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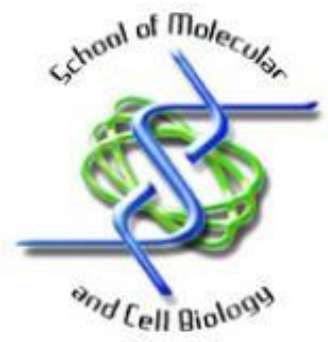



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DST Biocatalysis Initiative

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

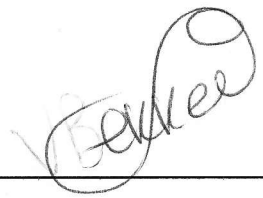
Metabolic engineering of *Streptomyces albulus* for polylysine production

MASTER'S DISSERTATION 2014

Valerie Bekker
7/17/2014

DECLARATION

I, Valerie Bekker, declare that this dissertation is my own unaided work. It is being submitted in full fulfilment of the Master of Science in Microbiology and Biotechnology at the University of Witwatersrand, Johannesburg. It has not been submitted for any other degree at the University.



Valerie Bekker

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17 day of July 2014

ABSTRACT

During the last few decades, *Streptomycetes* have shown to be an important and adaptable group of bacteria for the production of various beneficial secondary metabolites. One such secondary metabolite, epsilon polylysine (ε-PL), produced by *Streptomyces albulus* is of particular interest due to its antimicrobial activity.

This work aimed to study different facets surrounding ε-PL and its production. Firstly, to grow *S. albulus* CCRC 11814, using economically viable crude glycerol as a carbon source and subsequently measure ε-PL production using an anionic dye, trypan blue. Secondly, to evaluate the antimicrobial activity of ε-PL against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Penicillium simplissium*. Thirdly, to determine whether there is economic feasibility of ε-PL as a food preservative in South Africa. Lastly, to develop and optimise tools for metabolic engineering such as recombineering and group II introns to improve ε-PL production.

The results obtained in this study fall into three different areas:

In terms of growth studies, *S. albulus* grew in the presence of crude glycerol, although the growth was suboptimal, 0.48 g/L as compared to 1.04 g/L produced using pure glycerol or glucose. This is due to the pressures on the bacteria from the impurities of crude glycerol such as methanol and salts. ε-PL antimicrobial activity was effective at a concentration of 100 µg/ml against *S. aureus*, *E. coli* and *A. niger*. It was however, ineffective against *P. aeruginosa* owing to the low outer membrane permeability of the bacteria. Due to the ability of *S. albulus* to grow in crude glycerol, it could be used as a financially viable option to produce ε-PL as a natural food preservative in South Africa.

The economic feasibility of ε-PL as a food preservative in South Africa showed potential in terms of market research as well as the financial evaluations. However; the production volumes are low due to the use of the crude glycerol and may not cater for the large food industry in the country. For these reasons, metabolic engineering could be employed to improve these production volumes.

The first step to metabolic engineering was to develop novel tools which can be used for genetic modifications in *S. albulus*. The group II intron tools for gene knockouts were developed by the construction of a vector which subsequently requires sequencing and testing to perform gene knockouts. Based on current knowledge, this is the first experiment of its kind. In terms of introduction of genetic material post gene knockouts,

transformation was shown to be a more effective gene transfer technique as opposed to electroporation, producing 7.75 transformants/μg and 0.038 transformants/μg of DNA, respectively.

Future work would involve the use of biocatalysis for metabolic engineering of *S. albulus* by either removing genes inhibiting ε-PL or overexpressing the enzyme responsible for its production.

This research has developed the groundwork for future ε-PL production improvement using biocatalysis and economically viable crude glycerol as a carbon source for applications of the secondary metabolite as a food preservative.

ACKNOWLEDGEMENTS

Firstly, I would like to extend my deepest gratitude to my supervisor, Dr Karl Rumbold. Your assistance and open door policy throughout this project has been amazing and I couldn't have done it without you.

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A big thank you to the DST Biocatalysis Initiative the funding of this project on several occasions. In addition, thank you to GDARD for further financial assistance.

Thank you to Prof Bertolt Gust and Prof Mervyn Bibb who kindly provided the plasmids I required for this project.

Itumeleng Makhale and Ela Romonowska for their huge contribution to the economic feasibility chapter. As well as Kevin for the use of the CEBER modeller.

To all my fellow students who have shared the ups and the downs with me and your support and encouragement played a vital role in making this learning experience so wonderful.

Most importantly a huge thank you to all my loved ones (especially my mom and dad) who have been behind the scenes but have given me all their support throughout this year and dealing with my stress and making it that much easier for me.

Lastly, a huge thank you to University of Witwatersrand for this amazing opportunity. The amount which I have learnt throughout this degree is truly invaluable.

RESEARCH OUTPUTS

Publications from this dissertation:

A mini review article entitled “Tools for metabolic engineering in *Streptomyces*” has been accepted for publication in the Bioengineered Journal.

DEDICATION

This work is dedicated to my parents, Olga and Serge Bekker – For your constant love and support – It means the world to me.

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ABBREVIATIONS

$	dollars (American)
°C	degrees Celsius
A-domain	adenylation domain
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BACs	bacterial artificial chromosome
BLAST	basic local alignment search tool
C	cytosine
CD	circular dichroism
C-domain	condensation domain
DNA	deoxyribonucleic acid
dtt	dithiothreitol
EBS	exon binding site
G	guanine
G3P	glyceraldehyde-3-phosphate
GI	growth index
IBS	intron binding site
IR	infrared
kb	kilobase
kDA	kilodalton
kg	kilograms
LB	Luria broth
LTR	long-terminal-repeat
MB	megabases
mM	millimolar
NMR	nuclear magnetic resonance
NRPS	non-ribosomal peptide synthetase
PGA	polyglutamic acid
Pld	ε-PL degrading enzyme
Pls	ε-PL synthetase
RNP	ribonucleoprotein complex
RT	reverse transcriptase
SDS-PAGE	sodium dodecyl sulphate- poly-acrylamide gel electrophoresis
sp	species
ssp	sub species
T-domain	thiolation domain
TE-domain	thioesterase domain
TM-domain	transmembrane domain
UV	ultraviolet
w/v	weight per volume
α	alpha
α-PL	poly-α-lysine

β	beta
ε	epsilon
ε-PL	poly-ε-lysine
µl	microliter
Φ	phi

CHAPTER 1. LITERATURE REVIEW

1.1. *Streptomycetes*

Streptomycetes are gram positive, rod shaped bacteria which form the largest genus in the phylum Actinobacteria (Hodgson, 2000). These bacteria are soil saprophytes; have large genomes of ~ 8-10 MB and a high G-C content of >70%. *Streptomycetes* genomes consist of large linear chromosomes and uniquely, mainly linear plasmids, although circular ones are also found (Hopwood, 2006).

The morphology of *Streptomyces* cells are displayed by chains of extensively branched mycelia which share a similarity to filamentous fungi. The growth cycle of these bacteria is very complex and begins with filamentous vegetative growth and the formation of aerial hyphae followed by differentiation of the hyphae into spore chains (Kieser *et al*., 2000) (Figure 1.1).

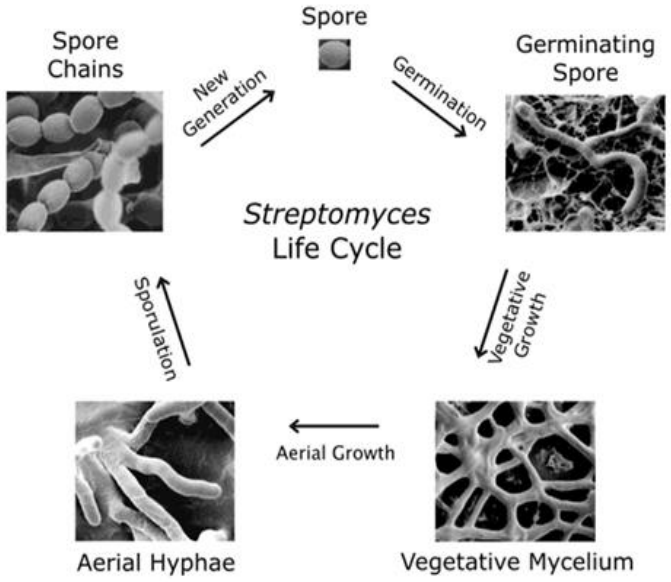


Figure 1.1. The lifecycle of *Streptomycetes* bacteria. A vegetative mycelium germinates from a single spore followed by aerial growth with the production of aerial hyphae. These undergo sporulation to form spore chains (Reproduced from Paradkar *et al*., 2003).

Another distinctive quality of *Streptomycetes* is their ability to synthesize many secondary metabolites/natural products, (Davies and Ryan, 2012) near the end of the microorganisms' growth phase (Hopwood, 2006).

1.1.1. *Streptomycetes* secondary metabolites

Among actinomycetes, around 2400 unique compounds are produced by *Streptomyces* species and are therefore known as the industrial source of secondary metabolites (Berdy, 2005; Lucas *et al*., 2012). Each secondary metabolite is normally encoded by large 20-30 biosynthetic gene clusters (Thompson *et al*., 2002; Ikeda *et al*., 2003; Oliynyk *et al*., 2007) which can reach up to 100 Kb in size (August *et al*., 1998; Donadio *et al*., 2002). These gene clusters are the main targets for the improvement of secondary metabolite production during engineering experiments.

The main types of secondary metabolites produced by *Streptomyces* are antibiotics, antifungal agents (Lee *et al*., 2009), food preservatives (Hiraki *et al*., 2003), anthelmintic and antitumour agents (Lee *et al*., 2009). Each of these categories of metabolites are important in industry and requires large scale production to cater for the growing market.

For the purpose of this research, one particular *Streptomyces* species, *Streptomyces albulus* has been selected for study in terms of its secondary metabolite production.

1.1.2. *Streptomyces albulus*

S. albulus is an aerobic, soil bacterium which grows in the same characteristic way as other *Streptomycetes* described in section 1.1. *S. albulus* grows fairly slowly at temperatures between 25-30°C and a neutral pH of 6.8-7.0 (Shepard *et al.*, 2010) (Figure 1.2).

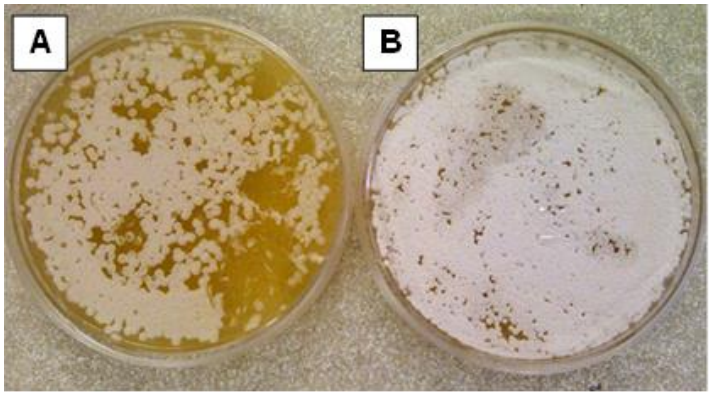


Figure 1.2. The growth of *S. albulus* on LB agar after different days of growth. (a) 3 days post spreading. (b) 5 days post spreading, showing a lawn of hyphae on the plate.

There is currently limited information on *S. albulus* and only recently, the genome was sequenced using the MiSeq platform from Illumina. The genome is 9.43 Mb in size and has a very high G-C content of 72.2%, which follows suit of other *Streptomycetes* genomes (Dodd *et al*., 2013).

S. albulus has been the subject of studies for several years due to its ability to produce a very important polyamino acid (Shima and Sakai, 1977).

1.2. Polyamino acids

Microorganisms have the ability to produce a wide range of biopolymers which are the most abundant molecules in living matter. There are several types of biopolymers which include polynucleotides, polysaccharides, polyesters, polyketides and polyamides (Hamano, 2011). Polyamides (proteins) are a class of biodegradable polymers which consist of repeating units linked by amide bonds. There are two main types of polyamides: homopolyamide which consist of one type of monomer and copolyamides (or proteins), which are made up of different kinds types of monomers. Subsets of polyamides are known as polyamino acids and these are different to regular proteins due to the way they are synthesized (Oppermann-Sanio and Steinbüchel, 2002). There are three naturally occuring polyamino acids which are polyglutamic acid (PGA), polylysine (Hamano, 2011), and cyanophycin (Oppermann-Sanio and Steinbüchel, 2002) (Figure 1.3).

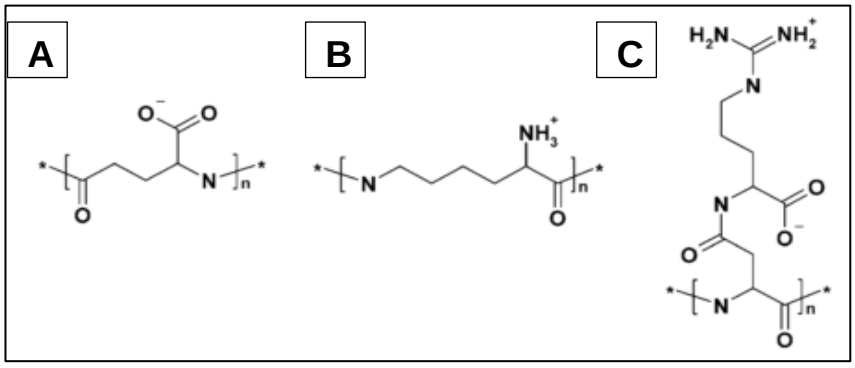


Figure 1.3. The structures of three naturally occuring polyamino acids. (a) polyglutamic, acid (b) polylysine and (c) cyanophycin. The figure shows the monomeric structure which can be repeated several times; represented by the n to form the polyamino acid. (Reproduced from Oppermann-Sanio and Steinbüchel, 2002).

This project focused on the polyamino acid, polylysine and therefore PGA and cyanophycin will not be discussed further.

1.3. Polylysine

Lysine, a positively charged proteogenic amino acid, is not produced by the human metabolism but is essential for growth and also plays a vital role in the production of carnitine, which is responsible for converting fatty acids into energy and lowering cholesterol. Lysine also helps the body absorb calcium, and therefore the formation of

collagen, a substance important for bones. The consumption of lysine occurs through the intake of foods (Tomé and Bos, 2007).

There are two main types of homopolyamides of lysine, poly-α-lysine (α-PL) (Sela and Katchalski, 1959) and poly-ε-lysine (ε-PL) (Shima and Sakai, 1977). The former is only chemically synthesized and has been investigated in several human drug delivery systems, however poly-α-amino acids are toxic and therefore its applications are limited (Sela and Katchalski, 1959). ε-PL on the other hand is naturally produced and is therefore of interest in many studies for its possible applications.

1.3.1. ε-PL

ε-PL was first discovered in 1977 by Shima and Sakai during an experiment involving screening for Dragendorrf's positive substances produced by *S. albulus* ssp *lysinopolymerus* 346 isolated from the soil. Further studies were performed to verify that the substance consisted of only the L isomer of the amino acid thus, when ε-PL is referred to, it is the L isomer (Shima and Sakai, 1981). ε-PL is a naturally occuring secondary metabolite which is a cationic, homopolymeric compound consisting of 25-35 lysine monomer repeats. There is a peptide bond between the ε-amino and the α- carboxyl groups (Figure 1.4).

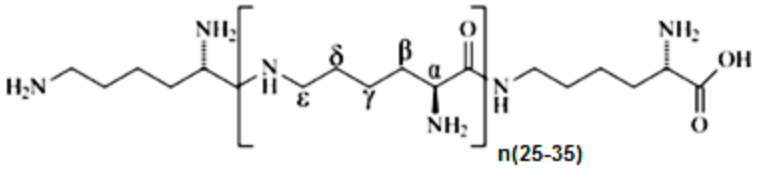


Figure 1.4. A schematic representation of the structure ε-PL. Polylysine is made up of 25-35 lysine monomer repeats and there is a peptide bond between the ε-amino and the α-carboxyl groups (Reproduced from Chen *et al*., 2013).

1.3.2. Occurrence of ε-PL in other microorganisms

Although *S. albulus* was the first microorganism in which ε-PL was discovered, recently other ε-PL producing strains were discovered. One study involved the use of a method of detecting basic polymers (such as polylysine) which may interact with a charged dye embedded in an agar plate This study by Nishikawa and Ogawa, 2002, involved the isolation of more than 10 ε-PL producing species from 300 soil samples. These organisms came from the genera *Streptomyces* and *Kitasatospora* mainly. Another study by Hirohara *et al.,* 2007 made use of a two stage culture method for screening ε-PL producers. Using this method, a further 10 strains and their ε-PLs were studied. It was found that the ε-PLs

showed variable lengths and were all from the genus *Streptomyces* and the longest chain consisted of 36 L-lysine residues which is the longest found to date.

1.3.3. Conformation of ε-PL

Several studies have investigated the molecular structure and the conformation of ε-PL produced by a strain of *S. albulus* in aqueous solution. These experiments employed nuclear magnetic resonance (NMR), infrared (IR) and circular dichroism (CD) spectroscopy (Kushwaha and Mathur, 1980; Lee *et al*., 1991, 1995; Fukushi *et al*., 1993). The results from all the experiments follow the consensus that ε-PL is in a β sheet conformation.

1.3.4. Production of ε-PL

Kawai *et al*., 2003 first reported that ε-PL may be produced by a non-ribosomal peptide synthetase (NRPS). NRPSs are multifunctional enzymes which have multiple semi-autonomous domains that have the ability to synthesize numerous secondary metabolites (Mootz *et al*., 2002; Schwarzer *et al.,* 2003; Strieker *et al*., 2010). These enzymes function using a thio-templated mechanism to activate, tether and modify amino-acid monomers creating the polypeptide chain. The sequence and length of the peptide product is determined by the order and number of modules of an NRPS.

In order to confirm or discard the above hypothesis, Yamanaka *et al*., (2008) attempted identifying an ε-PL synthetase (Pls). To do this a protein extraction method was used by growing cells of *S. albulus* NBRC14147, sonicating and centrifuging them to obtain a cell free extract. The presumptive Pls was purified by column chromatography and the molecular mass was estimated by gel filtration chromatography and sodium dodecyl sulphate- poly-acrylamide gel electrophoresis (SDS-PAGE). The estimated molecular mass was shown to be 270 kDa and 130 kDa for each method respectively, thus, suggesting that Pls is a homodimer.

To further prove that Pls is in fact responsible for ε-PL production, the purified enzyme was incubated with L-lysine and adenosine triphosphate (ATP). A polydisperse group of enzyme dependent polymer products were detected, supporting the fact that this enzyme is responsible for ε-PL production (Yamanaka *et al*., 2008).

Catalytic mechanism of Pls

From the studies performed on Pls it was discovered that the enzyme does not have a classical structure of a traditional NRPS. However, the enzyme showed the presence of a typical adenylation domain (A-domain) and thiolation domain (T-domain) at the N-terminal region. These domains are responsible for the activation of the amino acid substrate and the formation of an intermediate acylthioester as well as adenosine monophosphate (AMP) release respectively (Mootz *et al*., 2002; Schwarzer *et al.,* 2003). Pls does not possess domains with sequence similarity to the traditional condensation domains (C-domains) which are vital in peptide bond formation. Finally, there is no thioesterase domain (TE-domain), which functions to release the final secondary metabolite. Physiochemical analysis suggested the existence of six transmembrane domains (TM-domains) and three soluble C domains (Yamanaka *et al*., 2008; Kito *et al*., 2012) (Figure 1.5).

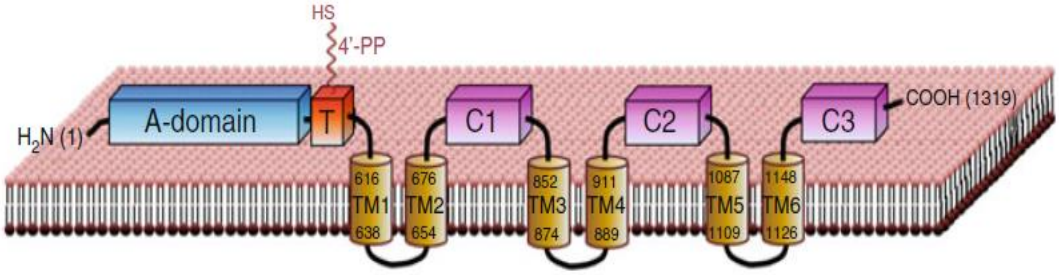


Figure 1.5. Domain architecture of the Pls protein. The A domain, T domain, six TM domains and three tandem domains (C1 domain, C2 domain and C3 domain) are shown schematically. The numbers on Pls are the amino acid residue numbers (Yamanaka *et al*., 2008).

1.3.5. Degradation of ε-PL

Apart from Pls, an ε-PL degrading enzyme (Pld) has been identified, purified and characterized (Kito *et al*., 2002). This enzyme functions at a pH of 7.0 and degrades ε- PL back to L-lysine through a process of depolymerisation (Kahar *et al*., 2001). The *pld* gene has been reported to play a role in self resistance against ε-PL (Kito *et al*., 2002; Hamano *et al*., 2006).

1.3.6. Measurement and purification of ε-PL

Several methods have been developed to measure the concentration of ε-PL, which are either colorimetric or enzymatic. Itzhaki, (1972) discovered a colorimetric method which uses the fact that ε-PL can interact with an anionic dye, methyl orange. This method works on estimating unbound ε-PL residues using spectrophotometry. The disadvantage

of using this technique is that ε-PL of only relatively high concentrations (>10 μg/ml) can be detected. Shen *et al*., (1984) then discovered that the same colorimetric method can be used with trypan blue. The absorbance of unbound dye in the supernatant is inversely proportional to the concentration of this polyamino acid. The advantage of using trypan blue is that ε-PL concentrations of between 1 μg/ml and 10 μg/ml can be detected. This is about 10-fold lower than that of the methyl orange precipitation method. Once a sufficient amount of ε-PL has been measured, for application purposes, it needs to be purified.

ε-PL can be purified by ion exchange chromatography from the culture broth due to the fact that it is a cationic polymer at a neutral pH (Shima and Sakai, 1977).

1.3.7. Function of ε-PL for the bacteria

Due to the fact that the *Streptomyces* are found in the soil, there are many other competing bacteria in their surroundings. In order to minimize the competition for themselves they produce ε-PL to serve as an antimicrobial agent in their habitat. This secondary metabolite offers a potentially significant selective advantage to the bacteria (Yoon and Nodwell, 2014).

1.3.8. Applications of ε-PL

ε-PL has been of interest in the past few years for a wide variety of industrial applications in food, medicine, environment and electronics. ε-PL can be utilized in many different ways such as a food preservative , emulsifying agent, dietary agent, biodegradable fibres, highly water absorbable hydrogels, drug carriers, anticancer agent enhancer and bio- chip coatings. These applications are summarized in Table 1.1.

Table 1.1. The applications of ε-PL in various fields.

Field of application	Application examples
Food Industry	Natural food preservative (Shima and Sakai, 1983) Dietary agent (Kido *et al*., 2003)
Medical Industry	Drug delivery carrier (Shen and Ryser, 1979, Shen and Ryser ,1981; Ryser and Shen, 1978) Lipopolysaccharides (LPS)-Endotoxin removal (Sakata *et al*., 2002) Non-viral gene delivery vectors (Choi *et al*., 2000)
Other applications	Hydrogels (Yoshida and Nagasava, 2003) Bioelectronics (Ostuni *et al*., 1999)

The function that is of interest in this study is the use of ε-PL as a natural and safe food preservative due to its antimicrobial properties.

Antimicrobial activity of ε-PL

ε-PL is unique as an antimicrobial agent as it has been shown to have activity against both bacteria and fungi, and even against bacteriophages. In addition, ε-PL is water soluble, biodegradable, edible and nontoxic towards humans and the environment (Hamano *et al*., 2011). The process behind the antimicrobial activity is adsorption to the cell surface of bacteria and fungi. Adsorption involves the binding of atoms or ions from a gas, liquid or dissolved solid on cell surface. This results in the attachment to the outer membrane resulting in the disruption of the cytoplasm and eventually leads to cell death (Shima *et al.*, 1984).

The chain length diversity of ε-PL is directly related to its antimicrobial activity. Shima *et al*., (1984) found that ε-PL with more than nine L-lysine residues has a high antimicrobial activity and therefore inhibits microbial growth. On the contrary, an ε-PL octamer showed negligible antimicrobial activity.

Due to relatively wide antimicrobial spectrum, it has been used as a preservative in Japan, China and the United States. It is considered to be a safe addition in several staple foods such as fish sushi, soups, cooked vegetables, noodles and boiled rice. Levels of up to 20 000 to 50 000 ppm of ε-PL can be added to the food with no notable adverse effects, according to toxicology studies performed in rats (Hiraki *et al*., 2003). Furthermore, lysine which is not naturally produced by the body is essential for growth and plays a vital role in the production of carnitine which is responsible for converting fatty acids into energy and lowering cholesterol. Lysine also helps the body absorb calcium, and therefore the formation of collagen, a substance important for bones (Singh *et al*., 2005).

Seeing that ε-PL has such huge economic potential, there has been recent interest in a by-product, crude glycerol during the production of a biodiesel, a biofuel, to lower the cost of ε-PL production.

Applications of ε-PL in an African context

The L-lysine residues found in ε-PL have application as feed additives in livestock. Only one producer of L-lysine was known in Africa and obtained the residues from molasses which is ten times as expensive as crude glycerol. The use of crude glycerol for L-lysine may be economically valuable for farmers and biodiesel producers. South Africa

consumes about 9000 tonnes of L-lysine annually, which is all imported as the L-lysine plant in Richards Bay was converted to a yeast production plant. Thus, there is a space to produce ε-PL more economically and in larger quantities (Zhou, 2010).

1.4. Biofuels

At present, fossil fuels are both costly and responsible for large amounts of pollution in the form of carbon emissions. Furthermore, their reserves are depleting due to over exploitation (Campbell and Laherrère, 1998). For these reasons the focus has moved to alternative bio-based fuel sources (Hill *et al*., 2006).

Biodiesel is an alternative fuel source which is derived from triglyceride oils and can substitute fossil diesel. Biodiesel is both non-toxic and biodegradable which has led to its commercialisation within several countries (Peterson and Reece, 1994). The production of biodiesel through the process of transesterfication generates approximately 10% of low-grade crude glycerol, which is considered a waste product (Figure 1.6) (Dasari, 2007). Currently crude glycerol's supply is greater than its demand due to cost implications for its disposal and purification. To bypass these costs, crude glycerol could be utilized in different applications such as chemical requirements for bacterial growth (Johnson and Taconi, 2007).

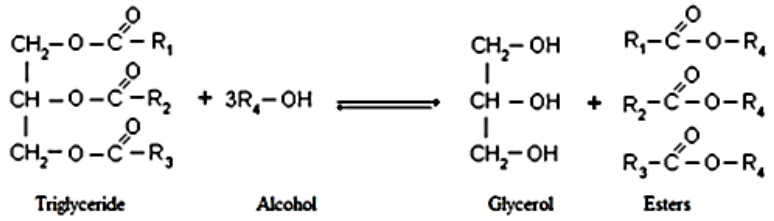


Figure 1.6. The process of transesterfication of triglyceride oil for production of biodiesel and glycerol. Biodiesel is produced using a triglyceride and an alcohol (such as methanol) in the presence of a catalyst to form glycerol (Reproduced from Chisti, 2007).

1.5. Crude glycerol

Glycerol, also known as glycerine, is a colourless, odourless liquid. Glycerol is nontoxic to human health and the environment and is therefore used in industrial cosmetic, food, pharmaceutical and domestic products. Glycerol can exist in two forms both crude and pure, whereby the former has impurities such as potassium salts and is about 70-80% pure (Ayoub and Abdullah, 2012).

Very few bacteria are able to make use of crude glycerol as a carbon source (Wilke and Vorlop, 2004). In a previous study in our laboratory, crude glycerol from six South African biodiesel producers was tested as a carbon source supplement for *S. albulus.* From this intial investigation it can be seen that there is a possibility for an effective use of crude glycerol as *S. albulus* is able to utilize crude glycerol although this has not been optimised.

One of the main advantages of using crude glycerol is the excess of it due to the increase in biodiesel production. This has reduced its price, thus, making it affordable in biotechnological applications. The current price of crude glycerol is low, $0.04–0.11/kg and biodiesel producers are storing it until alternative uses are identified (Bohon *et al*, 2011). From this it can be said that glycerol is a sustainable biological feedstock for the production of certain products due to its availability and economic viability. The challenges of using crude glycerol are the impurities associated which could potential lead to slow bacterial growth rate and hence, secondary metabolite production (Çelik *et al*., 2008). .

1.6. Economic feasibility of ε-PL production

In order to determine whether there is a market available ε-PL production in South Africa, several figures around its production and cost need to be taken into account. The average cost of ε-PL is $ 5000/kg and fermentations on average can produce 40 g/L using a 5 L jar fermenter (Kahar *et al*., 2001). Running the fermenter will require man power costing approximately $ 220 a day per individual and thus 3 individuals would cost $ 660 per day. There are currently many different preservatives available on the market and the cost price of one such preservative, sodium benzoate is US $ 1,340 - 1,355 / 1000 kg (Food preservatives, 2012). Although these preservatives appear more cost efficient, much larger quantities are required for effective use as compared to ε-PL.

Due to the high cost of ε-PL to optimise, ε-PL production through genetic or metabolic engineering in order to lower the price.

1.7. Genetic Engineering

A variety of high-value secondary metabolites are produced by microbial fermentation (Demain, 2006). In many cases, the production of these products has not been optimised to an industrial scale and the enhancement of metabolic reactions to increase the yield is the focus of much prior, on-going and future research (Baltz, 2011). Throughout the years, a wide range of engineering techniques have been employed for these purposes.

Previously, genetic engineering involved random mutagenesis without the targeting of a specific gene. More recently genetic engineering has moved to target specific genes. (Nielsen, 1998; Adrio and Demain, 2006). This is attributed to the recent advances in the development of modern technologies such as second and third generation sequencing, genomics, proteomics, metabolomics, transcription profiling and transcriptomics has allowed for more efficient ways to engineer microorganisms for secondary metabolite production (Bro and Nielsen, 2004).

Genetic engineering involves several steps: The first being to choose clinically or industrially relevant secondary metabolites that are amenable to engineering. This amenability is usually attributed to the host organism. The general steps in genetic engineering involve: (a) cloning and sequencing the biosynthetic genes responsible for the production of the secondary metabolite; (b) using efficient tools to engineer the biosynthetic pathway by random mutagenesis, extra chromosomal or whole genome engineering; (c) choosing or developing suitable gene transfer methods, expression hosts and methods to stably maintain and express the engineered biosynthetic gene(s) (Baltz, 2006).

1.7.1. Cloning and Engineering the biosynthetic gene(s)

In order to genetically engineer a gene, it needs to be produced in large amounts. This is usually performed by removing the gene from its natural location and ligating it onto a plasmid and cloning it into *Escherichia coli*. Through the growth of *E. coli*, the gene(s) will be expressed in large quantities and may be used for subsequent genetic engineering steps. Each genetic engineering experiment uses a different *E. coli* strain and plasmid for cloning and therefore these must be selected based on the experiment specifications (Baltz, 2006).

1.7.2. Random Mutagenesis

Random mutagenesis involves the use of radiation or chemical compounds to randomly produce changes in the DNA. This is followed by screening of the mutant strains for the single best performer for the product in question. This technique is used less frequently because it does not give information on genes and molecular mechanisms during the improvement and it is likely that some mutations may be detrimental or neutral in terms of production (Alduina and Gallo, 2012).

1.7.3. Extra chromosomal engineering

Extra chromosomal engineering involves the use of plasmids, bacterial artificial chromosomes (BACs) or cosmids which are inserted as a separate entity into a microorganism, containing the gene(s) required for the production of the target product, which is then expressed. Certain biosynthetic gene clusters are extremely large and in order to engineer them, artificial chromosomes that can be stably maintained in *Streptomyces* have been constructed. These vectors can allow for the mobility of genetic information from an original producer to a more genetically suitable and amenable host for the particular gene expression (Alduina and Gallo, 2012).

Plasmids are most commonly used for engineering strategies but are limited to being able to hold one or two genes. If the cloning of entire gene clusters is required, cosmids should be considered (Alduina and Gallo, 2012).

Cosmids may be used for gene clusters of up to 45kb in size and show high transformation efficiency. The cloning of biosynthetic gene clusters in *Streptomyces* has shown to be successful in several cases. These include the transfer of several antibiotic gene clusters such as blasticidin of *S. griseochromogenes* (Cone *et al*., 2003); validamycin from *S. hygroscopicus* (Singh *et al*., 2006) and lincomycin from *S. lincolnensis* (Koberská *et al*., 2008). The limitation of this strategy is that many biosynthetic gene clusters for natural products are larger than 45kb and therefore cosmid vectors are not suitable. In order to overcome this, bacterial artificial chromosomes (BACs) may be employed (Gust *et al*., 2004).

BACs may be used for the insertion of gene clusters which are larger than 100kb. These can be carried in *E. coli* but are not suitable for heterologous expression due to the G-C rich nature of *Streptomyces* genomes. Vectors have been designed that can be shuttled between *E. coli* and *Streptomyces*. The *E. coli* strain can be used for library construction and manipulation while the *Streptomyces* strain will be for gene expression and secondary metabolite production. Gene clusters can be introduced within a few days and then can be integrated into the *Streptomyces* chromosome for further analysis (Gust *et al*., 2004). To date two BACs have been constructed to host gene clusters for *Streptomyces* engineering: pSTREPTOBAC V (Miao *et al*., 2005) and pSBAC (Liu *et al*., 2009). These BACs have been used for the cloning of antibiotics daptomycin biosynthetic gene cluster in *S. roseosporus*. Both these vectors are able to replicative in *E. coli* and integrate into *Streptomyces*. Recombinant clones can then be selected by antibiotic resistant markers

found on the vectors. Both pSTREPTOBAC V and pSBAC contain an apramycin resistance selectable marker for recombinant clone selection (Alduina and Gallo, 2012).

Many studies avoid the use of extra chromosomal engineering as a method of manipulating the *Streptomyces* genome as more advanced and rapid methods (discussed in section 1.9) have been created. The reason for this is to avoid issues such as vector replication, stability, incompatibility and copy number variance (Heil *et al*., 2012). However, if an entire gene cluster is required to be expressed in a heterologous host this method cannot be avoided.

1.7.4. Whole genome engineering by PCR based methods

Whole genome engineering involves the editing of the genome of the microorganism by the insertion or deletion of specific genes or promoters. The latter methods require knowledge of the gene sequence and function and the type of techniques which may be used for genetic engineering. Previously, genetic engineering techniques were limited by restriction enzymes and availability of restriction sites which can obstruct the introduction of DNA into *Streptomyces*. Another limitation included the use of self-replicating plasmid vectors which can inhibit secondary metabolite production. Lastly, recombinant strains containing heterologous DNA may have effects on the regulation of the host genome. These hurdles were overcome by the use of intergeneric conjugation to avoid host restriction, and integration of cloned DNA into neutral genomic sites which prevents product inhibition by self-replicating plasmids, and has enabled construction of recombinant strains lacking heterologous DNA sequences (Baltz and Hosted, 1996).

Until recently, techniques for the introduction of defined changes into *Streptomyces* chromosomes were very time-consuming. In particular, manipulation of large DNA fragments has been challenging due to the absence of suitable restriction sites for restriction- and ligation-based techniques. The homologous recombination approach (Gust *et al*., 2003) and phage integration systems (Baltz, 2012) have greatly facilitated targeted genetic modifications of complex biosynthetic pathways in *Streptomyces* by eliminating many of the time-consuming and labour-intensive steps.

Although genetic engineering has been successful in improving secondary metabolite production the emergence of a new field may show further improvement. Metabolic engineering considers whole cellular systems and any genetic manipulations would change the efficiency of the overall bioprocess, thus distinguishing it from simple genetic engineering (Kern *et al*., 2007).

1.8. Metabolic Engineering

Metabolic engineering has been described as the “improvement of cellular activities by the manipulation of enzymatic, transport and regulatory functions of the cell with use of recombinant DNA technology” (Bailey, 1991). Metabolic engineering allows for defined and rationalised changes to be made to the organism to render a particular improvement (Kern *et al*., 2007).

There may be several different motives for the metabolic engineering of microorganisms which include (Kern *et al*., 2007): 1. Improvement of yield and productivity of a commercially viable product. 2. Extension of the substrate range of a product. 3. Removal or reduction of by-product formation 4. Introduction of innovative pathways for product production.

Metabolic engineering is a multistep process. Firstly, a target product and its wild type host are selected followed by computational analysis of metabolic pathways in its production. A genetic modification is proposed, based on analysis of metabolic pathways relating to the product of interest. After genetic modification, the recombinant (engineered) strain is analysed and the results are then used to identify the next target for genetic manipulation, if necessary. Lastly, fermentation studies and other downstream processes are performed for industrial scale production of the target product (Kern *et al*., 2007).

Metabolic engineering can be achieved through several different approaches which improve the efficiency of the secondary metabolite production pathway. These include: precursor engineering, engineering structural genes, engineering regulatory networks and genome shuffling. The necessity for metabolic engineering stems from the complex pathways used to synthesize secondary metabolites as opposed to single isolated genes. Due to this in depth analysis of the pathways are required followed by the targeting of possibly more than one gene in the pathway (Olano *et al*., 2008).

1.8.1. Precursor Flux Engineering

Precursors for secondary metabolite production are vital and are often the produced through the catabolism of carbon compounds during primary metabolism. This means precursors are found at the intersection of primary and secondary metabolism. In order to increase the production of secondary metabolites, the precursor should be readily available and in certain instances this is not the case. The engineering approach is therefore to genetically engineer certain enzymes in order to increase the availability of certain precursors in different pathways (Olano *et al*., 2008). An example of precursor engineering involved actinorhodin, a polyketides antibiotic produced by *S. coelicolor*, is

another example where increased precursor availability leads to enhancement of product production. Ryu *et al*., (2006) attempted overexpressing by adding an additional copy on a plasmid. *accA2*, *accB* and *ace* genes which code for the different subunits of the enzyme acetyl-CoA carboxylase (ACC). These genes are precursors for actinorhodin production. The results showed an enhancement of carbon flux to malonyl-CoA, which is also a precursor enzyme of actinorhodin resulting in a 6-fold increase in the production of the product.

1.8.2. Engineering structural genes

Structural genes found in the metabolic pathways responsible for the production of the secondary metabolites are usually attractive targets for metabolic engineering. In these cases, the gene dose may be increased; the gene may be inactivated, deleted or modified (Olano *et al*., 2008).

Gene dose increase

Increasing the dose of a particular gene would theoretically correspond to an increase of the target protein and thus, increase the production of secondary metabolites. Integration of the clavaminate synthase gene, *cas2* into the chromosome of *S. clavuligerus*, resulted in up to five-fold increase in clavulanic acid production. This was done using recombinant plasmids designed in the study. Plasmids were constructed for both overexpression and different plasmids for chromosomal integration and transformed into *S. clavuligerus* (Hung *et al*., 2007).

Inactivation or deletion of genes

Inactivation or deletion of genes can improve metabolite production by removing genes coding for activities that transform the metabolite into a different one. In addition it can also involve the removal of genes whose products inhibit or degrade the target product (Olano *et al*., 2008). Inactivation of the *nysF* gene by the insertion of a kanamycin resistance marker into its coding region, and by in-frame deletion improves nystatin production by 60% which suggests a negative role of the gene in nystatin biosynthesis (Zelyas *et al*., 2009).

1.8.3. Engineering regulatory networks

Genes for the biosynthesis of secondary metabolism pathways are commonly grouped together in clusters on the chromosome including their pathway-specific regulatory genes.

Pathway-specific regulators can have either positive (activators) or negative (repressors) effects on the expression of gene cluster elements. There are clusters containing a different number of pathway-specific positive regulatory genes. The best-known example is *S. coelicolor* that produces several antibiotics (actinorhodin, calcium-dependent antibiotic, undecylprodigiosin and methylenomycin) and where the onset of their biosynthesis is controlled by specific regulators (*actII-orf4*, *cdaR*, *redD* and *redZ*), while there are several pleiotropic genes (i.e. *afs*, *abs* and *bld*) affecting antibiotic production and, in addition, the morphological development of the bacteria (Huang *et al*., 2005). Taking into account all previous observations it seems obvious that deregulation of the expression of secondary metabolite pathways, by overexpression of pathway-specific positive regulators or by inactivation of pathway repressors, is the most intuitive approach for the improvement of their production.

1.8.4. Up-regulation

The up-regulation of genes is usually controlled by regulatory sequences on the DNA. In *Streptomyces*, there are such regulators that activate the transcription of biosynthetic genes. These are known as SARP (*Streptomyces* antibiotic regulatory proteins). One such SARP, AfsR-p from *S. peucetius* have been shown to enhance doxorubicin biosynthesis (Parajuli *et al*., 2005). In addition, overexpression of AfsR-p in *S. lividans*, *S. clavuligerus*, *S. griseus* and *S. venezuelae* on a plasmid leads to overproduction of actinorhodin, clavulanic acid, streptomycin and pikromycin, respectively (Parajuli *et al*., 2005 ; Maharjan *et al*., 2008).

1.8.5. Down-regulation

The down regulation of genes is also controlled by regulatory sequences in DNA, however, in this case these would be pathway-specific repressors. The inactivation of these can lead to overproduction of secondary metabolites. This is the case of chromomycin whose production is increased when a pathway- specific transcriptional repressor cmmRII is inactivated in *S. griseus* ssp. *griseus* using a plasmid (Menéndez *et al*., 2007).

A well-known pleiotropic repressor system related with phosphate regulation of secondary metabolite production is the two-component *phoR–phoP* system. Disruption of *phoR* or simultaneous deletion of both *phoR* and *phoP* has been recently shown to increase pimaricin production in *S. nataliensis*. Deletion of the same system in *S. lividans* was reported to boost actinorhodin and undecylprodigiosin production, 5- and 12-fold increase, respectively (Sola-Landa *et al*., 2003).

1.9. Genetic/metabolic engineering for ε-PL improvement in *S. albulus*

There have been several attempts to improve ε-PL production in *S. albulus* either through attempting to improve the fermentation culture conditions or by using genetic and/or metabolic engineering.

1.9.1. Improvement of ε-PL production using fermentation culture conditions

The first way to optimize the production of a secondary metabolite such as ε-PL would require the optimization of the growth and fermentation conditions of *S. albulus*. There have been various reports of such nature regarding the enhancement of ε-PL production. The first attempt was by Shima *et al*., 1983 which involved the optimization of ε-PL production using a two-step cultivation method. This method involved the growth of *S. albulus* in two different mineral media which improved the production of ε-PL to 4-5 g/L after 8 days (as compared to 0.2 and 0.5 g/L originally produced) (Shima and Sakai, 1977; Shima and Sakai, 1981).

Hiraki *et al.*, (1998) attempted to increase ε-PL by isolating a mutant of *S. albulus* (strain 346) by the use of nitrosoguanidine which is a mutagenic agent. This produced a 2-aminoethyl-L-cysteine (AEC) and glycine resistant mutant that was capable of producing four times greater amounts of ε-PL of 1.2 g/L after 96 hours in M3G medium.

Another attempt which aimed at pH control of the culture broth to maximize ε-PL production was attempted using glucose as a carbon source (Kahar *et al*., 2001). The results produced employ a two phase system of pH control in a continuous culture. In the first phase (day 1 and 2), the pH>5 in order to allow the growth of the bacteria optimally. In the second phase (day 3-8), the pH is maintained between 4.0 and 4.2 in order to allow for ε-PL production. Such maintenance can allow for ε-PL production as high as 48.3 g/L. It is vitally important to maintain a glucose concentration at 10 g/L because if glucose is consumed completely, its depletion causes a rapid increase in pH due to ammonia accumulation.

Chen *et al.,* (2011) used *Streptomyces* sp. M-Z18 to observe the production of ε-PL using glycerol as a carbon source. The media used was improved in order to optimize ε-PL production while the pH was initially 6.8 but was not controlled further in the experiment at a temperature of 30°C. The results showed that 2.27 ± 0.15 g/L of ε-PL was produced after 96 hours in a batch fermentation culture.

Most recently, Shukla and Mishra, 2012 studied optimisation of different parameters for ε-PL production by changing one factor at a time using the wild type *S. albulus* strain. According to this study, the carbon source which produces the highest amount of ε-PL (88 hours at 30°C) is a combination of glucose to glycerol (weight ratio 1:1), produced 1.2 g/L. In addition, the optimal inorganic and organic nitrogen sources were found to be ammonium sulphate and yeast extract respectively.

1.9.2. Improvement of ε-PL production using genetic or metabolic engineering

Another way to improve the production of ε- PL in *S. albulus* once growth and fermentation conditions have been optimized is the use of metabolic engineering.

In a previous study in this department, there was an attempt to clone the glycerol operon which contains three genes (*glp*D, *glp*F and *glp*K) from *E. coli*. This is because *E. coli* shows very efficient glycerol metabolism due to the presence of these genes and could subsequently enhance the glycerol utilization. The study however only showed successful cloning of the *glp*D. In the same study it was shown that there is a limit to the amount of carbon utilization as the amount of glycerol increases. In addition it was shown that there is a weak negative correlation between the amount of ε-PL produced and carbon utilization. This provides the motivation for metabolic engineering of *S. albulus* to improve glycerol utilization.

The L-lysine molecule is utilized to produce ε-PL and is biosynthesized by the aspartate pathway. The first two enzymes in the pathway are aspartokinase (Ask) and aspartate semialdehyde dehydrogenase (Asd). Ask is responsible for catalysing the phosphorylation L-aspartic acid to L-4-phospho aspartic acid. These enzymes are highly regulated and different bacteria have different patterns of their regulation. Many of the Ask genes in different bacteria are susceptible to feedback inhibitions. *S. albulus* Ask was found to be partially resistant to feedback-inhibition (Hamano *et al*, 2007). Hamano *et al* proceeded to create a mutant Ask, rAsk (M68V) whose feedback inhibition regulation was completely removed. The results showed that Ask produced a maximum of 15 g/L of ε-PL as opposed to 11 g/L in the wild type. This is an example of the concept of biocatalysis which can be defined as the use of biological molecules, usually enzymes to catalyse certain reactions for the production of natural products (Schmid *et al*., 2001).

Most recently, genome shuffling has been used as an engineering technique. Genome shuffling involves DNA fragmentation and ligation in order to create possible beneficial mutations. Genome shuffling has been described as a new method that can rapidly

enhance secondary metabolite production. The most recent example of this technique is for production of ε-PL in five *Streptomyces* species. In this case genome shuffling and interspecific hybridization (combining DNA of two species from the same genus), via ultraviolet (UV) irradiation allowed for the construction of hybrids which showed 3-fold improvements in ε-PL production (Li *et al*., 2013).

Lastly, the effects of precursor engineering, whereby external L-lysine on ε-PL production was evaluated in *Streptomyces* sp. M-Z18. In combination with glucose–glycerol co-fermentation ε-PL production, was shown to be 37.6 g/L, which was 6.2 % greater than without added L-lysine (35.14 g/L) (Chen *et al*., 2013).

1.10. Novel engineering mechanisms in *S. albulus*

There have been several fairly novel engineering techniques which have been used for genetic changes in different kinds of bacteria. Two examples of such methods include group II introns and recombineering. These methods have not been used with *S. albulus* and will therefore be discussed in detail below.

1.10.1. Group II Introns

Group II introns are a class of introns found in rRNA, tRNA, mRNA of organelles in fungi, plants, protists, and mRNA in bacteria (Dai and Zimmerly, 2003). They were first discovered in mitochondria and chloroplast genomes of eukaryotes and higher plants (Michel *et al.,* 1989). The first intron to be found in a prokaryote was a group I intron in a bacteriophage in 1984 (Chu *et al*, 1984). Following this, both group I and II introns have been found in different types of bacteria. Bacterial group II introns were initially identified by PCR screens (Ferat and Michel 1993) and then later by genome sequencing which revealed that they are very common in both Gram-negative and Gram-positive bacteria (Dai and Zimmerly*,* 2003).

Group II introns are catalytic RNAs which have the ability to catalyse their own splicing (Reinhold-Hurek and Shub, 1992). In addition they have the ability to function as mobile elements. The mobility characteristic of the introns resembles that of non-long-terminal-repeat (LTR) retrotransposable elements (Curcio and Belfort, 1996) and can be utilized as a method of gene disruption via their insertion into a desired DNA target (Karberg *et al.*, 2001). Their mobility is due to a mechanism called retrohoming. This a process whereby group II introns self splice and reverse transcribe due to the presence of their own reverse transcriptase (RT) and can subsequently insert into intronless genes. This is mediated by ribonucleoprotein complex (RNP).

The basic procedure on the use of group II intron for gene knockouts is shown in Figure 1.7. The procedure begins with the reverse splicing of the intron in the exon junction in the target DNA. This is followed by the cleavage of the antisense strand and cDNA synthesis using the intron RNA as the template. Lastly, recombination and repair activities complete the intron insertion process (Karberg *et al*., 2001).

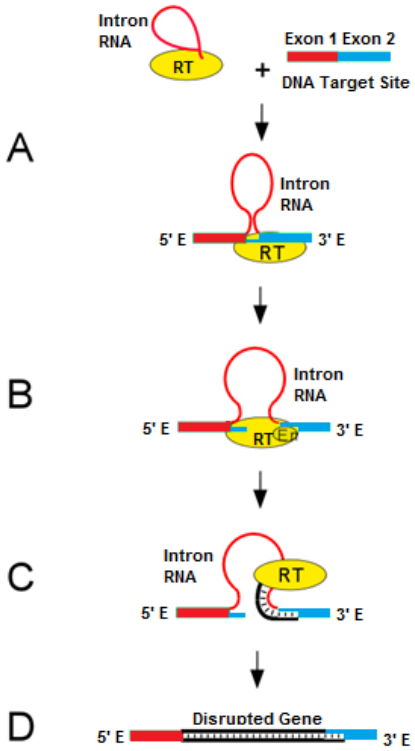


Figure 1.7. The basic mechanism of a gene knockout using group II introns. (a) The target DNA and intron RNA (containing the mobility and catalytic mechanism of the RT) are combined, followed by reverse splicing of the target DNA. (b) Cleavage of the antisense strand. (c) cDNA synthesis using the intron RNA as the template. (d) Repair of final disrupted gene. (Adapted from Karberg *et al*., 2001).

The retrohoming mechanism makes group II introns an attractive option for genetic engineering. Firstly because it has been shown to be both efficient and specific and retrohoming frequencies can approach 100%. Lastly the introns are not dependent on host factors and are thus applicable to a wide range of bacteria (Targetron® Gene Knockout System user guide, 2013).

Structure of group II introns

Group II introns are made up of 6 domains. Domain I, V, and VI are involved the splicing reaction, while domain II and III are involved in stabilization. Doman IV is involved in folding or catalytic activity. Domain I is the largest domain, in which the exon-binding

sequences sites (EBS)1 and EBS2 (see section 3.2.3.1) bind specifically to the sequences of intron-binding sites (IBS) 1 and IBS 2. Domain V is an essential catalytic component of the active ribozyme, and this domain can induce splicing reaction in special conditions (Qin and Pyle, 1998).

TargeTron system

The TargeTron® gene knockout system (Sigma-Aldrich) uses the group II intron principle (Figure 1.8) and provides reagents and protocols for the disruption of bacterial genes. Firstly, a computer algorithm is used to identify target sites in a gene of interest. A 1 kb gene can be anticipated to contain 5 to 11 group II intron insertion sites. Secondly, the computer algorithm outputs primer sequences, which are used to mutate (re-target) the intron by PCR. The mutated 350 bp PCR fragment is ligated into a linearized pACD4K-c vector that contains the remaining intron components. The ligation reaction is transformed into a host organism followed by expression of the re-targeted intron. Knockouts are then selected using a kanamycin marker that is activated upon chromosomal insertion. Lastly, gene specific primers are used to confirm insertion.

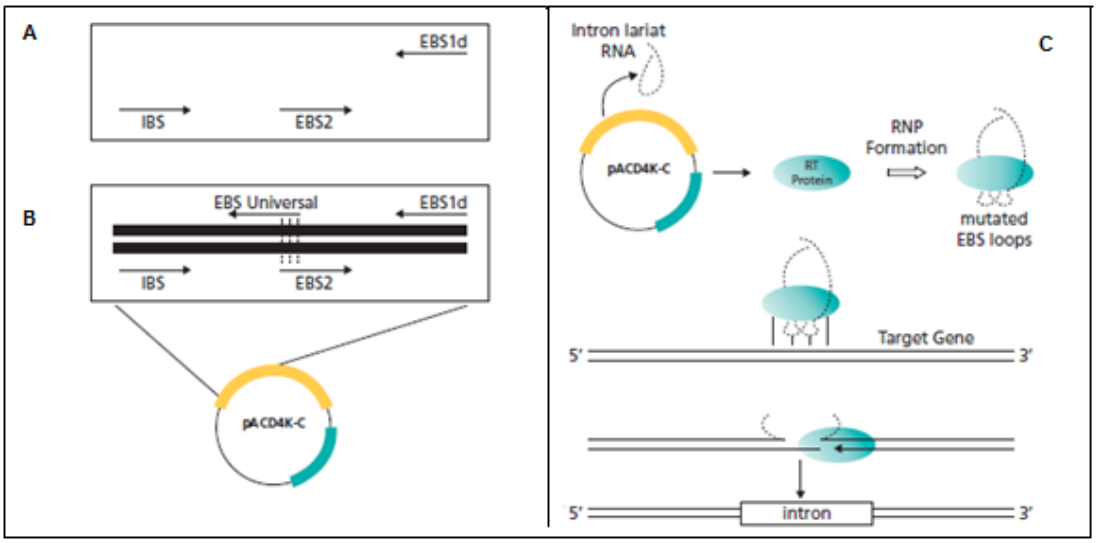


Figure 1.8. The mechanism of the Targetron gene knockout system (Sigma-Aldrich). (a) Selection of DNA target site from TargeTron Design Web site and primer ordering. (b) PCR for re-targeting of intron RNA (350 bp PCR product). (c) Cloning of mutated PCR fragment into an appropriate expression vector followed by host transformation and expression of RNA-protein complex (RNP). Lastly, the re-targeted RNP finds genomic target. RNP inserts RNA, reverse transcribes cDNA. (Reproduced from (TargeTron® Gene Knockout System user guide, 2013).

The use of this system has proven successful in a wide variety of bacteria shown in Table 1.2.

Table 1.2. List of bacteria which have been genetically engineered by group II introns.

Bacterium	Reference
Escherichia coli	Karberg *et al*., 2001; Zhong *et al*., 2003; Perutka *et al*., 2004;
Shigella flexneri	Karberg *et al*., 2001
Lactococcus lactis	Frazier *et al*., 2003
Clostridium perfringens	Chen *et al*., 2005
Staphylococcus aureus	Yao *et al*., 2006
Clostridium acetobutylicum	Shao *et al*., 2007
Pseudomonas aeruginosa	Yao and Lambowitz, 2007
Agrobacterium tumefaciens	Yao and Lambowitz, 2007
Francisella tularensis	Rodriguez *et al*., 2008
Proteus mirabilis	Pearson and Mobley, 2007
Azospirillum brasilense	Malhotra and Srivastava, 2008

Adapted from Rodriguez *et al*., 2008

There are several advantages of using the TargeTron® Gene Knockout System: Firstly the retrotransposon allows for insertion of up to 1.8 kb into the genome (Karberg *et al*., 2001). In addition possible sequences which can be used for insertion are found every few hundred bases which can allow for a large variety of genes to be disrupted (Perutka *et al*., 2004). Furthermore, the system allows for the site specific targeted disruption of genes.

There are also several disadvantages of using TargeTron® whereby intron retrotransposition efficiencies can vary depending on the species of bacteria as well as the site of targeted disruption (Perutka *et al*., 2004). Moreover, the system has neither been attempted nor optimized in *S. albulus* and thus could be challenging in practise.

1.10.2. Recombineering

Recombination-mediated genetic engineering (recombineering) is a method that makes use of homologous recombination and was first described in *E. coli* (Zhang *et al*., 1998). The main basis of recombineering is the introduction of a linear DNA construct which is

designed to contain the required mutation as well as small homologous sequences to the target DNA (Sawitzke *et al*., 2011). The recombination is mediated by bacteriophage proteins using either a RecET (Zhang *et al*., 1998) or a λ Red (Datsenko and Wanner, 2000) system and can be used to knockout genes and create deletions. Since the λ Red system is both newer and a more widely used model, it will be the focus of this literature review.

The λ Red system makes use of three phage proteins: Exo, Beta and Gam (Zhang *et al*., 1998). Exo is a monomer (M_r 24 000) and is a double stranded exonuclease (5'→3') which functions by digesting linear double stranded DNA (dsDNA), leaving a 3' single strand which acts as substrate for successive recombination. The Beta protein (M_r 28 000) is a recombinase which facilitates recombination by hybridizing the single strand to its linear strand complement. The Gam protein (M_r 16 000) is vital in inhibiting RecBCD exonucleases of *E. coli* which degrade dsDNA, thus allowing the insertion of the linear construct without degrading it (Maresca *et al*., 2010).

The basic procedure of the λ Red system involves the PCR amplification of a selectable marker. The amplification is performed by long primers (~60 nucleotides (nt)) which have the 5´end (39 nt) matching the sequence adjacent to the gene to be inactivated, and a 3´sequence (19 nt or 20 nt) matching the right or left end of the disruption cassette. There is then preparation of competent cells for recombineering, electrotransformation or conjugation of the selectable marker into the competent cells and selection and confirmation of knockout mutations (Datsenko and Wanner, 2000) (Figure 1.9).

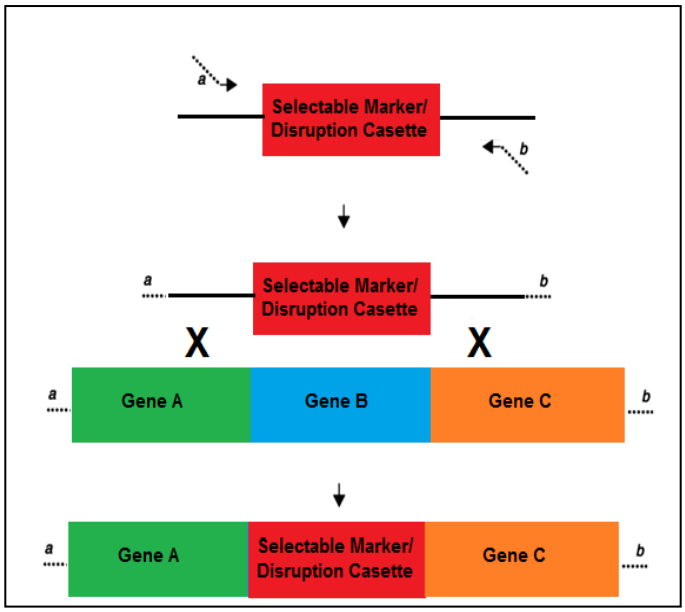


Figure 1.9. A crude representation of the basic principle of recombineering. The target gene to be knocked out is Gene B which is replaced by a selectable marker. Colonies which have lost their target gene can be selected using the selectable marker (reproduced from Sawitzke *et al*., 2011).

The differences between *E.coli* and *Streptomyces* recombineering is largely attributed to the difference in the genetic systems. The basic recombineering technique has been adapted for use in various *Streptomyces* species. In order to adapt recombineering to *Streptomyces*, a two-step strategy is required. In this case a λ-Red-expressing *E. coli* strain containing the target region (on a cosmid clone) is first targeted with a PCR-product to replace the *Streptomyces* gene of interest within the cosmid. The genetic exchange in *Streptomyces* is then achieved by homologous recombination between the chromosomal locus and the recombinant cosmid after transformation with cosmid DNA. The reason for this two-step strategy is the inability of the *E. coli* cloning vector to replicate in the *Streptomyces* (Gust *et al*., 2004). Moreover, there is a requirement for disruption cassettes that can be selected in both *E. coli* and *Streptomyces.* These disruption cassettes contain antibiotic resistance genes and are most commonly: *aac(3)IV* coding for apramycin resistance; *aadA* coding for spectinomycin resistance and *vph* coding for viomycin resistance. These disruption cassettes can be amplified from the plasmids pIJ773, pIJ778 and pIJ780, respectively (Gust *et al*., 2003).

Due to the use of a “proxy” cosmid, *Streptomyces* recombineering is time and labour intensity. Recombineering protocol in *E. coli* may take a maximum of 7 days (Sawitzke *et al*., 2011), while in *Streptomyces* on average it may take up to 3 weeks if the protocol has been optimised (Gust *et al*., 2004).

Nevertheless, an example of a successful recombineering technique for *S. coelicolor* has been described. The system makes use of several well characterized plasmids which contain the three phage recombineering proteins. Examples of such plasmids are pIJ790, pKD20 and pKD46. This method was utilized to create over 100 gene disruptions in *S. coelicolor* (Gust *et al*., 2003)

The λ Red system has also been used to make gene disruptions in other *Streptomyces.* Several examples include the genetic engineering of novobiocin derivatives in *S. coelicolor* (Eustaquio *et al*., 2004), daptomycin derivatives in *S. roseosporus* (Nguyen *et al*., 2006) and bleomycin gene cluster in *S. verticillus* (Galm *et al*., 2008). These experiments have showed the success of λ Red system by the increase in the production of the respective products.

There are several advantages of using recombineering as a gene disruption technique. Firstly, it is not limited to areas where there are restriction sites as would be with the use of restriction and ligation techniques. Secondly, the constructs are designed to the base pair and are therefore very specific to the target region. Thirdly, it can be used to create complete gene knockouts, point mutations, deletions, duplications and inversions thereby making it a versatile technique (Datsenko and Wanner, 2000).

1.11. AIMS AND OBJECTIVES

Through various work performed both abroad and in our research laboratory it can be seen that the growth of *S. albulus* is slow and the production of ε-PL is not optimal. For these reasons, metabolic engineering of the ε-PL production pathway may allow for the improvement of this secondary metabolite which would lead to commercialisation. Therefore the broad objective of the study was to determine wild type *S. albulus* parameters and develop tools which can be used to genetically and/or metabolically engineer *S. albulus* for the improvement of ε-PL production. These tools would then be used in our laboratory to perform gene knock-outs or knock-ins.

The specific aims for this study were:

1. *To determine S. albulus growth rate in different carbon sources, ε-PL production and antimicrobial activity.*

   The above parameters have been established previously in several studies; however these need to be set in our laboratory in order to use the data for downstream processes.

2. *To evaluate the commercial feasibility of ε-PL in terms of its use as a food preservative in Southern Africa.*

   ε-PL has been used as a food preservative for staple foods in Japan, Korea and the United States but has not been introduced into the Southern Hemisphere to date. In addition, production of ε-PL using glucose as a carbon source to grow *S. albulus* is expensive and crude glycerol may reduce these costs making it economically feasible.

3. *To develop tools and protocols for two engineering strategies namely the group II intron TargeTron® gene knockout system and λ Red mediated recombineering system in S. albulus.*

   Engineering systems have been developed in various *Streptomyces* species and more specifically in *S. albulus.* However these have been limited and the methods described above have not been attempted in *S. albulus*.

CHAPTER 2. *STREPTOMYCES ALBULUS* GROWTH AND ANTIMICROBIAL ACTIVITY

2.1. INTRODUCTION

ε-PL is a secondary metabolite produced naturally by *S*. *albulus*, a Gram positive, aerobic, filamentous and soil dwelling bacteria. It consists of 25-35 L-lysine residues, characterized by a peptide bond between the α-carboxyl and ε-amino group (Shima *et al*., 1984).

The sole method of ε-PL production is by aerobic fermentation (pathway in Appendix 7.5) (Hamano *et al*, 2007; Shukla *et al.*, 2012) and glucose is the conventional carbon source used by *S. albulus* for ε-PL biosynthesis (Shima *et al*., 1983). However, glucose is expensive and prices unstable; hence alternative carbon sources such as glycerol have been studied (da Silva *et al*., 2009; Chen *et al*., 2011).

Glycerol has shown to be feasible as an intermediate in a number of metabolic processes in microorganisms for its use as a carbon source. Shima and Sakai, 1977, reported that wild-type *S. albulus* produced 0.3 g/L ε-PL after 96 hours of flask culture in a medium containing glycerol. More recently it was shown that a combination of glucose to glycerol (weight ratio 1:1), produced 1.2 g/l of ε-PL (Shukla and Mishra, 2012). Nevertheless, the purifying costs of glycerol use is too high and therefore not economically viable (Johnson and Taconi., 2007).

Since glycerol is both a cell metabolite and a carbon substrate for growth, residual, crude glycerol from biodiesel could be postulated as a substrate for microbial growth. From every 100 kg of biodiesel produced 10 kg of crude glycerol is obtained via the transesterfication process. Crude glycerol (70-80 % pure) has little economic value because of the surplus in its production, lowering the price (Johnson and Taconi., 2007). To date, no effective use for crude glycerol exists. Therefore, new value added uses for crude glycerol are necessary, such as its use as a carbon source for bacterial growth.

ε-PL has recently attracted significant attention due to its wide antimicrobial spectrum and high safety. It shows strong antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and some viruses (Hiraki *et al*., 2003; Yoshida and Nagasawa, 2003). It is these properties that make it suitable for use in the food and medical

industries. The antimicrobial activity is of interest for it to be considered an alternative natural food preservative.

More specifically, it has shown antimicrobial activity against, *S. aureus*, *Bacillus spp*., *Listeria Monocytogenes*, *Salmonella typhimurium*, *E. coli*, *Pseudomonas spp*., *Camphylobacter jejuni*, *Micrococcus spp*., *Clostridium acetobutylicium* (Georgnaras *et al*., 2007), *Aspergillus niger*, *Fusarium oxysporum*, *Candida spp*. and *Penicillium* spp. (Shih *et al*., 2006) in broth culture media. These however have not been validated on solid media.

The aim of this chapter is to determine *S. albulus* CCRC 11814 growth rate in different carbon sources, ε-PL production and antimicrobial activity in our laboratory.

2.2. MATERIALS AND METHODS

2.2.1. Microorganisms

The bacterium *S. albulus* CCRC 11814 was used throughout the study. It was obtained as a gift from Ing–Lung Shih from Da-Yeh University in Japan. The strain was stored as a glycerol stock at -70°C and thawed when required.

2.2.2. Media and culture conditions

Minimum medium containing per litre: 8 g NH_4Cl, 0.5 g $(NH_4)_2SO_4$, 0.3 g $MgCl_2.6H_2O$, 40 mg EDTA, 2 mg $ZnSO_4.7H_2O$, 1 mg $CaCl_2.H_20$, 15 mg $FeSO_4.7H_2O$, 0.2 mg $Na_2MoO_4.2H_2O$, 2 mg $CuSO_4.5H_2O$, 0.4 mg $CoCl_2.6H_2O$ and 1 mg $MnCl_2.4H_2O$ was used for *S. albulus* growth. Media pH was adjusted to 6.8 (optimal pH for *S. albulus* growth) using 0.75 M K_2HPO_4 and 0.75 M NaH_2PO_4 buffers. pH was confirmed using a basic 20^+ pH meter (Crison instruments). Growth conditions were maintained at 25 $^{\circ}$C, which is the optimal temperature for general *Streptomyces* growth.

2.2.3. *S. albulus* growth in various carbon sources

Glucose (Merck chemicals), pure glycerol (Sigma-Aldrich) and crude glycerol were used as carbon sources to determine whether *S. albulus* has the ability to grow in their presence. The crude glycerol was obtained, as a gift, from a biodiesel producer in South Africa. Previously, in our laboratory, it was shown that *S. albulus* can grow on glucose (2 %), hence, it was used as a positive control. The strain was grown in 1 L Erlenmeyer flasks containing 200 ml of minimum medium, with various concentrations of pure glycerol (2 %, 5 % and 10 %) and crude glycerol (1 %, 2% and 10 %). Concentrations of crude glycerol were calculated based on the fact that the purity was 70 %. The experiments

were performed in triplicate and the flasks were incubated at 25^{0}C on a rotary shaker (Labcon) at 220 rpm.

The different glycerol concentrations in the medium were used to determine the optimal level of carbon source utilised by the strain. From each sample, 1 ml was drawn daily using a sterile pipette, for cell density determination. Growth was measured using optical density (OD) at 660 nm with a UV-Vis spectrophotometer (PG instruments).

2.2.4. Quantitative determination of ε-PL

ε-PL was quantified using an original colorimetric method developed by Itzhaki in 1972. This method was adapted based on the interaction of ε-PL with an acidic, anionic dye, trypan blue (Shen *et al*., 1984). The dye and ε-PL form an insoluble complex and the remaining trypan blue is collected as a supernatant after centrifugation. The unbound dye is used to estimate ε-PL production at an absorbance of 580 nm. From each sample, 1 ml was mixed with 2.88 ml of 0.1 M saline buffer at pH 7 and 0.12 ml of 1 mg/ml trypan blue (Sigma-Aldrich) to make up a total volume of 4 ml. The mixture was placed on a rotary shaker (Labcon) at 30 ^{0}C for 30 minutes to allow for the reaction to occur. The water-insoluble complex was centrifuged at 1600 x g for 10 minutes at 4 ^{0}C using a F15-8X50C rotor of a Sorvall RC 6+^{TM} centrifuge. The supernatant was collected and the absorbance was measured using a T60 UV-Vis spectrophotometer (PG instruments). Commercially supplied ε-PL was used to prepare a range of concentrations of 200 mg/ml to 1000 mg/ml to plot a standard curve. Absorbance results from the standard curve were used to determine the concentration of ε-PL.

2.2.5. Theoretical up-scaling: ε-PL production

Theoretical up-scaling was performed using the CEBER Modeller developed by Dr Kevin Harding. This is an excel model which uses algorithms in order to upscale the production of compounds in terms of the media requirements and energy consumption. The model works by inputting lab scale figures obtained from experiments. The following lab scale inputs were selected: product composition (including media and bacteria), dry cell weight (DCW), amount of product produced and amount of product required at the end. The up-scaling was performed in order to predict media requirements as well as energy consumption for bioreactor and industrial fermentations.

2.2.6. Assessing the antimicrobial capabilities of ε-PL

In order to assess the antimicrobial capabilities of ε-PL against both bacteria and fungi, two different experiments were set up. The ε-PL used for these experiments was obtained from a supplier.

Antimicrobial effects against bacteria

Nutrient agar (NA) plates containing different concentrations (50 µg/ml, 100 µg/ml and 200 µg/ml) of ε-PL were made. The ε-PL was spread onto the plates after the solidification of the agar. 100 µl of standardised S*. aureus*, *E. coli* or *P. aeruginosa* bacterial suspension (by cfu/ml) was spread onto the plates and they were incubated for 24 hours at 37°C.

Antimicrobial effects against fungi

Potato Dextrose Agar (PDA) (Merck Chemicals) containing different concentrations (50 µg/ml, 100 µg/ml and 200 µg/ml) of ε-PL were made. The ε-PL was spread onto the plates after the solidification of the agar. The plates were cross sectioned and an 8 mm diameter part of *Aspergillus niger* or *Penicillium simplicissimum* was placed in the centre using a sterile cork borer. The plates were incubated at 25°C until the growth of the control (0 µg/mL ε-PL) reached the end of the plate. Diameter measurements were taken daily using three replicates. Rates of extension of fungal growth were calculated for both the experiment and control. These were used to calculate the growth index (GI), which represents the promotion or inhibition of fungal growth. The GI formula is: (E/C x 100) – 100, where E and C represent the experiment and control rate of extension respectively (Chen and Jiang, 2001).

2.3. RESULTS

2.3.1. *S. albulus* growth

Carbon Source

S. albulus showed sigmoidal growth curves in the presence of pure and crude glycerol. Bacterial growth in the presence of glucose, was the fastest, followed by 5 %, 1 0% and 2 % pure glycerol respectively (Figure 2.1). Crude glycerol showed slower growth compared to pure glycerol but growth was fastest in 5 %, 10 % and 2 % respectively (Figure 2.2).

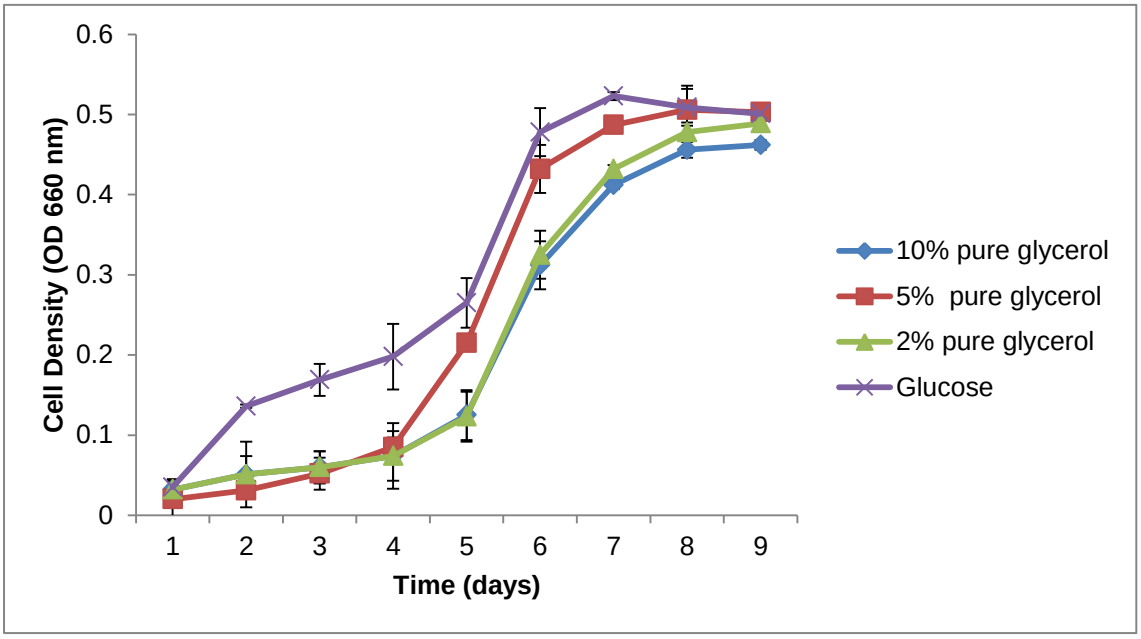


Figure 2.1. Cell density represented by OD readings of *S. albulus* grown in shake flasks with different concentrations of pure glycerol and glucose as a control. Glucose was the best utilized carbon source followed by 5%, 10% and 2% glycerol respectively. The 5% containing glycerol medium showed a typical bacterial growth curve and was the best utilized carbon source. Results represent the means of 3 replicates ± standard error of mean.

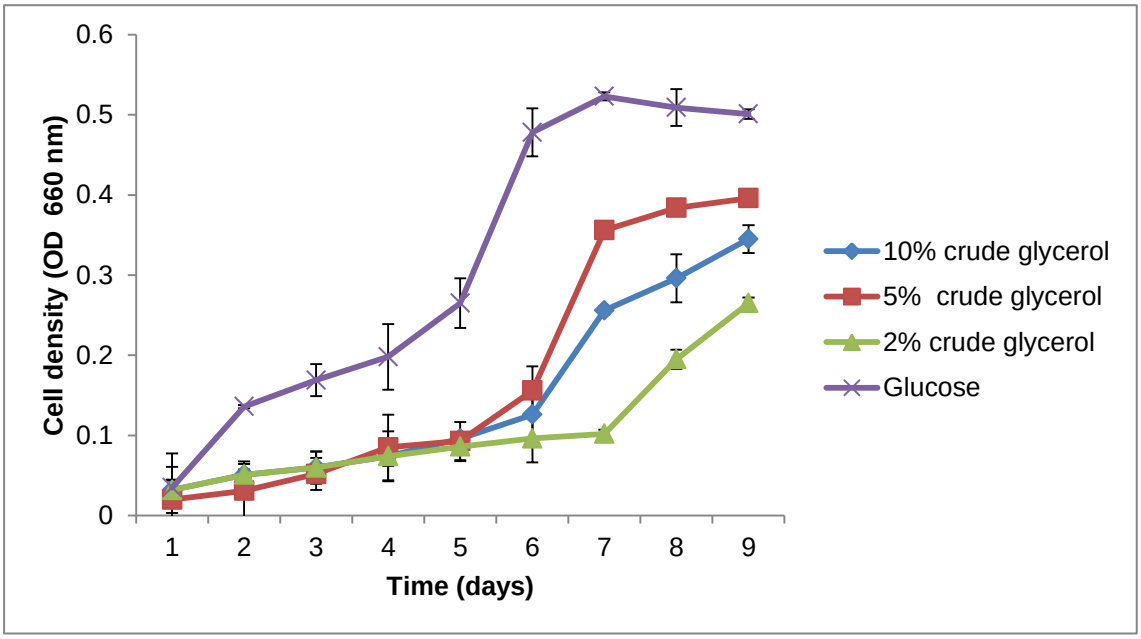


Figure 2.2. Cell density represented by OD readings of *S. albulus* grown in shake flasks with different concentrations of crude glycerol and glucose as a control. Glucose was the best utilized carbon source followed by 5%, 10% and 2% crude glycerol respectively. The 5% containing glycerol medium showed a typical bacterial growth curve and was the best utilized carbon source. Results represent the means of 3 replicates ± standard error of mean.

2.3.2. ε-PL quantification

The standard curve of absorbance and ε-PL concentration is shown in Figure 2.3. The trend shows that absorbance decreases with increasing concentration of ε-PL.

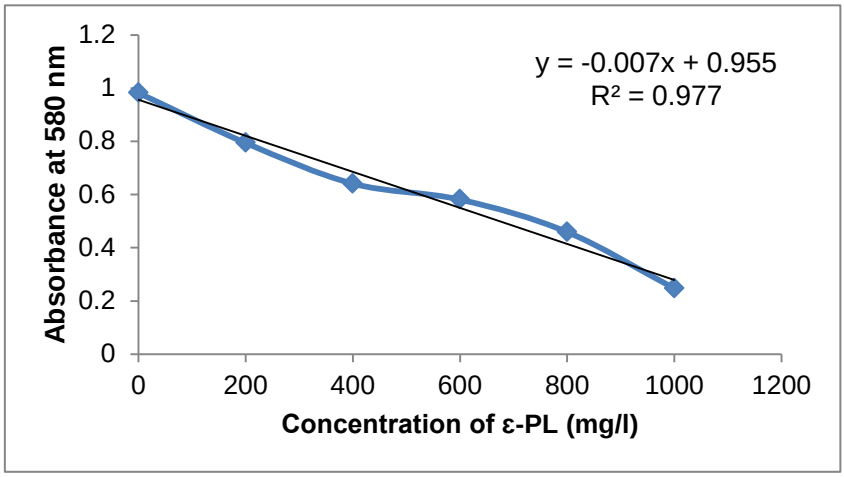


Figure 2.3. Calibration curve of mean absorbance vs concentration of ε-PL (mg/l). The graph shows that the absorbance decreases with increasing concentration of ε-PL.

ε-PL was produced in the presence of both pure and crude glycerol of all concentrations (Figure 2.4). The highest amount of ε-PL is produced using 2 % glucose as the carbon source followed by 10 % pure glycerol, 5 % pure glycerol and 2 % pure glycerol (blue bars). With regards to the crude glycerol (red bars), the highest concentration of ε-PL was produced at 10 %, followed by 2 % and 5 %.

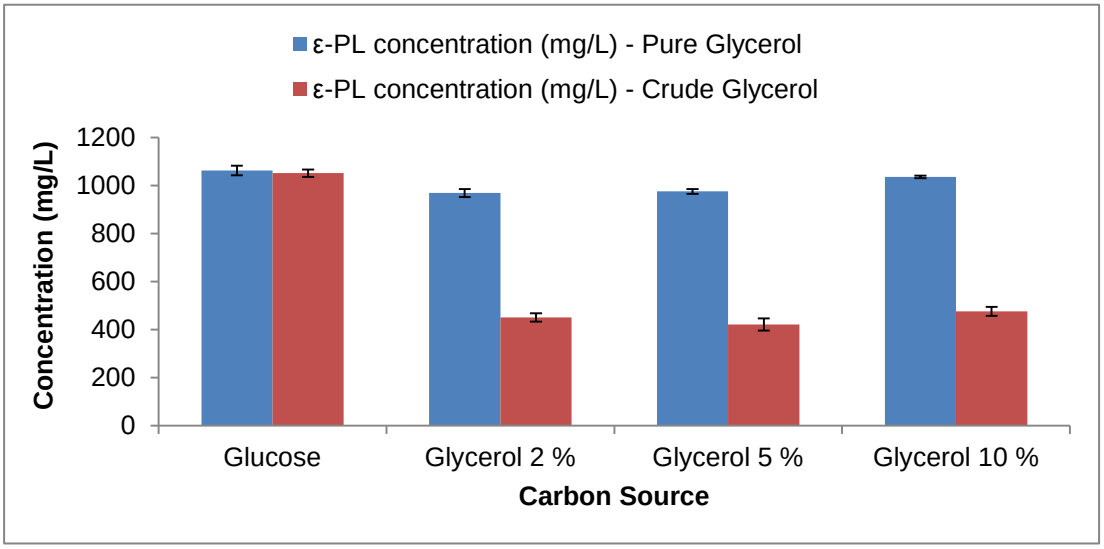


Figure 2.4. Amount of ε-PL produced by *S. albulus* bacteria. The carbon source utilised for this experiment was pure and crude glycerol. On average, half the amount of ε-PL is produced using crude glycerol compared with pure glycerol. Results represent the means of 3 replicates ± standard error of mean.

2.3.3. Up-scaling ε-PL production

The theoretical up-scaling of ε-PL production on an industrial scale will produce 41192.5 kg/year of crude product with a recovery rate of 92.18 % and a purity of 94.87 %. This will require the input of 1585 kg of *S. albulus* as well as several other media sources. This industrial upscale will produce a large amount of waste of which the majority attributed to bacteria (835800 kg) as well as large emissions of oxygen, nitrogen and carbon dioxide producing 18630 tons, 83000 tons and 4525 tons respectively (Table 2.1).

Table 2.1. The theoretical product output, input of materials and waste emitted for the up-scaling of ε-PL on an industrial scale.

	Material	Amount	Unit
Product out-put	Product Name	Lysine	
	Total Product Mass	41192	kg
	Purity	94.87	%
	Recovery	92.18	%
IN	Electricity	56810	GJ
	Steam	2614	ton
	Cooling water	596900	m^3
	Municipal water	272.9	m^3
	Distilled water	15850	m^3
	Antifoam	152300	kg
	Streptomyces albulus	1585	kg
	Carbon source	991100	kg
	Oxygen	22040000	kg
	Nitrogen	82920000	kg
	Nitrogen feed (Fertilizer)	708400	kg
	Ammonia sulphate	398500	kg
	Buffer	105100	kg
Waste	Water	18370	m^3
	Antifoam	152300	kg
	Streptomyces albulus	835800	kg
	Carbon source	9813	kg
	Nitrogen feed	33730	kg
	Ammonia sulphate	18980	kg
	Buffer	5003	kg
	Lysine	3316	kg
	K (ions)	44920	kg
Nett air emissions	Oxygen	18630000	kg
	Nitrogen	83000000	kg
	Carbon dioxide	4525000	kg
Solid waste	None	0	kg

Energy consumption results show that most of the energy (82 %) will be used up during the agitation process of microbial growth and product formation. The remainder of the energy is used for aeration (14 %) and post fermentation processes (4 %) such as product purification (Figure 2.5).

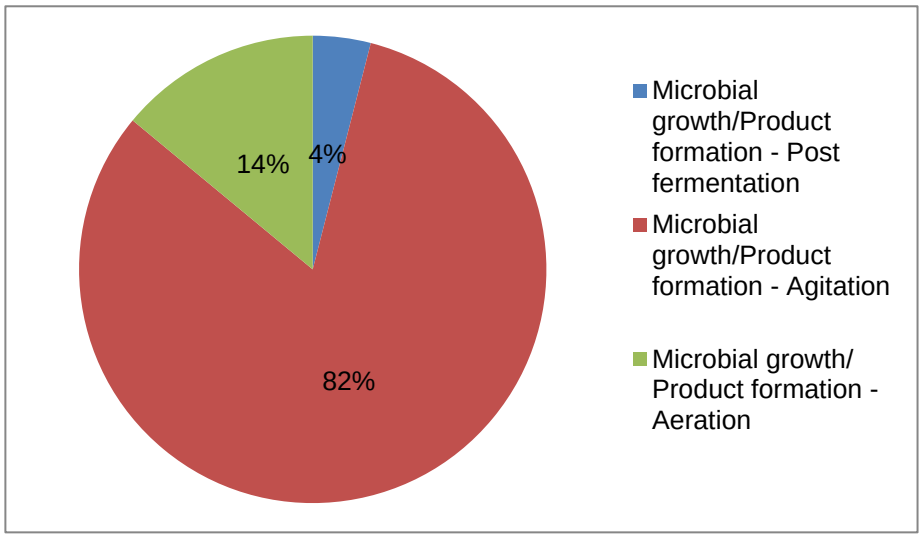


Figure 2.5. The energy consumption during the manufacturing of ε-PL on a large scale. The figure shows the energy allocations for several processes in microbial growth/product formation.

2.3.4. Antimicrobial activity of ε-PL

Antimicrobial effect against bacteria

ε-PL showed the inhibition of growth of both *S. aureus* and *E. coli* and had no effect on the growth *P. aeruginosa*.

The control *S. aureus* showed growth throughout the entire plate, while at 50 µg/ml only several isolated colonies were seen (indicated by the red circles). No growth was seen on the 100 µg/ml and 200 µg/ml plate (Figure 2.6). The murkiness of the plates is attributed to the ε-PL that was spread onto the plate after solidification.

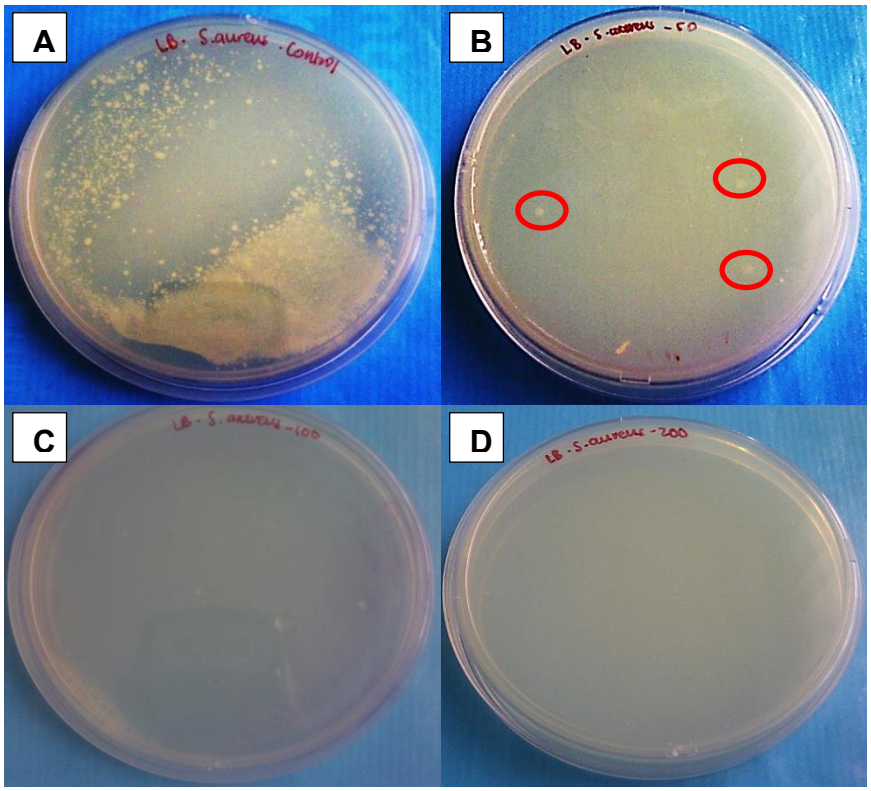


Figure 2.6. The growth of *S. aureus* in a solid model system containing varying concentrations of ε-PL. The bacteria was grown on NA plates. **(**a) No ε-PL. (b) 50 µl/ml. (c) 100 µl/ml. (d) 200 µl/ml. 100 µl of bacterial suspension was added to each plate and incubated at 37°C for 24 hours.

The plates containing *E. coli* showed a similar trend whereby the control plate and the 50 µg/ml was covered in a lawn of bacteria, whereas the plates containing ε-PL showed no growth (Figure 2.7).

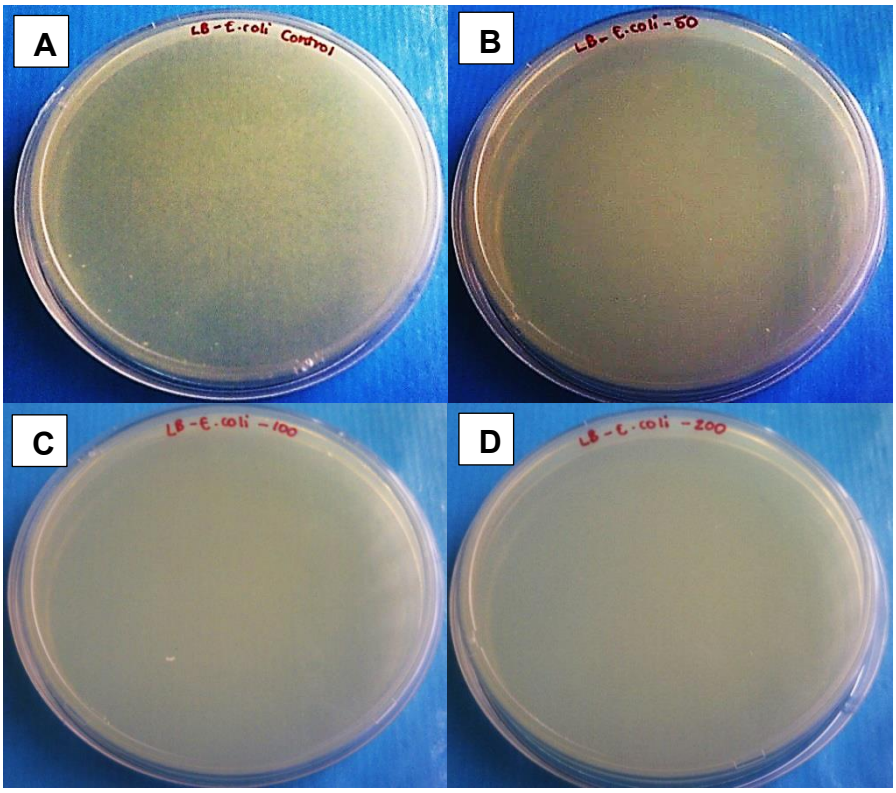


Figure 2.7. The growth of *E. coli* in a solid model sytem containing varying concentrations of ε-PL. The bacteria was grown on NA plates. (a) No ε-PL. (b) 50 µl/ml. (c) 100 µl/ml. (d) 200 µl/ml. 100 µl of bacterial suspension was added to each plate and incubated at 37°C for 24 hours.

P. aeruginosa showed a lawn of growth on the control plate as well as on the experiment plates for all three concentrations (Figure 2.8).

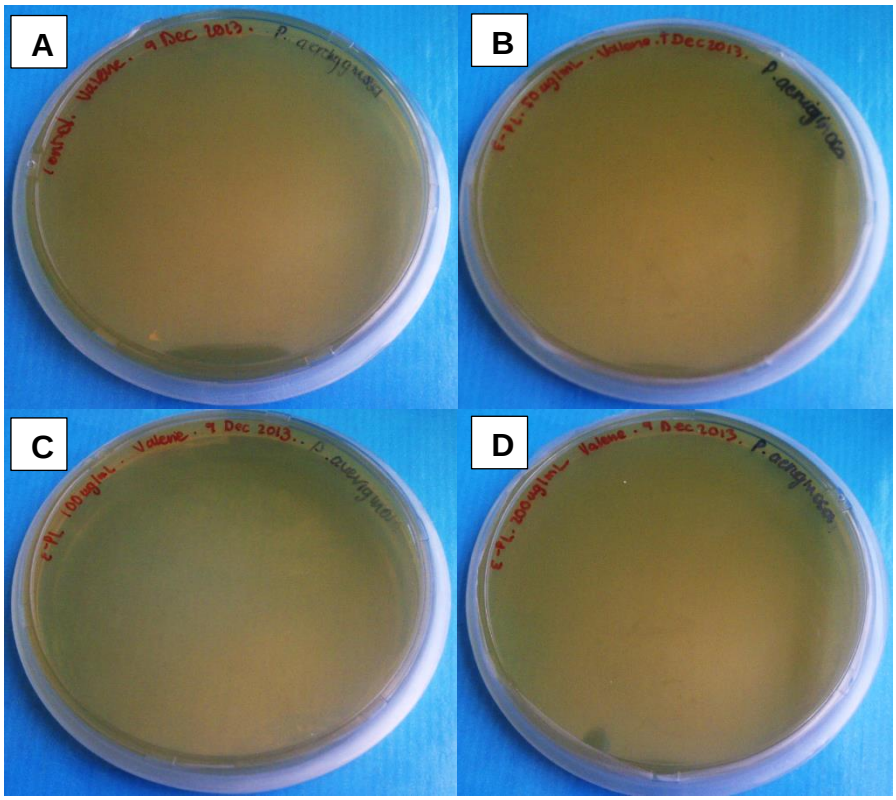


Figure 2.8. The growth of *P. aeruginosa* in a solid model system containing varying concentrations of ε-PL. The bacteria was grown on NA plates. (a) No ε-PL. (b) 50 µl/ml. (c) 100 µl/ml. (d) 200 µl/ml. 100 µl of bacterial suspension was added to each plate and incubated at 37°C for 24 hours.

Antimicrobial effect against fungi

ε-PL did not show significant inhibition of *A. niger* growth as compared with the control (Figure 2.9). The *A. niger* extension rates (R_p:R_C) were 1.25, 0.5 and 1.25 for ε-PL concentrations of 50 µg/ml, 100 µg/ml and 200 µg/ml respectively.

ε-PL showed some inhibition of *P. simplicissimum* growth as compared with the control (Figure 2.10). The *P. simplicissimum* extension rates (R_p:R_C) were 0.66, 0.13 and 0.2 for ε-PL concentrations of 50 µg/ml, 100 µg/ml and 200 µg/ml respectively.

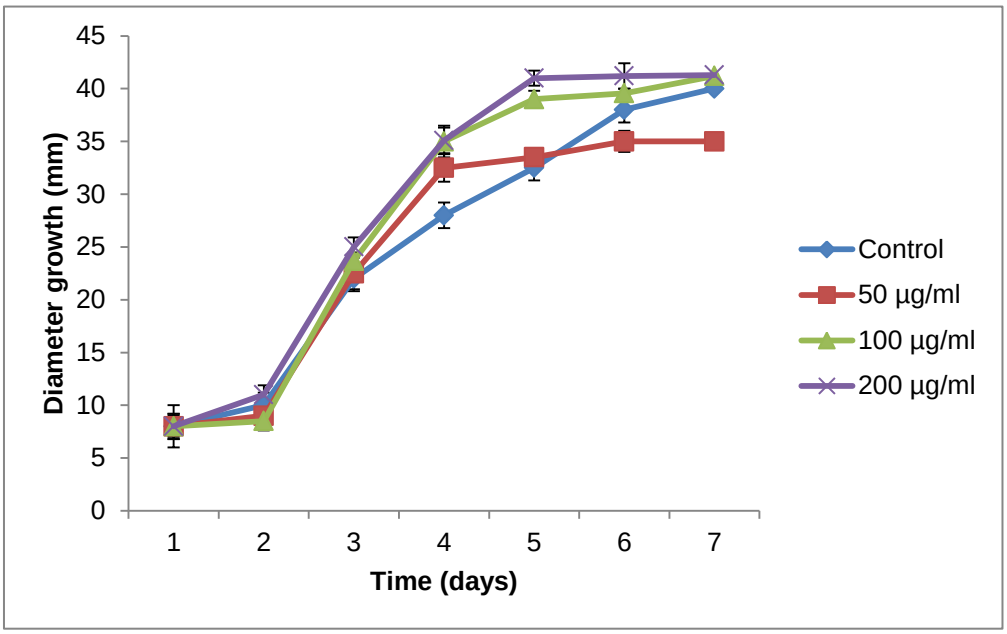


Figure 2.9. The growth (mm) of *A. niger* on PDA agar plates in the presence of different concentrations of ε-PL as well as a control over a period of time(days). Results represent means of 6 values and error bars indicate ± standard error of mean. The figure shows the growth of the fungus *A. niger* on PDA agar plates, containing different concentrations of ε-PL (50 µg/ml; 100 µg/ml and 200 µg/ml) as well as control plate containing no ε-PL.

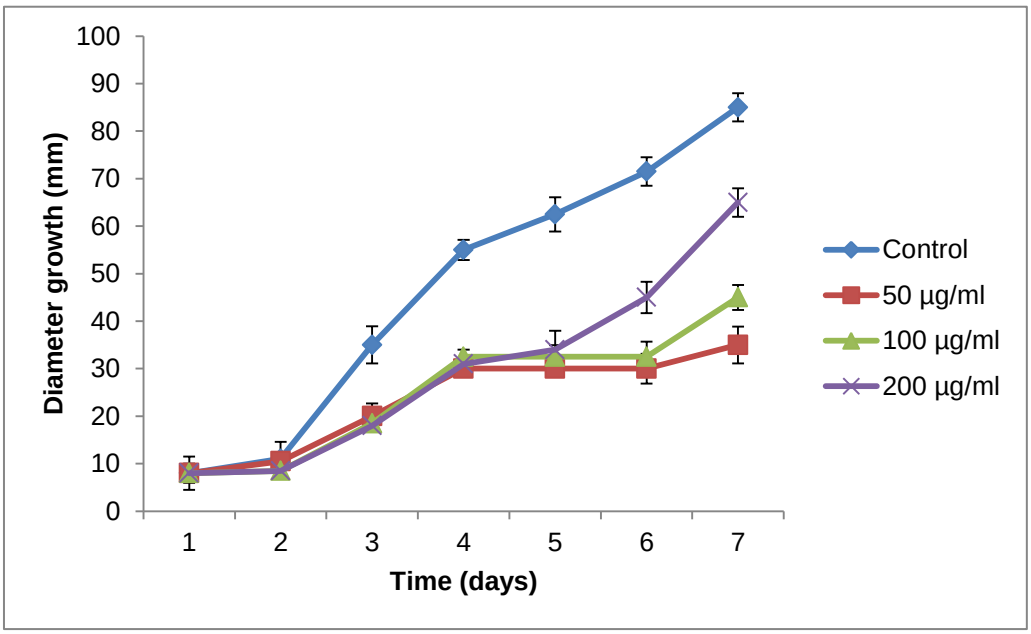


Figure 2.10. The growth (mm) of *P. simplicissimum* on PDA agar plates in the presence of different concentrations of ε-PL as well as a control over a period of time(days). Results represent means of 6 values and error bars indicate ± standard error of mean. The figure shows the growth of the fungus *P. simplicissimum* on PDA agar plates, containing different concentrations of ε-PL (50 µg/ml; 100 µg/ml and 200 µg/ml) as well as control plate containing no ε-PL.

The GI for *A. niger* and *P. simplissimum* is shown in Figure 2.11. The growth of *P. simplissimum* was inhibited by all concentrations of ε-PL, with the highest percentage at 100 µg/ml. *A. niger* growth was promoted at 50 µg/ml, 100 µg/ml and 200 µg/ml.

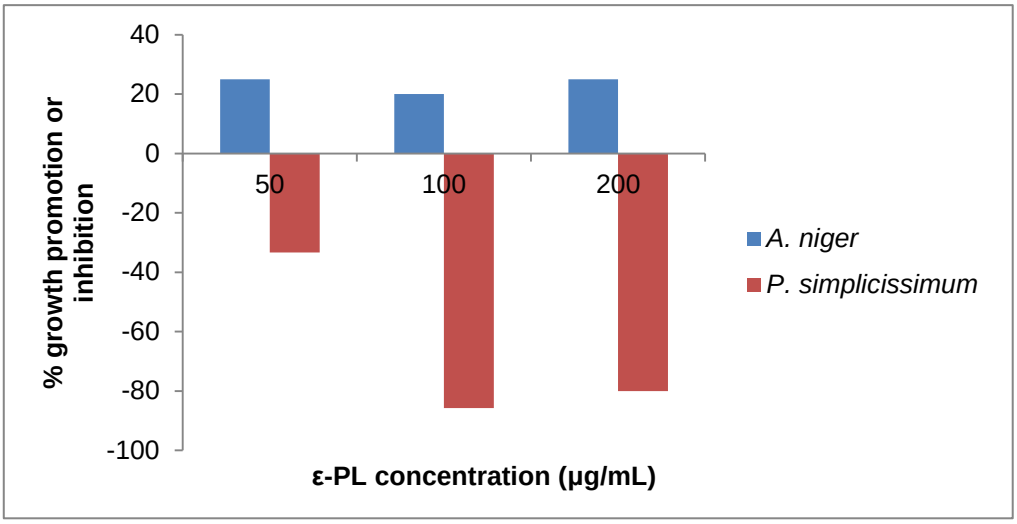


Figure 2.11. The growth promotion or inhibition of *A. niger* and *P. simplissimum* in the presence of different concentrations of ε-PL. The growth of *P. simplissimum* was inhibited by all concentrations of ε-PL, with the highest percentage at 100 µg/ml. *A. niger* growth was promoted at 50 µg/ml, 100 µg/ml and 200 µg/ml.

2.4. DISCUSSION

S. albulus growth

Based on the growth experiments, *S. albulus* shows faster growth in the presence of glucose, as opposed to pure and crude glycerol. There are several possibilities for this: Firstly, bacterial glucose metabolism is faster than glycerol due to its 6-carbon molecule and therefore, completes two cycles with one mole as compared to 2 moles of a 3-carbon molecule glycerol. Secondly, glycerol enters the microbial cells by facilitated diffusion and the rate at which the strain takes up this substrate is dependent on the effectiveness of the uptake process (Papanikolaou *et al*., 2008). *E. coli* has a glycerol facilitator protein, responsible for glycerol uptake whereas the uptake of glycerol by *Streptomyces spp* is not documented (Voegele *et al*., 1993). It is this non-selective glycerol facilitator protein that allows *E. coli* to utilise glycerol at higher rate as compared to *S. albulus.* Thirdly, glycerol viscosity hinders oxygen transfer, which reduces the growth rate because *S. albulus* is an aerobic organism (Pachauri and He, 2006). Lastly, there is a lack of information on the chemical composition of crude glycerol as a feedstock in industrial processes. Crude glycerol purity is dependent on the substances used in its production process (Gonzalez-Pajuelo *et al*., 2005).

S. albulus showed the ability to grow in the presence of crude glycerol. This result is very interesting as very few microorganisms can actually utilize crude glycerol (Rumbold *et al*., 2010). Growth in crude glycerol was slow as shown by the lower growth rates possibly due to impurities (Sneha *et al*., 2009). Crude glycerol contains between 15 – 30% impurities in the form of methanol, fat or oil remains and sodium or potassium hydroxide (Çelik *et al*., 2008). Such impurities would lengthen the lag phase as the strain adapts to the new environment. Growth inhibition is probably due to high levels of sodium and methanol used in biodiesel production (Rumbold *et al*., 2009).

ε-PL production

ε-PL produced in this study, using 2 % glucose was 1.06 g/L, which differs to the original amount of 0.2 g/L reported by Shima and Sakai, (1977), when a wild strain of *S. albulus* was grown in a basal medium containing glucose. However, it is similar to the amount produced by Hiraki *et al*., (1998) which was, 1.2 g/L. The slight differences in the amount of ε-PL produced may have been as a result of a lack of pH monitoring in this study as well as the strain used.

The concentration of ε-PL produced in the presence of 2 %, 5 % and 10 % pure glycerol, 0.97 g/L, 0.98 g/L and 1.04 g/L are similar to the 1.2 g/L reported by Hiraki *et al*., (1998) and 1-3 g/l reported by Hamano *et al*., (2007).

The ε-PL concentrations in the presence of crude glycerol were half of that obtained in glucose and pure glycerol. Due to the impurities found in crude glycerol, the bacterium is under stress and requires adaptation. Therefore more energy is used overcoming the stress than on secondary metabolite production.

Recently, an advantage of using glycerol as a carbon source for ε-PL production has been elucidated. Glycerol is beneficial to the production of low molecular weight ε-PL. ε -PL with fewer lysine residues produces a more pleasant taste when used as food preservative (Chen *et al*., 2013). In addition, recent research has suggested that a combination of glycerol and glucose in a 1:1 ratio produced 5.26 g/L (Chen *et al*., 2012), which is higher than 1.2 g/L reported previously (Hiraki *et al*., 1998)

Antimicrobial activity

On average, ε-PL has been shown to inhibit growth in a wide variety of organisms at concentrations of approximately 100 µg/mL (Yoshida and Nagasawa, 2003). This forms

part of the reasoning behind the selection of the three concentrations for the antimicrobial experiments.

S. aureus, a Gram positive bacteria was found to be completely inhibited by ε-PL at a concentrations of 100 µg/ml. The relevance of observing ε-PL activity against *S. aureus* is because it is a common cause of skin and soft tissue infections. Moreover, treatment of *S. aureus* infections in hospitals is ineffective because some of bacteria show resistance to multiple antibiotics. These pathogens are referred to as methicillin resistant *S. aureus* (MRSA) (Fridkin *et al*., 2005). Future work could involve obtaining samples of known MRSA and observe the antimicrobial activity of ε-PL against them.

Similarly *E. coli,* a Gram negative bacterium, growth was inhibited at a minimum concentration of 100 µg/ml. *E. coli* was selected due to the fact that it is such a common bacteria, it is a representative of the Gram negative group and may cause gastroenteritis.

P. aeruginosa, a bacterium responsible for responsible for causing chronic and fatal infections of lungs in cystic fibrosis patient's, growth was not inhibited by ε-PL. These bacteria are soil inhabitants and therefore require mechanisms of resistance to compete with *Streptomyces*. There has been evidence to suggest that a few isolates of *P. aeruginosa* are resistant to all reliable antibiotics due to low outer membrane permeability. (Livermore, 2002; Breidenstein *et al*., 2011). The permeability is 12–100 times less than that of *E. coli* (Hancock, 1998). The outer membrane of Gram negative bacteria acts as a selective barrier to uptake of antibiotics (Nicas and Hancock, 1983). *P. aeruginosa* was selected as it is important to look for new antibiotics that would have the ability to inhibit or reduce the growth of *P. aeruginosa.*

The bacterial antimicrobial studies were performed using solid media to imitate the texture of solid food products. It would also be beneficial to perform similar studies using liquid media and obtaining colony forming unit (cfu) counts (Zheng, 2014) to gain quantitative insight into ε-PL antimicrobial activity as opposed to only qualitative.

With regards to antimicrobial activity against fungi, the GI was used as a measure of fungal growth promotion or inhibition. The GI is expressed as a percentage, whereby negative values indicate fungal growth inhibition and positive values fungal growth promotion (Chen and Jiang, 2001). *A. niger* was selected as it is a common contaminant of food (Ramesh *et al*., 2013). This fungi was not inhibited by ε-PL but *P. simplissimum* showed inhibition at the lowest concentration of 50 µg/mL. A possible reason for ε-PL being ineffective against *A. niger* is its ability to depolymerise the polymer, thus changing

its structure and hence function. There have been studies showing that strains of *A. niger* are capable of producing proteases (Punt *et al*., 2008; Mukhtar and-Ul-Haq, 2009).

Significance of antimicrobial activity against both *A. niger* and *P. simplissimum* was evaluated using a student's T test. *P. simplissimum* were shown to be significant at a 95 % confidence level. T test results for *A. niger* were not shown to be significant, possibly due to the similarity of the growth rates between the control and treated samples (Data shown in Appendix 7.7).

The results shown in these experiments coincide with original minimum inhibitory concentration (MIC) performed by Shima *et al*., (1984). However, the experiments are based on the fact that the antimicrobial activity of ε-PL is dependent on its molecular size, which was unknown in these experiments.

Upscaling

The significance of the results obtained from the theoretical up-scaling model, demonstrate the need for extensive waste removal operations when producing a product on an industrial scale. These results would be applicable for media costing and waste removal activities in Chapter 4.

An area of growing concern worldwide is global warming which places greater demands on reducing CO_2 emissions. Due to the high emissions of CO_2, in the production of ε-PL process there is a need to introduce energy and cost efficient removal methods. Most recently, adsorption and membrane separation have been studied as the best method of CO_2 removal in industry (Ebner and Ritter, 2009).

2.5. CONCLUSION

In conclusion, based on these experiments, *S. albulus* has the ability to grow and produce ε-PL in the presence of crude glycerol. This growth and ε-PL production in the presence of crude glycerol is not optimal. ε-PL showed antimicrobial activity against two bacteria, *S. aureus*, *E. coli* as well as a fungi, *P. simplissium.* The secondary metabolite did not inhibit the growth of *P. aeruginosa* or *A. niger*.

Due to the fact that *S. albulus* has the ability to grow in crude glycerol, it could be used as an economically viable way to produce ε-PL for use as a food preservative in South Africa.

CHAPTER 3. THE COMMERCIAL FEASIBILITY OF EPSILON POLYLYSINE AS A FOOD PRESERVATIVE

3.1. INTRODUCTION

In order to determine whether there is a market available for ε-PL production in South Africa, several crude figures around the production and cost of ε-PL need to be taken into account. The average cost of polylysine is $ 3000/kg and on an industrial scale, fermentations on average can produce 40 g/L using a 5 L jar fermenter (Kahar *et al*., 2002). Running the fermenter will require man power costing approximately $ 220 a day per individual and thus 3 individuals would cost $ 660 per day. There are currently many different preservatives available on the market and, for example, the cost of a single one such as sodium benzoate is US $ 1,340 - 1,355 / 1000 kg (Food preservatives, 2012). Thus the ε-PL production market has an opportunity to create profit in terms of its use as a preservative compared with the use of current preservatives although a much deeper analysis of the commercial and economic feasibility of the product is required.

The aim of this chapter is to evaluate the commercial feasibility of ε-PL in terms of its use as a food preservative in Southern Africa. This will be achieved through the study of a hypothetical company called PεL Biosciences which will manufacture and sell the ε-PL under a product name of FoodSafe. The commercial feasibility will be presented in the form of a business plan.

3.2. Executive summary

PεL Biosciences (pronounced Pel) is being established to manufacture and distribute a natural food preservative known as FoodSafe, initially for fresh prepared chicken in the South African market. FoodSafe is a safe, non-allergenic, odourless, tasteless and colourless natural peptide derived from bacteria (Hiraki *et al*., 2003). It significantly extends the shelf-life of fresh produce, including meat, fruit and vegetables, whilst at the same time maintaining important consumer valued characteristics of smell, colour, texture, taste and the overall appearance of product freshness (JNC, 2013).

FoodSafe offers the following key benefits (JNC, 2013):

- Consumers of chicken and fresh produce value the freshness characteristics especially at the time of purchase.

- The ability to extend the lifespan of such produce adds value to retailers and consumers by reducing the waste of valuable produce stock by retailers, and by consumers after purchase.
- Since FoodSafe is a natural preservative, it is preferred by many consumers who perceive health risks associated with chemical preservatives
- FoodSafe has proven to maintain nutritional value, texture and flavour and is thus an extremely attractive product for food preservation. It acts by eliminating the growth of micro-organisms that typically cause spoilage and extends the typical shelf life of refrigerated chicken (which is cheaper to store than frozen chicken) and avocados by at least twice over for both products, or by an additional 4 days for chicken and an additional week for avocados.

Initially, the focus will be on the development and marketing of FoodSafe for chicken producers, specifically RCL Foods Ltd. (formerly Rainbow Chickens). Chicken has been shown to contain the highest microbial content of all meats and it has further been shown that extensive processing further increases the microbial population (under uncontrolled conditions). The focus on the company's Farmer Brown brand as RCL Foods has indicated a need to increase the shelf life of refrigerated chicken. This brand is wholly focused on supplying premium quality refrigerated fresh chicken that is extensively processed, i.e. trimmed of excess skin, fat and bone. There is a large local market opportunity (19 million chickens produced per week by RCL Foods Ltd) which we hope to corner with the use of FoodSafe.

Engagement and involvement with chicken producers at this early stage will enable us to tailor the FoodSafe product application during routine processing either via sprays, or dips, which is an established part of their production processes. This will determine how best it should be produced and supplied at the scale relevant to a particular client's food and packaging production process needs. Once established in this large volume market, we then intend to expand to other meats as well as fruits and vegetables (for example high value produce such as avocados which producers have indicated interest for shelf life extension, using a natural product for export markets). The application of the powder requires tailoring and testing for each type of produce, and this will be a key service we will provide to initially the Southern African, and in time southern hemisphere, products and produce, where similar products are not available.

3.3. Description of proposed product

FoodSafe is a natural polypeptide produced by bacteria and would typically be sold in the form of a white powder. It can be applied to fresh or processed food by either directly spraying a solution of FoodSafe and water onto the food product, or briefly immersing the food product into a solution of FoodSafe and water of appropriate concentration as part of the production process. Alternatively, FoodSafe can be added to the dry ingredients of a food prior to baking, cooking or other form of product finalisation. FoodSafe can also be used alone or in conjunction with other preservatives, and has been shown to have synergistic effects when combined with other preservatives (a concept known as hurdle technology). The combination of preservatives improves FoodSafe's antimicrobial activity significantly.

FoodSafe has several desirable features and benefits:

- Water soluble – can easily combine with water at an appropriate concentration for the application, and either sprayed onto food or have food immersed into the solution (Hiraki *et al*., 2003);
- Heat stable – food cooked at high temperatures would not cause removal or degradation of the preservative as it will remain on the food-product surface and maintain its action (Hiraki *et al*., 2003);
- Non-toxic and biodegradable – has no negative effects on human and animal health as well as the environment (Hiraki *et al*., 2003);
- Tasteless – the product would not influence the taste of the food product to which it is applied (Zheng, 2014);
- Healthy – contains the essential amino acid lysine which is not naturally produced by the body. Lysine is essential for growth and plays a vital role in the production of carnitine which is responsible for converting fatty acids into energy and lowering cholesterol. Lysine also helps the body absorb calcium, and therefore the formation of collagen, a substance important for bones (Singh *et al*., 2005);

3.4. Technology Solution and readiness

The following work has been conducted to demonstrate that the offering has potential to work and is practically implementable:

1. The results of experiments conducted in our laboratory have shown that the bacteria can grow using a waste feedstock (crude glycerol) to improve the economic feasibility of ε-PL production (results shown in Chapter 2).

2. The results of experiments conducted in our laboratory have shown that the current strain, *S. albulus* 11814 can produce ε-PL (results shown in Chapter 2).

3. Experiments conducted by JNC Corporation, provide a clear indication that at appropriate concentrations, the levels of bacteria (*E. coli* – 1.1 x 10^4 cfu/g) present in fresh meat can be reduced over time thereby improving shelf life and food safety. Acceptable levels of bacteria are 1000 bacteria per ml (JNC Corporation Data, 2013). The graph below shows that ε-PL is successful in maintaining declining bacterial numbers when using a ε-PL concentration of 400 ppm (Figure 3.1).

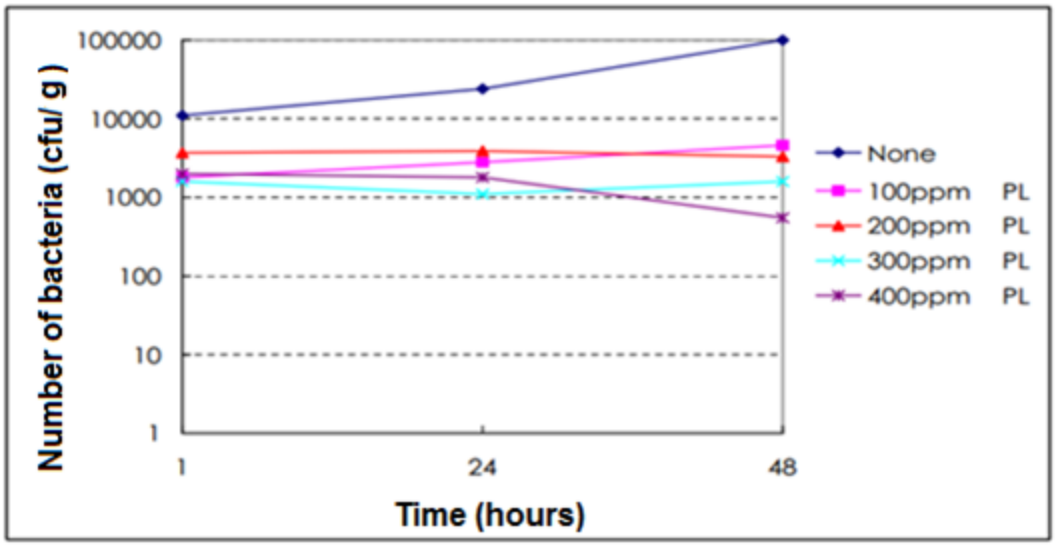


Figure 3.1. The effectiveness of ε-PL against *E. coli* on meat products. The figure shows the number of bacteria (cfu/ml) on meat decreasing after 48 hours in the presence of ε-PL of 400 parts per million (ppm) at an original bacterial concentration of 1.1 x 104 cfu/g. In the absence of ε-PL there is an increase in the number of bacteria (JNC data, 2013).

We intend to conduct further experiments to show the effectiveness of our product on chicken, and further wish to validate that our product is similar and comparable in performance to that of JNC Corporation. The necessary studies required to obtain regulatory approval will be conducted, and these will be performed during the first year to

achieve product registration as a safe human food additive in terms of Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Beyond this we will establish a commercial scale production facility that will produce FoodSafe using waste glycerol as a feedstock at sufficient volumes to support our sales drive. We also intend to undertake work aimed at improving yields of FoodSafe via genetic modifications of the production bacterium, and therefore lowering the cost of production. This modification would only improve the yield of product and will in no way change its properties.

3.5. Competitor analysis and competitive advantage

As far as we are aware, the biggest competitor is JNC Corporation, who claims to be the sole provider of ε-PL for food products in the Northern hemisphere. There are also a few other sellers of the product in China, however these are for small scale use such as research or other medical applications of the product. These include: Hangzhou Uniwise International Co., Ltd and Zhengzhou Sigma Chemical Co., Ltd. A summary of different factors of our company and the three known competitors (Table 3.1).

Table 3.1. A summary of the competitive factors between our company and three other identified competitors.

Factor	Companies			
	PεL Biosciences	**JNC Corporation**	**Zhengzhou Sigma Chemical Co., Ltd.**	**Hangzhou Uniwise International Co., Ltd**
Low Price	R3500/kg (including delivery)	Price on Request	R2500/kg (excluding delivery and import taxes)	Price on request
Superior Quality	The same product with the same properties			
Customisable product	Have the ability to customise to different foods based on reasearch	Have the ability to customise to different foods based on reasearch	Purchase Powder Only	Purchase Powder Only
Unique Features	Method of production Unique	Unknown	Unknown	Unknown
Rapid Product Delivery	Yes - manufactured in South Africa	No - Import from abroad	No - Import from abroad	No - Import from abroad

The table summarises PεL Biosciences and three competitors in terms of ε-PL and its sale. The main factors compared are price, quality, product features and delivery. PεL Biosciences is both competitive in price as well as rapid product delivery due to market proximity as opposed to the competitors.

The competitive advantage:

- Proximity to market and the types and packaging of fresh produce in the local markets;
- Ability to tailor specific products in collaboration with our major clients both for local sales and high value produce for export;
- In time, increasing price competitiveness as volumes increase. While with JNC, price information is only available through a consultation process specific to the customer's application of poly-lysine, our product is available at a fixed price per kilogram and the product formulation consultation will be done free of charge on an exclusivity basis and;
- Lastly we aim to offer rapid product delivery as opposed to competitors who are located overseas.

3.6. National benefit

Promoting postgraduate involvement in our business. In order to promote this initiative we would like to outsource the skills of outstanding current postgraduate and graduate students. This would involve post graduate students to participate in the research aspect of the business and assistance in the diversification of products

In addition FoodSafe aims to promote food security throughout rural areas in South Africa to decrease food loss as well as foodbourne diseases.

Lastly a large scale production facility producing large volumes of our product after several years will promote job creation for both very skilled and less skilled individuals.

3.7. Market Analysis

3.7.1. Basic global statistics

On average one-third of edible foods produced are lost or wasted globally, which is equal to about 1.3 billion tonnes per year. Food is wasted from initial agricultural production through to final household consumption. Significant food loss and waste usually occurs early in the food supply chain. In low-income countries food is mainly lost during the early and middle stages of the food supply chain and much less food is wasted at the consumer level. (Gustavsson *et al*., 2011). This provides the opportunity for intervention in these early and middle stages by introducing a suitable food preservative.

3.7.2. Basic South African statistics

According to recent statistics produced by the CSIR, annual food production in South Africa amounts to 28.8 million tons. Of that 9.04 million tons goes to waste which equates to 31.4% of food wastage per annum. That equals to 177 kg of waste per capita per annum amounting to R61.5 billion loss in revenues.

The majority of food is lost during the processing and packaging stage of production accounting for 27 %. This is followed by agricultural production and post harvest handling and storage which both account for 26 %. The least amount of food is lost during the consumer consumption process accounting for 4 % (Figure 3.2) (Oelofse and Nahman, 2007).

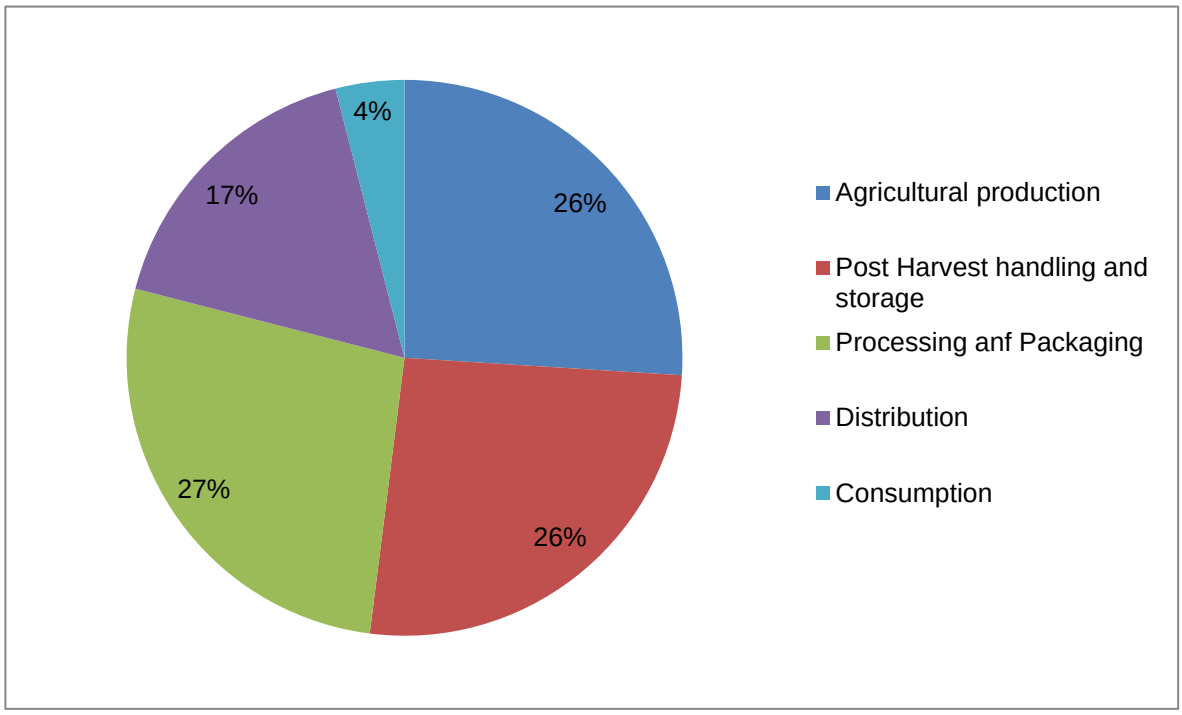


Figure 3.2. Percentage contribution of each step in the food supply chain to total average food waste in South Africa. The majority of food is lost during processing and packaging (27 %), followed by agricultural and post harvest handling and storage which both account for 26 % (Oelofse and Nahman, 2007).

The major food commodity contributing to most loss are fruit and vegetables (47 %). This is followed by cereals, roots and tubers, milk and meat contributing 28 %, 10 %, 8% and 5 % respectively (Figure 3.3) (Oelofse and Nahman, 2007).

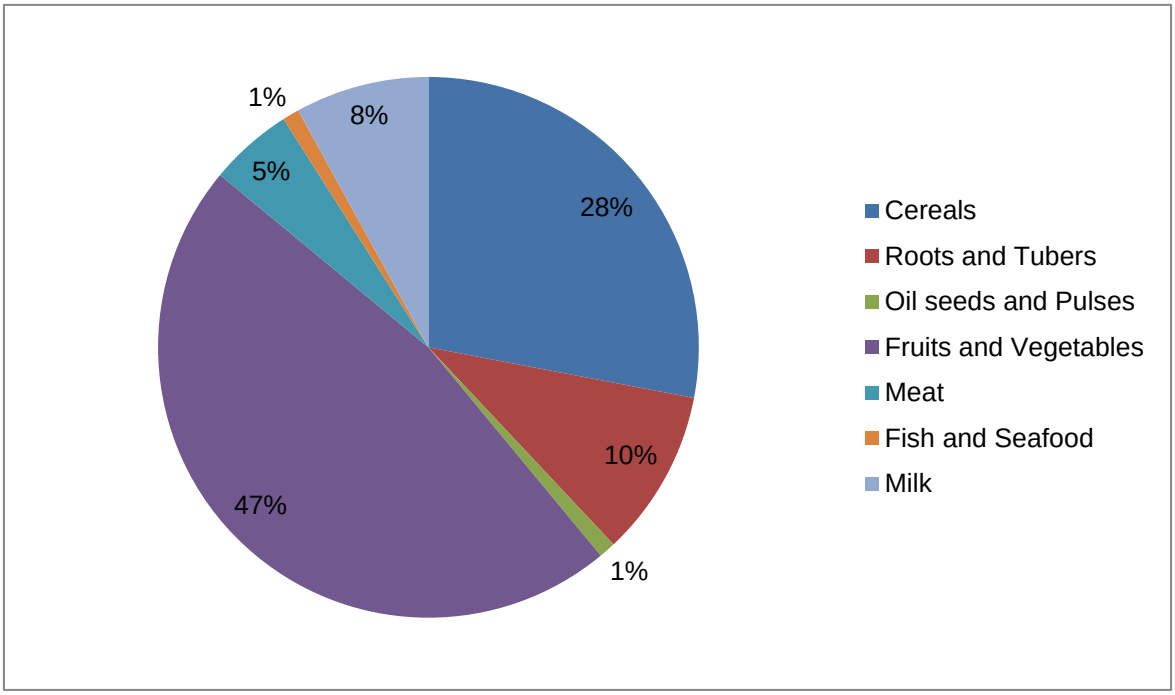


Figure 3.3. Percentage contribution of each commodity group to total average food loss in South Africa. Fruits and vegetables show the highest percentage lost, 47 %, followed by cereals which account for 28 % (Oelofse and Nahman, 2007).

3.8. Value creation

In order to understand, where the value of the product lies, the chain from the supplier of the product all the way through to the consumer needs to be studied. The value of ε-PL lies in the wholesaler because the earlier the intervention for preservation the higher the quality of the product at the customer level as majority of food is lost during packaging and distribution. Figure 3.4 shows the value chain specifically for the chicken industry in South Africa.



Figure 3.4. The chicken industry production chain from the supplier to the customer. The addition of ε-PL would be most valuable at the wholesaler stage. This allows early intervention and the maintenance of high quality food for the consumer.

3.9. Proposed route-to-market/commercialisation strategy

The first step in the route to market is the registration of the food product in the South African market:

3.9.1. Registration of FoodSafe

The first step for our business is to get the food preservative registered. Our product has not been listed in the regulations under the Foodstuffs, Cosmetics and Disinfectants (FCD) Act of 1972. In SA, only preservatives that have been evaluated by the JECFA can be considered as safe and used commercially. This is because there is not enough expertise in S.A. to scrutinise new additives for use in food.

South Africa uses the Codex Alimentarius as reference in determining the acceptability of a food additive. The list of codex specifications for food additives document from 2013 was read thoroughly and poly-lysine was not found. For an additive (other than a processing aid) not listed by Codex, a lengthy process to request inclusion in the Codex lists for food additives is followed. Two documents need to be submitted during a 'call for data' procedure to the JECFA. These documents include:

- Guidelines for the preparation of toxicological working papers for the Joint FAO/WHO Expert Committee on Food Additives and
- Guidelines for the preparation of working papers on intake of food additives for the Joint FAO/WHO Expert Committee on Food Additives.

The compilation of documents to be submitted to JECFA is underway and the evaluation process will take approximately 2 months. The people responsible for the compilation of information will be Valerie Bekker and Itumeleng Makhale, as well as other members of Dr Karl Rumbold's lab who participated in any experimental work performed towards the business. After this, the panel will evaluate whether the product will be accepted or not. Our advantage in this case is that the product has already been registered with the U.S. Food and Drug Administration (FDA). The price and the total time for the registration of food additives could not be obtained and was estimated in the financial model based on other food registration committee's prices.

3.9.2. Antimicrobial Studies

Preliminary antimicrobial studies using total cell counts and cell viability counts on skinned and skinless chicken have shown a decrease of microorganisms in the presence of 100 µg/mL of ε-PL.

3.9.3. Pilot Manufacturing

Contract Manufacturing

For the first year of selling the business will make use of contract manufacturing available through the CSIR. The Bio-manufacturing Industry Development Centre (BIDC) is a CSIR initiative, in partnership with the eGoliBio Incubator and is supported by the Development Bank of South Africa (DBSA) through the Jobs Fund programme. The BIDC will utilise the funds made available by the Jobs Fund programme to provide product and process development support to small and medium enterprises (SMEs) in the bio-manufacturing sector, with the aim of creating or expanding bio-manufacturing activity and associated job creation. Support will be provided to any qualifying biotech-based enterprises and/or entrepreneurs. The Centre's unique infrastructure is housed at a new facility on the CSIR campus in Pretoria and offers bioprocess development infrastructure from lab to pilot scale, including upstream reactors from 1 L to 200 L, as well as the necessary downstream processing, formulation and analytical support for process development. Below are some of the key offerings of the BIDC:

- Bio-manufacturing infrastructure and skilled human resource support (scientists, technologists, process engineers);
- Technical and business incubation support;
- Bio-based product development, formulation, and packaging;
- Support in developing bankable business plans; and
- Mentoring and training in business development and manufacturing skills.

An application has been sent to the CSIR and is currently under evaluation. If our business is selected by the advisory panel, the support will be provided free, but the cost of the support will be tracked and will be recovered from the business only if there will be a cash positive business (on a levy-on-sales basis). The amount to be recovered will only be discussed if the business is selected.

3.9.4. Full Scale Production

Once the business is established building a larger facility can be considered. Based on current output, competitor demand, the success of the product and business projections, this upscale would be realistic after 4 years. This phase will involve our own manufacturing, where we will hire one production manager as well as two technicians to run it. This plant will use a 5000 L bioreactor able to produce 600 kg of the product per month, and will involve an average of 4 runs of the reactor per month. The product will

then be packaged on site and delivered to RCL Foods' main distribution centre in Gauteng, who would re-distribute FoodSafe to their facilities. An agreement will be signed on the annual amounts of FoodSafe which will be purchased by RCL Foods.

3.9.5. GMP Certification and Approval

Prior to building a production facility, this would need GMP approval (Good Manufacturing Practices). Thereafter the large-scale facility will be built to produce FoodSafe as a food additive.

3.10. Economic and financial returns

There will be no revenues or profit during first year, as product registration and generating appropriate testing data for FoodSafe will be the on-going tasks. During that time, the product will be obtained as a gift from the Rumbold Lab at Wits University, for testing purposes.

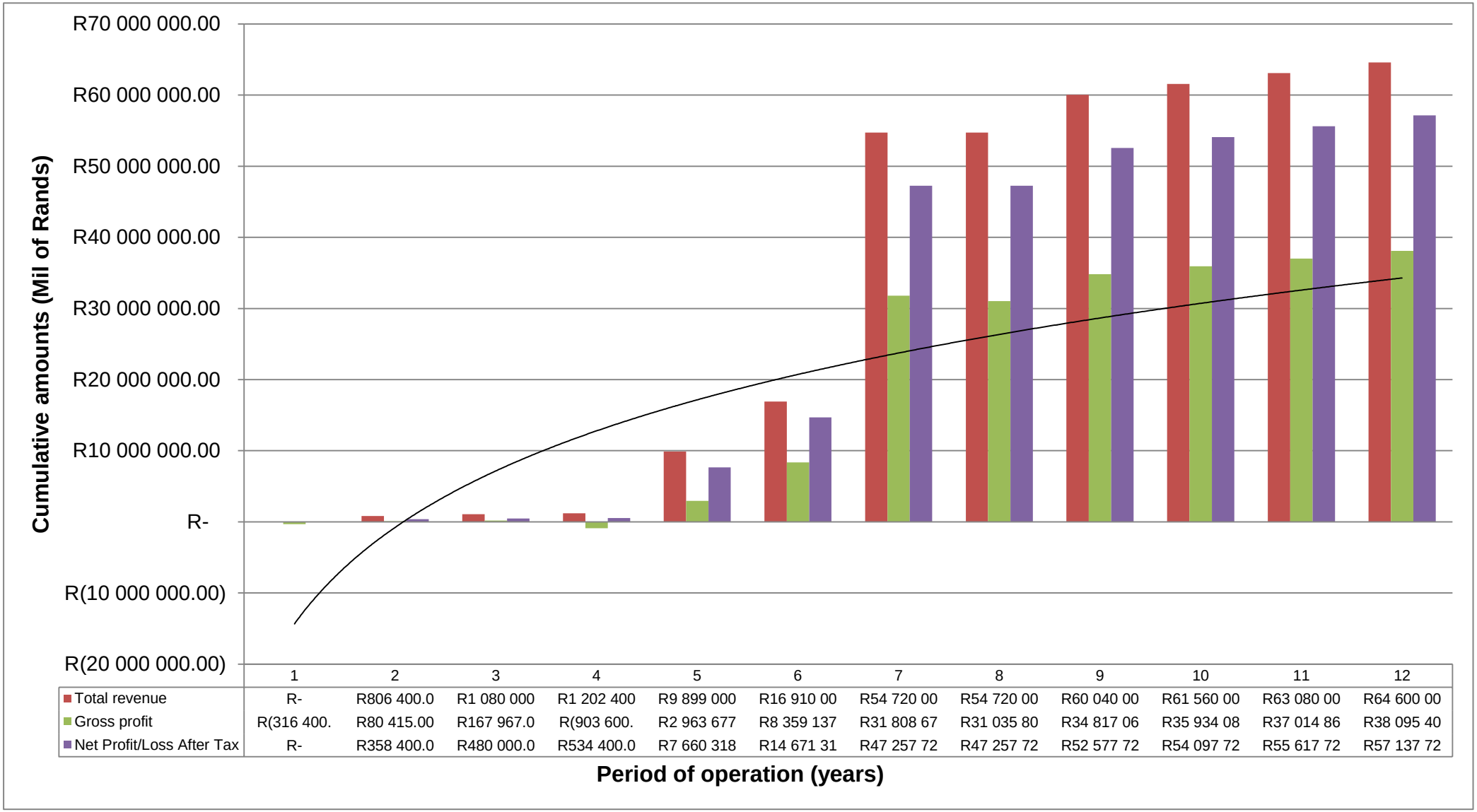
During the second year, we anticipate that the CSIR, or another contract manufacturer, will be producing FoodSafe at pilot scale at an assumed cost of R2 000 per kilogram, in order to decrease our initial capital costs significantly. In our analysis this is the more cost effective way of introducing the product, and as off take volumes increase, we will be build a full scale facility. In years 2 to 4, our model assumes that the majority of the product will be sold to RCL Foods Ltd, and in due course other fresh meat producers at a cost of R3 600 per kilogram. Our analysis is that one chicken requires ~0.4g of FoodSafe powder. Since RCL Foods sells 20 million chickens per week, our full scale production facility that can produce 7 200kg of FoodSafe per year, addresses only a small fraction of that market per year. Therefore we believe our volume growth predictions are extremely conservative. Fruit & Vegetable suppliers sales (see Production Volumes tab, Financial Model) will start in low volumes (i.e. the remainder of sales after RCL Foods) for evaluation purposes, and from year 4 these volumes are expected to ramp up.

We anticipate that sales to RCL Foods will steadily increase as we project that uptake of the product by the organisation will be in low volumes initially. Our contract pilot facility will have the capacity to match the increasing demand.

We will request the majority of the investment cost to construct the full-scale facility at the beginning of the 4th year. This is in order to reduce the impact on profits, and to make the investment a performance incentive over the pilot phases (Year 2 – Year 4).

FoodSafe prices will increase from the 5th year as the value of the product is fully realised in the market, and price increases are likely to be well tolerated

Below is a figure summarizing the income statement by showing the projected revenues, gross profits and net profits after tax over a 12 year period, for the above described conservative scenario, the single reactor production facility (Figure 3.5).



	1	2	3	4	5	6	7	8	9	10	11	12
Total revenue	R-	R806 400.0	R1 080 000	R1 202 400	R9 899 000	R16 910 00	R54 720 00	R54 720 00	R60 040 00	R61 560 00	R63 080 00	R64 600 00
Gross profit	R(316 400.	R80 415.00	R167 967.0	R(903 600.	R2 963 677	R8 359 137	R31 808 67	R31 035 80	R34 817 06	R35 934 08	R37 014 86	R38 095 40
Net Profit/Loss After Tax	R-	R358 400.0	R480 000.0	R534 400.0	R7 660 318	R14 671 31	R47 257 72	R47 257 72	R52 577 72	R54 097 72	R55 617 72	R57 137 72

Figure 3.5. Projected revenues and profits of PɛL Biosciences over a 12 year period. Amounts presented are in millions of Rands. A logarithmic trend of Net profit/loss after tax is indicated by the black line. Breakeven will occur in year five of business operation.

It should be noted that in light of the low volumes of chickens that will be treated with FoodSafe, and the above production facility assumptions, there is a more optimistic scenario that could be sketched. Taking into account the positive cash flows available in the business, we could double our production capability to meet potentially higher demand, without requiring any significant additional equity investment (Year 7).

3.10.1. Breakeven analysis

The business will be profitable by year 5, showing a working capital of R16 418 459, and a retained income of R1 992 059.

Although there is evidence of profit for the above scenarios, the amount of product produced, based on these models suggests that only 10 % of RCL chickens can be serviced.

3.11. CONCLUSION

Based on the above economic feasibility study, there is a space in the market for ε-PL use as a food preservative in South Africa. The main area of concern is the low production volumes to cater for the very large chicken industry in South Africa. For this main reason, metabolic engineering could be employed in order to increase yields.

CHAPTER 4. METABOLIC ENGINEERING

4.1. INTRODUCTION

ε-PL is a natural secondary metabolite produced naturally by *S*. *albulus*, a Gram positive, aerobic, filamentous bacteria found in the soil. It consists of 25-35 L-lysine residues, characterized by a peptide bond between the α-carboxyl and ε-amino group (Shima and Sakai, 1984). ε-PL has attracted significant attention due to its wide antimicrobial spectrum and high safety allowing its use as a food preservative in Japan and Korea (Hiraki *et al*., 2003). Based on this, there is a need for improvement of ε-PL production to meet the demand.

Strain improvement strategies have included various culture optimisation attempts to enhance ε-PL production. Although these have shown improvement of ε-PL yield, the medium cost contributes a very high percentage of overall production costs, more specifically, 40-60% of cost (Demain, 2006). As an alternative option, metabolic engineering can be used for strain improvement.

Metabolic engineering can been described as the "improvement of cellular activities by the manipulation of enzymatic, transport and regulatory functions of the cell with use of recombinant DNA technology" (Bailey, 1991). There may be several different motives for the metabolic engineering of microorganisms which can include: extension of the substrate range of a product; removal or reduction of by-product formation; introduction of innovative pathways for product production and improvement of yield and productivity of commercially valuable products (Kern *et al.*, 2007).

There has been limited metabolic engineering work on *S. albulus* to improve ε-PL production. In a previous study in our laboratory, there was an attempt to clone the glycerol operon, which contains three genes (*glp*D, *glp*F and *glp*K), from *E. coli* (Weissenborn *et al*., 1992). This is because *E. coli* shows very efficient glycerol metabolism and the operon could subsequently enhance the glycerol utilisation in *S. albulus*. The study however only showed successful cloning of the *glp*D which did not amount to improved ε-PL production (Unpublished data)

Another study used the fact that, L-lysine molecule is utilised to produce ε- PL and is biosynthesized by the aspartate pathway. The first two enzymes in the pathway are aspartokinase (Ask) and aspartate semialdehyde dehydrogenase (Asd) (Appendix 7.5). Ask is responsible for catalysing the phosphorylation L-aspartic acid to L-4-phospho

aspartic acid. These enzymes are highly regulated and different bacteria have different patterns of their regulation. Many of the Ask genes in different bacteria are susceptible to feedback inhibitions. *S. albulus,* Ask was found to be partially resistant to feedback-inhibition. A mutant, rAsk (M68V) was created whose feedback inhibition regulation was completely removed. The results showed that the rAsk produced a maximum of 15 g/L of ε- PL as opposed to 11g/L in the wild type (Hamano *et al*, 2007).

Lastly, the effects of precursor engineering, whereby addition of external L-lysine on ε-PL production was evaluated in *Streptomyces* sp. M-Z18. In combination with glucose–glycerol co-fermentation ε-PL production, was shown to be 37.6 g/L, which was 6.2 % greater than without L-lysine (35.14 g/L) (Chen *et al*., 2013).

Due to this limited work done both in our laboratory as well as abroad, there is a space in order to pioneer metabolic engineering in *S. albulus*. However, modern day tools have not been developed for such experiments in *S. albulus*. Currently, two efficient engineering methods have been used to create mutations in bacteria. The first, makes use of group II introns to perform gene knockouts in bacteria. This method has been successful in a wide variety of bacteria but has not been developed for *Streptomyces* (Karberg *et al.*, 2001). The second, λ-red recombineering has been a well-developed technique for knocking out gene in *E. coli.* This method can be used to perform several knockouts in one week and has been successful in *S. coelicolor,* although the method is both time consuming and laborious (Gust *et al*., 2004)

The rationale of this chapter is to develop tools and protocols for metabolic engineering of *S. albulus* CCRC 11814 in order to increase the metabolic flux, growth and ε-PL production. More specifically, it aims to optimise two different techniques, namely group II introns and recombineering. To our knowledge, this is the first experiment of its kind in *S. albulus*.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial strains, plasmids, and media

The *S. albulus*, *E. coli* strains and plasmids used in this study are listed in Table 4.1 below.

Table 4.1. The strains and plasmids used and their relevant characteristics to the study.

Strain or plasmid	Relevant characteristics and uses	Reference or source
***S. albulus* strains**		
CCRC 11814	Containing cryptic plasmid pNO33	Provided from the University of Japan
CCRC 11814*	Cryptic plasmid (pNO33) free	This study
***E. coli* strains**		
DH5α	For cloning experiments	
BW25113	Contains plasmid for recombineering	A gift from Prof Bertolt Gust (Department of Pharmaceutical Biology – Tübingen University)
Plasmids		
pLAE001	Transformation experiments	
pKD20	Genes for recombineering	A gift from Prof Bertolt Gust
pACD4K-C (linear)	Group II introns	Targetron System (Sigma)
pACD4K-C (circular)	Plasmid design	This study
pIJ12551	Source of ermE* promoter	A gift from Prof Mervyn Bibb (John Innes Centre, Norwich)

* Plasmid was removed from the strain through the creation of protoplasts.

4.2.2. Gene Knockout - λ-Red mediated recombineering

The current recombineering procedure to perform gene knockouts in *S. coelicolor* is laborious and time consuming. In an attempt to reduce the complexity around this method, the recombineering proteins were isolated from the plasmid pIJ790 for use in gene knockouts.

Protein expression and isolation

E. coli BW2511 containing pIJ790 was first grown in LB (Appendix 7.1) as a 5 mL preculture followed by growth in 50 mL flasks in the presence of L-arabinose (Sigma-Aldrich) of varying concentrations (10%, 1%, 0.1%, 0.01% and 0.001%). L-arabinose was used to induce expression of the paraBAD promoter (3 hours), which expresses the three genes. The cells were then centrifuged (Sorvall RC 6+^{TM} centrifuge) at 5000 x g for 10 min and the supernatant was discarded. The cells were resuspended in 5 mL of protein lysis buffer (Appendix 7.1) and sonicated in order to disrupt the cell

membrane (five times for 15 s). The mixture was then centrifuged (16 000 x g for 20 min) using a F15-8x50C Sorvall RC 6+™ centrifuge in order to bring all the cell debris to the bottom and the protein (if soluble) would be found in the supernatant. The proteins were then quantified using the Qubit® 2.0 Fluorometer (Life Technologies™) [1]. The supernatant samples were then run on a 12% SDS gel and viewed using the ChemiDoc™ MP Imaging System (Biorad).

4.2.3. Gene Knockout - Group II introns

The group II intron technique was initially performed using the TargeTron® Gene Knockout System (Sigma-Aldrich). Several steps are involved in this system which included: target site selection and primer ordering, primer preparation and PCR, gel electrophoresis, PCR product clean-up, restriction digest and purification, ligation, transformation and blue-white colony selection. Each of these steps is described in more detail below. In order to establish these techniques in our laboratory, the *pld* gene of *S. albulus* was selected (described in section 1.3.5).

Target site selection and primer ordering

The sequence of the *pld* gene (accession no. AB243405) of *S. albulus* was downloaded from GenBank and used in designing primers for retargeting the group II intron with the aid of the TargeTron Design Site software (www.sigmaaldrich.com/targetron). The primers were designed based on a specific algorithm which searches for potential LtrA recognition sites within a sequence, and then provides the sequence of the appropriate oligonucleotides to retarget Ll.LtrB. The primer sets (IBS, EBS2, and EBS1d) were ordered from Inqaba Biotech, South Africa. The primer sequences are shown in Table 4.2.

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[1] The Qubit® 2.0 Fluorometer functions through the use of Molecular Probes® dyes to quantify biomolecules of interest. The fluorescent dyes will only emit signals when they are bound to specific target molecules, even at low concentrations. The Qubit® 2.0 Fluorometer was used as opposed to the nanodrop because it has been shown to be more accurate (The Qubit® 2.0 Fluorometer user guide, 2012).

Table 4.2. The primer sequences used to retarget the intron region for gene knockout

Name	Primer Sequence (5' to 3' direction)	Size	PCR product size (bp)
IBS	AAAAAAGCTTATAATTATCCTTACCACCCCACCGCGTGCGCCCAGATAGGGTG	53	350
EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCACCGCGGTAACTTACCTTTCTTTGT	60	
EBS2	TGAACGCAAGTTTCTAATTTCGGTTGGTGGTCGATAGAGGAAAGTGTCT	49	
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC	28	

Primer preparation and PCR

The stock solutions of the IBS and EBS1d primers were used (100 µM), and the EBS2 primer was diluted to 20 µM, in nuclease free water. These three primers were mixed, with the fourth universal primer, EBS universal primer, to prepare a mix for overlapping PCR (Table 4.3). A PCR was performed using a PCR mix shown in Table 4.4.

Table 4.3. Four primer master mix for use in the PCR reaction

Reagent	Volume (µL)	Concentration (µM)
IBS Primer	2	100
EBS1d Primer	2	100
EBS2 Primer	2	20
EBS Universal Primer	2	20
Nuclease free water	12	
Total Volume	20	

Table 4.4. PCR mix used for the re-targeting of the intron

Reagent	Volume (µL)	Concentration (µM)
Nuclease free water	23	
Four Primer Mix (Gene specific or LacZ positive control)	1	
Intron PCR template	1	
JumpStart REDTaq ReadyMix	25	2.5 units/reaction
Total Volume	50	

The PCR was performed using the T100™ Thermocycler (Biorad) with the conditions shown in Table 4.5.

Table 4.5. PCR conditions for the re-targeting of the intron

Step	Temperature (°C)	Time (seconds)	No. of cycles
Initial Denaturation	94	30	1
Denaturation	94	15	30
Annealing	Gradient (52-65)	30	
Extension	72	30	
Final Extension	72	120	1
Hold	4	∞	

The primers orientation to retarget the intron in the PCR are shown in Figure 4.1.

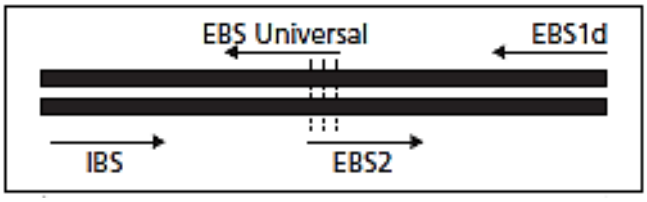

Figure 4.1. Primer orientation for intron retargeting in PCR. (TargeTron® Gene Knockout System user manual, 2013).

Gel Electrophoresis

A 3% agarose gel (Bioline) (Appendix 7.1) was used to visualize if the correctly sized fragment was amplified during PCR and to check for spurious amplification. The PCR product sizes were determined using a GeneRuler™ 1kb Plus DNA Ladder (Fermentas). Ethidium bromide (10 µg/ml) (Bioline) was added to the gel during preparation. This chemical intercalates between the DNA nucleotides and when exposed to UV light, fluoresces, thus allowing the DNA to be visualized. The gel was visualized using the ChemiDoc™ MP Imaging System (Biorad).

PCR product clean-up

Following successful PCR amplification, products were confirmed and cleaned using a NucleoSpin ® Extract II Cleanup kit (Macherey-Nagel, Clontech Laboratories, Inc). This kit removes the excess primers and other contaminants, such as salts, from the PCR reaction mix to prevent them from interfering in the downstream applications of ligation and transformation. This clean-up involves the use of a column and is a three step process. Firstly, the DNA binds to the column and is trapped on it. The remaining contaminants are eluted through several wash steps. Elution buffer is then used to elute the DNA from the column (NucleoSpin ® Extract II Cleanup kit manual). The protocol followed is given in Appendix 7.2.

Restriction Digest and purification

A restriction digest was performed on the PCR product in order to create sticky end to allow for the ligation into the vector. A double digestion was performed using the restriction enzymes Hind III and BsrG I. Hind III recognizes a 6 bp site AAGCTT/TTCGAA and the cleavage of this sequence between the AA's results in 5' overhangs producing sticky ends. BsrG I recognizes a 6 bp site TGTACA/ACATGT and also creates sticky ends. The setup for the restriction digest is shown in Table 4.6.

Table 4.6. Restriction digest setup

Reagent	Volume (µl)	Concentration
Purified PCR product	8	~ 200 ng
10x Restriction Enzyme Buffer	2	10x
Hind III	1	20 U/µl
BsrG I	1	10 U/µl
Nucelase free water	8	
Total volume	20	

Reagents used in this setup were provided in the TargeTron® gene knockout system (Sigma-Aldrich).

The restriction products were purified using the NucleoSpin ® Extract II Cleanup kit (Macherey-Nagel, Clontech Laboratories, Inc) in order to remove excess restriction enzymes and small DNA fragments.

Ligation

The restricted intron PCR product was ligated into a pACD4K-c. The ratio of insert to vector was 3:1 in order to allow for an optimum ligation reaction. The reaction mixture for the ligation setup is outlined in Table 4.7 below.

Table 4.7. The reaction mixture for the ligation setup

Reagent	Volume (µl)	Concentration
pACD4K-C linear vector	2	40ng
Restriction digest intron PCR product	6	350bp
T4 DNA ligase buffer	2	10X
T4 DNA ligase	1	
Nuclease free water	9	
Total volume	20	

pACD4K-c linear vector was provided in the TargeTron® gene knockout system (Sigma-Aldrich). T4 DNA ligase buffer and T4 DNA ligase were obtained from Thermo Scientific.

Transformation and blue-white colony selection

Competent *E. coli* DH5α cells were transformed with the pACD4K-c vector containing the restricted intron PCR product. The cells were made competent using the detailed procedure outlined in Appendix 7.4. In summary, the cells and ligation reaction were kept on ice followed by a brief heat shocking step and the addition of SOC media. Lastly, the cells were incubated on a rotary shaker (Labcon) at 220 rpm and 37°C. 100 µl of the reaction was spread onto LB agar plates containing chloramphenicol (25 mg/ml) (Sigma-Aldrich), IPTG (100 mM) (Sigma-Aldrich) and X-Gal (20 mg/mL) (Sigma-Aldrich). The plates were incubated at 37°C overnight, after which blue-white colony selection was used in order to identify recombinant cells. Colony PCR was used to confirm the presence of the insert.

Plasmid isolation

The pACD4K-c plasmid containing the insert was purified using the Nucleospin® Plasmid QuickPure (Macherey-Nagel, Clontech Laboratories, Inc). The detailed procedure is outlined in Appendix 7.3.

The vector however was not able to be stably transformed into *S. albulus* because it does not contain the correct replicon and promoter and therefore a construct was required to be designed.

Construct design

Through the above described steps it was founded that a novel shuttle vector (construct) would need to be designed in order to complete a gene knockout using the group II intron method. This construct was designed using three plasmids: pLAE001, pACD4K-C and pIJ12551. Several portions of each of the plasmids were required in order to make a functional shuttle vector between *S. albulus* and *E. coli* (Table 4.8).

Table 4.8. Plasmid regions and functions required for pVBKR construction

Plasmid	Section required	Size	Function
pIJ12551	ermE* promoter	82 bp	Expression
pLAE001	pNO33 replicon	4.1 kb	Successful replication in *S. albulus* and *E. coli*
	bla	861 bp	For selection in *S. albulus* (*tsr*) and *E. coli* (*tsr*)
	tsr	421 bp	
pACD4K-c	Intron RNA region	2.3 kb	Functional expression of the group II intron RNP
	LtrA ORF	2 kb	
pVBKR		**~8.6 kb**	

In order to transfer the functional components of the TargeTron system to an alternative vector backbone, sub-clone the sequence from the promoter region to the end of the LtrA ORF (~4.5 kb). This region contains all the required features for functional expression of the group II intron RNP. Sub-cloning of the region may be performed by PCR amplification or restriction digestion.

The plasmid maps used for the construct were derived using VectorNTI Advance 10. All plasmid maps used for pVBKR construction are shown in Appendix 7.6.

The first step to plasmid construction was to join the ermE* promoter (82 bp) (from pIJ12551) to a portion of the pACD4K-C (from 1802 bp to 6161 bp = ~ 4.3 kb). This was

performed using overlap extension PCR. An intial PCR was performed in order to amplify the 4.3 kb region of pACD4K-c plasmid using the pACD4K-c F and R primers. The pACD4K-c vector was first linearised by ligating the two blunt ends. A second PCR was performed in order to amplify the promoter ermE*. The two PCR products were then joined together using overlap extension PCR. The primer sequences used are shown in Table 4.9.

Table 4.9. The primer sequences used to amplify the ermE* promoter and pACD4K-c for plasmid construction.

Name	Primer Sequence (5' to 3' direction)	Size
pIJ12551 - F	GACCCGAGCACGCGCCGGCA	20
pIJ12551 - R	TATTTTGCATCGCATGGACGTCCCCTTC	28
pACD4K-c - F	TCCATGCGATGCAAAATAACGGCGCTGCT	29
pACD4K-c - R	AGCTTATCATCGATAAGCT	19

In order to splice two DNA molecules, special primers were used at the ends of the pieces that are to be joined. For each molecule, the primer at the end to be joined is constructed so that the 5' overhang complementary to the end of the other molecule (Figure 4.2). Once both DNA molecules are extended in such a manner, they are mixed and a PCR is carried out with only the primers for the far ends. The overlapping complementary sequences introduced will serve as primers and the two sequences will be fused (Bryksin and Matsumura, 2010).

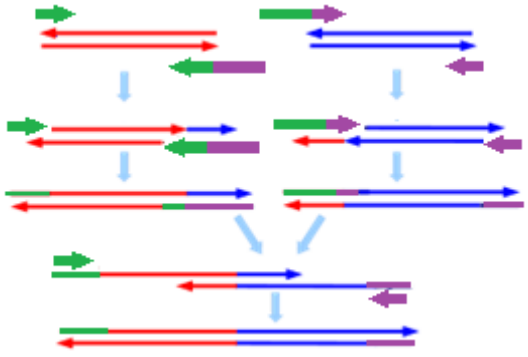

Figure 4.2. Overlap extension PCR process (Reproduced from Bryksin and Matsumura, 2010).

4.2.4. Introduction of DNA into *S. albulus*

The plasmid pLAE001 was used for insertion into *S. albulus* for the below described experiments and was designed as a shuttle vector between *S. albulus* and *E. coli* (Hamano *et al*., 2005).

Chemical transformation

In order to introduce a plasmid into *S. albulus*, protoplast transformation of cells was performed in order to cure the strain of the cryptic plasmid pNO33 which is found naturally in the bacteria. This is because pNO33 and pLAE001 have the same replicon and if both plasmids are inserted into the bacteria there would be an incompatibility problem causing one of the plasmids to be 'knocked out'.

In order to generate protoplasts, a 4 mL preculture of LB media (Appendix 7.1) was placed into a 50 mL Erlenmeyer flask containing 10 glass beads and inoculated with a single *S. albulus* colony from a fresh LB agar streak plate containing glucose (2 %). This was incubated at 30°C for 48 hours. 0.5 mL of the preculture was transferred to a 250 mL Erlenmeyer flask containing 50 mL LB and 2 % glycine. The cells were then centrifuged at 1500 x g for 10 minutes in 50 mL falcon tubes. The supernatant was removed and 10 mL of Protoplast (P) buffer (Appendix 7.1) was added to the cells in order to wash them by centrifugation at 1500 x g for 10 minutes. The supernatant was removed and the cells were resuspended in 10 mL of P Buffer again at 1500 x g for 10 minutes. 30 mL of P buffer containing 5 mg/mL of lysozyme was added to the cells and they were incubated at 30°C for 3 hours. During this incubation the tubes were inverted every 10 – 20 minutes. After the incubation, 10 mL of cold P buffer was added after the incubation and cells were harvested by centrifugation followed by two washes with 10 ml P buffer (cells were resuspended between each wash). Finally, protoplasts were resuspended in 2 mL P buffer and 500 µl aliquots were stored at -70°C.

Protoplasts were then regenerated on a *Rhodococcus* regeneration media previously described by Dabbs *et al*., 1990 (Appendix 6.1). A 100 µl suspension of the protoplasts were spread onto the regeneration media and allowed to grow for 24 hours at 25°C. Isolated colonies were placed in 10 µl LB broth and used as a DNA source in a colony PCR. The primers used for this PCR were designed to bind to the replicon region of pNO33 (Table 4.10). Colonies showing no amplicon and therefore the loss of the plasmid were selected.

Table 4.10. The primer sequences used to identify the loss of the pNO33 plasmid.

Name	Primer Sequence (5' to 3' direction)	Size	PCR product size (bp)
pNO33 - F	CTGTACACGCGAACTGTCGT	20	600
pNO33 - R	TTTTGTGGCATGATCCTTGA	20	

These were then used to culture an *S. albulus* strain which is missing the pNO33 plasmid. The pLAE001 plasmid was then inserted into the strain by thawing protoplasts and transferring 100 µl into an Eppendorf to which 5 µl of plasmid was added (100 ng/µl). The contents were mixed and the tube was placed on ice for 1 minute followed by the addition of 400 µl of P buffer. 100 µl of the mixture was then spread on *Rhodococcus* regeneration media. The plates were sealed with parafilm and incubated at 30°C for 24 hours. The following day, 3.5 ML of soft nutrient agar overlay was added containing thiostrepton (5 µg/mL) (Sigma-Aldrich). The plates were sealed with parafilm and incubated for a further 5 days at 30°C.

Electroporation

Electroporation involves the application of a short, high voltage to cells which creates transient pores in the membrane which facilitates the uptake of DNA (Pigac and Schrempf, 2005). Electroporation was performed using the ECM® 830 Electroporation System (BTX Harvard Apparatus) following the protocol outlined below (Figure 4.3). The electroporation cuvette used was a 2 mm Gene Pulser® Cuvette (Biorad).

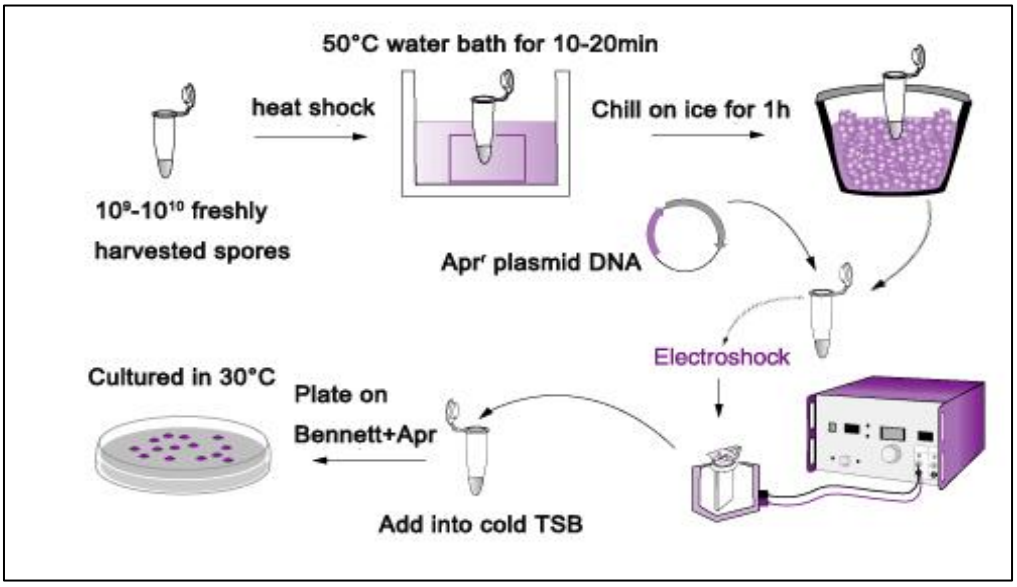

Figure 4.3. A summarised electroporation procedure for *Streptomyces* bacteria. The basic procedure of electroporation in *Streptomyces* requires freshly harvested spores which are heat shocked and chilled on ice. The DNA (plasmid) is added and the mixture is electroshocked. The cells are then grown on an agar plate and colonies are screened for the presence of the plasmid.

Optimization of electroporation parameters were required because to our knowledge, electroporation has never been attempted in *S. albulus*. The different parameters attempted are shown in the Table 4.11 below:

Table 4.11. The different parameters attempted for electroporation of *S. albulus* bacteria

Parameter	Attempt 1	Attempt 2	Attempt 3
Mode	High Voltage	High Voltage	High Voltage
Voltage	2500 V	2500 V	2000 V
Pulse Length	100	120	100
No. of pulses	1	2	2
Interval	100	120	100
Polarity	Unipolar	Unipolar	Unipolar

The different attempts to perform electroporation for *S. albulus* using different parameters in terms of voltage, pulse number and length and intervals between pulses.

Confirmation of gene transfer techniques

Transformation and regeneration efficiencies were calculated for both gene transfer methods. Regeneration efficiency was calculated by dividing the number of regenerated colonies by the original number of cells used in the transformation (Katsumata *et al*., 1984). In order to confirm if the bacteria contained the pLAE001 plasmid, a single colony from the regeneration media/NA overlay was selected. This single colony was resuspended in 10 µl of LB and was used as the template DNA in a PCR setup as shown in the Table 4.12 below.

Table 4.12. PCR mix used for the confirmation of pLAE001 transfer to *S. albulus.*

Reagent	Volume (µL)	Concentration (µM)
Dream Taq Green PCR Master Mix	10	1 x
Forward Primer	0.5	10
Reverse Primer	0.5	10
Template DNA	2	
Nuclease free water	7	
Total Volume	20	

4.3. RESULTS

In order to improve ε-PL production while using economically viable crude glycerol, two engineering strategies were attempted, recombineering and group II introns.

4.3.1. Recombineering – Protein expression

The first method, recombineering make use of three phage proteins and has been successfully used in *E. coli*. This process has been transferred to a member of the *Streptomyces* species, *S. coelicolor;* however it is lengthy, laborious and costly. For these reasons, a more simplified method was attempted by expressing these proteins *in vitro*.

The SDS page gel shown in Figure 4.4 shows an attempt to express the three phage proteins from the pIJ790 plasmid using different concentrations of L-arabinose. There was no difference between induced (lanes 2-6) and non-induced (lane 7) *E. coli* cells containing the pIJ790 plasmid.

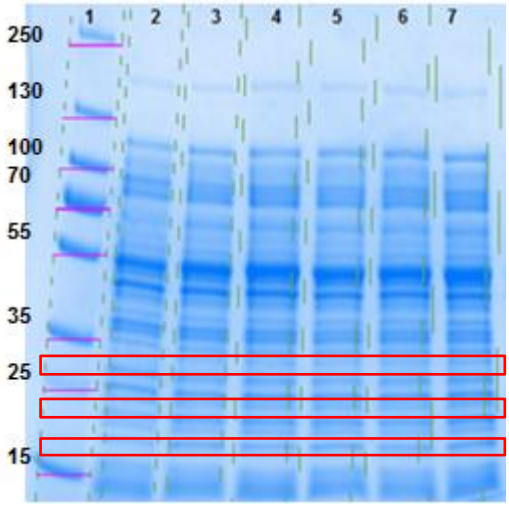

Figure 4.4. An SDS-Page for the extraction of three recombineering proteins: gamma, beta and exo from *E. coli* BW25113 containing the pIJ790 plasmid induced with different concentrations of L-arabinose. (1) Page RulerTM Plus Prestained Protein Ladder (Fermentas); (2) 10 % L-arabinose; (3) 1 % L-arabinose; (4) 0.1 % L-arabinose; (5) 0.01 % L-arabinose; (6) 0.001 % L-arabinose (7) Un-induced. The red rectangles represent the proteins of interest of 28 kDa; 24 kDa and 16 kDa going down, respectively.

The simplification of the recombineering method was shown to be unsuccessful as shown by the inability to express the proteins *in vitro*. The recombineering method was not attempted further and a different engineering method, namely group II introns was studied.

4.3.2. Group II introns

The group II intron method was attempted using the TargeTron® gene knockout kit from Sigma Aldrich.

Gel electrophoresis for viewing PCR products

The first step involves a PCR using specific primers in order to retarget the intron to the target DNA. The results show the experiment as well as both positive and negative control. The gel produces three distinct bands (Figure 4.5). The uppermost 350 bp band is the desired and more prominent band at suitable temperatures (lanes 6-9). Even though the lower two bands are visible, they do not affect downstream applications significantly and therefore do not need to be removed by agarose gel purification (TargeTron® Gene Knockout System).The optimal temperature for the PCR products was found to be between 52°C and 55°C corresponding to lanes 6-9.

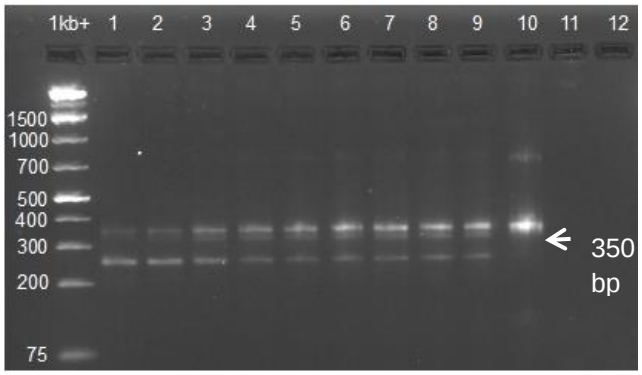

Figure 4.5. PCR for intron re-targeting of the pld gene on a 4% agarose gel. The first lane shows the 1 kb+ DNA ladder. Lanes 1-9 shows the different temperatures of the experiment ranging from 65°C in lane 1 to 52°C in lane 9. Lane 10 shows the positive control of the LacZ gene knockout. Lane 11 and 12 show the negative control (random DNA template) and non-template control respectively.

Transformation and blue-white colony screening

The next steps involve the ligation of the PCR product into a linearized pACD4K-c vector that contains the remaining intron components. The ligation reaction is then transformed into a host organism followed by expression of the re-targeted intron.

The PCR product which was ligated into the linear pACD4K-c vector was transformed into competent *E. coli* DH5α cells and grown on LB plates (Figure 4.6). The experiment and positive control plates (Figure 4.6 A and B), both showed the presence of blue and white

colonies. The plate containing the vector (Figure 4.6 C), only showed blue colonies, while plates containing only the insert or neither the vector or the insert (Figure 4.6 D and E respectively).

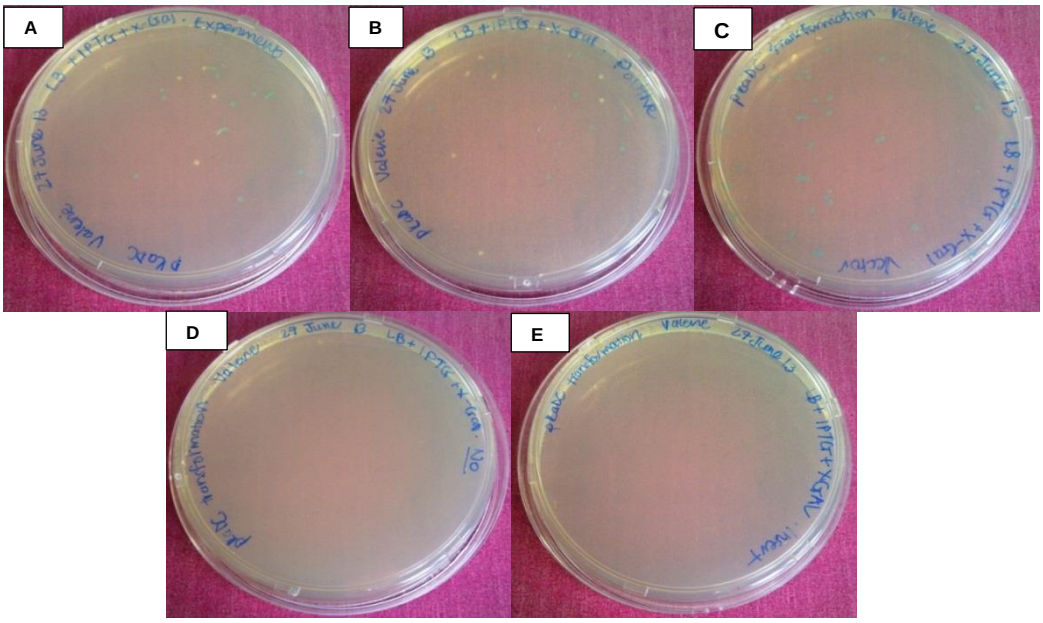

Figure 4.6. Blue-white colony selection plates. (a) Experiment plate (*Pld* gene knockout) containing IPTG, X-GAL and Amp with both blue and white colonies present. (b) Positive control plate (LacZ gene knockout) plate containing IPTG, X-GAL and Amp with both blue and white colonies present. (c) Vector only containing IPTG, X-GAL and Amp with only blue colonies present. (d) Insert only containing IPTG, X-GAL and Amp with no colonies present. (e) Plate containing competent cells only.

The recombinant plasmid obtained from the above (Figure 4.6), was not transformed into *S. albulus* successfully, as it does not have the correct replicon. For these reasons, a new plasmid was constructed in order to create gene knockouts in *S. albulus*.

In silico plasmid construction

An *in silico* construct model was designed using SnapGene Viewer Version 2.2.1 and is shown in Figure 4.7. This plasmid contains all required components in order to use group II introns for gene knockouts in *S. albulus*. The ermE* promoter (yellow) is required for constitutive gene expression of the intron RNA (purple - including the 3' exon) which is required to perform gene knockouts using group II introns. The LtrA ORF (green) contains the genes for catalytic and mobile activity of the intron. The replicon (dark blue) is required for plasmid replication in both *E. coli* and *S. albulus*. The resistance genes: AmpR (light blue) and thiostrepton (red) to allow for selection in *E. coli* and *S. albulus*, respectively.

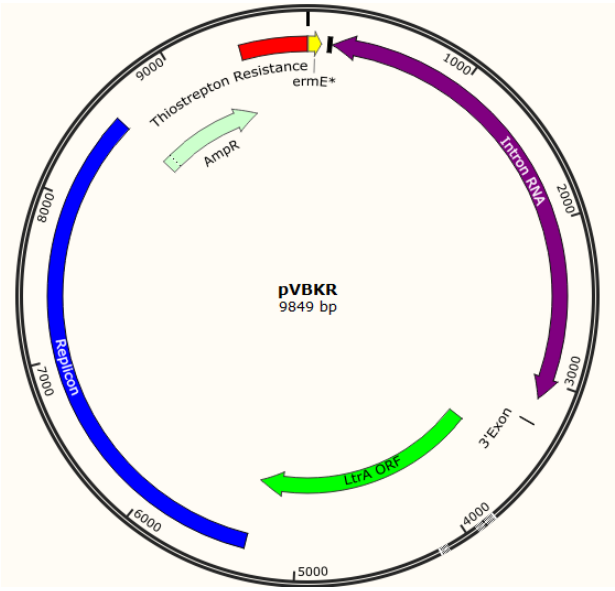

Figure 4.7. An *in silico* design of the pVBKR plasmid. The plasmid shows the necessary regions required to perform gene knockout experiments. The ermE* promoter (yellow) is required for constitutive gene expression of the intron RNA (purple - including the 3' exon) which is required to perform gene knockouts using group II introns. The LtrA ORF (green) is responsible for the catalytic and retrohoming activity of the intron . The replicon (dark blue) is required for plasmid replication in both *E. coli* and *S. albulus*. The resistance genes: AmpR (light blue) and thiostrepton (red) to allow for selection in *E. coli* and *S. albulus*, respectively.

In order to construct the plasmid, the amplification of several of the required regions was performed with specially designed primers to ultimately perform an overlap PCR. The ermE* promoter was successfully amplified from the pIJ12551 plasmid. The 4% agarose gel below shows successful amplification of the ermE* promoter which is 82 bp (Figure 4.8)

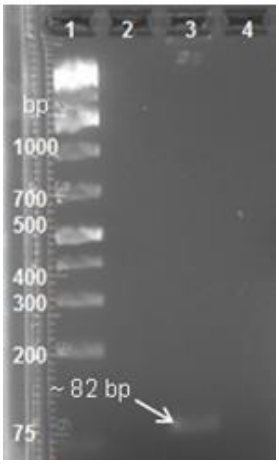

Figure 4.8. A 4% agarose gel showing the successful amplification of the ermE* promoter. (1) GeneRuler™ 1kb Plus DNA Ladder (Fermentas). (2) ermE* concentration 1. (3) ermE* concentration 2. (4) NTC.

Secondly, the intron RNA region as well as the LtrA region required for the gene knockout from the circular pACD4K-c plasmid. The 1% agarose gel below shows successful amplification of the pACD4K-c region which is ~4500 bp (Figure 4.9)

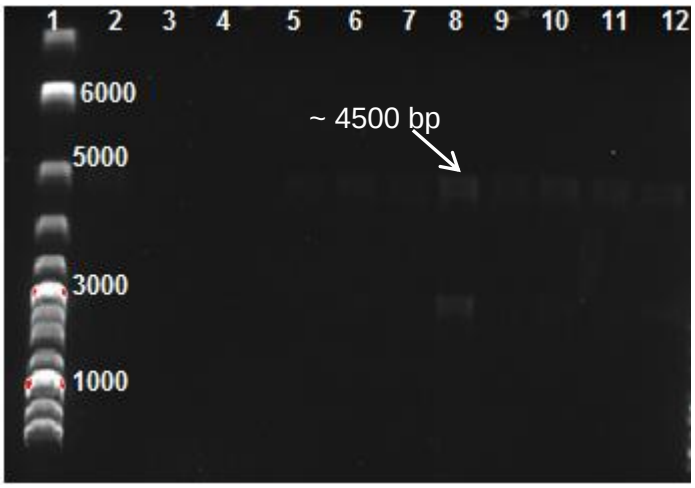

Figure 4.9. A 1% agarose gel showing the successful amplification of the ~ 4.5 kb region on the pACD4K-c plasmid. (1) GeneRuler^{TM} 1kb DNA Ladder (Fermentas). (2) NTC. (3-12) pACD4K-c region amplification replicates.

Lastly, the replicon region required for stable transformation into *E. coli* and *Streptomyces* was PCR amplified from the pLAE001 plasmid. The 1% agarose gel below shows successful amplification of the amplicon is ~4100 bp (Figure 4.10)

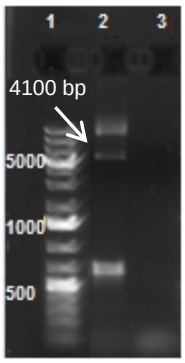

Figure 4.10. A 1% agarose gel showing the successful amplification of the replicon. (1) GeneRuler^{TM} 1kb Plus DNA Ladder (Fermentas). (2) 4.1 kb replicon region. (3) NTC.

4.3.3. Introduction of genetic material

Once a gene knockout has been successful on a plasmid which is in *E. coli*, it must be transferred to *S. albulus*. This can be performed by either chemical or physical

transformation. The prior requires the formation of protoplast in order to ease the process by removing the bacterial cell wall as well as removing the cryptic plasmid pNO33 found in *S. albulus*.

Protoplasts were made and observed under a compound microscope and compared with normal *S. albulus* in order to confirm successful protoplast creation. According to the images, protoplasts were made under optimal conditions of growing the cells at 30 °C for 48 hours (Figure 4.11).

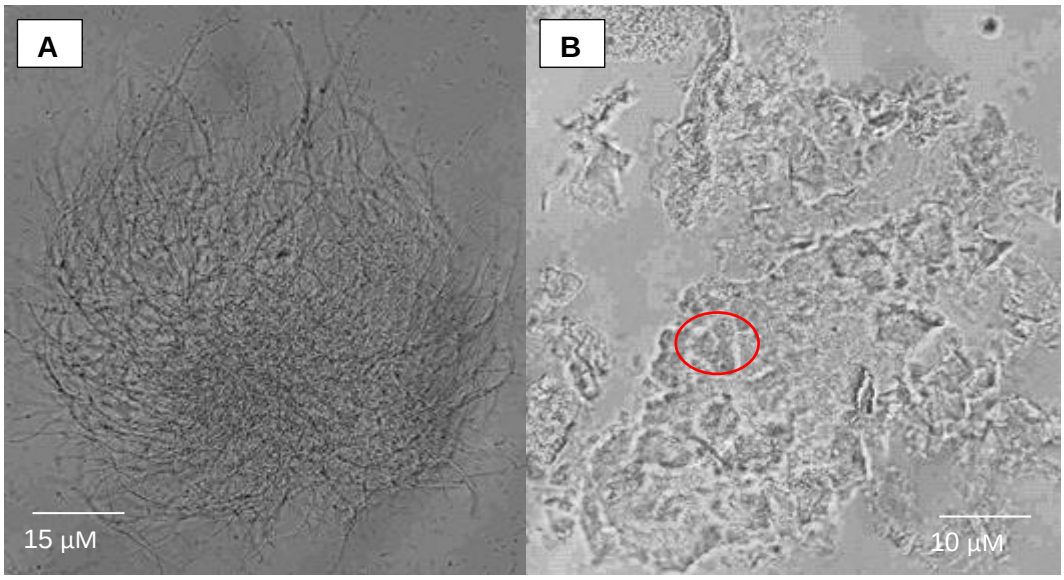

Figure 4.11. *S. albulus* cells and protoplasts under a compound microscope. (a) The filamentous structure of *S. albulus.* (b) Protoplasts which have lost their filamentous shape and appear circular shaped (shown in red) amongst the cell debris. 400 X magnification.

A colony PCR was performed on regenerated protoplasts in order to select for those which had lost their pNO33 plasmid. Primers were designed to amplify a ~650 bp region on the replicon. The presence of a band indicates the presence of a pNO33 plasmid and the absence shows the loss of the pNO33 plasmid. A total of 5 bands were visible on the gel showing the presence of the pNO33 plasmid, while the remainder showed no band (Figure 4.12).

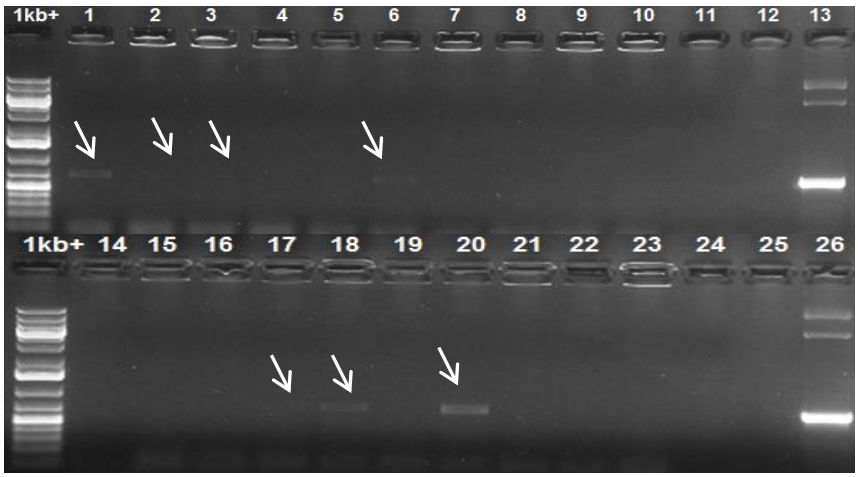

Figure 4.12. Detection of the cryptic plasmid pNO33 in *S. albulus* regenerated protoplasts. (1kb+) GeneRuler™ 1kb Plus DNA Ladder (Fermentas); (1-12) Different colonies on *Rhodococcus media* tested for the presence of the pNO33 plasmid; (13) pNO33 plasmid; (14-24) Different colonies on *Rhodococcus* media tested for the presence of the pNO33 plasmid; (25) NTC; (26) pNO33 plasmid. The arrows show the presence of a band indicating the presence of the pNO33 plasmid.

S. albulus cells that had lost their pNO33 were then transformed with pLAE001 extracted from *E. coli* DH5α using protoplasts and their growth on the regeneration media. The transformation efficiency was at its highest level when the mycelia were grown for 24 h at 30°C on a rotary shaker at 220 rpm.

4.3.3.1. pLAE001 introduction into *S. albulus* via chemical transformation

The pLAE001 plasmid was introduced into *S. albulus* via chemical transformation and the cells were then grown on media containing the selective marker. Chemical transformation showed many colonies on the *Rhodococcus* regeneration media containing a soft nutrient overlay as shown in Figure 4.13 below, after a total of 6 days at 25°C.

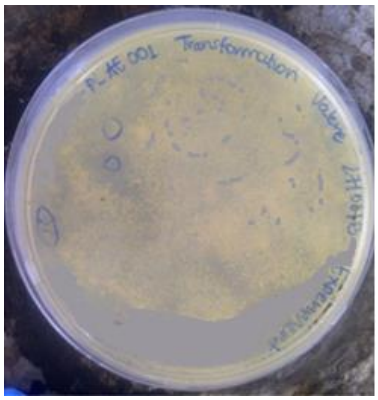

Figure 4.13. Growth of *S. albulus* on *Rhodococcus* regeneration media containing a soft nutrient overlay after transformation. The conditions for growth were 25°C for a total of 6 days. Growth can be seen throughout the entire plate.

Nine colonies were selected to test for the presence of the pLAE001 plasmid. The nine colonies showed the presence of a ~ 650 bp band after colony PCR (Figure 4.14). This shows the success of the chemical transformation of *S. albulus* with the pLAE001 plasmid.

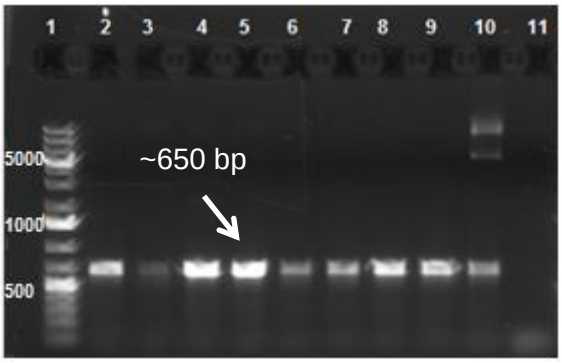

Figure 4.14. A 1% agarose gel showing the successful amplification of a portion pLAE001 from *S. albulus*. (1) GeneRuler™ 1kb Plus DNA Ladder (Fermentas). (2-9) pLAE001 region amplification replicates. (10) pLAE001 plasmid. (11) NTC. The nine selected colonies showed the successful amplification of a region of the pLAE001 plasmid.

4.3.3.2. pLAE001 introduction after electroporation

The pLAE001 plasmid was introduced into *S. albulus* via physical transformation and grown on selective media. Electroporation showed few colonies on the *Rhodococcus* regeneration media containing a soft nutrient overlay as shown in Figure 4.15 below, after a total of 7 days at 25°C.



Figure 4.15. Growth of *S. albulus* on *Rhodococcus* regeneration media containing a soft nutrient overlay after electroporation. There are several colonies visible on the plate indicated by the red circle. The conditions for growth were 25°C for a total of 7 days.

After electroporation, 12 colonies were selected for a colony PCR, which showed no amplification of a portion of the pLAE001 plasmid. There were no transformants containing the plasmid found using all the parameters tested as well as different protocols for creating electrocompetent cells (Figure 4.16).

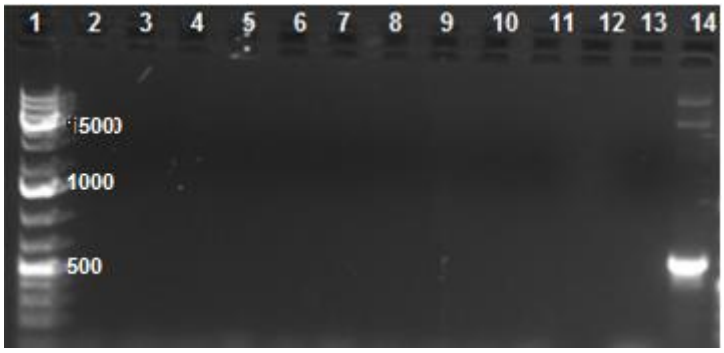

Figure 4.16. A 1% agarose gel showing a colony PCR for the confirmation of the pLAE001 plasmid after electroporation. (1) GeneRuler^{TM} 1kb Plus DNA Ladder (Fermentas); (2-12) pLAE001 region amplification replicates; (13) NTC; (14) pLAE001 plasmid. The 11 selected colonies did no show the amplification of a region of the pLAE001 plasmid.

The two procedures were then compared in order to determine the most optimal one to use for further applications. Transformation is a longer procedure as compared with electroporation; however it yielded a larger amount of transformants. In addition the regeneration efficiency of transformation, 62 % was higher as compared with electroporation, 2.3 %. Comparison of the two gene transfer techniques is shown in Table 4.13.

Table 4.13. Comparison of two gene transfer methods in *S. albulus*

Gene Transfer Method	Organism	Regeneration Effciency (%)	Transformation Efficiency (Transformants / µg DNA)	Type of molecule delivered	Time required
Chemical transformation	Bacteria	62	7.75 ± 3.2	Plasmid	5-7 days
Electroporation	Bacteria	2.3	$3.77 \times 10^{-1} \pm 1.1$	Plasmid	3 days

The table compares two gene transfer methods in *S. albulus* in terms of the regeneration efficiency, which was higher with the use of chemical transformation (62 %) as compared with electroporation (2.3 %). Transformation efficiency was also higher using chemical transformation, 7.75 ± 3.2 transformants/µg, as opposed to $3.77 \times 10^{-1} \pm 1.1$ transformants/µg with electroporation. Lastly, the time required which is longer using chemical transformation of 5-7 days and 3 days for electroporation.

4.4. DISCUSSION

The ultimate goal of this project was to improve ε-PL production; this however was not possible due to two main reasons: 1. There is very little research done on the metabolic pathway of ε-PL production and more specifically on the genes involved in it. In addition, the genome was only recently sequenced (Dodd *et al*., 2013) and during this project the sequence was not known. This made it very difficult to identify targets for selection to perform metabolic engineering. 2. New, metabolic engineering tools are required to quickly and effectively introduce changes into a microorganism and these newer tools have not been developed for *S. albulus.*

For these reasons, the goal of this project shifted to the development of these tools such as group II introns and recombineering for the future of metabolic engineering in *S. albulus*.

Group II introns

The Targetron® Gene Knockout System was shown to be successful in knocking out LacZ in *E. coli*. This was performed in order to confirm the success of the system.

In order to use the system in *Streptomyces*, a plasmid was constructed in order to contain all the required components for the gene knockout and subsequent replication and maintenance in *Streptomyces*. This plasmid could potentially be used in accordance with the components originally found in the kit. According to current literature, this is the first experiment of its kind.

Similar experiments have been conducted, using the same principles as shown above. The plasmid pNL9164 was constructed for gene knockouts in *S. aureus*. Furthermore, the pJIR750ai plasmid was designed for gene knockouts in *Clostridium perfringens.* Both these studies showed successful gene knockouts of *hsa* (Yao *et al*., 2006) and *plc* (Chen *et al*., 2005) gene respectively.

Several concerns may arise through the use of group II introns as a gene knockout method. Firstly, group II introns may be used for any DNA sequence, even those found on a plasmid. Secondly, there is a concern of polar effects on adjacent genes which could reduce the viability of the bacteria. In order to overcome this, the gene of interest should be cloned out of the operon and onto a plasmid. Lastly, there is the issue of whether the knockout will be stable in subsequent generations. A study was performed in *Lactococcus*

lactis in order to test this. Mutants passaged for 80 generations illustrated that 100% of the mutants were stable (Frazier *et al*., 2003).

For future work, to confirm that all the components of the plasmid have been assembled, it should be sequenced. Following this, the plasmid will be tested to perform a gene knockout in *S. albulus*. The first step would be to select a gene based on transcriptomics analysis currently undergoing in our laboratory.

Recombineering

This experiment was unsuccessful in improving the current laborious recombineering method. Based on the study of current literature there is no information on function of the Exo, Beta and Gam proteins *in vitro*. Genes which are placed under the control of the arabinose-inducible paraBAD promoter (PBAD) of *E. coli* are expressed in an "all-or-none fashion" (Khlebnikov *et al*., 2001). The promoter is also referred to as a high level of expression promoter of vectors which possess it (Guzman *et al*., 1995). Even though this is the case, there are several possible explanations for the lack of expression. Firstly, the plasmid could be a low copy number plasmid. Secondly, the genes are being induced but not to a level detectable on a gel because they only function *in vivo* and are not meant for over expression or use *in vitro*. Lastly, the pBAD promoter may be leaky. There however, is no literature to confirm these theories.

A study by Tyurin, (2013) suggested the use of exogenous recombineering proteins to perform gene knockouts and was shown to be successful in *Clostridium* sp. A similar approach can be attempted for use in *S. albulus*. Furthermore, a Western blot could be used in order to confirm the presence of the proteins; however it was not possible to purchase antibodies in time for its completion. Future work could involve the use of a different high expression vector or perhaps a different strain of *E. coli*.

Gene transfer methods

Protoplast formation is important in Gram positive bacteria, because of the thickness of their cell wall, due to the peptidoglycan layer. It is difficult for genetic material to cross this layer and therefore the removal of the cell wall can ease this movement. Protoplast formation is dependent on the age or physiological state of the mycelium. In some cases the older mycelium makes for more successful protoplasts, while in other cases, younger mycelium does. This is species dependent (Kieser *et al*., 2000). High concentrations of glycine can cause loss of the cell wall; this effect, at least in some bacteria, is now known to be due to replacement of D-alanine residues by glycine in the peptidoglycan, thereby

interfering with cross-linking (Hopwood, 1981). Protoplasts were regenerated using sucrose, because sugars are known to be good stabilizers for regeneration as they favour the osmotic stability for protoplast viability (Ishikawa *et al*., 2010).

There is debate on which gene transfer technique is best suited to a particular microorganism. According to literature, electroporation is less laborious than chemical transformation and generally produces higher transformation efficiencies (Hamano *et al*., 2005). This however was not the case in this study, whereby the transformation efficiency was significantly lower and the obtained colonies did not contain the pLAE001 plasmid. There are several explanations for this: The ECM® 830 Electroporation System was mainly but not exclusively designed for mammalian and plant engineering strategies. However, this was the only machine available for use during the course of the experiment. In addition it is also sensitive to salt, and samples can be lost during the electroporation process if traces of salt are detected. This however was minimised due to the elution of the plasmid using nuclease free water. Furthermore, electroporation is also expensive in terms of the requirement of apparatus and special cuvettes.

Further attempts to improve the electroporation procedure for *S. albulus* can be performed by verifying and optimising physical, biological, and chemical parameters.

Transformation, although a longer method, was shown to be very successful in terms of regeneration frequency and transformation efficiency. This coincides with other studies in which *S. albulus* was successfully transformed with a slightly higher transformation efficiency of 10^2 transformants per µg DNA (Hamano *et al*., 2005).

Apart from chemical transformation and electroporation, conjugation has been of interest as it can bypass the need for protoplast formation and regeneration. In an experiment using *S. albulus*, the conjugation efficiency was very low (exconjugants per recipient spores: 9.0×10^{-8}) (Hamano *et al*., 2005) and therefore also requires optimisation before it can be used as a successful method of gene transfer.

Newer engineering techniques

In a recent study, *pls* was cloned from strain a newly identified *S. albulus* strain NK660 by genome walking. The *pls* gene was heterologously expressed in *S. lividans* ZX7. This recombinant strain was shown to synthesise ε-PL. This was the first experiment showing heterologous expression of the *pls* gene in *S. lividans*. This would allow the possibility of

working with a model *Streptomyces* organism, for which engineering strategies have been developed, for improvement of ε-PL production (Geng *et al*., 2014).

Furthermore, as previously mentioned, the metabolic engineering relates to the concept of biocatalysis (Shen and Thorson, 2012). Biocatalysis for industrial biotechnology has shown significant growth in the last decade (Bornscheuer, 2012). It is therefore of interest to apply biocatalysis to the metabolic engineering of *S. albulus* to improve ε-PL production as previously attempted (section 1.9.2). One such way is through the use of lipases in order to decrease the amounts of salts and methanol from the crude glycerol and therefore increase ε-PL yield. Lipases are an attractive alternative to attempt as they have been described as the most important group of biocatalysts for biotechnological applications (Hasan *et al*., 2006). Future work can also involve increasing surface expression of Pls, by the addition of lysine, which would promote a pure biocatalysis route to ε-PL yield improvement. This will allow the use of natural catalysts to improve ε-PL yield and therefore make its use as a natural food preservative applicable.

4.5. CONCLUSION

Metabolic engineering techniques are only applicable with the knowledge of well characterized biosynthetic pathways and to organisms for which molecular tools are available and optimized to some extent. In addition, an extensive knowledge of the biochemistry and physiology of the producing bacteria in order to select appropriate metabolic alterations (Paradkar *et al*., 2003; Li *et al*., 2013). This however, was not the case with *S. albulus* which could partially account for the difficulty in developing techniques. Furthermore, finding a target and increasing ε-PL production in a short period was not possible owing to lack of genetic backgrounds (Li *et al*., 2013).

This study has acted as a stepping stone for research to commence in this field, primarily since the recent sequencing of the *S. albulus* genome. Such research could include the study of metabolic flux of both precursors and intermediates and to identify rate limiting steps which can act as targets for genetic manipulations. Once the genetic techniques have been established potential gene targets for knockout to improve ε-PL production should be looked at.

CHAPTER 5. FINAL CONCLUSION AND FUTURE WORK

S. albulus has the ability to grow and produce ε-PL in the presence of crude glycerol, although at a suboptimal level as compared with glucose and pure glycerol. Due to the fact that crude glycerol is not costly, it provides an economically feasible method of growing and producing ε-PL for its use as a natural food preservative. However, *S. albulus* grows slowly and the production process is not optimal. Hence, the need to develop tools and protocols for metabolic engineering of *S. albulus* in order to increase ε-PL production.

Recently, there has been research performed on *S. albulus*, which will allow for further characterisation of genes and proteins. These would be able to create a more detailed picture on the metabolome and allow for the improvement of techniques for gene-knockouts or knock-ins. In addition it will allow for the selection of targets to knock-out/in in order to improve glycerol uptake and ε-PL production.

In general, *Streptromyces* compared to other microbial production hosts, are not a very popular target for metabolic engineering, because they are not as well characterized as for example *E. coli* and therefore requires more elaborate and time consuming procedures. With the current rate at which the field of biotechnology and genetic engineering is developing, however, the possibility of newer and more efficient techniques is not far off.

There has been a large amount of work in the field of biotechnology in terms of secondary metabolite improvement during the last decade. Different methods used for genetic and metabolic engineering have their advantages and limitations and each may be suited to a particular *Streptomyces* species. The growth potential in the bio-based economy is a strong pulling force for bioprocessing and biocatalysis, especially for high-value compounds produced by microorganisms such as *Streptomyces*. This can be employed in the case of engineering *S. albulus*.

This work has laid the foundation for the future of ε-PL production improvement using economically viable crude glycerol as a carbon source for potential use of the secondary metabolite as a food preservative.

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7. APPENDICES

7.1. Recipes

Luria Broth (LB) agar (1litre)

Bacto-tryptone (Merck)	10 g
Yeast Extract (Merck)	5 g
Sodium Chloride (Merck)	10 g
Bacto-agar (Merck)	15 g
2% glucose (Merck)	7.5g

All components are mixed together and autoclaved (Labotech) for 20min at 121°C. Once cooled slightly, 20ml of LB is poured into 88mm diameter petri dish until solidified.

Luria Broth (LB) medium (1litre)

Bacto-tryptone (Merck)	10 g
Yeast Extract (Merck)	5 g
Sodium Chloride (Merck)	10 g
2% glucose (Merck)	

All components are mixed together and autoclaved for 20min at 121°C.

Defined Media (1 litre) (Rumbold *et al*., 2009)

NH_4Cl	8 g
$(NH_4)_2SO_4$	0.5 g
$MgCl_2•6H_2O$	0.3 g
EDTA	40 mg
$ZnSO_4•7H_2O$	2 mg
$CaCl_2•2H_2O$	1 mg
$FeSO_4•7H_2O$	15 mg
$Na_2MoO_4•2H_2O$	0.2 mg
$CuSO_4•5H_2O$	2 mg
$CoCl_2•6H_2O$	0.4 mg
$MnCl_2•4H_2O$	1 mg

All components are mixed together; the pH is adjusted to pH7.3 using phosphate buffers: 0.75M K_2HPO_4 and 0.75M KH_2PO_4 and the media is purified using sterile syringe 0.45µm pore filters.

10x PBS (250mL)

Sodium Chloride (NaCl)	8 g
Potassium Chloride (KCl)	0.2 g
Na_2HPO_4	1.78 g
KH_2PO_4	0.24 g

All components are mixed together and autoclaved for 20min at 121°C.

10x TAE (1litre)

Tris base	48.4 g
Glacial acetic acid (17.4 M)	11.4 mL
EDTA, disodium salt	3.7 g

Add up to 1 litre with distilled water and mix all components together.

.**1 % Agarose gel**

Add 1 g of agarose (Bioline) to 100 mL 1x TAE. The powder is then melted and cooled and 3µl of ethidium bromide (10 µg/ml) (Bioline) is added to the solution.

4% Agarose Gel

Add 4 g of agarose (Bioline) to 100 mL 1x TAE. The powder is then melted and cooled and 3µl of ethidium bromide (10 µg/ml) (Bioline) is added to the solution.

Rhodococcus regeneration media

35 g of sucrose was dissolved in 280 mL of dH_20. After which the following was added:

Agar	4.5 g
Tryptone	3 g
Yeast Extract	1.5 g
Sodium Chloride	0.9 g

The solution is autoclaved for 20 minutes at 120°C.

After autoclaving, the following (filter sterilized – 0.22 µm) was added in this order:

0.25 M TES	10 mL
1 M Calcium Chloride	6 mL

SOB (1 litre)

2% Peptone
0.5% Yeast Extract
10 mM NaCl
2.5 mM KCl
10 mM each $MgCl_2$ and $MgSO_4$

Pipes buffer

10 mmol/l $CaCl_2$,
10 mmol/l PIPES-HCl,
15% glycerol v/v

pH to 7 using sodium hydroxide

Protein lysis buffer

The following components are mixed together in 1 litre and autoclaved:

KH_2PO_4 (the buffer)	6.8 g
$MgCl_2.6H_2O$	0.61 g
DTT*	0.77 g
EDTA	0.37 g

*DTT is added after autoclaving in order to prevent its degradation.

The protein can be stored in the buffer until further use.

7.2. Clean up protocols

7.2.1. NucleoSpin ® Extract II Cleanup (From PCR product, if there are no spurious bands)

1. 2 volumes of NT buffer with 1 volume PCR product are mixed (45µl PCR product and 90µl NT buffer).

2. A column is placed in a collection tube and the sample is loaded onto the column and centrifuged for 1 minute at 11 000 g. The flow through is discarded and the column is placed back in the collection tube.

3. 600µl of NT3 buffer (to which 70% ethanol is added to before use) is loaded onto the column and centrifuged at 1 minute at 11 000 g. The flow through is discarded and the column is placed back in the collection tube.

4. The column and collection tube are then dry centrifuged for another 2 minutes at 11 000 g. The samples are incubated for 2-5 minutes at 70°C to evaporate the ethanol. The flow through is discarded and the column is placed in a clean Eppendorf tube.

5. 20µl of elution buffer (at 70°C) is loaded onto the column and the samples are incubated for 1 minute at room temperature to increase the yield. The samples are centrifuged at 1 minute at 11 000 g. The column is discarded and the cleaned PCR product is found in the Eppendorf tube.

7.3. Plasmid Isolation

1. Bacterial suspension of 3 mL is centrifuged using a benchtop microcentrifuge for 30 s at 11 000 x g. The supernatant is discarded.

2. The cells are resuspended in 250 µl of buffer A1 by vortexing. This is followed by the addition of 250 µl of buffer A2. The mixture is then inverted 6-8 times and handled with care to avoid shearing of genomic DNA. The suspension is then incubated for 5 min at room temperature, after which 300 µl of buffer A3 followed by inverting the tube 6-8 times.

3. The tube is then centrifuged for 5 min at 11,000 x g at room temperature.

4. A NucleoSpin® Plasmid QuickPure Column is placed in a collection Tube (2 mL) and 750 µL of the supernatant is pipetted onto it. The tube is centrifuged for 1 min at

11,000 x g. The flow-through is discarded and the NucleoSpin® Plasmid QuickPure Column is placed back into the collection tube.

5. Buffer AQ is added to the tube (450 µl) and it is centrifuged for 3 min at 11,000 x g.

6. The NucleoSpin® Plasmid QuickPure Column is placed in a 1.5 mL microcentrifuge tube and pure plasmid is eluted by the addition of 50 µL of Buffer AE. The tube is then incubated for 1 min at room temperature and centrifuged for 1 min at 11,000 x g.

7.4. Creating Competent Cells

1. Streak DH5α cells unto a SOB agar plate and allow to grow overnight (16-20 hours) at 37˚C.

2. Inoculate a single colony in 5 mL of SOB medium and grow the culture overnight (16-20 hours).

3. The following day, subculture the 5mL overnight culture into 50 mL of LB and allowed to for 90 min or until the culture reached OD600 of 0.3 to 0.5.

4. Centrifuge the 50 mL culture for 15 min at 3000 g, 4˚C, discard the supernatant and resuspend the pellet gently in in ice cold 5 ml PIPES buffer.

5. Incubate on ice for 20 min, centrifuge at 1000 g for 10 min at 4˚C, discard the supernatant and gently resuspend the pellet in 2 mL PIPES buffer

Store the competent in 100µl aliquots

7.5. L-Lysine production pathway

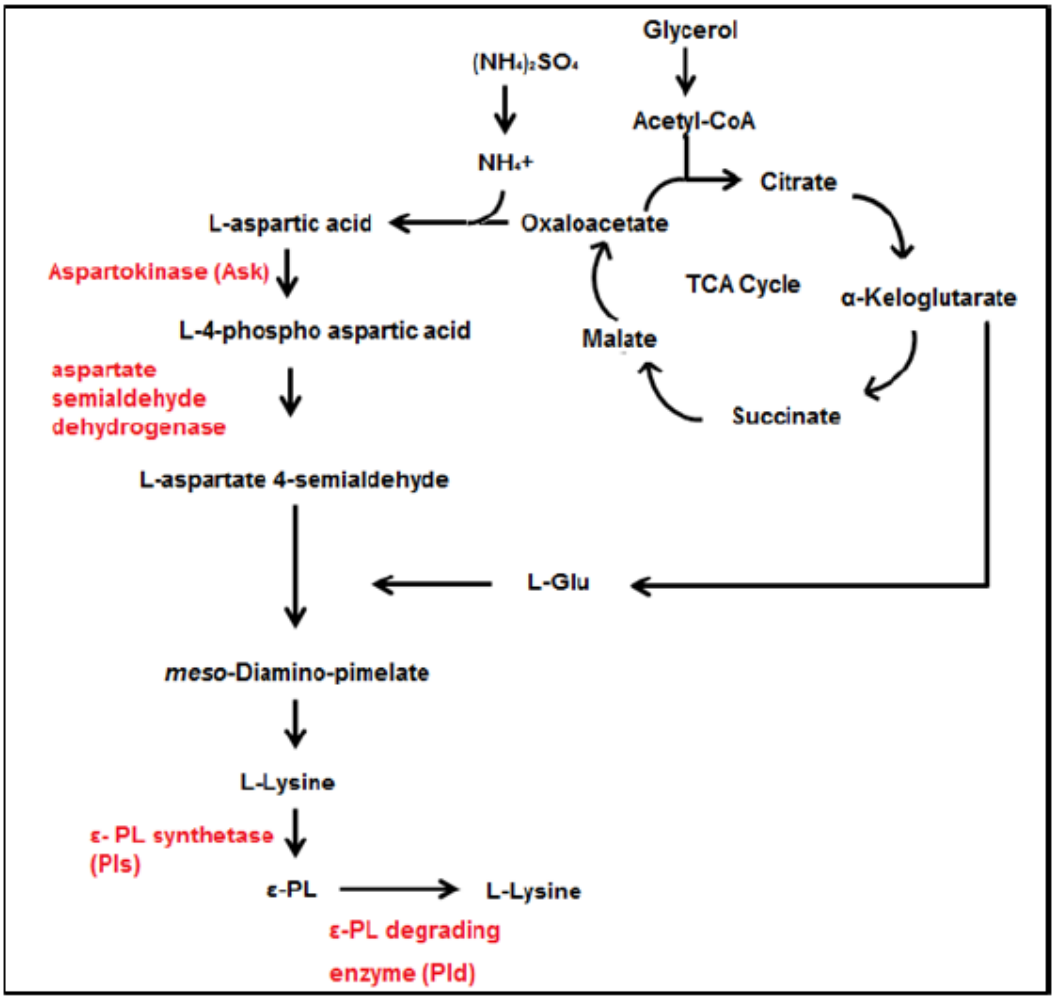

Figure A.1. A simplified metabolic pathway of glycerol utilization to ε-PL production in *S. albulus*. The pathway involves the TCA which the carbon source such as glycerol enters. This is then converted into oxaloacetate, followed by L-aspartic acid. The L-aspartic acid then undergoes a series of reactions to form L-lysine, whereby the Pls enzyme is involved in converting it into ε-PL (adapted from Hamano et al., 2007; Hamano et al., 2010).

7.6. Plasmid Maps

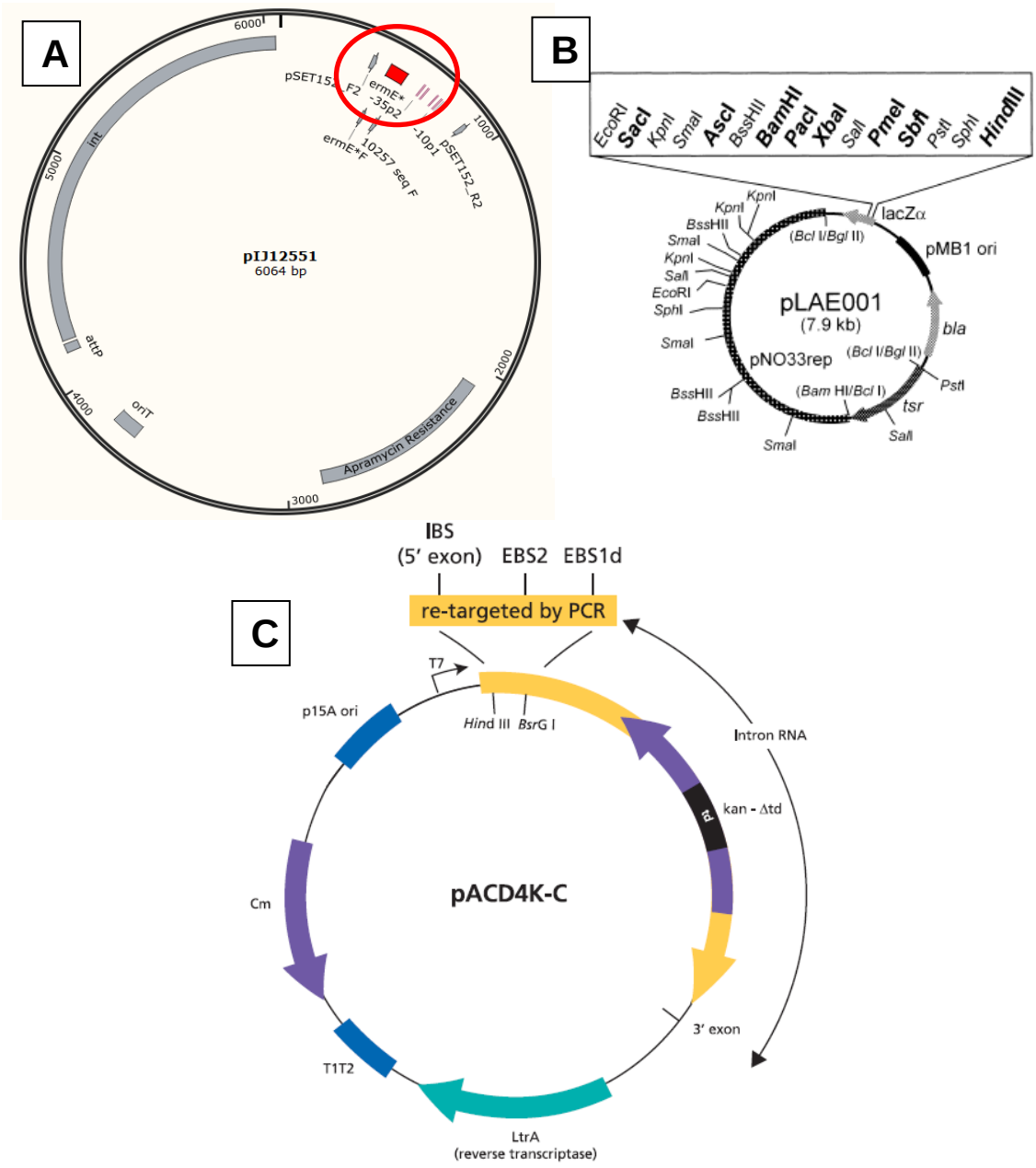

Figure A.2. The plasmids used to create a novel vector for gene knockouts in *S. albulus*. (a) pIJ12551 plasmid containing the ermE* promoter. (b) pLAE001 plasmid containing the replicon region. (c) pACD4K-c containing the genes required for gene knockouts using group II introns. (Hamano et al., 2005; TargeTron® Gene knockout system user guide, 2013).

7.7. Statistical analysis

Table A.1. Students t test for the significance of growth studies with *P. simplissimum*.

	50 µg/mL	100 µg/mL	200 µg/mL
Mean	46.85714286	46.85714286	46.85714286
Variance	884.8928571	884.8928571	884.8928571
Observations	7	7	7
Pearson Correlation	0.980407575	0.984575526	0.961654685
Hypothesized Mean Difference	0	0	0
df	6	6	6
t Stat	3.201475853	3.516521515	3.928956404
P(T<=t) one-tail	0.009282732	0.006285745	0.003861002
t Critical one-tail	1.943180281	1.943180281	1.943180281
P(T<=t) two-tail	0.018565465	0.012571491	0.007722005
t Critical two-tail	2.446911851	2.446911851	2.446911851

The t stat value is greater than the t test which indicates a significant value at 95 % confidence level.

Table A.2. Students t test for the significance of growth studies with *A. niger*.

	50 µg/mL	100 µg/mL	200 µg/mL
Mean	25.5	25.5	25.5
Variance	163.4166667	163.4166667	163.4166667
Observations	7	7	7
Pearson Correlation	0.971745614	0.981218494	0.980296505
Hypothesized Mean Difference	0	0	0
df	6	6	6
t Stat	0.373785225	-1.942314387	-2.842450232
P(T<=t) one-tail	0.360708078	0.050060061	0.014736016
t Critical one-tail	1.943180281	1.943180281	1.943180281
P(T<=t) two-tail	0.721416156	0.100120123	0.029472032
t Critical two-tail	2.446911851	2.446911851	2.446911851

The t stat value is less than the t test which indicates no significant value at 95 % confidence level.