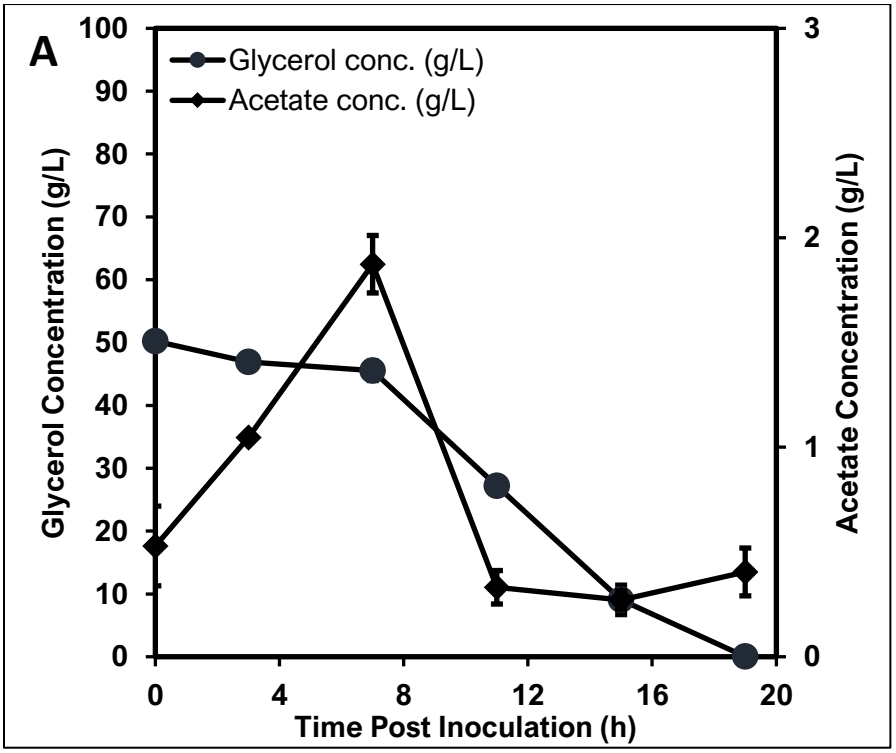


Figure 5.1: Chart showing the growth curve of fermentation with media containing (A) 50 g/L glycerol and 10 g/L casamino acid and (B) 50 g/L glycerol only. Plots are mean of duplicate independent fermentations (n=2).

Surprisingly, the presence of the casamino acid does not seem to influence the final biomass yield as there was no significant increase in the final biomass yield of the casamino acid-containing media over glycerol-only media. It would be expected that the presence of casamino acid will provide additional carbon source for situations when the first carbon source becomes completely exhausted (J. Berg, Tymoczko and Stryer, 2002).

To confirm if this was the case, the glycerol consumption was monitored throughout the fermentation run. Figure 5.2 shows the glycerol consumption profile for both the SDCAS (Semi-Defined Casamino acid) media and non-SDCAS media. In the SDCAS media fermentation, there was complete glycerol consumption by the end of the fermentation run with a final glycerol concentration of 0.04 g/L and an acetate accumulation of 0.4 g/L. in addition to measuring the final glycerol concentration, the complete consumption of oxygen was signified by the spike in the dissolved oxygen tension (% DOT) and an increase in the pH. However, for the non-SDCAS media, the glycerol was not completely consumed with a final glycerol concentration of 6.5 g/L and an acetate accumulation of about 3 g/L. The acetate accumulation observed is similar to what was reported by Korz *et al.,* (1995) when the maximum specific growth rate exceeds 0.2 h^{-1} for fermentation where the carbon source is glycerol.



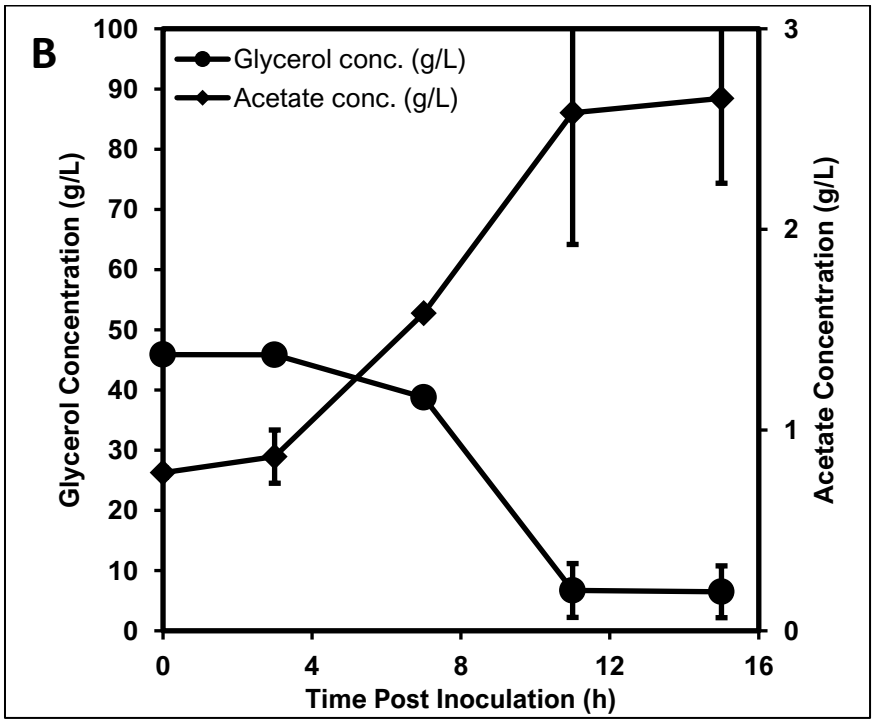


Figure 5.2: Glycerol consumption and acetate accumulation during batch cultures using media containing (A) 50 g/L glycerol with 10 g/L casamino acid and (B) without casamino acid. Results are mean of duplicate independent fermentations (n=2)

One possible explanation for the incomplete consumption of glycerol is the accumulation of metabolites like acetate. Though not as high as would expect with glucose, the accumulation of acetate is enough to cause inhibition. In SDCAS media, final acetate concentration could have been lowered due to the presence of amino acids. Zawada and Schwatz (2005) reported the suppression of acetate accumulation by addition of amino acid to *E coli* fermentations. In another study, methionine and glycine were reported to suppress acetate accumulation in fermentations with glucose as carbon source (Han, Hong and Lim, 1993).

Another possible explanation as to why there was incomplete consumption of glycerol in the glycerol-only media could be due to nitrogen limitation since the only source of nitrogen in the media is from ammonium salt which makes up the defined media. However, this is insignificant to the final biomass yield as there was no statistically significant difference between the final biomass yield of the SDCAS media and non-SDCAS media. Although there was a statistically significant difference in the final OD600nm between the two strategies, it is thought that the high OD in SDCAS media could have been due to the increase in turbidity associated with the addition of casamino acids to the media.

5.2.1.2 Supercoiled Plasmid Yield Semi-Defined Casamino Acid and Glycerol

The effect of SDCAS/Glycerol media on the supercoiled plasmid yield was also investigated. Figure 5.3 shows the result of the plasmid yield and supercoiled content of SDCAS media and non-SDCAS media. In non-SDCAS media which contains only glycerol as the carbon source, the final plasmid yield observed was about 10 mg/L, which increased throughout the course of the fermentation. This is much lower than the plasmid yield observed in the SDCAS media. The final plasmid yield observed in the fermentation with the SDCAS media was 33 g/L which is three

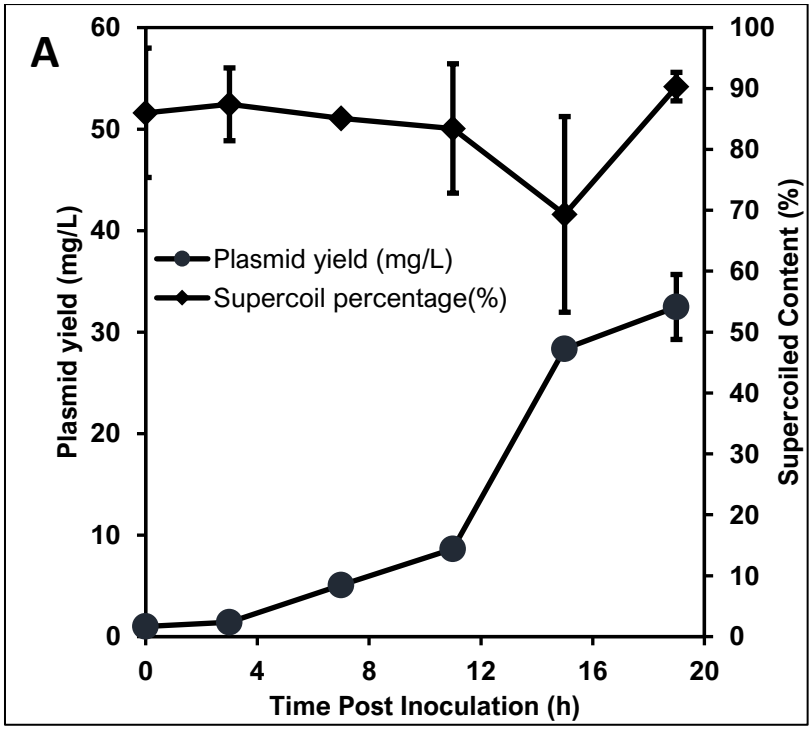
times more than observed with glycerol only media, despite having the same initial glycerol concentration. In both cases, the plasmid yield increased with an increase in biomass, but a much more significant increase observed in SDCAS media.

This observation is not surprising as amino acids have been reported to boost plasmid yield (O'Kennedy et al., 2003; Zawada and Swartz, 2005). The nitrogen atoms in the amino acids are precursors for the synthesis of nucleotides which in turn are required for RNA and DNA synthesis (Wang *et al.*, 2001). In addition, some amino acids also contain functional groups/rings which can be incorporated directly into DNA synthesis, hence increasing the rate of plasmid synthesis (J. Berg, Tymoczko and Stryer, 2002).

There are several reports that support this observation of high plasmid yield with semi-defined media, some of which have used tryptone, peptone, and yeast extract (Williams, Carnes and Hodgson; Lahijani *et al.*, 1996; Phue *et al.*, 2008; Islas-Lugo *et al.*, 2016). Majority of these reports have employed a fed-batch fermentation strategy to achieve the high plasmid yield in addition to supplementing the media. In one report, batch fermentation was carried out 50 g/L glycerol, and 20% yeast extract and a plasmid yield of 57 mg/L achieved (Carnes, Hodgson and Williams, 2006). O'Kennedy *et al*., (2003) reported a plasmid yield of about 8 g/L with SDCAS media and glucose as carbon source. This result has shown that a high plasmid yield could be achieved with SDCAS media and glycerol as the carbon source.

The supercoiled content of the plasmid preparation from fermentation with SDCAS medium was maintained throughout the fermentation with a final supercoiled content of 90%. Although there was a dip in the supercoiled content at the beginning of the stationary phase, it quickly rose to 90% at the time of harvest. This was not the case in plasmid preparation from fermentation with glycerol-only media. The

supercoiled content was maintained at around 85% until towards the end of the exponential phase when it dropped down to 30% supercoiled, further decreasing the overall supercoiled plasmid yield (Figure 5.3). This further reiterates the influence of amino acid on the overall yield of supercoiled plasmid DNA.



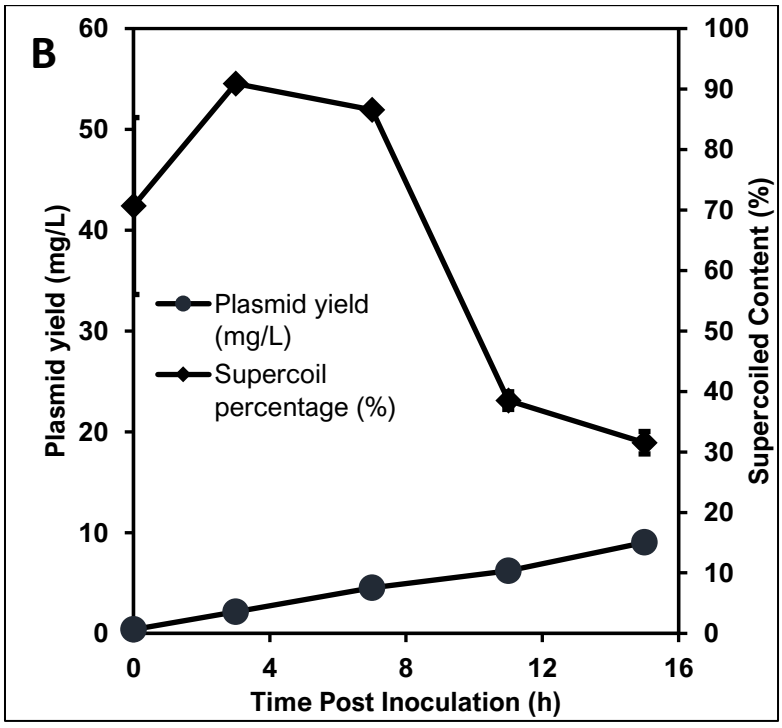


Figure 5.3: Total plasmid yield (mg/L) and supercoiled content monitored during fermentation for media containing (A) 50 g/L glycerol supplemented with casamino acid and (B) 50 g/L glycerol only. Results are mean of duplicate independent fermentations (n=2)

5.2.2 Casamino Acid-supplemented media in the presence of High Glycerol Concentration

In order to achieve high cell density for higher plasmid yield than observed with using SDCAS media with 50 g/L glycerol, the initial glycerol concentration was increased to 100 g/L, the casamino acid remained 10%, and the fermentation carried out in similar conditions as in section 5.2.1.1.

5.2.2.1 Biomass Yield on Semi-Defined Casamino Acid and High Initial Glycerol Concentration

Two parallel independent fermentations were carried out. The growth curve showing the biomass yield in terms of OD600nm and g/L DCW is shown in Figure 5.4. The final biomass increased with increasing glycerol concentration. The final OD observed was 106 and DCW of 33.3 g/L DCW, both of which are higher with when compared with SDCAS media with 50 g/L initial glycerol concentration. The growth pattern observed was similar in both cases with the exponential phase picking up at after 7 h of lag phase compared to 4 hours when the initial glycerol concentration was 50 g/L.

There was a total consumption of glycerol by the end of fermentation with a final glycerol concentration of 0.2 g/L. Although it was observed that most of the glycerol was consumed during the final stages of the exponential phase, this could be due to the fact that the cell density was higher at this point. The glycerol consumption trend was similar for glycerol concentration of 50 g/L until the 15 h post-inoculation at which the glycerol was almost completely consumed in SDCAS media with 50 g/L glycerol.

With the initial glycerol concentration doubled, the final biomass yield (OD and DCW) was almost doubled. This shows that increasing the glycerol concentration leads to a linear increase in the final biomass yield, thereby making it possible to achieve the high cell density for high plasmid yield. The effect of increasing glycerol in SDCAS media on growth characteristics is shown in Table 3.1.

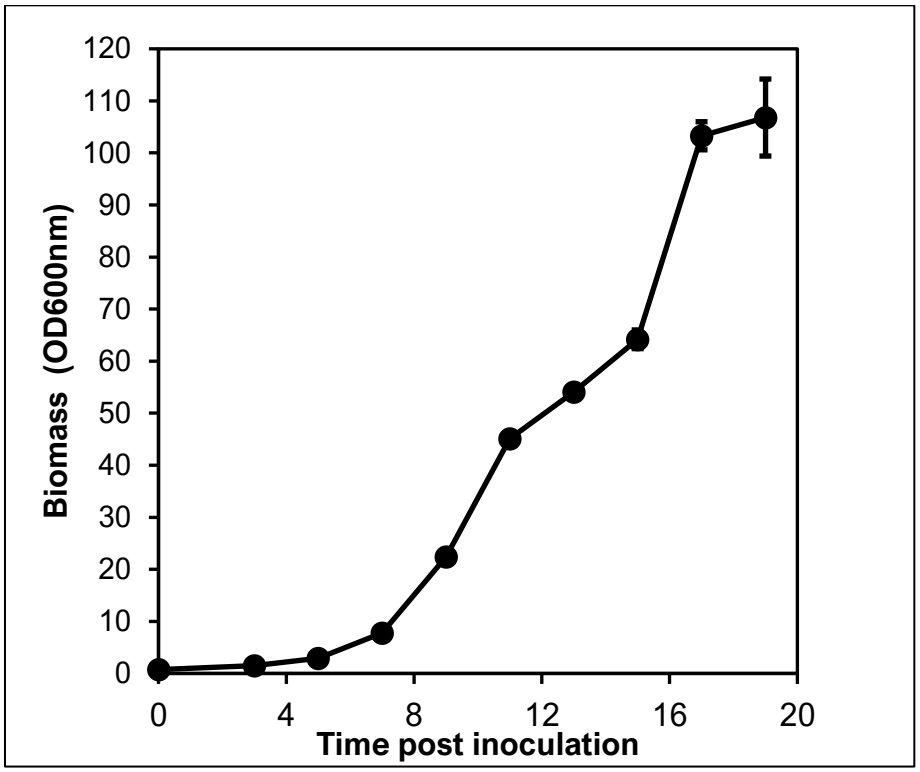


Figure 5.4: Growth curve of fermentation with media containing 100 g/L glycerol and 10 g/L casamino acid. Plots are mean of duplicate independent fermentations.

To further compare the effect of SDCAS in high glycerol media, a parallel set of fermentations were carried out with a glycerol-only media containing 100 g/L glycerol. The fermentation growth curve is shown in Figure 5.5. There was inhibition of growth observed with a final OD600nm of 27 and a final biomass yield of 12 g/L. The observed biomass yield is lower than observed when the initial glycerol concentration of 50 g/L was used. The growth rate was also lower, with a maximum growth rate (μ_{max}) of 0.17 h-1. It has been reported that by increasing glycerol

concentration beyond 50 g/L, the biomass yield reduces due to inhibition caused by the initial high glycerol concentration (Szymanowska-PowałOwska, 2015), hence the reduction in the biomass yield and growth rate could be due to substrate inhibition metabolite inhibition and/or osmotic pressure (Knabben *et al.*, 2010; Borja *et al.*, 2012; Gonçalves *et al.*, 2014).

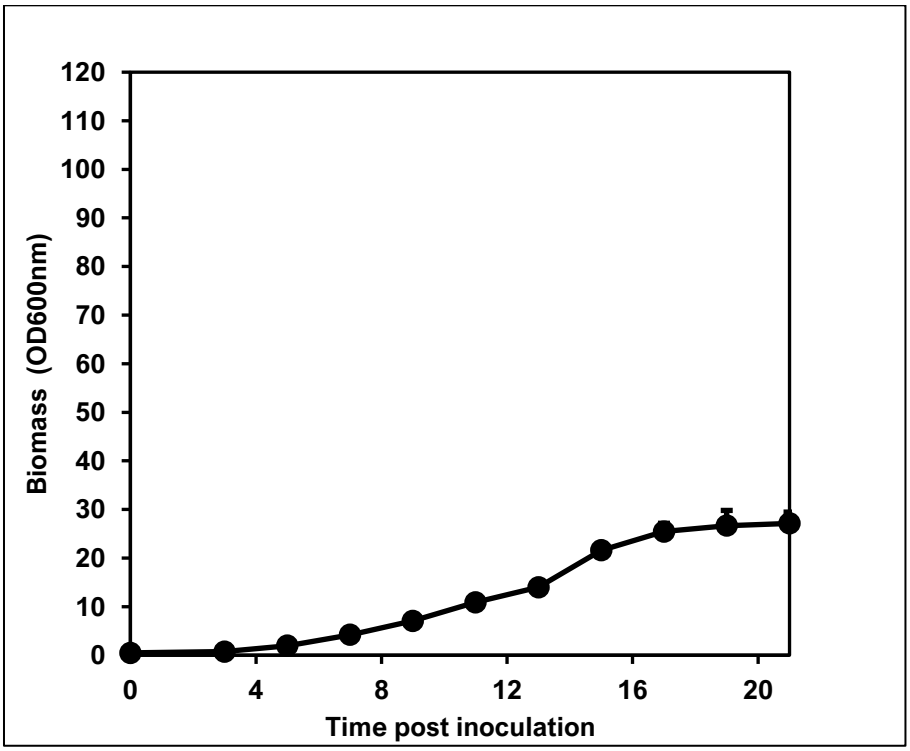


Figure 5.5: Growth curve of fermentation with media containing 100 g/L glycerol with no casamino acid supplementation. Plots are mean of duplicate independent fermentations.

The glycerol concentration was monitored throughout the course of the fermentation and it was observed that there was incomplete consumption of glycerol with three-quarters of the initial glycerol concentration unused at the end of the fermentation (Figure 5.6). Despite the high final glycerol concentration in the media, there was an acetate accumulation of 1.5 g/L; more than was observed in both SDCAS media, but lower than observed in glycerol-only media with 50 g/L initial glycerol concentration. Again, the probable explanation why the inhibitory effect was

not observed in SDCAS media with 100 g/L glycerol is the presence of the different amino acids in casamino acid supplements (Han, Hong and Lim, 1993; Zawada and Swartz, 2005).

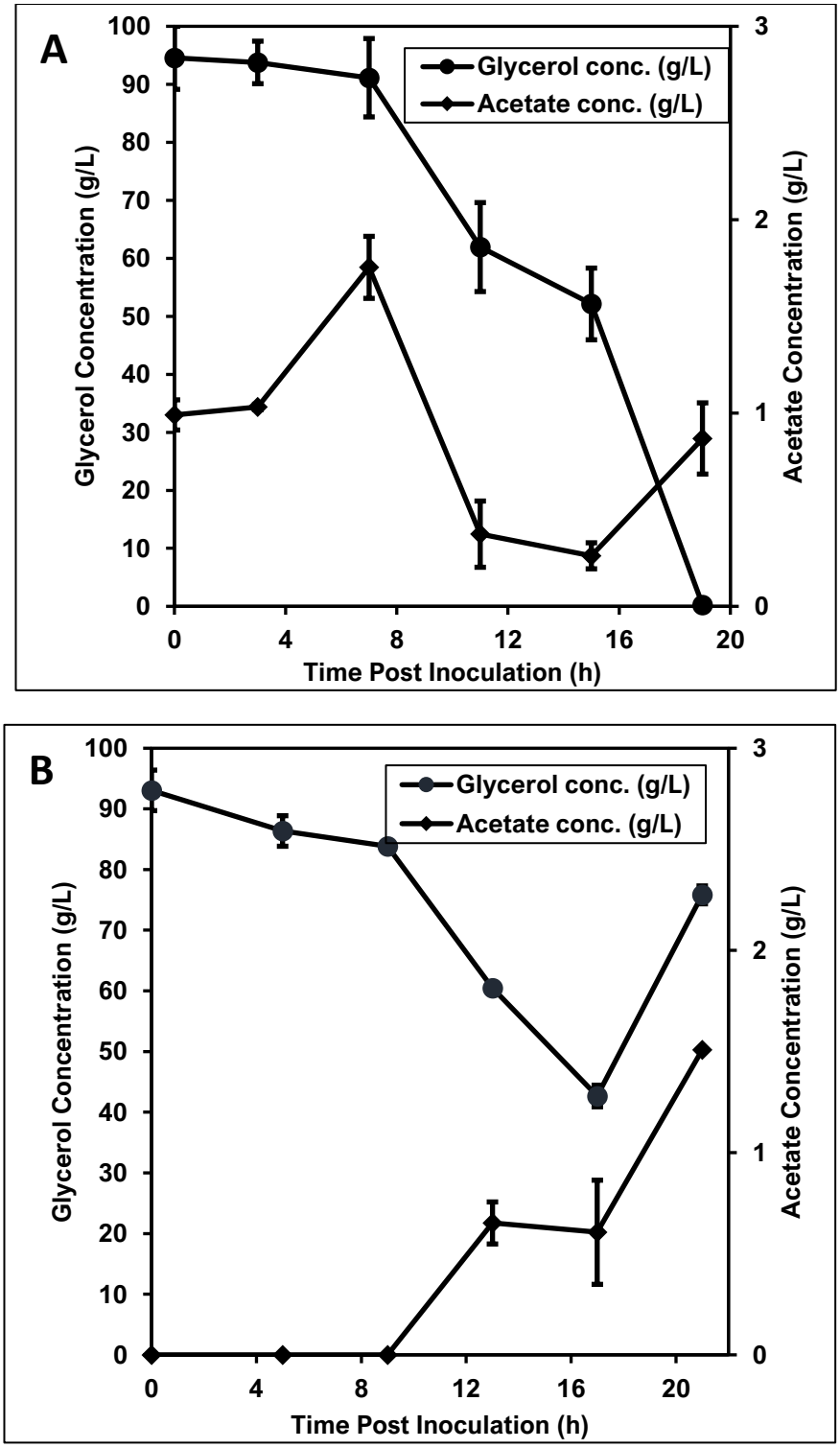


Figure 5.6: Glycerol consumption and acetate accumulation during batch cultures using media containing (A) 100 g/L glycerol with 10 g/L casamino acid and (B) 100 g/L without casamino acid.

5.2.2.2 Supercoiled Plasmid Yield Semi-Defined Casamino Acid and High Initial Glycerol Concentration

The effect of SDCAS media with high glycerol concentration on the plasmid yield and supercoiled content are shown in Figure 5.7. A final volumetric plasmid yield of 53 mg/L was observed in SDCAS with 100 g/L glycerol. This was almost doubled the volumetric plasmid yield in SDCAS media with 50 g/L glycerol. Islas-Lugo *et al.* (2016)reported a similar observation, where yeast extract supplemented-media with initial glycerol concentration of 70 g/L led to an increase in the plasmid yield, compared to the use of 50 g/L. The increase in biomass yield observed translate to a similar increase in plasmid yield. This shows that by maintaining a high cell density culture, a high plasmid yield can be achieved.

In fermentation involving the use of 100 g/L glycerol only media, the final volumetric plasmid yield was observed to be 13.8 mg/L; similar to what was observed in the glycerol only media with 50 g/L initial glycerol concentration. Apart from amino acid being involved in biomass yield, amino acids are also essential for improving plasmid yield (O'Kennedy, Baldwin and Keshavarz-Moore, 2000).

The supercoiled content of plasmid preparation extracted from fermentation with SDCAS media with 100 g/L glycerol was maintained above 85% throughout the fermentation, with a final supercoiled content of 90%. This is similar to what was observed when the initial glycerol concentration was 50 g/L, where 90% supercoiled content was also observed in the final plasmid preparation. The supercoiled content of plasmid preparation in glycerol-only media with 100 g/L glycerol dropped during the fermentation. The final supercoiled content of plasmid preparation on harvest was 68%. This observation shows that supplementation of media with casamino acid maintains the supercoiled content of plasmid preparation and by increasing the glycerol concentration, the total supercoiled plasmid can be increased.

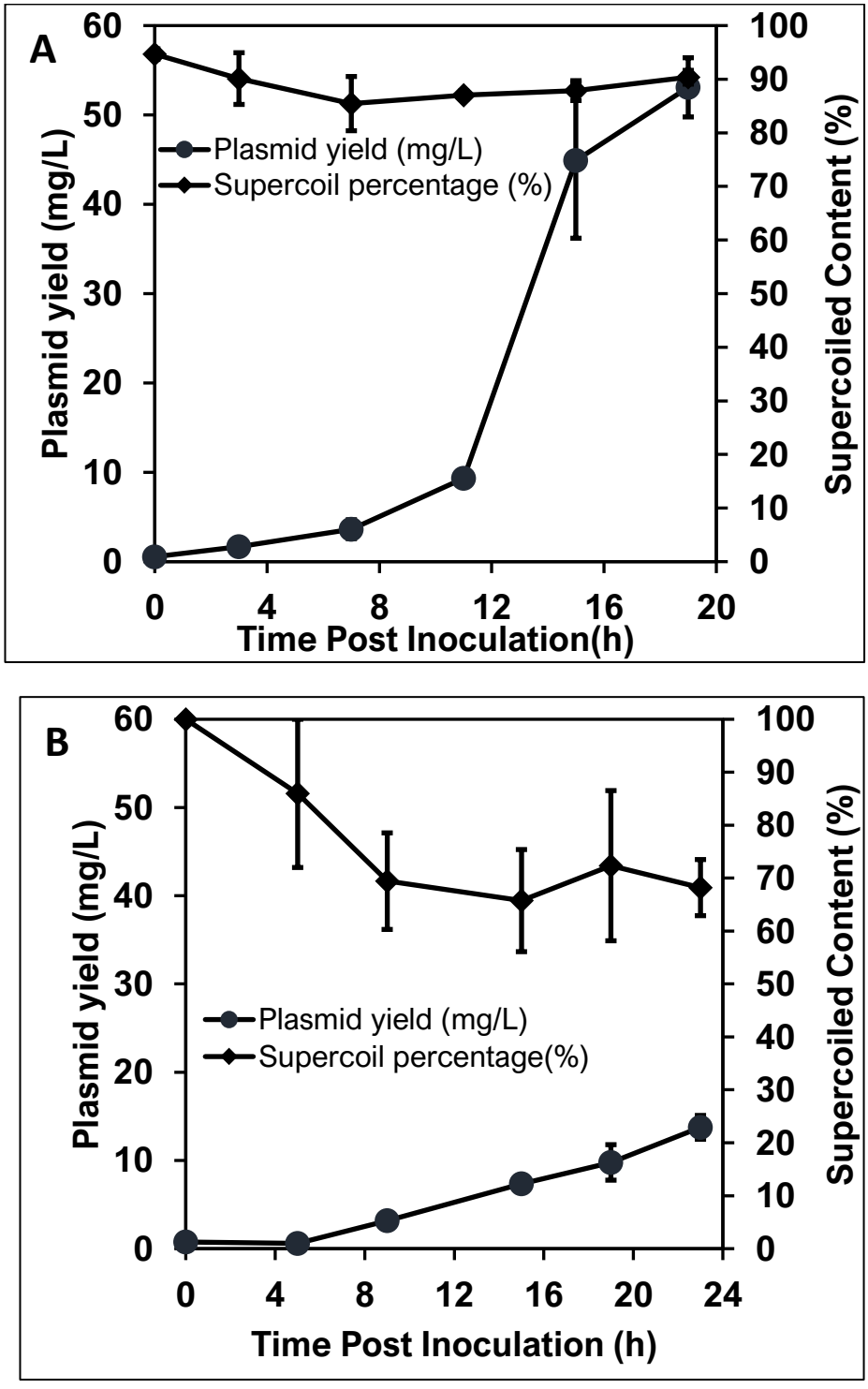


Figure 5.7: Total plasmid yield (mg/L) and supercoiled content monitored during fermentation for media containing (A) 100 g/L glycerol supplemented with casamino acid and (B) 100 g/L glycerol only. Results are mean +/- SD of duplicate fermentations.

There was no significant difference between the specific plasmid yield of SDCAS media with 50 g/L glycerol and 100 g/L (Figure 5.8) which suggest that the increase in plasmid yield was due to the increase in biomass yield observed in media with glycerol concentration. Although it is worth noting that high cell density can be achieved by increasing the glycerol concentration without affecting the supercoiled plasmid yield negatively.

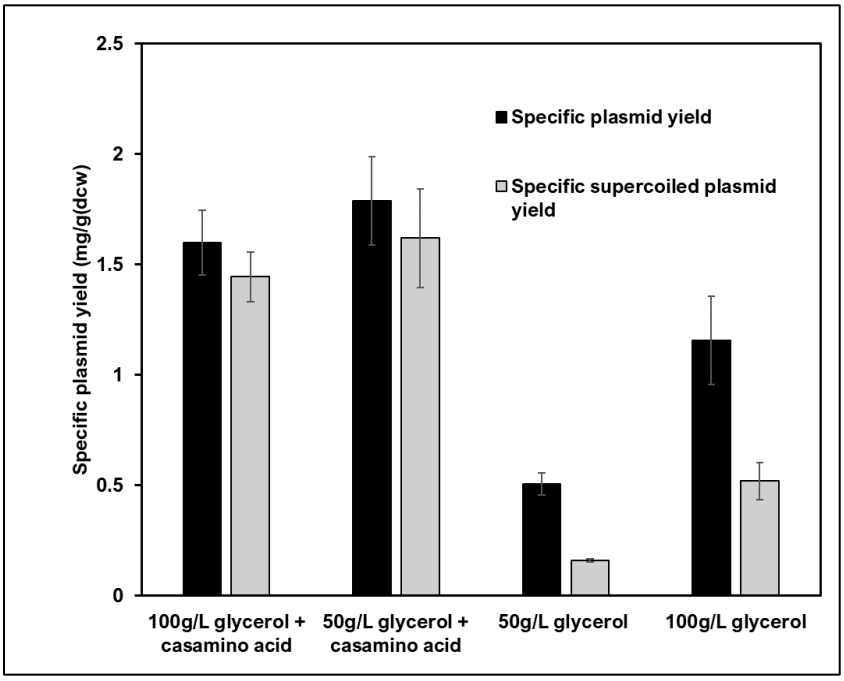


Figure 5.8: Specific (total and supercoiled) plasmid yield (mg/g DCW) of cultures with an initial glycerol concentration of 50 g/L and 100 g/L and supplemented with casamino acid. Results are mean of duplicate independent fermentations.

5.2.3 Effect of Media Supplementation with Amino Acids and Glycerol as Carbon Source

Although the observation shows that supplementing fermentation medium with casamino acid improves the overall yield of plasmid DNA, the challenge here is that it is difficult to be consistent as the casamino acid used in the media is a complex nutrient source with the components tending to change from batch to batch

(Prazeres, 2011). It is, therefore, necessary to develop media that is truly defined and reproducible to allow for lot consistency during production.

To overcome this challenge, it is essential to develop a medium that is capable of achieving high cell density as observed with the SDCAS-supplemented high glycerol concentration media and can be defined to allow batch and lot consistencies. Dorward *et al.,* (2019) identified glycine, histidine and leucine as important for increased biomass but plasmid amplification is activated in the absence of leucine. Although this approach was implemented as part of a feeding strategy, the effect of the addition of the amino acids to batch culture was not investigated.

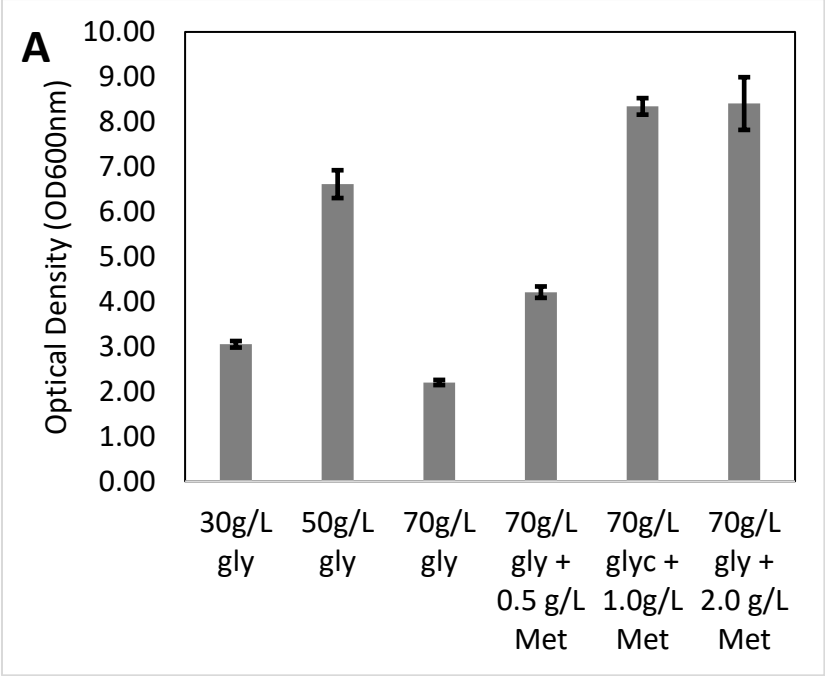
In addition, methionine and glycine have been implicated in relieving overflow metabolism in *E. coli* fermentations both in batch ad fed-batch mode (Han, Hong and Lim, 1993). Since one of the challenges of achieving high cell density is inhibition of growth due to high initial carbon source concentration, a high cell density culture with increased plasmid amplification can be achieved by developing an approach involving amino acids supplementation.

5.2.3.1 Selection of Optimum Methionine for amino acid supplementation

To determine the optimum methionine concentration for high cell density culture, small scale fermentations (10 mL cultures) were carried out with different methionine concentration and high initial glycerol concentration. Figure 5.9 shows the final biomass yield in terms of optical density (OD600nm) and the plasmid yield of the different methionine concentration and glycerol. Results are shown as analyses of triplicate culture.

As expected, the cultures with high initial glycerol concentration had a low biomass yield of all samples. This is due to the high glycerol concentration leading

to overflow metabolism. However, the supplementation of the media with methionine seems to allow efficient consumption of glycerol by the cells. This observation agrees with the report of Han *et al.,* (1993) that feed supplementation with methionine tends to support cultures with a high initial carbon source. To fully exploit the effect of methionine, different concentrations of methionine were investigated. It was observed that increasing the methionine concentration beyond 1 g/L had no special effect on the biomass yield.



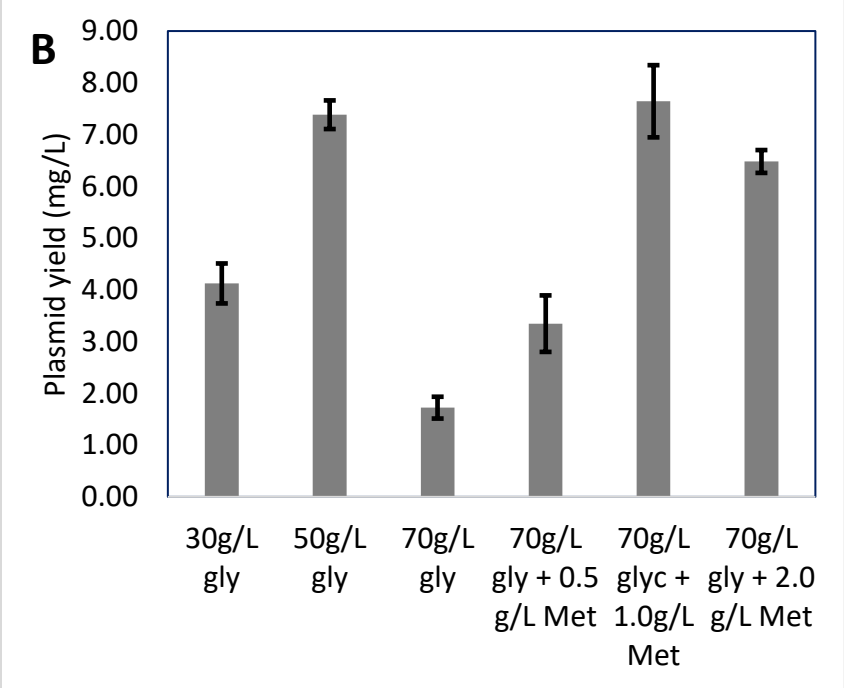


Figure 5.9: Plots showing the effect of methionine supplementation on (A) the biomass yield and (B) the plasmid yield on cultures with high initial glycerol concentrations. Yields are expressed as Mean +/- S.D of triplicate samples.

The effect of methionine supplementation on the plasmid yield was also investigated. Like biomass yield, methionine addition leads to an increase in the plasmid yield. This could be due to the high biomass in cultures containing

methionine. There seems to be no significant statistical difference between media supplemented with 1 g/L methionine and 2 g/L methionine (*p-value >0.05*). However, the difference observed between media supplemented with 1 g/L methionine and no methionine is statistically significant (p-value < 0.05). Due to this observation, it was decided that the media be supplemented with 1 g/L methionine.

5.2.3.2 Effect of Media- Supplementation on Biomass Yield

Batch fermentations were carried out in 1L Applikon Biotechnology reactors with glycerol and selected amino acids as the nutrient sources. The media was supplemented with 0.17 g/L histidine, 0.56 g/L glycine and 1 g/L methionine. High glycerol concentration of 100 g/L was used in the media and duplicate independent batch fermentations were carried out. The details of the fermentation can be found in the Materials and Method section.

The biomass yield (g/L DCW) observed was similar to what was observed with casamino acid supplemented media with similar glycerol concentration achieving a biomass yield of 32 g/L DCW and higher than media with 50 g/L glycerol and casamino acid supplementation. The growth curve was similar to the use of casamino acid except for a reduced maximum growth rate. There was also a more prolonged lag phase that is similar to the observation in non-supplemented media. (Figure 5.10).

The glycerol concentration was monitored, and it was observed to be completely consumed, similar to what was observed with casamino acid supplementation. However, there was no significant acetate accumulation during the fermentation, with the final acetate concentration being 0.03 g/L which is less than both glycerol-only medium and casamino acid supplemented medium (Figure 5.11).

This effect can be attributed to the presence of the amino acids relieving the accumulation of acetate, therefore, preventing growth inhibition (Han, Hong and Lim, 1993; Roe *et al.*, 2002).

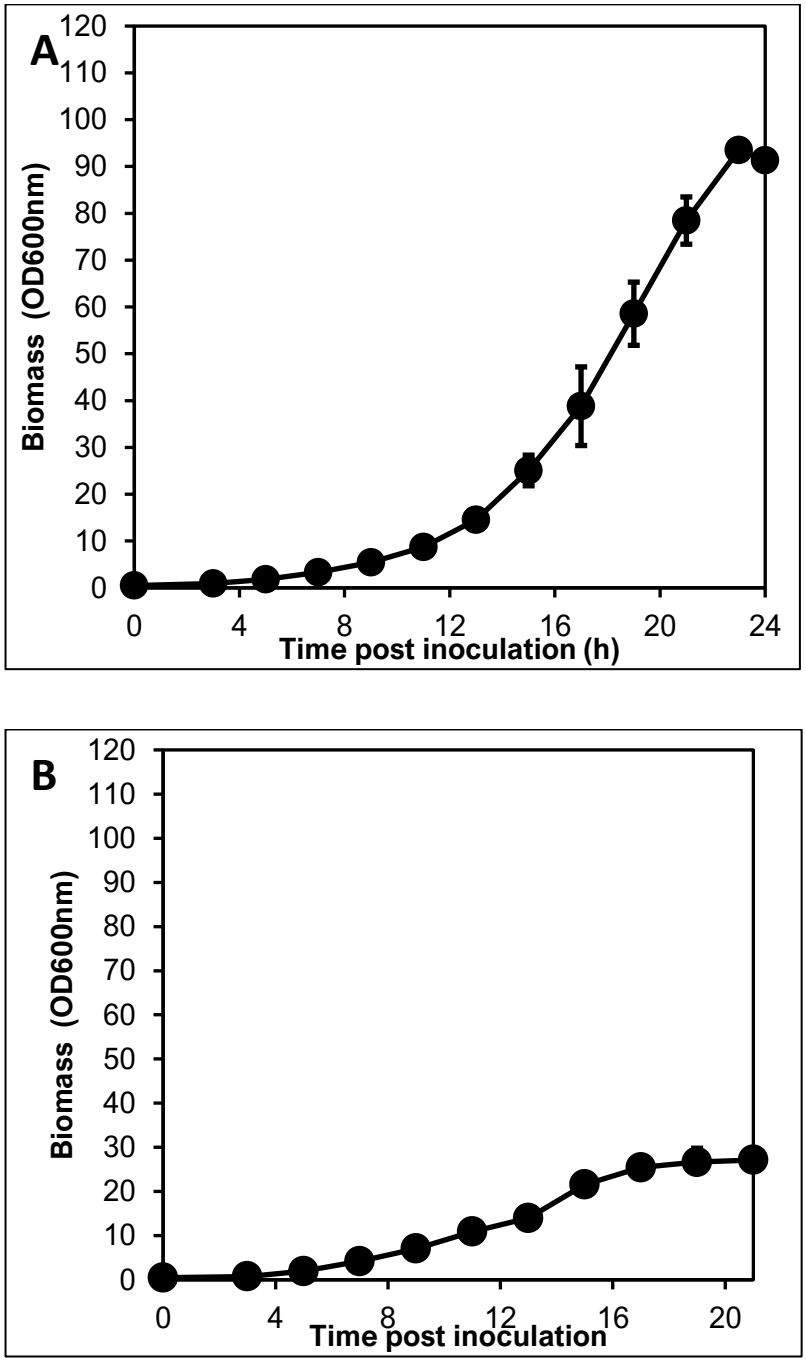


Figure 5.10: Growth curve of batch fermentations with media containing (A)100 g/L glycerol supplemented with the selected amino acids and (B) media containing 100 g/L with no supplementation. Values shown are mean of duplicate independent fermentations.

The increase in acetate accumulation in media with casamino acid could be attributed to the presence of some glucogenic amino acids, particularly those that are converted to pyruvate which is a precursor to acetate formation (J. Berg, Tymoczko and Stryer, 2002). The amino acids used for supplementation do not belong to this group; hence, there are no other sources of acetate accumulation. This reiterates the positive effect of supplementing media with specific amino acids for high biomass yield.

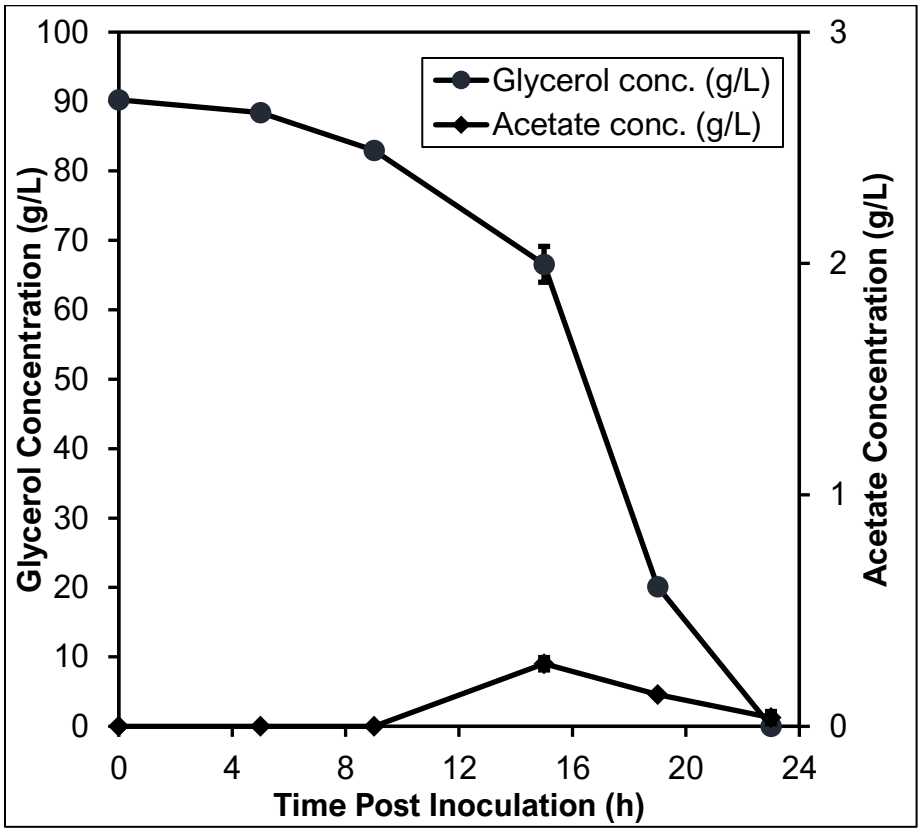


Figure 5.11: Glycerol consumption and acetate accumulation during batch cultures using media containing 100 g/L glycerol with selected amino acid supplements.

5.2.3.3 Effect of Media-Supplementation on Supercoiled Plasmid Yield

The effect of supplementation with amino acid was also investigated on the yield and supercoiling of plasmid. Some reports have suggested that amino acid starvation is can induce plasmid amplification (Wróbel and Węgrzyn, 1997; Sandén

et al., 2003). It is, therefore, necessary to investigate how the supplementation will affect the plasmid yield.

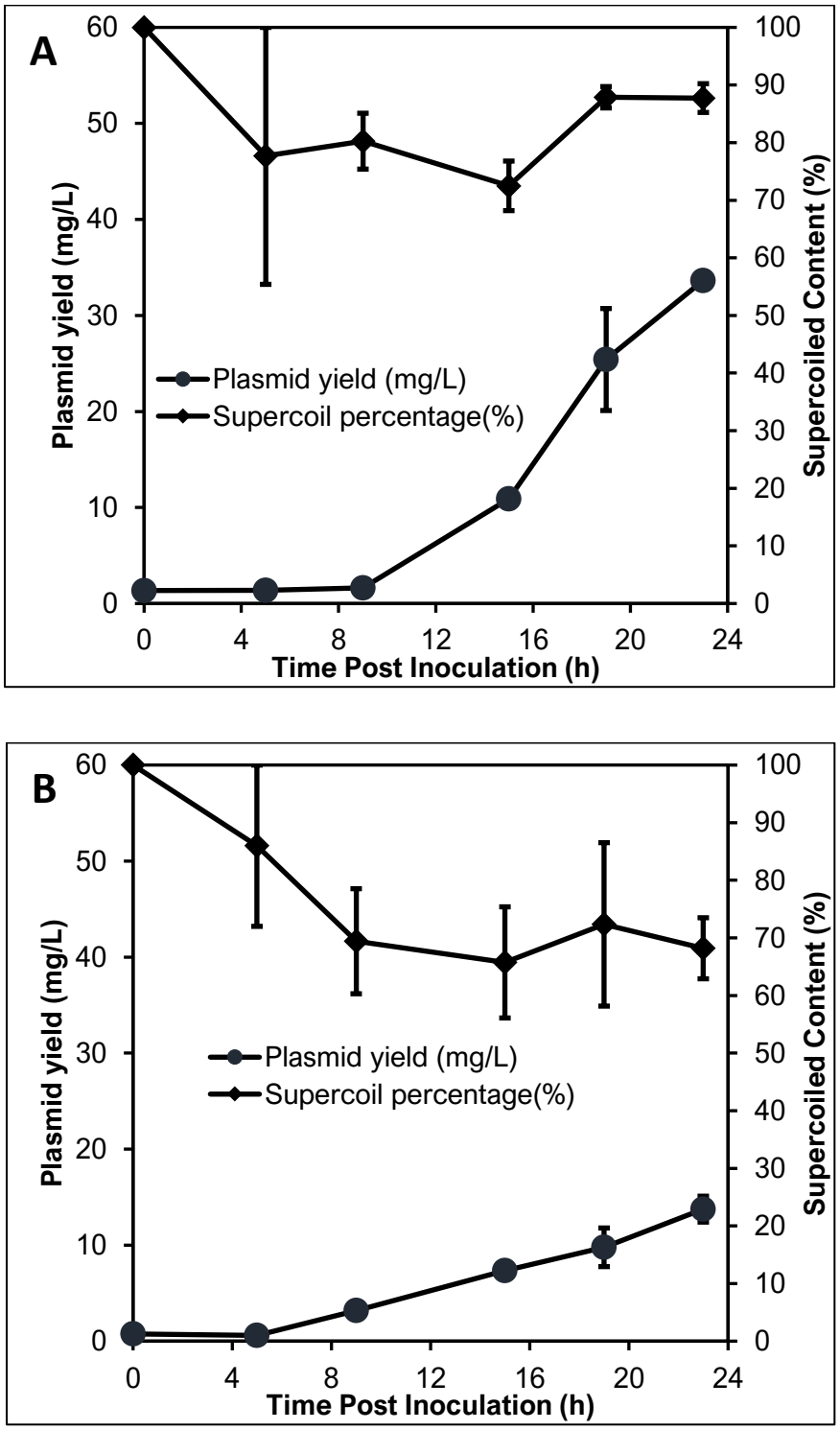


Figure 5.12: Total plasmid yield (mg/L) and supercoiled content monitored during fermentation for media containing (A) 100 g/L glycerol supplemented with selected amino acids and (B) 100 g/L glycerol only.

Figure 5.12 (above) shows the result of plasmid yield and supercoiled content measured during fermentation. The plasmid yield increases with increase in biomass as would be expected. However, the final total plasmid yield was 34 mg/L, which was significantly lower than the final total plasmid yield for casamino acid supplemented media. This could suggest that there are some nutrients present in the casamino acid that might be influencing the plasmid yield in addition to the selected amino acids. Also, since the casamino acid is a complex casein hydrolysate, there is also the possibility of additional carbon source or nutrient unaccounted for that might be contributing to the plasmid yield.

Table 5.1: Effect of high glycerol concentration and amino acids supercoiled plasmid yield, biomass yield and growth characteristics in media containing 50 g/L glycerol **(A)**, 50 g/L glycerol plus casamino acid **(B)**, 100 g/L glycerol **(C)**, 100 g/L glycerol plus casamino acid **(D)** and 100 g/L glycerol plus selected amino acids **(E)**. Plasmid and Biomass yield reported were at harvest.

	A	**B**	**C**	**D**	**E**
Glycerol Conc. (g/L)	50	50	100	100	100
Casmino Acid conc. (g/L)	0	10	0	10	0
Selected Amino acids	N	N	N	N	Y
DCW (g/L)	17.95	18.2	12.08	33.28	31.88
Volumetric plasmid yield (mg/L)	9.02	32.48	13.75	53.08	33.65
Supercoiled Content (%)	31.5	90.3	68.2	90.37	87.74
Supercoiled plasmid yield (mg/L)	2.88	29.23	9.44	47.93	29.54
Specific Plasmid yield (mg/g)	0.50	1.78	1.14	1.59	1.06
$\mu max(h^{-1})$	0.33	0.17	0.22	0.16	0.13
$Y_{x/Glycerol}(g/g)$	0.45	0.46	0.15	0.42	0.40

However, when compared to media with no supplementation, there was a significant improvement in the plasmid yield with over two-fold increase in the

plasmid yield (33.65 g/L to 13.75 g/L). Figure 5.13 shows a plot comparing the plasmid yield of the three media preparations.

The supercoiled plasmid content was also monitored. Although there was a dip in the supercoiled content in the early stage of the fermentation, it rose to about 90% supercoiled during the early stationary phase and was maintained at harvest. The same phenomenon was observed with the casamino acid supplementation, where there was a dip in the supercoiled content and rose to 90% at harvest. This observation shows the beneficial effect of supplementation with specific amino acids and hence moving towards a defined medium for plasmid bioprocessing. Overall, the fraction of supercoiled plasmid yield to total plasmid yield was similar for both casamino acid- supplemented media and specific amino acids-supplemented media. Table 5.1 shows a summary of the fermentation characteristics for all investigations carried out in this study.

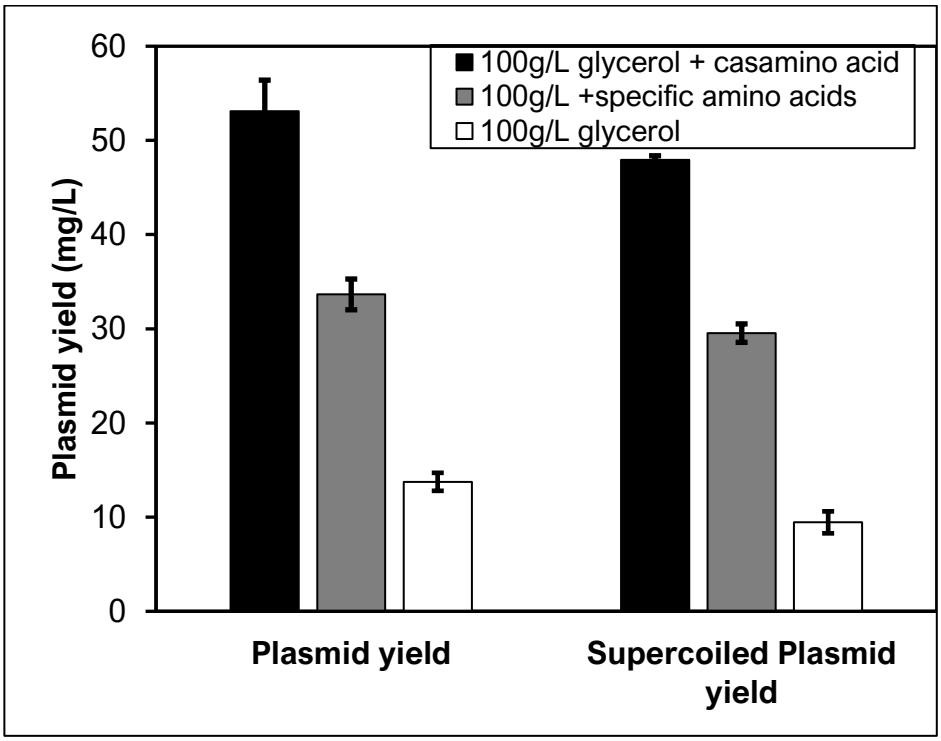


Figure 5.13: Plots of comparing total plasmid yield (mg/L) and supercoiled plasmid yield (mg/L) of the three high initial carbon source concentration media. Values are mean +/- SD of duplicate independent fermentations.

5.3 Conclusion

There is a need to develop a defined medium for the bioprocessing of plasmid DNA for biotherapeutics application. Current media preparations are either complex media or semi-defined media where the complex sources include tryptone, casamino acids and peptone. This poses a challenge of reproducibility and consistency of lots during manufacture. Also, since the plasmid are produced intracellular, a high cell density culture is advantageous to have a high plasmid yield. However, achieving a high cell density in batch mode is a challenge due to overflow metabolism associated with using a high carbon source.

A high cell density culture was shown to be possible by using a high glycerol concentration as the carbon source supplemented with casamino acid. High biomass was observed when the high glycerol concentration media was supplemented with casamino acid compared to with no casamino acid. The increase in biomass yield also translated to an increase in plasmid yield with a three-fold increase when compared to high glycerol media with no supplementation. This shows that it is possible to achieve a high cell density and subsequently, high plasmid yield by developing a media supplementation strategy.

However, the addition of casamino acid to media leads to the development of a semi defined media. Hence, specific amino acids were selected for supplementation. Methionine was explicitly selected, and the concentration determined to relieve the effect of acetate on growth inhibition. The addition of histidine and glycine are due to report of these amino acids improving the yield of plasmid production.

Supplementation with the specific amino acids has a similar effect on the biomass yield like it was observed with casamino acid supplementation. However,

the plasmid yield was lower than observed with casamino acid supplementation. This could suggest that there were some extra nutrients present in the casamino acid that have not been identified yet. Furthermore, the presence of the specific amino acid leads to the maintenance of the supercoiled content during the course of the fermentation with a final supercoiled content of 90%.

The investigation in this chapter shows that it is possible to achieve a high cell density culture by supplementing media with growth inhibition relieving nutrient at the same time improving the plasmid yield while using a defined media at the same time. The significance of these results is that it is possible use a defined media to allow for reproducibility and maintenance of lot consistency during batch fermentation for plasmid production.

CHAPTER 6. Large scale batch-fermentation of 27kb Plasmid using Amino Acid Supplemented Medium and High Initial Carbon Source.

6.1 Introduction

The challenges for growing a large amount of plasmid in a short period of time while the plasmid maintains a high quality is still not tackled completely. With the potential possibility of increasing the plasmid size for gene therapy and vaccine application, there is a need to develop a bioprocessing approach to manufacture large plasmid at the rate of a small-sized plasmid. Typically, strategies for developing improved bioprocessing approaches are carried out in bench-scale bioreactors, and then this can be further scaled to large bioreactors for reproducibility.

Approaches for bioprocessing of large plasmid in batch mode have been developed during this work. However, like other strategies, these have been carried out in bench-scale reactors. It is therefore important to test the reproducibility of these improved approaches at a large scale as the growing demand for plasmid use cannot be met using bench-scale reactors.

Batch fermentation can be a preferred strategy for producing plasmid DNA because it reduces the chances of contamination which can happen when feeding into a fed-batch fermentation or continuous. Although some limitations to batch fermentation exist which include the restriction on the biomass than can be achieved and also limitations on the primary carbon source used. In previous chapters, it has been shown that these limitations can be ameliorated by developing an approach that allows using a high initial carbon source without limiting the plasmid yield (Dorward *et al.*, 2019).

This chapter aims to investigate the effect of scale-up on:

- The biomass yield when using a high concentration of initial carbon source and supplementation with the specific amino acids.
- The growth characteristics of *E. coli* strain HB101 at scale.
- The plasmid yield and supercoiled content of the final plasmid yield.

6.2 Results and Discussion

6.2.1 HB101 as a Potential Candidate for Plasmid Production

E. coli strain HB101 is one of the commonly studied *E. coli* strain for industrial purposes. However, it is not the strain of choice when it comes to plasmid bioprocessing. The *E. coli* strain that has been adopted widely for plasmid bioprocessing is the strain DH5α (Lara and Ramírez, 2012). This is in part due to its *relA, endA* and *recA* mutations (Huber et al., 2005; Bower and Prather, 2009).

Although there are few studies that have employed HB101 for plasmid bioprocessing (Yau, Keshavarz-Moore and Ward, 2008), it is not the strain of choice. One possible reason is because of the high carbohydrate content in the strain, which becomes a challenge during downstream processing. However, with the advances in downstream processing of plasmid bioprocessing, this challenge can be tackled efficiently (Xenopoulos and Pattnaik, 2014). Figure 6.1 shows plasmid yield and biomass yield when *E. coli* strains HB101 and DH5α were used for amplification of a 27kb plasmid. The two strains were transformed with the same sets of plasmids with the same protocols and fermentations carried out under the same conditions. In both plasmid cases used, the strain HB101 had a higher biomass yield and plasmid yield than the strain DH5α.

With these observations, it is suggested that strain HB101 could be an excellent candidate strain for plasmid bioprocessing, behaving better than DH5α which is the popular strain.

To further characterize this strain, batch fermentation was carried out in a 20L reactor with the strain amplifying the 27kb plasmid pSTFλ-SGS.

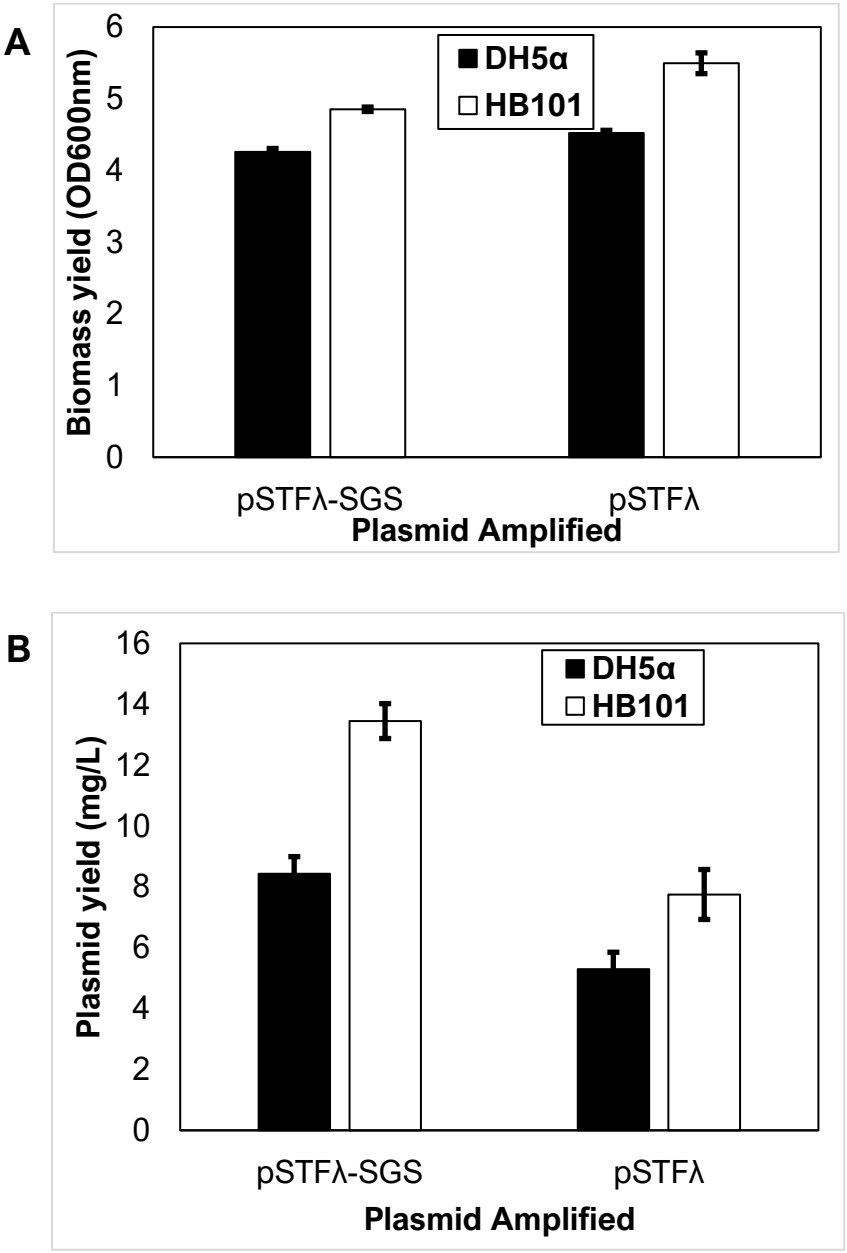


Figure 6.1: Comparison of *E. coli* strain DH5α and HB101 with respect to biomass and plasmid yield when amplifying a 27kb plasmid pSTFλ-SGS.

6.2.2 Cell Growth Characteristics

Batch fermentation was carried out in a 20 L reactor (11 L) working volume. Defined media was used with initial glycerol concentration of 100 g/L and supplementation with amino acids methionine, glycine and histidine. Air mixed with oxygen was sparged into the reactor at 2vvm, and the dissolved oxygen tension (DOT) maintained at 30%.

To prepare the starter culture, 5 mL LB media was inoculated from the working cell bank (WCB). This was used to inoculate the 200 mL defined media in Shake Flask for 24 hours. The content of the shake flask was used to inoculate the reactor with a starting OD600nm of 0.6. The fermentation reached the exponential phase after 7 hours post-inoculation. The oxygen consumption increased during the exponential phase with the dissolved oxygen tension (DOT) dropping below the set point.

To maintain the DOT set point of 30%, the stirrer speed increased accordingly. Figure 6.2 shows the growth curve of the fermentation. The fermentation reached stationary phase after 13 hours post-inoculation and the culture reached an OD of 62.15 and final biomass yield of 24 g/L. Although a higher OD was expected due to the high initial glycerol concentration used, the reduced biomass yield could be due to the size of the plasmid which means it may confer additional metabolic stress on the cells (Cunningham, Koepsel, *et al.*, 2009). Another possible explanation could be due to the differences in the hydrodynamic properties of the reactors, despite scaling using power-per-unit Volume (P/V).

However, the growth rate was similar to what was observed when the same conditions and fermentation media was used in the amplification of a smaller plasmid.

This observation shows that in terms of the biomass yield, the fermentation media strategy can be used at scale for improved biomass yield and reproducibility.

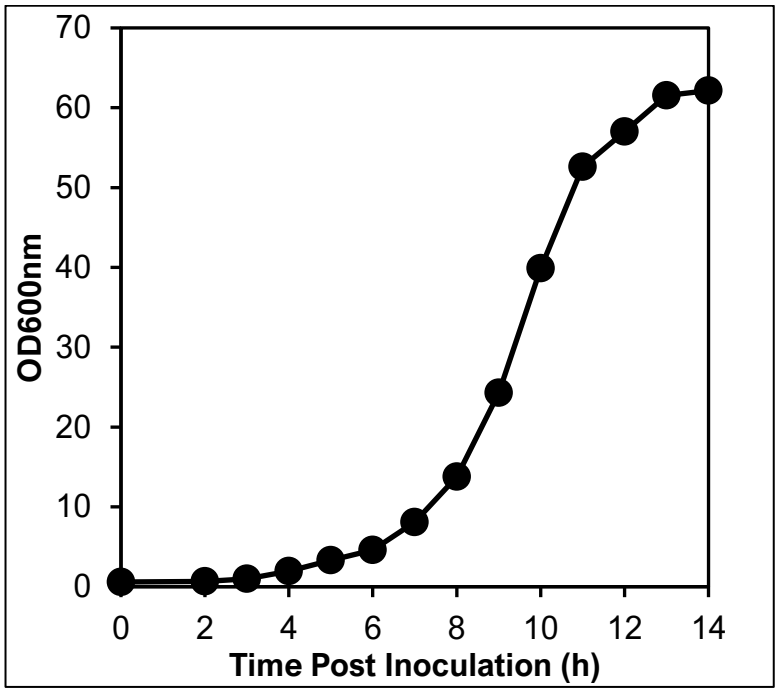


Figure 6.2: Chart showing the fermentation growth curve of HB101 amplifying plasmid pSTFλ-SGS in a 20 L reactor.

6.2.3 Supercoiled DNA yield

The plasmid yield was also monitored during the course of the fermentation (Figure 6.3). It is expected with the increase observed in the biomass during fermentation, that there would be a corresponding increase in plasmid yield as it is intracellular. This expectation was met as the final plasmid yield of 33.4 mg/L was observed. The biomass yield observed was similar to what was observed when the strategy was implemented in a 1 L bioreactor. Although the biomass yield was lower compared to the 1 L reactor this did not seem to affect the plasmid yield with a final specific plasmid yield of 1.4 mg/g (DCW).

The final supercoiled content was also maintained at 90%. Although the supercoiled content was lower during the early exponential phase, it rose during the stationary phase and was maintained at harvest. Again, the supercoiled content was similar to what was observed in the 1 L bench scale reactor.

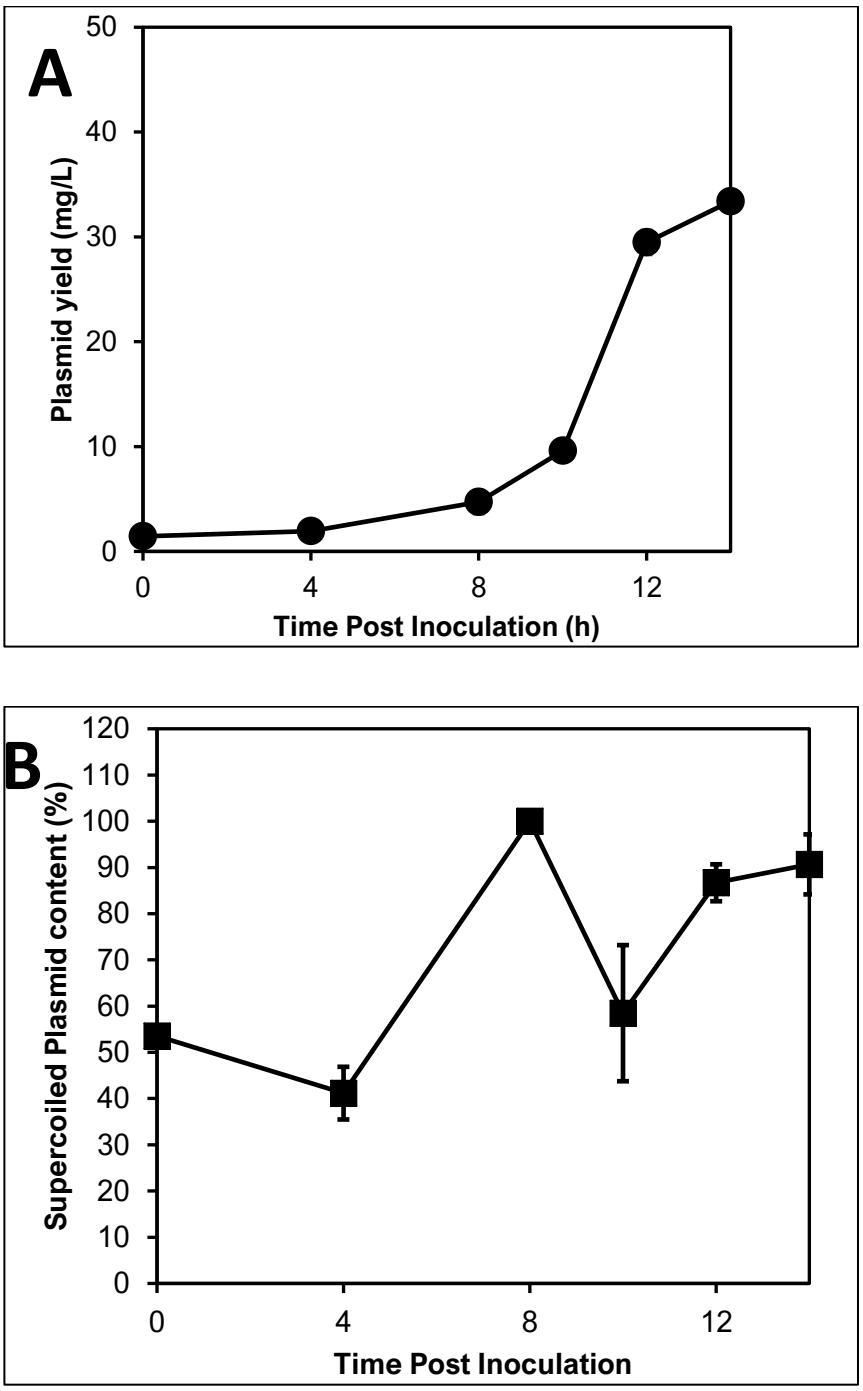


Figure 6.3: The plasmid yield (mg/L) (TOP) and supercoiled content (BOTTOM) monitored during fermentation. Results are expressed as mean of triplicate analyses.

6.3 Conclusion

There is a huge market potential for plasmid therapeutics which would see large scale bioprocessing of plasmid very crucial to meet the market demands. With the current advances in the developing plasmid therapeutics and the number of clinical trials that are ongoing, kilograms of plasmid per year would be required. Therefore, any approach developed to improve the yield and/or quality must be scalable and reproducible in large scale.

The approach that has been developed was tested on a 20L reactor. A batch scale fermentation was run in the reactor and strain HB101 was used as the amplifying *E. coli* strain. Although the strain DH5α has been the most popular strain for amplification, the strain HB101 serves as a better alternative for upstream processing of plasmid DNA. The fermentation reached an OD of 62 and biomass yield of 24 g/L. the ratio of OD to biomass yield in g/L was similar to what was observed in a bench-scale reactor. However, the final biomass yield was lower than observed in a bench-scale reactor, but this is not surprising as the plasmid being amplified is larger which could cause higher metabolic stress on the cells.

The final plasmid yield observed was around 33 g/L. This was also comparable to the final volumetric plasmid yield observed in a bench-scale reactor under the same conditions. The supercoiled content peaked 100% after 8 hours which is the early exponential phase. However, this is not the optimum harvest period as the yield during this stage of the fermentation is low. Harvesting at the stationary phase seems to be the best harvest point as the highest yield was observed at this phase and the supercoiled content was maintained at 90%.

This shows that at scale, it is possible to develop a batch fermentation using a high concentration of initial carbon source and by supplementing with amino acids,

a high yield process can for amplifying plasmid with size >20kb be achieved. Although a 20L reactor was used here, it will be interesting to investigate if the process can be reproducible on a larger scale.

Finally, with the recent advances in the downstream processing of plasmid DNA, it is high time focus is shifted to HB101 as the choice of *E. coli* strain for plasmid amplification.

CHAPTER 7. Conclusion and Future Works

This section aims to review the objectives of the thesis outlined in the introductory chapter, summarise the key research findings and provide some future recommendations for this work.

7.1 Effect of Mu Strong Gyrase Binding Site on the Overall Yield and Quality of Plasmid

A 398 bp strong gyrase binding sequence was inserted into the multiple cloning site of plasmids in order to investigate the effect on the yield and quality of the plasmid produced during bioprocessing. A 7.2 kb plasmid and a 27 kb plasmid were constructed for the purpose of this investigation. Four different host cells were selected to amplify the plasmids and the yield produced from these host strains were compared.

Two of the strains (HB101 and DH5α) selected amplified the plasmids effectively compared to the other two strains (BL21 and W3110). The yield of plasmid bearing the Mu SGS was higher than without the SGS in both strains used. The quality of the plasmid was also measured by determining the percentage supercoiled plasmid present in the final plasmid preparation. It was also observed that plasmid constructs bearing the Mu SGS had higher supercoiled content in the final preparation than constructs without the SGS. Although they both reached a supercoiled content of 90%, the SGS containing constructed were able to reach as high as 96% supercoiled content which showed that the SGS had an effect on the supercoiling of the plasmid.

In order to further measure the level of supercoiling in the amplified plasmids, the superhelical density was determined using the 2D chloroquine agarose gel electrophoresis, which is able to resolve topoisomers based on the twist and knots present in them. The superhelical density measurement also showed that the plasmid constructs bearing the SGS are more compact that constructs without the SGS. This was only possible with the smaller 7.2 kb plasmid as all attempt to measure the superhelical density of the large 27 kb plasmid was unsuccessful.

In order to test the integrity of the plasmid during downstream processing, the plasmid preparation was subjected to shear conditions similar to what is observed during downstream processing of plasmids. Ultra-scale down technology was employed by using the USD shear device. The plasmid preparation was subjected to different shear rate and it was observed that the effect of shear on the smaller plasmid is minimal as there was no significant difference observed between non-sheared samples and sheared samples. However, for the 27 kb plasmid, the effect of shear on the supercoiled content was significant. Plasmid preparation with constructs bearing the SGS was also subjected to shear, and it was observed that they are able to maintain their integrity better than plasmids without the SGS. This also highlights the significant effect of SGS on plasmid integrity.

7.2 Amino Acid Supplementation-Based High Cell Density Fermentation for Supercoiled plasmid

An attempt was made to develop a high cell density batch fermentation using defined media supplemented with specific amino acids. High initial glycerol as carbon source and its effect on the biomass yield was investigated. When 50 g/L initial glycerol concentration was used, there was accumulated biomass which

agrees with previous reports. However, it was observed that the accumulated biomass does not directly translate to corresponding plasmid yield when compared to a batch fermentation where semi-defined casamino acid media was used. It was observed that the presence of casamino acid in the media stimulates the amplification of plasmid.

By using a higher glycerol concentration of 100 g/L and casamino acid, a higher cell density was achieved compared to when 50 g/L glycerol was used. The increase in biomass observed was translated to an increase in plasmid yield which was similar to what was observed when 50 g/L glycerol with casamino acid. However, when 100 g/L glycerol only was used in the media, there was growth inhibition observed which agrees with what has been reported about overflow metabolism. Despite the use of 100 g/L with casamino acid producing the best yield, the objective is to develop a defined media to allow for reproducibility.

Amino acids identified from previous work was used to supplement defined media prepared with 100 g/L glycerol as the initial carbon source. The amino acids added were able to prevent overflow metabolism while improving the proportionate increase in the plasmid yield. However, the plasmid yield observed was lower than when casamino acid was used. This signifies that there might still be some nutrients that have not been identified yet that could be a major player in the induction of plasmid.

The findings show that it is possible to achieve a high cell density in batch fermentation strategy by supplementing with growth inhibition-relieving nutrients and at the same time improving the overall plasmid yield while allowing reproducibility due to the defined nature of the medium.

7.3 Large Scale Fermentation of 27 kb Plasmid using Amino acid Supplemented Medium

By selecting the best strain, the 27 kb plasmid constructed was amplified by employing batch fermentation in a 20 L reactor. 100 g/L glycerol was used as the primary carbon source and the medium was supplemented with amino acids that would improve the yield and inhibit overflow metabolism. The final plasmid yield was comparable to what was observed in a 1 L bench-scale reactor showing that the approaches investigated in the research could be scaled up and implemented on a large scale.

7.4 Future Work and Recommendations

The effect of SGS on plasmid yield and quality has been investigated on two different-sized plasmids. Although it is expected that these effects will be observed for any other kind of plasmid if the Mu SGS is introduced, it is essential to verify using other plasmids irrespective of size.

The impact of SGS on the integrity of the plasmid was also investigated on the final plasmid preparation, while it shows that plasmid constructs bearing the SGS were able to maintain their integrity compared to constructs without the SGS, a future work will be to test the integrity of these plasmids during clarification of the lysate before purification is carried out.

One of the potential benefits of having a highly compact plasmid is an increase in transfection efficiency; hence the effect of the increase in compactness on the transfection efficiency should be investigated, both in tissue cultures and cell

cultures. This would further add to proof on why plasmid supercoiled content should be at a certain level to be effective as therapeutics.

In addition to investigating the effect of SGS on the maintenance of integrity using USD shear device, the effect should also be tested on filtration and chromatography setup. This work has only explored the effect of physical conditions on plasmid integrity. It is worth investigating the effect of chemical conditions too as plasmid get exposed to harsh chemicals especially during lysis stage (alkaline lysis)

Attempt to determine the superhelical density of the 27 kb plasmid proved unsuccessful. It is recommended that the 2D chloroquine agarose electrophoresis be combined with pulse-field electrophoresis in order to measure the superhelical density of the plasmid.

Other future works include investigating the use of Atomic Force Microscopy (AFM) to measure the compactness of the plasmid construct. Superhelical density measurement involves detecting the number of knots are present in the plasmid. AFM is able to produce a clear image of the knots in a single plasmid. By developing an image analysis tools, it would be possible to automate the counting of the knots in the image.

Further investigation is required to determine what other nutrients in casamino acid mixture contribute to an increase in the plasmid yield. Fed-batch fermentation with a high initial carbon source should also be investigated in order to further increase the plasmid yield and overall productivity of plasmid bioprocessing. HB101 was observed to be the best strains for plasmid bioprocessing but it is not the strain of choice in the industry. There should be a further characterisation of this strain for industrial acceptability.

Semi-densitometry was adopted for the supercoiled content quantification. It is worth noting that this is not an optimal method, and other methods such as capillary electrophoresis and HPLC should be explored, especially as an optimal method that can be controlled is required for manufacturing purposes.

7.5 Conferences Attended

BioProcess UK, Newcastle, United Kingdom

22 – 24 November 2016

Poster Presentation: *A New Approach to Supercoiled Plasmid DNA Upstream Processing for Vaccines and Gene Therapy Applications.*

Microbial Engineering, Santa Fe, New Mexico, USA

4 – 8 March 2018

Poster Presentation: *Enhancing the Yield and Quality of Supercoiled Plasmid through Plasmid Engineering.*

Oral Presentation: *Enhancing the Yield and Quality of Supercoiled Plasmid through Plasmid Engineering.*

7.6 Peer-Reviewed Publications

1. Folarin, O., Nesbeth, D., Ward, J.M. and Keshavarz-Moore, E., 2019. Application of Plasmid Engineering to Enhance Yield and Quality of Plasmid for Vaccine and Gene Therapy. *Bioengineering*, *6*(2), p.54.

2. Lara, A.R., Jaén, K.E., Folarin, O., Keshavarz-Moore, E. and Büchs, J., 2019. Effect of the oxygen transfer rate on oxygen-limited production of plasmid DNA by Escherichia coli. *Biochemical Engineering Journal*, *150*, p.107303.

3. Dorward, A., O'Kennedy, R.D., Folarin, O., Ward, J.M. and Keshavarz-Moore, E., 2019. The role of amino acids in the amplification and quality of DNA vectors for industrial applications. *Biotechnology progress*.

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Appendix A: Migration of Large Plasmid Topoisomers is Affected by Varying Gel Concentration

Separation of DNA fragments on gel is made possible due to their charges and sizes. In addition to the size, the electric voltage used, buffer types, buffer concentration, gel concentration, type of agarose, staining method used and DNA topology also determine the resolution of the fragments on gel. When the sizes of two fragments being separated are similar (difference by few bp), increasing the gel concentration can improve the resolution of separation of the two fragments. Agarose is a polysaccharide consisting of repeating subunits of agarobiose (L- and D-galactose).

During gelation, agarose polymers associate non-covalently and form a network of bundles whose pore sizes determine a gel's molecular sieving properties (Lee *et al.*, 2012). DNA fragments move through the agarose by a model known as 'biased reptation' where leading edges move and pull the rest of the molecule along. The effects of el concentration on the mobility of DNA have been extensively reviewed by (Stellwagen and Stellwagen, 2009).

For DNA topoisomers, the migration pattern also vary. For DNA with the same number of base pairs, the supercoiled DNA migrates faster on a gel followed by linearized DNA and the nicked circular DNA migrating the slowest. The migration pattern exhibited by supercoiled DNA is due to the compactness of the DNA, allowing it to squeeze through the pores of the agarose (Mickel, Arena and Bauer, 1977; Stellwagen and Stellwagen, 2009). Nicked or open circular DNA get trapped in pores and so their migration is slower than the other two topoisomers with the same (Levene and Zimm, 1987).

For small-sized plasmids (< 20 kb), the migration pattern observed for the three different topoisomers is the same irrespective of the agarose gel concentration. Nicked plasmids will always migrate the slowest and supercoiled the fastest. This allows to monitor the different topoisomers as supercoiled and linear markers can be used to determine which topoisomers are present. However, for large sized DNA ($\geq$ 20 kb in size), the migration pattern varies with the gel concentration.

An investigation was carried as to how migration of topoisomers are affected by the gel concentration specifically the supercoiled and linear topoisomers. A 27 kb plasmid preparation containing nicked, linear and supercoiled topoisomers was separated on agarose gel with concentration of 0.4%, 0.6%, 0.8% and 1%. The electrophoresis was carried out in a large gel component with a migration distance of 15 cm. The gel wells were loaded with the same amount of DNA and the same buffer type and buffer concentrations was used. The distance travelled was measured relative to the starting well and this was used to compute the band migration in mm/V·min.

As expected, increasing the gel concentration leads to a decrease in band migration (Figure A.0.1). This is due to the network of polysaccharides in the agarose gel inhibiting the movement through. The supercoiled retards the highest with the reduction in band migration of 0.04 $mmV^{-1}min^{-1}$ for every 0.2% increase in gel concentration. This is followed by the nicked plasmid with a reduction of 0.03 $mmV^{-1}min^{-1}$ for every 0.2% increase in gel concentration. There seems to be a very slight change in the band migration for linear plasmid with a reduction of 0.01 $mmV^{-1}min^{-1}$ for every 0.2% increase in gel concentration. This shows that supercoiled plasmids are more sensitive to gel concentration than nicked and linear with linearized DNA being the last sensitive to the change in gel concentration.

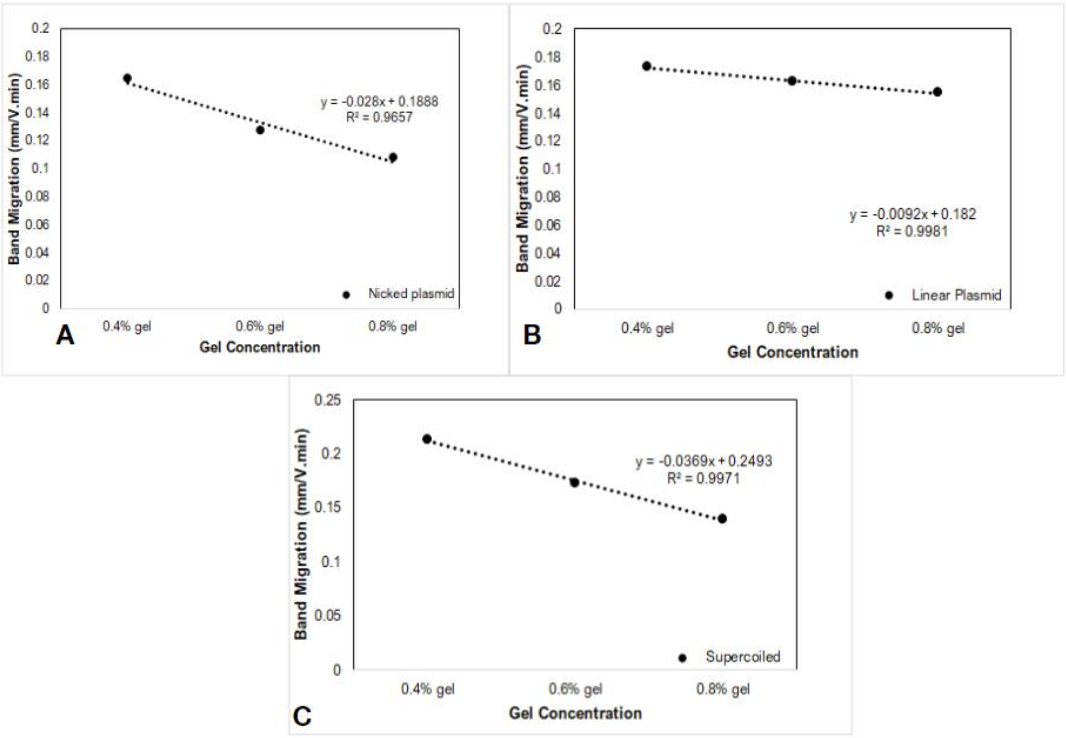

Figure A.0.1: Band migration for different plasmid topoisomers (A) Nicked plasmid, (B), Linear Plasmid (C) Supercoiled plasmid; on gels with agarose gel concentrations of 0.4%, 0.6% and 0.8%. All bands migrations followed the expected pattern

However, the band migration of supercoiled DNA at high gel concentration ($\geq$ 0.8%) is lower than linearized plasmid. This is because the linearized plasmid is less sensitive to change in gel concentration. The sensitivity means that during gel electrophoresis to separate these topoisomers at a gel concentration of 0.8%, linearized plasmid would migrate faster than supercoiled plasmid in contrast to what is expected.

This phenomenon is only observed for plasmid larger than 20 kb. For smaller plasmid, irrespective of the gel concentration, the supercoiled plasmid will always migrate faster than linearized and nicked plasmid. An explanation for this observation could be that supercoiled plasmids still assume a globular shape despite the compact nature, this makes them trapped in the pores of agarose at high concentration

(especially for large plasmids). The edges of linearized DNA can easily move through the pores hence migrating faster.

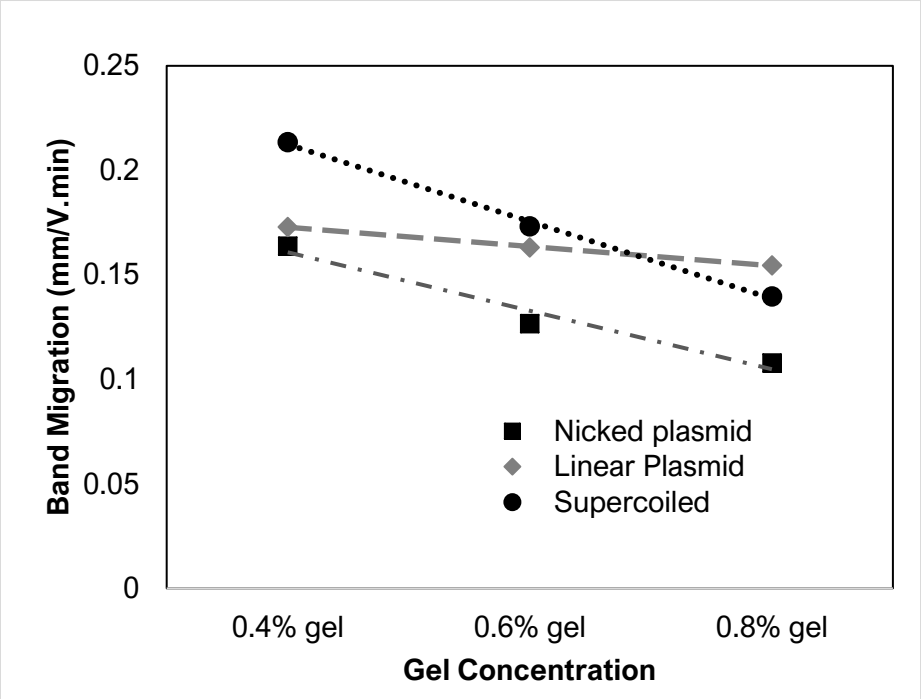

Figure A.0.2: Comparing the band migration pattern of the topoisomers at gel concentration of 0.4%, 0.6% and 0.8%. At 0.8% gel concentration, linearised plasmid migrate faster on gel than supercoiled while supercoiled plasmid migrate faster at 0.4% and 0.6%. Band migration is the mean of duplicate gel electrophoresis.

Appendix B: SOP for Sterilization and Operation of the Applikon Fermenters

SOP No __

Title:

Sterilisation and Operation of Applikon 1L X4 Fermenters

WRITTEN BY	DATE	OPERATING HEAD	DATE	DOC. REF.
O. Folarin	01/05/19		01/05/19	SOP __
Signature		Signature		

1.0 Introduction

1.1 This document outlines the procedures to follow for setting up and sterilization of the vessel prior to inoculation, running, and fermenter close-down at the end of the fermentation, and cleaning the vessels. The Applikon 1L x4 is an Applikon (Applikon Biotechnology) 1L parallel fermenter system including 4 vessels and series instrumentation control modules.

1.2 This fermenter is not sterilisable in situ, and thus requires an available autoclave. Refer to SOP No.30 for operation of the Getinge autoclaves in the ACBE.

1.3 Acid, base, antifoam and feed bottles are not attached to the vessels so care must be taken when moving the vessels as each set of bottles supply two vessels.

1.4 Please note that after removal of vessels from autoclave the DOT probe needs to be polarized for a period of at least 2-6 hrs (preferably 12 hours) before use.

2.0 Pre-Sterilization Set-Up

2.1 Refer to technician or previous fermenter checklist to ensure that no technical faults encountered with this fermenter from the previous run, and if any faults noted, verify with a member of the technical staff that these have been rectified.

2.2 Ensure the vessel is clean. If vessel is not clean, carry out appropriate cleaning procedure as outlined below (step 5.11.1 to 5.11.8).

2.3 Check condition of all 'O'-rings and replace any if damaged.

2.4 Check that the impellers are tightened properly as they sometimes come loose during cleaning from the previous run. Also check that the height of the impellers are the right height for your fermentation run.

2.5 Fill the vessel with the media to the required working volume (possibly with a 10% over-dilution to allow for losses during autoclaving).

2.6 Re-fit the top plate (ensuring that the o-ring is in position and forms a good seal) loosely attaching the nuts that hold the top plate to the vessel.

NOTE: do not over-tighten the nuts, as this may cause the glass pot to break during the autoclave cycle.

2.7 Switch the my-Control bio controller on (the switch can be found at the rear of the controller) and start the Internet Browser (Internet Explorer). The IP-address comes with a preset IP-address and navigation to this IP-address is automatically done when you open Internet Explorer. Four tabs are opened, one for each controller. The IP-address can be customized but it is advisable to NOT tamper with it.

2.8 The my-Control bio controller is passworded, so you must be authorized to use it.

2.9 Rinse off KCl solution from the the ends of the pH probes with RO water.

2.10 Check that the membrane of DOT probe is intact. Verify response - O2/N2 to verify that probe is responsive to Sparge nitrogen through air inlet filter to check DOT probe response

2.11 Fit the pH and DOT probes, checking the O-ring seals (maximum of 3 for each probe in this order: red/black-white-red/black) and replacing if necessary. (Push the O-ring through the glass probe- not vice versa!). Hand tighten only! Do not

use tools! Cover the ends of the pH and DO probes with its designated cap (black or green).

2.12 Calibrate pH probes - see SOP No.?. Place probe in fermenter and tighten the cap

2.13 Ensure that at least bottom third of probe is immersed in liquid.

2.14 Ensure that at least bottom third of DO probe is immersed in liquid.

2.15 Fit the sampling port, check the O-ring seal and replace as necessary. Place a clean glass bottle (for autoclaving only) in the bottle holder and a clean filter on the sample port vent. Clip off the line using a gate clip.

2.11 Fit septum seals in the remaining ports replacing any that require replacement, and tighten the blank ports down.

2.12 Fit a clean 0.22 micron air filter (red line on filter) to the air inlet (top of the sparger pipe) and close the line using a gate clip. Note the side of the filter marked INLET should be on the top, facing away from the vessel.

2.13 Fit the condenser and place a clean air filter on its exhaust. Note this filter must be clear to vent. (Filters: 0.2μm filter (inlet) is red, and 0.2 μm filter (outlet) is green).

2.14 Push in the condenser to tighten and keep in position. (It is easier to take out an O-ring – this doesn't need to be finger tight as just need to form a seal).

3.0 Preparing for Autoclave Sterilization of Vessel and Contents

3.1 Cover all filters (inlet and outlet of condenser) and those on the 250mL feed, acid, antifoam and base reagent bottles and on the sample port with cotton wool and aluminum foil.

3.2 Acid addition reservoir (this should be empty – DO NOT AUTOCLAVE ACIDS) and ensure that the reagent bottle's lid is loose.

3.3 Alkali addition reservoir (this should be empty – DO NOT AUTOCLAVE ACIDS) and ensure that the reagent bottle's lid is loose.

3.4 Fill in the antifoam bottle with the required amount. This can be autoclaved alongside the vessels.

3.5 It is recommended that all silicone tubing contain RO water when autoclaving.

3.6 Inoculum port – ensure there is a 12 mm septum inside (wide side down) and seal with its screw collar and port fitting using the supplied hexagon wrench. Replace O-rings as necessary and wet with alcohol before refitting.

3.7 Loosen the cap of the sample tube and of any other reagent addition bottle.

3.8 Ensure that the nuts on the top plate are loosened off slightly.

3.9 Cover the electrical contacts of the DOT probe, with the provided cap. Make sure it is tightened.

3.10 Ensure air inlet line is clamped off. Seal the filter with cotton wool, foil and autoclave tape.

3.4 Adjust foam probe if used to lowest depth in the port so that the precise height can be set after sterilization.

3.5 Ensure sample port line is clamped off. Seal the filter with cotton wool, foil and autoclave tape.

3.6 Ensure that the tubing on the air exit line will not get bent over or kinked. Cover the air exit filter outlet with cotton wool, foil and autoclave tape and ensure the line remains open. (Clamp off ALL lines except air outlet on condenser).

3.7 Remove the heating blanket and fold on the velcrow. DO NOT place flat on the control unit as the heating may be on.

3.8 Transfer fermenter vessels and ancillary bottles in their frame in one of the Large Getinge autoclaves and sterilize on Fluids cycle with load probe in equal volume of water. It is better to transfer two vessels at a time as they are connected to a single set of addition bottles.

3.9 Refer to checklist for autoclaving – SOP.

4.0 Set-up for Fermenter Operation

4.1 Open autoclave once cycle has finished.

4.2 The fermenter and ancillaries should now be sterile. Allow the vessel to cool enough for handling before proceeding.

4.3 Once cooled, retighten the screw clamps on the top plate to hand tight

4.4 Retighten the nuts that hold the top plate to the vessel and the caps of all reagent bottles and replace the glass sample tube with a sterile universal tube.

4.5 Remove all foil, cotton wool and autoclave tape from the filters, electrical connections and probes.

4.6 Attach the heating blanket to the glass vessel

4.7 Attach the stirrer motor to the head of the reactor (make sure it is properly fixed).

4.8 Attach both pH and DOT leads to respective probes and ensure modules are switched on (the pH probe is connected to the lead with the red end. The connector should be pushed down onto the electrode, the collar pulled down and the screwed into place).

NOTE: DOT probe must be polarized by connecting to a powered module for at least 2-6 h (12 hours recommended).

4.9 Switch on the aside chiller (it is recommended that ”soft” water (up to 1.5 mmol/L calcium carbonate is used). Attach water inlet and outlet lines to condenser for each fermenter that will be used. “Water in” means water in from base unit, into the condenser (at the bottom) (to the exit gas cooler). “Water out” means water out from base unit (always connect water in first).

4.10 Turn the toggle switches beneath this fully in the anticlockwise direction to allow water in.

4.11 Connect the silicon tubing from the addition bottles to the pumps on the bio controller making sure that flow is in the right direction (i.e. from the acid and base bottles to the vessel).

4.12 Start with everything off except gas mixing:

4.12.1 Put the compressed air on (blue line) at services panel by turning the tap 45degrees to the left.

4.12.2 Attach air-line to the air inlet filter. Adjust airflow using the black rotameter to the desired setting for each (if gas analysis is required, switch on the Tandem mass flow meter (to the left of the parallel fermenter systems) first, using the black switch on the bottom left hand side of the Tandem.

4.12.3 Ensure that the top plate is sealed and clamp off all tubing so that gas in = gas out.

4.12.4 Switch on the unit system for air flow into the reactor.

4.12.5 Start gas mixing (there should be visual upward movement of the ball on the rotameter which can be adjusted to the desired setting e.g. 1.6vvm).

4.12.6 Pressure test on outlet filter- (press with thumb and release – should hear a hiss sound).

4.12.7 On the Tandem, you should notice that the reading rotates sequentially between the 4 fermenters. If necessary, twist white clamp on tubing connection to set at 1.6 – you should not have to do again unless someone moves this – this is not to do with calibration, just setting up. Check that the gas analyzer reading matches that of the rotameter of the fermenter systems.

4.13 Set the stirrer to the desired speed using the control panel and switch it on (it is advisable to switch this on first, before the temperature control as stirring will affect the temperature). To do this, follow the guideline below. You should then be able to enter the desired stirrer speed.

Guidelines for using the control module/display panel:

- Make sure the my-Control display is on the ‘Home’ tab. You might need to provide a passcode to access it

- Select ‘Overview’ from the screen. This shows all the Process Parameters to the left of the screen.

- Select the Process parameter you are trying to adjust and input the required value for each process parameter.

- Select the check to save. You may start individual process parameter by selecting the start/stop button to the right of each process parameter in the View Mode. Alternatively, you can start all process parameters by selecting the Start All button at the top of the ‘Overview’ panel.

- When the Process Parameters are running, it should display Green, and Yellow if the parameter is not started.

- If the color changes to red, this implies that the parameter is out of the set range and requires attention.

4.14 Place the temperature probe for each vessel all the way down (until metal to metal contact can be felt) into its 10mm pocket on the top plate. You may add some water into the pocket for adequate contact.

4.15 Set the temperature to the desired settings from the View Mode as explained in the Guideline above.

4.16 If cascade control is desired: Stirrer first then gas mix (air flow rate second). E.g. stirrer: 600-1000 (setpoint 600); air flowrate 0-10 (setpoint 0).

4.17 For setting up pH control, aseptically connect acid/base reagent bottles to the appropriate green lids. Fill the 250 mL reagent bottles approximately 2/3 full. Do not autoclave these- avoid a high concentration of NH3 as this will damage the tubing and perhaps use NaOH instead. For acid, use H2SO4 (20%) (do not use HCl – as this attacks the plastic tubing). Note that these reservoirs serve 2 vessel base units.

4.18 Feed the tubing through the relevant pump heads (if this was previously disconnected). The pump heads are labelled for acid, base, antifoam and feed. Open any gate clips on the tubing.

4.19 Set the pH on the control module to the desired value.

4.20 Prime the addition lines by running the pump. This can be found in the list by selecting the ‘Actuator’ tab (just right of the ‘Overview’ tab). Start the lines you want to prime and keep an eye on the tube so you know when to stop priming

4.21 Ensure that the foam probe is at the desired level – note that after sterilization, the probe can only be pulled out of the vessel to adjust height – not down into the

vessel! (be careful not to pull this probe out whilst the side screw is screwed in! Once the height is fixed, however, screw in to fix into position). If the fermenter is fitted with the foam probe and foam control is required, aseptically connect the antifoam reservoir to a steri-connector on an addition port. Feed the tubing through the antifoam pump head. Open any gate clips on the silicone tubing.

4.22 Prime the antifoam line as explained in section 4.20 above, stopping when the liquid level is close to the addition port.

4.23 Ensure that the foam probe is connected up (black and red banana connections – the red banana connector should be fitted to the hole in the top of the electrode and the black one should be fitted into the small hole in the top plate made to accept the connector. DO NOT push the probe any further down into the vessel at this stage.

4.24 Take a sample by attaching a syringe to the sample port filter, loosening the gate clamp on the line and sucking out the sample (into a universal) with the syringe. When 1/4 full, push the syringe plunger down to blow the contents of the sample line back into the vessel. Re-clamp the line, remove the bottle and cap it aseptically. Fit a new bottle to the cap and screw into place. Repeat this procedure and use the second sample of the desired volume, discarding the first one.

4.25 With stirrer and airflow at desired settings, check DOT probe is reading 99.9%. Refer to SOP No. for adjustment of DOT probe and calibration at this stage. Sparge nitrogen through air inlet filter to check DOT probe response and to check readings on Tandem. Once the probe response has been checked, reconnect fermenter air supply to air inlet filter and ensure airflow is at the correct setting.

4.26 Before Inoculation, start the logging software. Refer to SOP ?? for logging data software setup.

4.27 Once any other remaining media additions have been added, the fermenter can now be inoculated through the inoculation port (represented by the single tallest port fitting). The inoculation can be carried out using a Luer-lock syringe on the inoculation port. Use 70% ethanol to sterilize the surface opening.

5.0 Stopping/shutting down the fermenter

5.1 Shut off the services supplies (i.e. gas and cool water) and release any overpressure on the lines. (Take care that no medium can enter into the gas supply e.g. from the sparger).

5.2 Switch off the stirrer speed and control of temperature, pH, airflow, etc. one by one. Turn off chilled water to and from condenser, and disconnect lines.

5.3 Using the control module/display panel, go to the 'Home' tab and under 'Overview', select the global STOP button to stop all Process Parameters. Also, go on the BioXpert (Data logging) and end the fermentation runs started.

5.4 Turn off the device at the main power switches (one for each pair of vessels).

5.5 Harvest cells, if required, by removing the top plate. Alternatively, aseptically connect a "harvest pump" to the sample pipe and pump the culture over into a sterile container. Ensure good containment practices are followed and harvest into dedicated contained harvest Nalgene.

5.6 Once cells are harvested, refill to the same working volume with RO water

5.7 Empty contents of acid, alkali and antifoam cylinders into dedicated storage containers. Ensure any residual left in lines is drained back in the storage containers. (For acid, base and antifoam lines, it is possible to press the switch above each pump to the left or right to move any residual liquids to the vessel or the reservoirs respectively.

5.8 Withdraw the tubes from the corrective reagent bottles of any acid, base, antifoam or other addition reservoirs (without dismantling the pump heads of the bottles) and rinse the tubing thoroughly with RO water.

5.9 Prepare all addition reservoirs for kill/resterilisation in Getinge autoclave (it is recommended for the sample port to add RO water to the universal, open slightly, and turn upside down, so that the water falls by gravity into the vessel. For acid and base reservoirs, it is recommended to fill the relevant reservoirs with RO water and use pumps (manual control) to rinse these lines) (thorough rinsing to remove acid and base residues is ESSENTIAL before autoclaving).

5.10 If cells are not to be harvested they should be killed off by sterilization. Follow the same procedure (steps 3.0 to 3.9) as for sterilizing the media.

5.11 After harvesting/killing off, clean vessel as outlined in SOP No_

5.12 After cleaning, check all O-rings and replace if necessary.

5.13 After sterilization kill, remove pH probes immediately and place ends back into designated plastic caps filled with 3M KCl solution.

5.14 Remove DOT probes, cover the sensitive membrane at its end with its designated plastic cap. (Cover the end of the DOT lead on the base unit with its designated cap).

5.15 Close off all lines.

5.16 Ensure that all control switches are switched off, and no water is left on.

5.17 To save the logged data on the laptop that was recorded by the BioXpert software:

Safety notes:

- Contact manufacturer first before using unusual applications e.g. higher temperatures or pressure control.
- Never use the mains switch as an operational ON/OFF.
- Always turn parameters off individually before switching off.
- Do not autoclave the reagent bottle lines filled with corrosive agents as this will damage the pump head and tubing.