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Upgrading of biodiesel-derived glycerol in the biosynthesis of e poly L lysine an integrated biorefinery approach

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Upgrading of biodiesel-derived glycerol in the biosynthesis of ε-Poly-L-lysine: an integrated biorefinery approach.

MSc Biotechnology Research Report

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A report submitted to the School of Molecular and Cell Biology in partial fulfillment of the requirements for the Masters of Science Degree in Biotechnology

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ORIGINALITY DECLARATION

I, the undersigned, hereby declare that the work contained in this research report is my own original work, except as acknowledged in the report and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:  Date: 23 July 2010

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DEDICATION

I would like to dedicate this research report to my family for instilling in me the good and hard works values and sticking to their principles in stressing the value of education. Their encouragement, sacrifices and pride in my work and the zeal for education has always been my motivation to further my studies.

ABSTRACT

Crude glycerol, a by-product and major drawback of biodiesel, 10% by weight, once a valuable product is now considered a waste associated with disposal costs because of salts and methanol impurities. With an exponential increase of the biodiesel producers in South Africa and beyond, excess crude glycerol of concern as its supply exceeds its demand, creating excess glycerol of less value. Although purification is an option, South Africa does not have purification plants and exporting such negatively-valued glycerol is not economically viable. Innovative ways to dispose of crude glycerol are clearly necessary.

The study investigated the feasibility of upgrading crude glycerol as a carbon source in the aerobic production of ε-Poly-L-lysine using *Streptomyces albulus* (CCRC 11814). The polymer has a number of applications in food, biomedical and agricultural industries which exploit its water solubility, polycationic, biodegradability, edibility and non-toxicity to humans and the environment.

A total of 8 crude glycerol samples from 6 biodiesel producers around South Africa were analysed of elemental composition, as well as glycerol and methanol using an Inductively Coupled Plasma – Optical Emission Spectrometry and an HPLC respectively. The results of the only work of such analysis in South Africa showed that potassium salts were the main impurity, glycerol and methanol concentrations varied from one producer to the next.

This work compared growth and polymer production when *S. albulus* was grown in pure and crude glycerol. This work is important as it is the first to report *Streptomyces albulus* growing on crude glycerol (20g/l) (after reports that *Escherichia coli* and *Clostridium glutamicum* do not grow at all, with *Sachoromyces. cerevisiae* growing slowly) with remarkable biomass accumulating comparable to pure glycerol. Although, *S. albulus* grew slowly in methanol-containing glycerol (≤ 30g/l), the attribute could negate the costly methanol removal step. 0.219 g/l of ε-Poly-L-lysine produced is similar to 0.2 g/l from a wild strain reported in the literature.

These results show that biodiesel-derived crude glycerol is a promising, accessible and lowly-priced alternative carbon source for the industrial production of ε-Poly-L-lysine and its integration to an existing biodiesel business to reduce the production costs and hence increase its profitability and sustainability.

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CHAPTER 1

1.1 INTRODUCTION

The world's economy powered by petroleum fuels as the main energy source is facing a gradual decline and the move towards a bio-based economy has been touted a noblest interest. The fossil reserves are projected to be completely depleted by the year 2050 (Campbell and Laherrère, 1998; Sheehan *et al*., 1998) and assuming the current alarming population growth rate, the post-petroleum unsustainable period is clearly in sight.

With the advent of technology in the modern era, there is more dependence on petroleum energy for food and lifestyle enhancement forcing markets to respond by increasing costs (Nowicki *et al*., 2008). In addition, petroleum deposits have become more and more difficult to extract as resources have been overexploited (Gallan *et al*., 2009). Climate change and other negative environmental effects from exhaust gases and industrial petroleum usage have ameliorated the search for renewable alternatives (Hill *et al*., 2006). Biodiesel has attracted attention as a mitigation effort for anthropogenic carbon dioxide emissions which have led to the current soaring global warming and subsequent climate change (Banholzer *et al*., 2008).

Biodiesel is an attractive alternative (Vasudevan and Briggs, 2008; Adhikari *et al*., 2008), clean burning, non-toxic and biodegradable (Petersen and Reece, 1994) fatty acid methyl ester compound produced by a transesterification process of animal or plant oils with methanol in the presence of a catalyst (Lemke, 2006; Wirawan and Tambunan, 2006; Marchetti *et al*., 2007). Research has proved that renewable fuels reduce green house gas emissions through complete combustion and carbon dioxide sequestration by plants and algae (Askew, 2005).The process of production and use of biodiesel reduces carbon dioxide (78%) and carbon monoxide (50%) and nearly 100% less of sulphur dioxide (Sheehan *et al*. 1998).

Above all, biofuels popularity is driven by the upsurge of petroleum fuels coupled with the governments' support in the renewable fuels through incentives (Cole *et al.*, 2008).

Biodiesel has several advantages but the major challenge is the by-product of biodiesel production, glycerol also known as glycerin, produced in large quantities (Jonhson and Taconi, 2007; Thompson and He, 2006). Approximately, 10% by weight crude glycerol is formed via transesterification of oils (Dasari *et al.*, 2005), for example, for every 100kg of biodiesel, 10 kg of glycerol is produced. Unfortunately, the by-product is generated by the main reaction which is unavoidable (Gallan *et al.*, 2009).The dramatic increase in biodiesel production has led to surplus crude glycerol on the market lowering the prices of glycerol and hence the need to reconsider it as an industrial feedstock for bioprocesses. In addition, the cost to purify this low grade glycerol for conventional uses is prohibitively too high (Johnson and Taconi, 2007), therefore new value added uses for this crude glycerol are necessary.

Although, biodiesel by-product, glycerol conversion into value-added products has been widely touted the best approach to profitability and sustainability of biorefineries, very few studies are undergoing in the literature, to the best of our knowledge, documenting the use of crude glycerol as a sole carbon source in industrial bioprocesses as large volume outlets of the waste stream. In addition, most documented studies are putting more emphasis on the production of fuels and a few have focused on the production of biopolymers which are a "multi-punch" in a number of industries serving different markets thereby stabilising the biodiesel industry. Economically, it will be more advantageous to use strains that utilise crude glycerol to negate the costs associated with its purification.

The objective of this study is to explore the potential of using crude glycerol in industrial bioprocesses, more specifically in the biosynthesis of ε-Poly-L- lysine (ε-PL) using

Streptomyces albulus (CCRC 11814). ε-PL production using *S. albulus* (Shima and Sakai, 1977), *S. lydicus* and other *Streptomyces* sp (Hirohara *et al*., 2006) using glucose as a carbon source was studied. However, an opportunity exists to utilise this abundant low grade glycerol in the production of such biopolymers as a new way of disposing of this waste stream to develop new markets in an integrated biorefinery approach. ε-PL is a polyamino acid made of L-lysine monomers with a variety of biological and chemical functionalities, and therefore has multitude of uses in the food, biomedical, environment and other industries that have been identified by Shih *et al*., 2006.

The present study also seeks to characterise the elemental composition, as well as glycerol and methanol content from different sources of crude glycerol from biodiesel producers around South Africa. This allows the investigation of the effects of the impurities in the growth of *S. albulus* and therefore production of the biopolymers. In addition, to the creation of a new and safe way to dispose of crude glycerol, the study entails producing a polymer for the chemical, foods and feeds industries in South Africa and beyond. This integrated biorefinery approach creates a symbiosis between the food industry and the biodiesel industry other than creating the competition which partly solves the contentious food versus fuel issue.

1.2 The glycerol challenge

The rapid increase in the demand for renewable alternatives of petroleum fuels has led to an excess of glycerol in the market (Whittall and McClean, 2007; Hirschmann *et al*., 2005), making the epichlorohydrin process no longer economical (Miller *et al*., 2008; Dharmadi *et al*., 2006; Deckwer, 1995) forcing closure of existing glycerol production plants (McCoy, 2006).

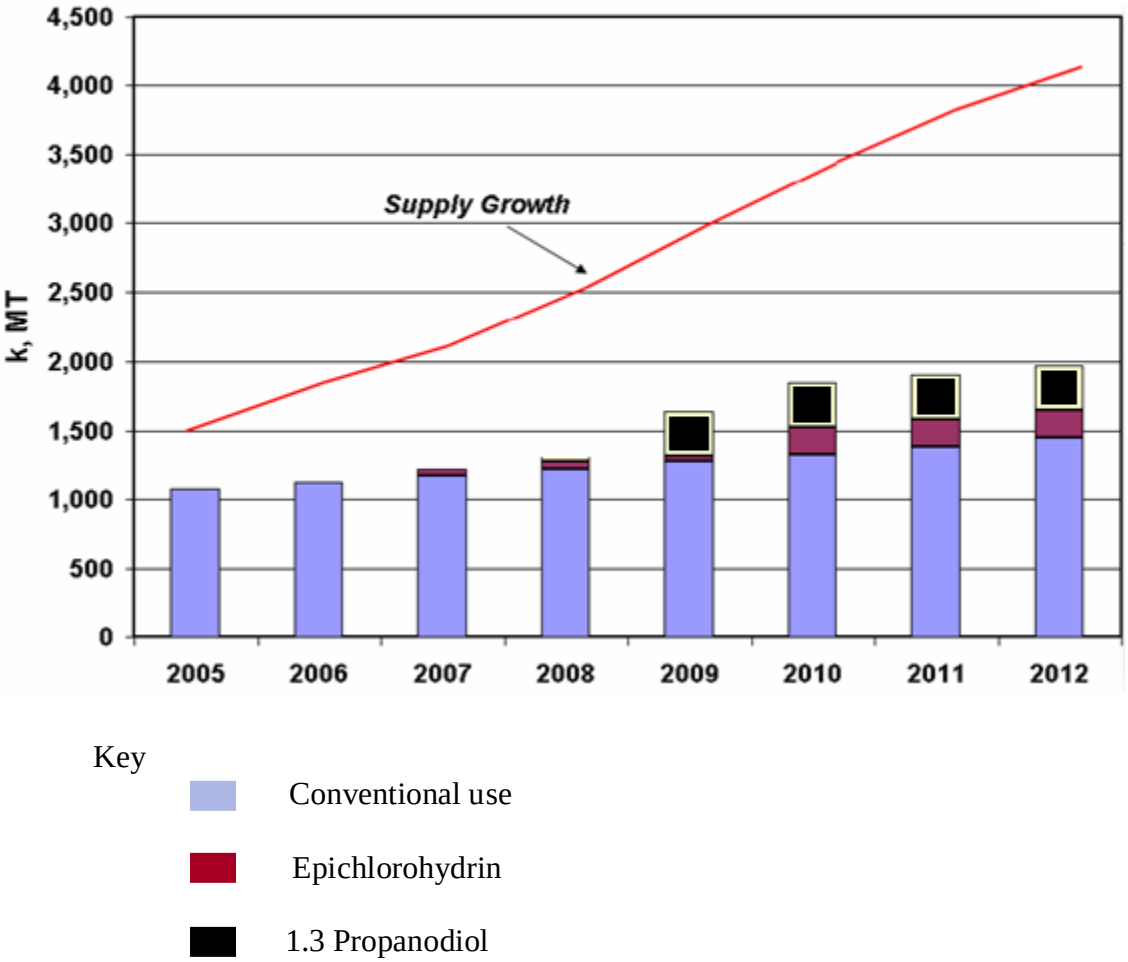


Figure1. Glycerol uses and growth demand estimation (Adapted from, PIRA, Cargill, 2007)

Crude glycerol, whose supply is greater than its demand (Figure 1), and once considered a valuable commodity that could increase biodiesel competence on the market is now considered a waste stream entailing a cost for disposal (Yazdani and Gonzalez, 2007; Wilke and Vorlop, 2004). Glycerol conventional uses in foods, pharmaceutical and the epichlorohydrin process from which it was made from can longer accommodate the surplus.

The number of biodiesel producers in the world has increased dramatically, with Germany producing around 2.5 billion litres per year and expected to grow by 5% in 2013 (Banse and Gerther, 2008; Macedo *et al.*, 2004). Brazil, in their 2% decrease in gasoline usage is set to reach 8 billion litres by 2020 (Pousa *et al.*, 2007). Canada produced over 2.1 billion litres in the year 2008 (Canada Biofuel Annual Report, 2008). South Africa injected R1.5 billion to

produce biodiesel from soya beans for its 5% projected figure replacement for petroleum fuels by 2013 (Parallax Report, 2006). South Africa consumed about 24 billion litres in the year 2008 and its consumption is projected to increase exponentially by 5 % every year (SAPIA, 2008). As does the increase in the production of biodiesel, so does the by-product, glycerol.

Pure glycerol had a stable price in the 1970s ranging at most $2 000 per tonne, until the last decades of arrival of biodiesel making the market volatile (Miller-Klein Associates, 2006). In 2005 the price fell to approximately $300 per tonne and falling dramatically until then (Miller-Klein Associates, 2006). In 2006, the global market reached about a million tonnes of glycerol, with two thirds from biodiesel production, lowering the price of glycerol to just below the 2005 value (Pagliaro and Rossi, 2008). However, biodiesel-derived glycerol contains between 15 – 30% residual methanol, fat or oil remains, sodium or potassium hydroxide, esters, low amounts of sulphur and other elements, proteins and minerals and other impurities making it of less economic value and toxic for conventional uses before purification (Elik *et al.*, 2008; Buffington and Steinman, 2007; Thompson and He, 2006). Excess methanol is used in the transesterification reaction and much is not recovered. Fatty acids remaining in the reaction react with bases to form soaps which are soluble in the glycerol layer. Crude glycerol concentrations produced from various feedstocks in the biodiesel production process range from 60 - 80 % (Thompson and He, 2006).

Crude glycerol costs $0.10 /kg whereas its purification costs range from $0.10/kg to $0.15/kg of which much is associated with freight costs and hence purification for industrial grades is not a viable option (Johnson and Taconi, 2007). The Glycerol Price Report in Europe reported at most $80 per tonne in 2008 (Horlock, 2008). However, current prices of the

abundant waste stream have not been publicised in the year 2009 possibly due to the scramble for markets.

Pure glycerol has a variety of uses such as food and feeds in the animal industry (Johnson and Taconi, 2007), pharmaceuticals as an ingredient in drugs (Sneha *et al*., 2009), cosmetic and toiletries as a humectant, softener in tobacco, paper and textile industries (Wang *et al*., 2001). Crude glycerol alternative uses other than its purification are being sought because of its global glut. Johnson and Taconi, 2007 reported the use of crude glycerol in combustion as a way of disposal whereas Brady, 2008 looked into the creation of renewable energy. Some researchers have suggested the use of this crude glycerol in composting and feeding of anaerobic digesters to produce biogas (Holm-Nielsen *et al*., 2008; Brown, 2007). DeFrain *et al*., 2004; Cerrate *et al*., 2006; Lammers *et al*., 2008 and others have documented the use of this waste stream in animal feeding with promising results but the authors still warn of the unknown negative effects associated with the impurities in the glycerol, specifically methanol.

Although a multitude of industrial uses of glycerol have been existent, they cannot absorb this excess which has exceeded the market demand forcing the prices to be negligible (Whittall and McClean, 2007). This has led to large amounts of glycerol waste stream in the hands of the biodiesel producers increasing their costs of disposal and thus incurring costs in the business (Brady, 2008). Unbelievably, surplus glycerol is currently disposed of by incineration (Whittall and McClean, 2007; O'Driscoll, 2007), dust suppression on dusty roads and even as a landfill (Buffington and Steinmann, 2007). However, land application with glycerol-containing methanol is prohibited by the Kyoto Protocol, incineration increases gaseous emissions aggravating the problem of gaseous emissions at hand whereas glycerol stored in fryer oil jugs leaks over a period of time, and methanol in it is a fire hazard because

it is very flammable (Buffington and Steinman, 2007). Biodiesel producers boast their environmentally friendly petroleum fuel alternative but avoid the question of disposal of waste glycerol which is equally damaging to the environment.

1.3 Glycerol as a feedstock for biorefineries

The utilisation of pure glycerol for industrial processes has been limited by the high market prices in the last decades (Johnson and Taconi, 2007). However, use of pure glycerol in bioprocesses has been shown to be feasible as it is an intermediate in a number of metabolic processes in microorganisms (Homann *et al*., 1990; Wang *et al*., 2001; Johnson and Taconi, 2007) such as *Klebsiella pneumoniae* (Xiu *et al*., 2004), *Lactobacillus reuteri* (Talarico *et al*., 1990), *Yarrowia* spp, *Candida* spp, *Rodhodotura* spp (Ashby *et al*., 2005; Papanikolaou and Aggelis, 2002; Papanikolaou *et al*., 2002) and *Clostridium spp* (Papanikolaou and Aggelis, 2003; Macis *et al*., 1998, Biebl *et al*., 1992), under anaerobic conditions. Only *Clostridium spp* have been reported to utilise glycerol as a sole carbon source independent of the presence of Vitamin B12 as a co-factor (NADH source) without altering the 1, 3-propanediol quality (Petitdemange *et al.*, 1995; Raynaud *et al*., 2003).

A number of organic acids have been produced from the fermentation of glycerol as a carbon source using *E. coli* (Darmadi *et al*., 2006). Very few microbes have been reported to metabolise glycerol in the absence of hydrogen acceptors (fermentatively), for example, *Enterobacteriaceae* family which has been studied in great detail (Bouvet *et al*., 1995). Glycerol can be metabolised either via the oxidative pathway or the reductive pathway (Bouvet *et al.*, 1995). The former involves the dehydrogenation of glycerol by the NAD-linked glycerol dehydrogenase to form dihydroxyacetone which is then phosphorylated by the ATP-dependent dihydroxyacetone kinase. The latter reaction, a reductive pathway,

involve the coenzyme B_{12}- dependent glycerol dehydratase which dehydrates glycerol to produce 3- hydroxypropionaldehyde before the formation of 1.3- propanediol (PDO) in the presence of the NADH-linked 1,3-PDO dehydrogenase (Figure 2).

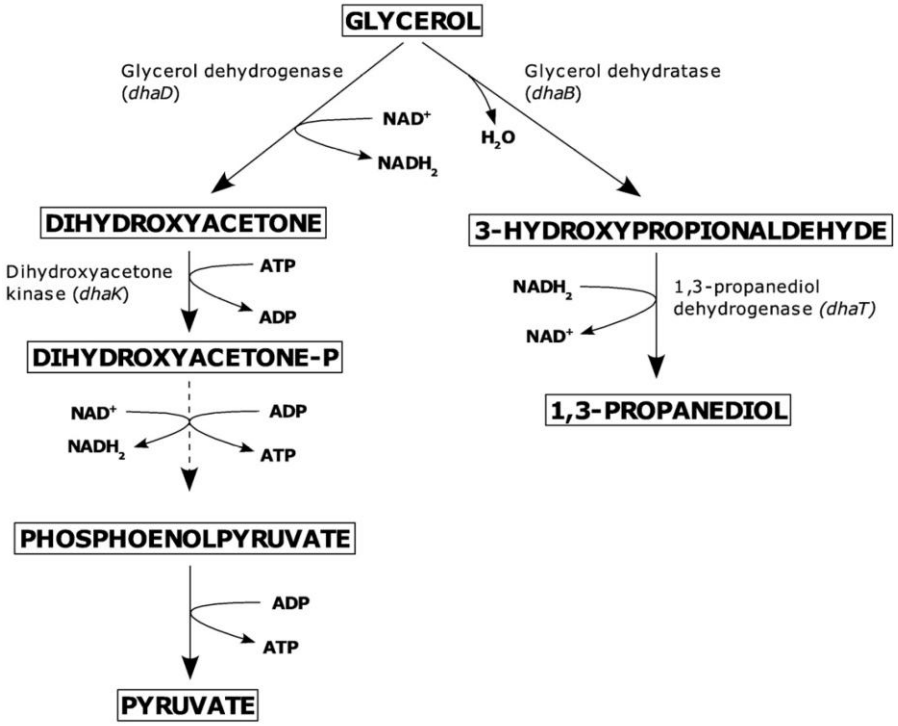


Figure2. Glycerol metabolism pathway in the *Enterobacteriaceae*. The enzymes and steps involved in glycerol metabolism are shown. NAD+ is regenerated when pyruvate is reduced to different organic compounds depending on the fermentation conditions. (Adapted from Bouvet *et al.*, 1995).

Aerobic processes utilising glycerol are limited because of the energy involved in aeration as glycerol is syrupy impeding the oxygen transfer (Pachauri and He, 2006). However, the current technological advancement allows use of bioreactors with powerful spargers and dilute glycerol to be used in such processes. In comparison with an optimal substrate, glucose, glycerol metabolism is in short fall of NAD required in its metabolism (Johnson and Taconi, 2007), but when an external hydrogen acceptor is added, cells are able utilise glycerol at a lag phase about the same rate to glucose (Magasanik, *et al*. 1953). The two carbon sources are structurally similar carbohydrates and thus they can possibly be used interchangeably in industrial bioprocesses. Given the current excess amounts on the market

(availability and accessibility) and projected negative prices (affordability), which presents ideal lowly-priced, renewable and accessible, industrial feedstocks, glycerol is a neglected carbon source (Yazdani and Gonzalez, 2007). However, conventional industrial processes utilise glucose as a sole carbon source which can be attributed to the markets when it was adopted.

1.4 The biorefinery concept

The concept of producing multiple products from biodiesel wastes, raw materials and by products is a new approach similar to the petroleum industry refineries where fossil fuels are used as inputs in the production of a wide range of products (Zwart, 2006). Biorefineries are an attempt to produce bio-based chemicals and foods simultaneously with liquid fuels: an economic option through integration of technologies which increase the energy efficiency of biofuels production (Dermirbas, 2009). The integration entails the conversion of biomass and upgrading of wastes leading to a large diversity of intermediate products, co-products and feedstock availability (Dermibas and Dincer, 2008). A generalisation of a biorefinery concept is a complete flow of wastes or by-products optimised between different industrial units, in which such by-products are a raw material of the next unit and their wastes being raw materials for the third one (Zwart, 2006).

The concept allows the use of every component in the processing of biodiesel, from the raw materials right to the wastes produced to produce high-value/low-volume useable products which increase productivity of the resource and hence improving economics and decreasing the environmental burden associated with waste disposal (Taylor, 2008). The approach allows products from the biodiesel industry to be able to compete cost-wise with those from the oil industry (Puhan *et al.*, 2005). The level of integration within the biodiesel processing

industry brings economic and production advantages and increases the potential of replacing petroleum with renewable bio-products.

Yuste and Dorado (2006), reported that at least 90% of the costs incurred in the biodiesel process are associated with feedstocks, on the other hand process profitability and sustainability is determined by how the by-products and their derivatives are used (Morrison, 2008). Alternative uses of glycerol to produce higher value-added products could play an important role in the establishment of an integrated biorefinery concept where co-products and wastes are used to make valuable products that can defray expenses in the production processes. The conversion of such waste streams in the modern world of dwindling resources, presents an opportunity to identify new and economically viable processes to produce value added products from limited resources available. It is desirable to produce a commodity with a higher price and large market to increase profitability and sustainability of biodiesel plants (Johnson and Taconi, 2007).

A number of researchers are investigating crude glycerol uses as a feedstock in the catalytic production of chemicals and industrial fermentation, (Figure 3), (Wang *et al*., 2001, Xiu and Zeng, 2008). These processes involve biologically mediated and biochemical processes which are widely recognised as energy efficient and environmentally friendly as compared to non-specific, energy intensive chemical processes (Papanikolou *et al*., 2008).

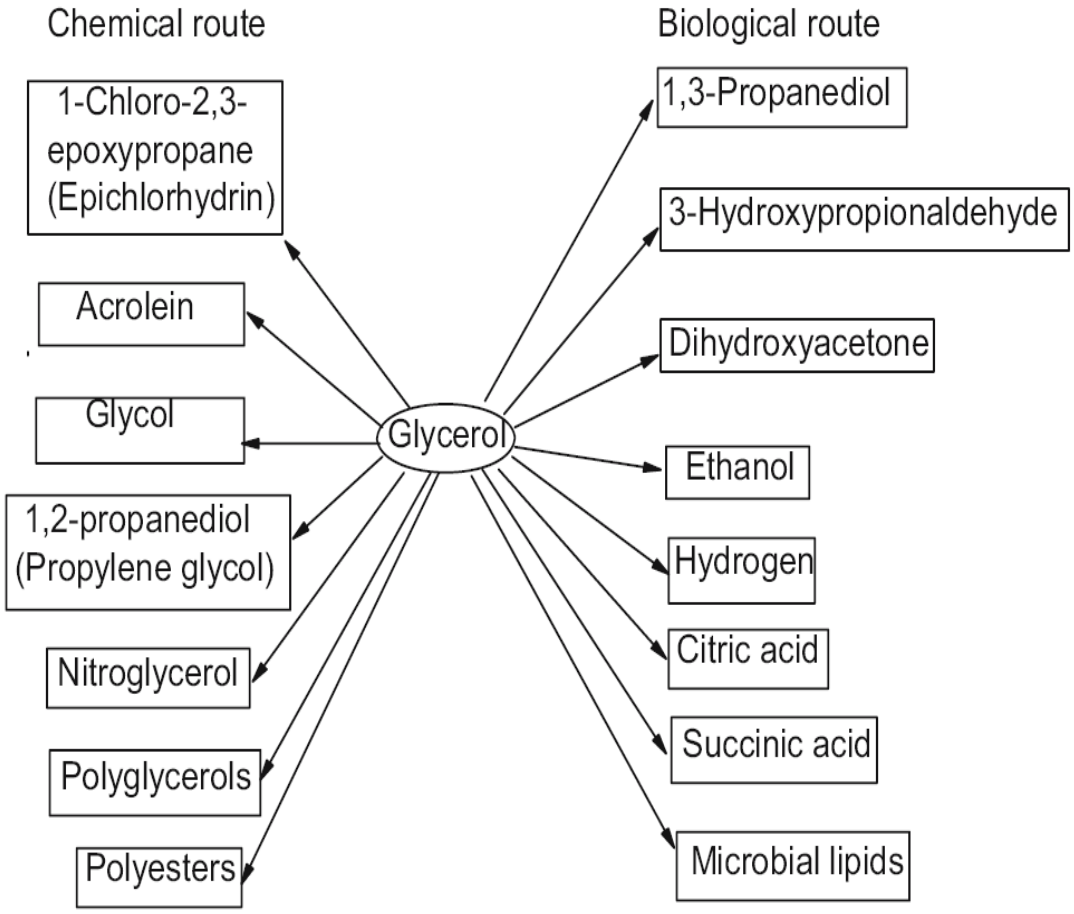


Figure 3. Glycerol utilisation by chemical and biological routes. Examples of potential products for both routes are shown (Adapted from Xiu and Zeng, 2008).

1.5 ε-Poly-L-Lysine

ε-PL is a cationic, basic homopolymer made of L-lysine residues accidentally discovered in the search for Dragendorff's positive substances by Shima and Sakai (1977), produced by *Streptomyces albulus* 346. The polymer consists of approximately 25-30 lysine residues (Figure 4) (Shima *et al.*, 1983), where ε-amino group forms an amide bond with a α-carboxyl group (Shima *et al.*, 1984).

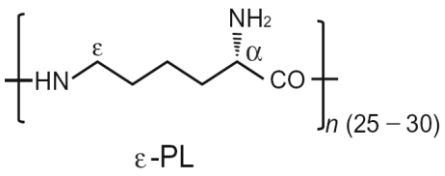


Figure 4. Repeating units of ε-PL (Adapted from, Shima *et al*., 1984).

ε-PL was reported to be produced non-ribosomally where the L-lysine molecule synthesised by the amino acid pathway from the aspartic acid (Figure 5) is directly polymerised (Kawai *et al*., 2003; Shima *et al*., 1983). L-aspartic acid formed from oxaloacetate by transamination is phosphorylated to produce L-4- phospho aspartic acid by aspartokinase and reduced to its semialdehyde by aspartate semialdehyde dehydrogenase. L-lysine is then formed from the decarboxylation of this semialdehyde (Girodeau *et al*., 1986; Hamano *et al*., 2007). Activated L-lysine then undergoes a condensation reaction to produce ε-PL (Kawai *et al*., 2003). SO_4^{2-} is responsible for the adenylation of lysine and is used as part of the co-substrate in polymerisation process (Kawai *et al*., 2003). However, enzymes responsible for the polymerisation process are still unknown (Kawai *et al*., 2003; Yoshida and Nagasawa, 2003).

ε-PL is a polymer that has its applications as a food preservative; because it is cationic it is adsorbed by bacterial cell surfaces in ionic interactions facilitating cell lysis (Hiraki, 1995; Shima *et al*., 1984). This antimicrobial activity makes it relevant to be considered an alternative natural food preservative to reduce the unknown health problems associated with conventional chemical preservatives (Hiraki, 2000). Neda *et al*., 1999 reported that ε-PL is non-mutagenic and non-toxic in his experiments with rats to high doses. Hiraki *et al*., 2003 documented that up to 20 000 ppm of ε-PL was given to rats in the diet experiments and no adverse effects were witnessed. In addition, ε-PL was found to be non-toxic to reproduction and development of rats up to the second generation (Neda *et al*., 1999). In Japan, ε-PL has been approved as generally safe as a food preservative in rice, noodles and other traditional foods (Hiraki *et al*., 2000). It can also be used in combination with other food additives to enhance its activity through synergistic effects (Hiraki, 2000).

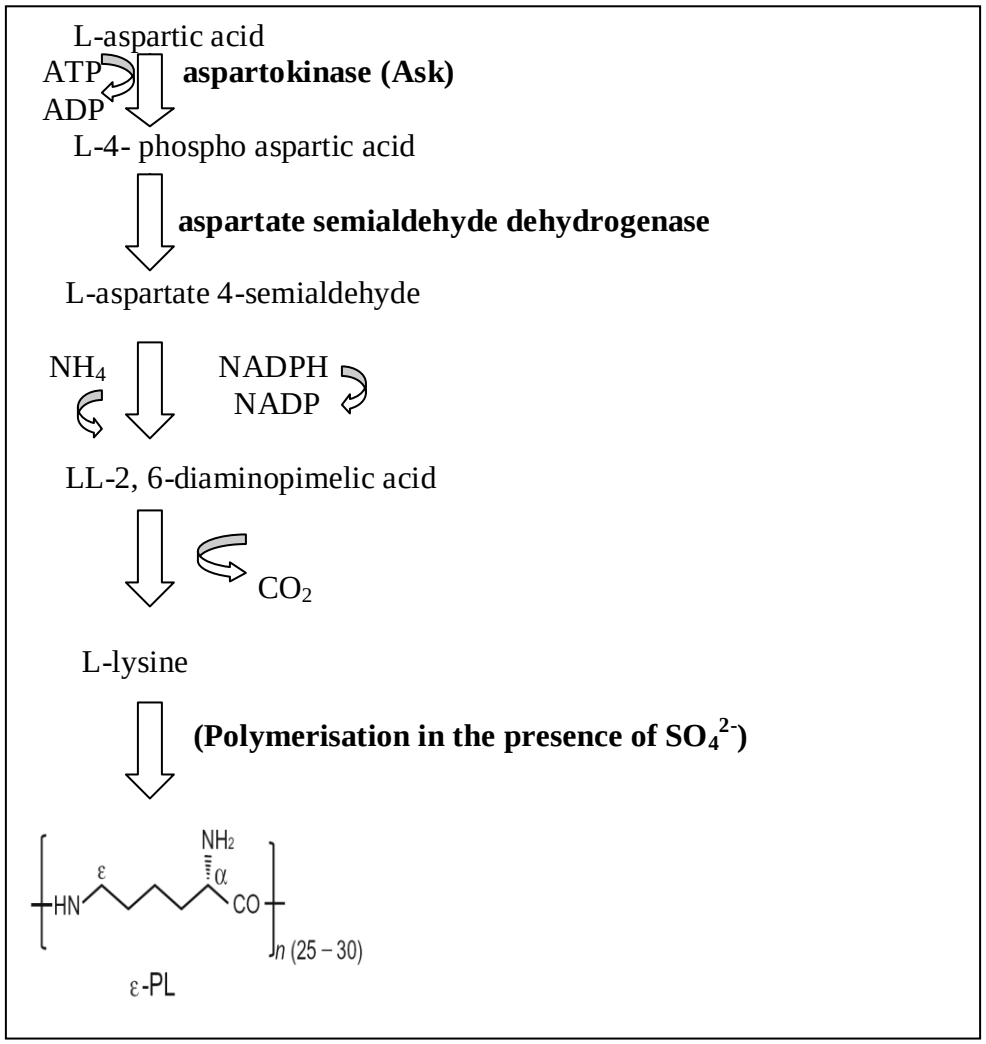


Figure 5. ε-PL biosynthetic pathway via the aspartate pathway (Adapted from Hamano *et al.*, 2007).

Nishikori, (2000) documented that the formulations of ε-PL and citrus fruit seeds extract were effective against disease causing bacteria such as *Salmonella typhirium*, *Vibrio Cholerae*, *Staphylococcus aureaus* , *E. coli* O-157:H7, *Candida* spp and inhibits yeasts, moulds and a wide range of gram positive and gram negative bacteria (Yoshida and Nagasawa, 2003)

ε-PL has been used as a dietary agent due to its ability to suppress pancreatic lipases (Kido *et al*., 2003; Tsujita *et al*., 2006). These lipases are responsible for the absorption of lipids from the intestine (Duan, 2000). If these lipases are inhibited, serious health problems including obesity leading to hypertension and diabetes can be avoided (Hill *et al*., 2000). In addition, it

has been linked as drug delivery carrier where a drug is covalently bonded to one of its many functional amino groups (Gad *et al*., 1982; Ryser and Shen, 1978), water absorbent hydrogels (Choi *et al*., 1995), and other applications in food, biomedical and agricultural industries which exploit its water solubility, polycationic, biodegradability, edibility and non-toxicity to humans and the environment (Shih *et al.*, 2004; Shih *et al.*, 2006).

In addition, ε-PL is water soluble disintegrating into L-lysine residues which have an application as feed additives in livestock production which forms the basis of the African economy. There is currently only one producer of L-lysine in Africa which is producing it from molasses, a feedstock ten times as expensive as the crude glycerol. Production of L-lysine from crude glycerol innovation could be well received by both farmers and biodiesel producers as the product is both environmentally and economically valuable offsetting production costs of biodiesel which are significantly higher than its competitor, non-renewable diesel, as well as reducing meat and meat products prices that are rising each year in South Africa (Baseline Report, 2008). This study of conversion of waste streams into feed additives comes at a time when South Africa and other parts of the continent are battling upsurges of animal feed prices. South Africa consumes about 9000 tonnes of L-lysine every year in feed additives of which 4500 tonnes are imported and thus the need to avail L-lysine in the country to reduce the costs associated with feeds and feed additives (Webber Wentzel, 2007).

The innovation at hand could be a link between the uses of cellulosic materials as feedstocks for second generation fuels, enabling the replacement of the use of crop remains as animal feeds which has been the basis of "food versus fuel" debates reducing biofuels acceptance in South Africa. Instead of using nutrient deficient, pathogen infested, difficult to digest crop remains, the innovation aims to avail high quality nutrient feed additives which have far

much greater economic benefits. The study also aims to investigate the potential of utilising crude glycerol from South African biodiesel plants, without any purification as a potential to negate purification costs by eliminating the methanol and soap removal steps.

1.6 Aim of the study

To investigate the feasibility of using biodiesel-derived glycerol as a feedstock in industrial processes in a biorefinery approach to increase profitability and sustainability of biodiesel.

1.6.1 Objectives

1. To analyse the biodiesel and ε-Poly-L-lysine markets in South Africa.
2. To analyse crude glycerol composition from different biodiesel producers.
3. To produce ε-Poly-L-Lysine using *Streptomyces albulus* grown on crude glycerol as feedstock.
4. To investigate the effects of impurities in glycerol on the fermentation process.

CHAPTER 2

MATERIALS AND METHODS

2.1 Crude glycerol analysis

Crude glycerol was acquired from 6 different biodiesel producers around South Africa. The elemental analysis of 8 different crude glycerol samples was conducted at the Environmental Chemistry Laboratory at the University of the Witwatersrand. Metals were extracted from about 2g of sample during acid digestion. The digested solution was diluted to 100 ml and analyzed in triplicate on Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). The average results obtained (ppm) were converted into the amount in the sample then expressed in term of mg per kilogram of sample (equivalent to ppm).

Expression used for calculations: Y.105.D/M

Y = value obtained from the run of the solution (ppm)

D = dilution factor of the solution to be run on ICP-OES (required to be in the range of detection)

M = mass of sample weighed (mg)

Crude glycerol and methanol were analysed at the School of Chemical Engineering at the University of the Witwatersrand using an Agilent 1200 series HPLC with Refractive Index Detector (RID temp 40 ºC) on a Bio-rad Aminex Fermentation monitoring column (Particle size 9µm; 150x7.8mm) at 60 ºC using 0.001M H_2SO_4 in bi-distilled water as the mobile phase . The flow rate was maintained at 0.8ml/minute. A calibration curve of methanol and glycerol against the area under the curve was used to estimate their concentration.

2.2 Strain and culture conditions

The bacterium *Streptomyces albulus* (CCRC 11814) used throughout this study was a gift from Bernille Verster at the University of Capetown who acquired it from Ing-Lung Shih, Da-Yeh University in Japan. The strain was maintained in yeast-starch agar medium containing, per litre: 2.0 g yeast extract, 10.0 g soluble starch, 15.0 g agar. 1 M NaOH was used to adjust the pH of the medium to 7.3.

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 $CaCl_2.2H_2O$, 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 $MnCl_2.4H_2O$ (Sigma-Aldrich) was used for both the seed and culture production throughout the study. The initial pH was adjusted with 0.75 M K_2HPO_4 and 0.75 M NaH_2PO_4 (Merck SA) and the media was not buffered thereafter. The carbon source was added to these media as described below.

The media was autoclaved as separate stock solutions at 120°C for 20 minutes and mixed after cooling. Growth conditions (temperature; 30°C, pH; 7.3) were maintained as previously reported (Hirohara *et al*., 2006).

2.3 Growth of *S. albulus* in glycerol

Analytical grade glycerol (Merck, South Africa) was used as a carbon source to determine the utilisation of glycerol by *Streptomyces albulus*. To investigate the optimum pH at which *S. albulus* grew, the strain was grown on solidified medium at pH 3, 4, 6, 7 and 9. The strain was then grown on solidified media (1.5% agar) containing different concentrations of glycerol (1%, 2%, 5%, and 10%) using the dilution plate technique. The different concentrations of glycerol in the medium were to determine the optimum level of glycerol

utilisation by the strain. A series of dilution tubes containing 9ml of sterile saline were prepared. A loopful of culture colonies was placed in a dilution tube and mixed thoroughly. Serial dilutions were performed, 0.1ml of the final dilution (third) were inoculated onto the solidified medium and spread with a sterile, bent glass rod. Growth of the bacterial strain was measured by colony counts after incubation at 30°C where numbers of colony forming units (CFU) were counted using a Reichert® Darkfield Quebec colony counter (Reichert Analytical Instruments Inc, USA) for 12 days. Commercially supplied starch (Merck, South Africa) was used as a control. With the colony counts obtained in CFU per millilitre, maximum glycerol utilisation percentage was noted.

The cells were also grown in 1L Erlenmeyer flasks containing 200ml of minimum medium containing different concentrations of glycerol and incubated at 30°C in an orbital shaker at 220 rpm. For seed culture, a loopful of CCRC 11814 was inoculated into a 250-ml Erlenmeyer flask containing 50 ml of minimum medium and precultured at the same temperature and rotation speed overnight. 10ml of seed culture were inoculated into each flask and cultured up to 7 days. 1ml samples from each flask were drawn daily using sterile pipettes and used for cell density and pH determination. Cell growth was monitored by OD (Optical Density) readings at 660 nm using a Novaspec II UV-Vis spectrophotometer (Pharmacia Biotech, England) with minimal medium as a blank and a Crison BASIC 20^{+} pH meter (Lasec, South Africa) was used for pH measurements. A growing curve was constructed from the cell density readings and later used as a standard percentage carbon source in subsequent experiments.

The procedure was repeated with crude glycerol samples acquired from different biodiesel producers in South Africa to investigate the effects of impurities in the growth of S. *albulus* CCRC 11814. 20g/l of each crude glycerol sample was used to grow the strain and the best

utilised sample was chosen for subsequent experiments. The chosen sample was diluted to 20g/l with distilled water so as to investigate the feasibility of using a diluted sample in industrial applications. However, this time cell density was difficult to determine given the turbidity of crude glycerol. Measurement of cell concentration using a spectrophotometer requires obtaining a clear solution. Culture broth was centrifuged at 2500 rpm for 10 minutes using an Eppendorf minispin centrifuge (Eppendorf AG, Germany) and supernatant discarded leaving cells and salts. Cells and the salts mixture pellet was resuspended in saline solution for 3 times and subsequent centrifugation to remain with cells only. Cell density was then determined with the saline solution as a blank using the same procedure with samples from pure glycerol. The best utilised glycerol sample was chosen for subsequent experiments.

Methanol in crude glycerol is one of the microbial growth impeding impurities (Çelik *et al*., 2008) whose effects in growth of *S. albulus* were determined. Using the same minimum medium and conditions, methanol in different concentrations (0.5%, 1%, 2%, and 3%) was added and cell density was compared to the medium without methanol. Autoclaving methanol at 120 $^{\circ}$C for 15 minutes completely evaporates it (Pyle *et al*, 2008), thus to study the effects of methanol on the growth of *S. albulus*, methanol was sterile filtered using 0.2µm filter and added to the medium after autoclaving. For each experimental condition, three replicates were used, and the standard deviation was calculated.

To investigate the feasibility of using the abundant crude glycerol as an industrial carbon source, cell growth in pure and crude glycerol were compared to an experiment with glucose as a carbon source.

2.4 Production of ε-Poly-L-Lysine (ε-PL)

Using a two stage process described by Hirohara *et al*., (2006), and Shima *et al*., (1983), *S.albulus* was grown in petri-dishes with solid media (solidified by 1.5% agar) containing minimum medium and different concentrations of pure glycerol (1%, 2%, 5%, 10%, 20%) as a carbon source. The first stage was done at a neutral pH for cell growth and the second at an acidic pH for ε-PL production because ε-PL is a basic polymer that is produced by cells under acid conditions (Shima *et al*., 1984).

The *Streptomyces* culture was then suspended in a saline solution (0.09% NaCl) and streaked on prepared media plates and incubated at 30°C for 24 hours for cell growth. Colonies that grew were transferred to agar plates containing the same carbon source and medium with a basic dye methylene blue and incubated to track the extracellular polymer diffusion. The method developed by Nishikawa and Ogawa, (2002) assumes that colonies that produce ε-PL will condense an acidic dye or exclude a basic dye (a halo is seen around the colony) because ε-PL is cationic; the dyes will be attracted or repelled respectively. The method is biased as any basic polymer can produce the same results (Shih *et al*., 2006). However, the structure can be defined by further analysis.

Shake flask fermentations were carried out in 1l Erlenmeyer flasks as outlined in section 3.1.1 as a small scale application of the positive experiments. The method of production was used to allow higher growth rates of the strain due to enough aeration possible with shakers as glycerol is viscous impeding the oxygen transfer rate. All other subsequent growth and production experiments were then done in shake flasks.

2.5 Isolation and Purification of ε-PL

After 7 days of incubation at 30°C, a halo around each colony was observed (see Appendix E). The area around each colony was excised and washed to remove impurities from the media and then heated at 100°C for 30 minutes to destroy the agar. After cultivation in shake flasks fermentations, culture broth was centrifuged at 10 000g for 30minutes using a Beckman J2-21 centrifuge (DJB Labcare Ltd, England) to remove cells and the supernatant containing the polymer was transferred to test tubes. ε-PL was then precipitated by an anionic dye, methyl orange (MeO) (Merck Chemicals (Pty) Ltd, South Africa), as previously described by Wetlaufer and Stahmann, (1952). This is a solvent extraction method in which the principle is ionic interactions of oppositely charged molecules. The solvent will bind to the polymer with polycationic sites as it is negatively charged forming a water-insoluble complex and thus precipitating it out of the solution (Reed *et al*., 1994). To separate the polymer from the dye, the complex was dissolved by boiling in 1M hydrochloric acid and then filtered using filter paper. The pH of the filtrate was adjusted to 8.5 using 0.1M NaOH and then separated using cation exchange as reported by Lee *et al*., 1991 and Shima and Sakai, 1977. The method, reported by Hirohara *et al*., 2006 is based on charged molecules binding with oppositely charged molecules on the matrix. ε-PL has its pI at pH 9, hence the elution buffer was run at pH 8 - 8.5 assuming that the further the working pH is from the pI of ε-PL, the tighter the binding of ε-PL and the last to elute using a buffer of increasing concentrations (Hirohara *et al.*, 2006).

The filtrate was applied on an OMNIFIT column (Sigma-Aldrich) with carboxymethyl (CM) cepharose resin (Merck Chemicals (Pty) Ltd, South Africa) and washed successively with 0.2N acetic acid and water under a constant gravity flow. Positively charged molecules at the elution pH are retained on the column whereas negatively charged molecules are washed out. Positively charged molecules which included our product, ε-Poly-L-lysine, were eluted with

0.1N hydrochloric acid as hydrogen ions displaced them. The eluent was neutralised with 6M NaOH to pH 6.5 and then decolourised with active charcoal. A colourless product was concentrated by evaporation. The elution profile was monitored using a JASCO UV-Visible spectrophotometer: V530 (JASCO Corporation, Japan) at 215 nm.

2.6 Analysis of ε-PL

2.6.1 Thin Layer Chromatography

The concentrated product from cation exchange was run on a thin layer chromatography plates (Merck Chemicals (Pty) Ltd) as a pilot procedure before HPLC analysis. Thin layer chromatography is based on a multistage distribution process of separated substances between a mobile phase and a stationary phase (Stahl, 1969). As each component is different in physical and chemical composition, the interaction between the mobile and the stationary phase is different and therefore individual bands corresponding to the distance migrated will be evident. Depending on the solubility of the side chain each amino acid will have a different migration rate. 90:10 ethanol and acetic acid was used as a mobile phase which carries the samples up the plate by capillary action. Samples to be analysed from the cation exchange and commercially acquired ε-Poly-L-lysine hydrobromide and lysine hydrobromide (Sigma Aldrich Co) were spotted on the TLC plate using a thin capillary tube and dried using a hair dryer. The spotted TLC plate was placed sample side down in a beaker containing the mobile phase and solvent was left to travel about 85% to the top. The TLC plate was carefully removed and immediately a pencil line was drawn at the solvent front and the chromatogram was allowed to dry completely on a hot plate stove. To develop the plates, the plates were taken to a fume hood and sprayed with 0.2% ninhydrin in ethanol. The principle of the assay is the formation of a purple compound when free alpha amino acids react with

ninhydrin (Jones *et al*., 2002). The distance migrated by the samples was compared with that of standards as a qualitative analysis of the samples

2.6.2 HPLC analysis

Further analysis of ε-PL was done using a High Performance Liquid Chromatography (HPLC) on an Ascentis® Express HILIC column (Supelco ™) (10cm x 4.6, 2.7µm). Commercially sourced ε-PL (Sigma-Aldrich Co, South Africa), run as a standard was monitored using a PDA (diode-array detector) detector at a range of 210-215 nm. A mobile phase, 88% acetonitrile water (Merck Chemicals (Pty) Ltd, South Africa) 12% ammonium formate (Merck Chemicals (Pty) Ltd, South Africa) prepared was adjusted to pH 3 with 98% formic acid (Sigma-Aldrich, South Africa). First, an isocratic run, where the same concentration of the mobile phase prepared was passed through the column at a flow rate of 1.0ml/minute was done. 1 µl of the standard was injected for HPLC analysis which eluted a retention time of 20 minutes at 25ºC. The flow rate was decreased to 0.6ml/minute for the isocratic run to increase separation efficiency. The procedure was repeated with a gradient run, with buffer A: 60% methanol and 40% HPLC grade water and buffer B: 95% methanol: 5% HPLC grade water.

2.6.3 Quantitative determination of ε-PL

A colorimetric method of Itzhaki, (1972), based on the interaction of ε-PL with an acidic and anionic dye, methyl orange (MeO), was used to determine the concentration of ε-PL produced. The stochiometric reaction forms an insoluble complex and the remaining MeO dye is collected as a supernatant after centrifuging and then concentration of ε-PL is estimated at an absorbance of 465nm using MeO as a standard. A known concentration of commercially supplied ε-PL, 1000mg/l was used to prepare a range of concentrations up to 7.8mg/l by two-fold dilutions. 0.1ml of each sample was added to 1.9ml of 0.1M phosphate

buffer, pH 6.5 and then 2ml of 1% MeO (Merck Chemicals (Pty) Ltd, South Africa) was added to make a total volume of 4ml in a 10ml test tube. The mixture was then shaken for 20 minutes on a rotary shaker at 30°C to allow a complete reaction to occur. The water-insoluble complex was centrifuged at 2500rpm for 3 minutes using a Beckman J2-21 centrifuge (DJB Labcare Ltd, England) and the supernatant was harvested for absorbance estimation using 0.1 M phosphate buffer (1.9ml) + 1% MeO (2ml) as a blank. Absorbance results from different concentrations were used to plot a standard curve from which the concentration of ε-PL produced could be interpolated.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Markets

3.1.1 Biodiesel market – glycerol market

South Africa, a fast-growing economy on the African continent is highly dependent on petroleum fuels for transport, lifestyle enhancement and industry despite its lack of crude oil reserves. Crude oil has remained its largest imported commodity, about 10% of its total imports (Singh, 2006). The need for biodiesel as an alternative in the South African perspective is clearly necessary because the potential of the market of biodiesel is determined by the existence of the fossil fuel market (Nolte, 2007).

The biofuels technology in South Africa has matured, with well established second generation processes that do not require food crops (Nolte, 2007). South Africa has the potential to produce about 1.4 billion litres of biodiesel per year from small scale farmers who use sunflower oils (Wilson *et al*., 2005; DST, 2003). As a result of high prices of seed oils in South Africa, commercial production of biodiesel can only be viable when the government puts in place favourable policies (Baseline Report , 2008). About 63 million litres is estimated to be produced from combining soybean and sunflower (contributing about 33 and 30 million litres respectively) for year ending 2010 (Figure 6). Sunflower prices are projected to increase from less than R9 a litre to about R10 a litre in the year 2015 whereas soybean prices are expected to decrease to less than R9. Biodiesel production prices have always been higher than petroleum diesel and the trend is expected to continue up to the projected 5 years from now (a sharp increase from 2010 to 2014). Despite the price increases, investors have expressed interest in investing into biofuels because of the soaring prices of

petroleum fuels and climate change. Integrated biorefinery strategies would be one opportunity to create a favourable market for biodiesel.

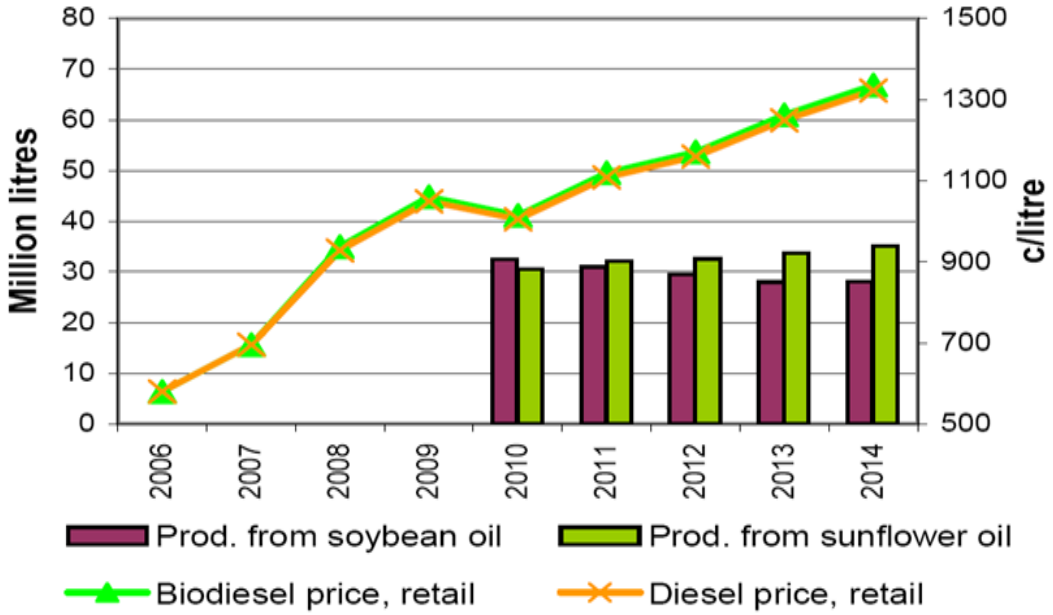


Figure 6. Biodiesel production and price. In general, biodiesel price is higher than petroleum diesel price as a result of high oil seeds prices. Given the maturity of the industry in South Africa production should be coupled by other biorefinery strategies (Adapted from South African Agriculture Baseline Report, 2008)

South Africa has a huge potential in setting up biodiesel processing plants given the number of towns situated in the farming areas where arable land is available to plant non-food crops (remembering the contentious food versus fuel issue). Such communities and local municipalities are keen to allow outside investments that will alleviate poverty and create employment for the community at hand. In addition, the government has ratified its much awaited biofuel policy draft and biodiesel is being produced from used oils and other farm projects using soybean than sunflower seeds catapulting the biodiesel market (Baseline report, 2008).

Today's biodiesel market share is less than 1% , but it is projected to increase exponentially by 2% every year reaching about 9% of the petroleum diesel in the market for year ending 2025 (Figure 7) (Winkler, 2005). The early stages show a higher biodiesel growth rate but it

is projected to slow down by 2024 as other factors become limiting such as the land available for cultivation.

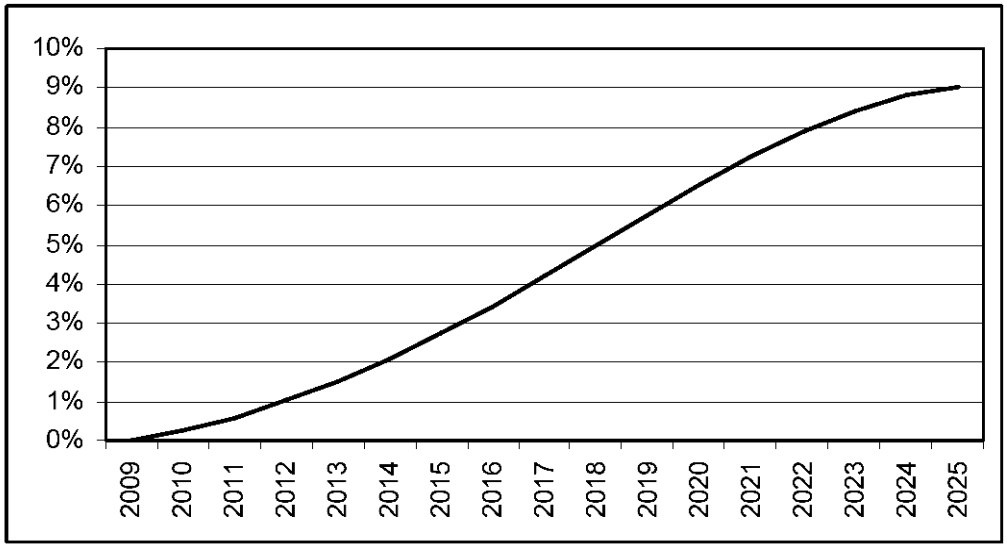


Figure 7. Share of biodiesel in transport diesel in South Africa. The biodiesel market share is projected to increase exponentially in year 2012 and thereafter (adapted from Energy Research Centre, Winkler *et al*., 2005).

Due to its fast growing economy, South Africa has seen the number of fuel users increasing exponentially thereby increasing the amounts of diesel being consumed per year. The South African Petroleum Industry Association (SAPIA) reported in 2008 that the product demand of diesel was 24,357 billion litres per year. Table 1, is a projection of product demand calculated, assuming an annual increase of 5% from the reported figure year ending 2008. By year 2018, about 40 billion litres of diesel are expected to be consumed per year following an annual 5% increase (SAPIA, 2008).

South Africa projected a 4.5% replacement figure by 2013 (Meyer *et al*., 2008). According to the calculations tabled (Table 1), about 1.9 billion litres of biodiesel will be needed to replace petroleum diesel by 2018 if a 4.5% replacement figure is not reversed. However, South Africa will have to produce about 4 billion litres of biodiesel in order to replace the 10% of its petroleum fuel consumption by year 2018. Assuming that for every 10kg of biodiesel

produced, 1kg of glycerol is produced (Dasari *et al*., 2005), and 400 million kilograms of glycerol will be availed in South Africa by 2018. This will increase the glycerol glut unless ways of disposal are sought.

Table 1. South African past and future consumption of diesel (million litres). A percentage biodiesel replacement figure was used to calculate the quantity of biodiesel needed given the petroleum diesel consumption figures (SAPIA, 2008).

Year	**Petroleum Diesel**	**Biodiesel**		
		1%	5%	10%
2008	24357	243	1218	2436
2010	26854	269	1343	2685
2012	29606	296	1480	2961
2014	32641	326	1632	3264
2016	35987	360	1799	3599
2018	39676	391	1984	3968

To my knowledge glycerol markets in South Africa have not been explored. This glycerol glut is currently and is expected to continue because there is an exponential growth of numbers biodiesel producers in South Africa because of the Biofuels Strategy Policy (2007) and incentives put in place and hence oversupply of crude glycerol globally forcing the prices to drop close to zero. In addition, established purification plants for this waste stream are non-existent increasing the burden to biodiesel producers to pay for transportation for incineration (negative price) (Miller-Klein Associates, 2006). It is therefore not economically feasible for South African producers to export this crude glycerol to European markets (Nolte, 2007).The abundance of this waste stream for the industrial applications being investigated in this study clearly shows a positive market outlook. The stability and predictability of crude glycerol as a raw material is motivational in the search for alternative uses of this abundant and neglected carbon source.

3.1.2 ε-PL and L-lysine market

The study entails the upgrading of the biodiesel derived glycerol in the commercially production of ε-PL for use as an antimicrobial polymer and as single residues of its monomers, L-lysine in animal feeds. There is no ε-Poly-L-Lysine producer on the continent but the product is imported and sells at US$ 880 per gram (Alamanda Polymers, 2009). This market is open because such an innovation to produce such a multi-purpose polymer will be the first of its own kind in South Africa and on the continent.

South Africa consumed about 9 000 tonnes of lysine in 2008 (possibly doubled by 2009) of which 50% was imported, with China's share at 2 500 tonnes (Department of Trade and Industry, 2007). The commodity sells at R13.50 per kg (SA BioProducts, 2009).

3.2 Analysis of crude glycerol

The lack of information of chemical composition is limiting the use of crude glycerol as a feedstock in industrial processes. Crude glycerol purity is a function of the raw materials used as a source of oils (Gonzalez-Pajuelo *et al*., 2005). Figure, 8 show different viscosity and colour of the crude glycerol samples analysed. Crude glycerol concentration in a number of waste streams ranges from about 62% to 76% as a result of varying purification processes (Mu *et al*., 2006; Thompson and He, 2006). However, our results (Table 2) show less concentrations of glycerol than reported in the literature, with only samples 5 and 7 with 60% and above, the rest had a glycerol percentage lower than 50%. However, Ooi *et al*., 2001 reported that crude glycerol from kernel oil methyl ester plant contained, on average, 20.2% glycerol. Samples 2, 3, and 4 were from the same biodiesel producer and it was interesting to



Figure 8. Crude glycerol samples variations in color.

note that sample 2 contained about 140g/l more than the two samples showing inconsistency with the biodiesel processes adopted by the producer. Samples 5 and 8 were solid at room temperature probably as a result of long fatty acids that solidify at room temperature and only the liquid part was collected for these analyses and the results show that most of the glycerol was in this part. However the lower compositions of glycerol as compared to those reported by Thomspon and He (2006) could be attributed to the South African biodiesel producers leaving lots of water in the washing process which then lowers the concentration of the glycerol waste stream as well as different purification processes as compared to European producers (Thompson and He, 2006).

Most biodiesel producers utilise methanol in its excess to drive the reaction to completion, and most of it will end up in the glycerol layer as a contaminant (Zappi *et al*., 2003). As shown in Table 2, sample 4 contained the highest amount of methanol (62.786 g/l) with sample 5 with the lowest (14.849 g/l) indicating more methanol being used in the former than the latter. The fact that sample 5, as mentioned before, was in solid form and the available methanol could have been exposed that high to evaporate from the container. All other samples, contained between 31 - 43 g/l of methanol. Thompson and He, (2006) reported between 12% and 28% of methanol remaining in the crude glycerol streams.

As observed in the glycerol analyses, samples 2, 3, and 4 again differed in the amounts of methanol observed with sample 4 containing about 20g/l more than the two samples which contained almost the same amounts of methanol. The results were not as accurate as they could be because methanol evaporates from samples kept for too long before analysis reducing the levels that could have been reported (Pyle *et al.*, 2008). The results did not match the expected concentrations the supplier reported possibly due to the variations of concentration estimation by the HPLC column and evaporation of methanol. The column used was not optimised for detection of methanol although it was capable of detecting it. It is therefore suggested to keep the time between sampling and sample analysis as short as possible.

Table 2. Elemental, glycerol and methanol composition of crude glycerol streams as detected by ICP-OES and HPLC analysis (extremes are in bold).

Sample	**K**	**Na**	**P**	**S**	**Pb**	**Zn**	**Glycerol**	**Methanol**
	$mgKg^{-1}$	**$mgKg^{-1}$**	**$mgKg^{-1}$**	**$mgKg^{-1}$**	**$mgKg^{-1}$**	**$mgKg^{-1}$**	**(g/l)**	**(g/l)**
Ref blank	0.094	0	0.637	0	3.084	3.764	2	2
1	**486.900**	10.470	**0.805**	0.750	4.581	6.693	251.285	42.618
2	173.400	48.990	1.338	**0**	7.081	6.014	349.208	42.692
3	387.200	34.530	3.977	0.805	6.324	8.094	**207.042**	43.190
4	449.000	28.470	**4.104**	**0.917**	5.765	7.308	219.050	**62.786**
5	324.700	**68.170**	0.908	0.342	**1.580**	**2.380**	628.356	**14.849**
6	175.700	50.580	1.174	0.181	4.535	5.877	342.600	44.378
7	373.200	6.440	0.803	**0**	**9.826**	5.131	**606.560**	48.521
8	**7.767**	**27.050**	1.512	2.030	0.479	**35.990**	289.960	31.274

Elemental analyses of the samples (Table, 2) show that potassium was the most abundant element in most of the samples ranging from 173.4 $mgKg^{-1}$ to 486.9 $mgKg^{-1}$ except sample 8 with 7.7 $mgKg^{-1}$. This could be attributed by the fact that the biodiesel producers use KOH as a catalyst and sodium methylate ($NaOCH_3$) respectively (Thompson and He, 2006). Samples 2, 3, and 4 again showed that there was no consistency in the process as evidenced by the

differences in the elements analysed. Trace elements, phosphorous, sulphur, lead, and zinc were also found. Thompson and He, (2006) suggested that these trace elements were from the soil on which the oil seeds grown.

The results of the analysis of crude glycerol samples showed that these waste streams varied from biodiesel producer to another possibly as a result of different processes adopted by the different producers. However, variation of the samples from the same biodiesel producer shows inconsistence in the quality control during biodiesel production. The differences in trace elements can be attributed to the differences in chemical composition of the soils in the South African soils meant for agriculture as well as the difference in source of the oils i.e. the actual oil crop (Thompson and He, 2006).

3.3 Pure glycerol utilisation by *S. albulus*

3.3.1 Preliminary screening on solidified medium

Preliminary studies to investigate growth conditions of *S. albulus* as a function of pH were performed and the results showed that the optimum pH condition was a neutral pH (Table 3). There were no colonies evident on the agar plates in which the strain was streaked on glycerol medium at pH 3 showing that the strain did not grow on such acidic conditions whereas pH 4 and 6 had significantly the same number of colonies but thrice as lower as the colonies evident on the medium at a neutral pH. Alkaline pH of 9 showed that the strain was able to utilise the medium at this pH.

The preliminary study, which showed that *S. albulus* grew in pure glycerol (at maximum 10%) on the second day of incubation, was a step towards crude glycerol utilisation. The experiment was repeated this time to estimate the growth rate over a period of 12 days by counting the number of colony forming units.

Table 3. Growth of *S. albulus* as a function of pH and percentage glycerol. Growth of *S. albulus* increased with increasing pH with its optimum at a neutral pH whereas its growth decreased with increasing concentrations of glycerol.

Growth of *S. albulus*			
pH		**% glycerol**	
3	-	1	+++
4	+	2	++
6	+	5	++
7	+++	10	+
9	++	>10	-

Key: Degree of growth

+++	Very fast growth
++	fast growth
+	slow growth
-	no growth

The results show that the highest CFU, 161 x 10^4 per milliliter, was observed when the strain was grown for 12 days in medium containing 10% of glycerol. Figure 9 shows that there were less number of colonies visible in 10% glycerol-containing medium on the second day of culturing but the strain produced more colonies on the last day of culture (12^{th} day). Exponential growth of the strain was observed between the first and the third day at 48.8 x 10^4 CFU per day and leveling off afterwards. Starch was the least utilised carbon source with less than 20 x 10^4 CFU counts within the first six days increasing to approximately less than half the other carbon sources. The low numbers of colonies visible on starch-containing medium could be attributed to the strain's inability to utilise starch effectively.

Medium containing 2% glycerol was the second best to be utilised, with 5% within the same limits (about 100 x 10^4 CFU counts) and error bars overlapping but with a longer lag phase. A longer lag phase was observed in medium containing 10% of glycerol, recording less than 20 x 10^4 CFU counts within the first three days but an exponential increase in the growth of the strain was observed thereafter with a growth rate equal to the 1% of glycerol containing

medium. Growth exceeded a 2% glycerol-containing medium on the sixth day and exceeded the best utilised, 1%, after 12 days.

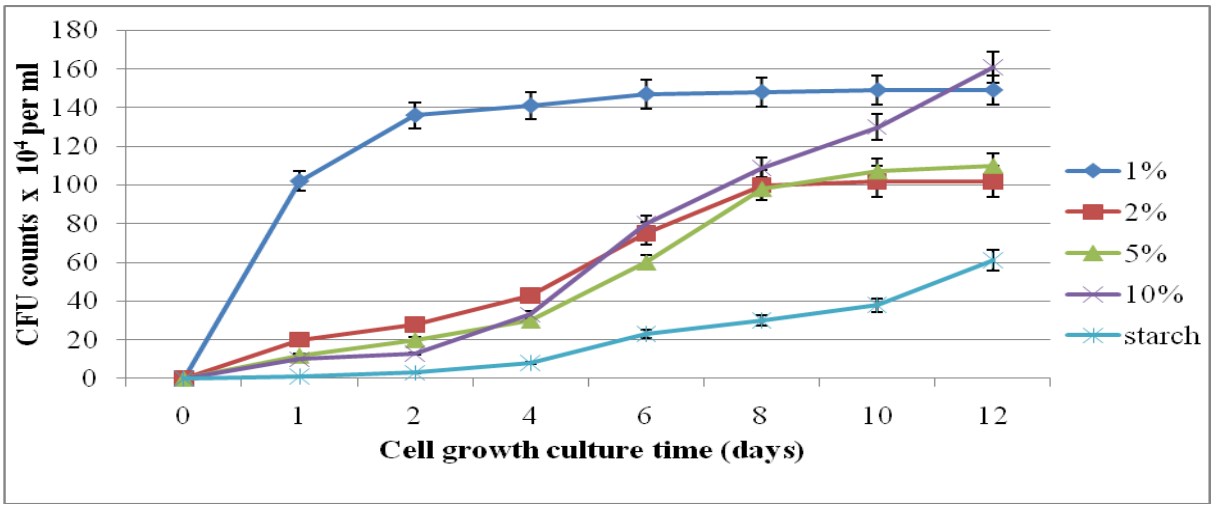


Figure 9. CFU counts on solid media with different concentrations of pure glycerol and 1% starch. 1% glycerol containing medium was the best utilised whereas starch containg medium was the least utilised. The strain grew well on 2% glycerol as compared to 5% and 10%, and therefore was chosen for use as a standard.

2% glycerol-containing medium was chosen for use as a standard glycerol utilisation percentage because it followed an exponential growth with a short lag phase from day one with a higher number of colony forming units appearing each day as compared to higher concentrations of glycerol (5% and 10%). In addition, 2% glycerol was chosen before 1% taking into account that the lower carbon source can be depleted before product formation.

In order to test the production capacities, shake flasks are used as the solid medium experimental results are not a true representation of the industrial bioprocesses. Shake flasks, small-scale liquid fermentations, were then carried out to determine the best concentrations the strain could utilise and their ability to produce ε-PL.

3.3.2 Shake flask fermentation

Preliminary experiments (3.3.1) showed that *S. albulus* was able to utilise glycerol as a carbon source and investigations that it would grow in the liquid medium in shake flask fermentation systems were necessary because conventional industrial production of biopolymers use submerged fermentation. To this date, industrial ε-PL production, glucose from molasses is used as a carbon source.

Glycerol viscosity impedes oxygen transfer and hence reduces the growth rate (Pachauri and He, 2006). Growth of the bacterial strain in shake flask to determine the best concentration of glycerol for further experiments was performed. This time, glucose was used instead of starch as a control used in conventional industrial applications and cell growth was followed by measuring cell density using a spectrophotometer.

Culture pH decreased from its initial value of 7.3 to 3.8 after 24 hrs and slowly increased to about 6 thereafter because the media was not buffered. The decrease in pH can be explained by the buildup of carbon dioxide produced when L-lysine is produced (see Figure 5) and subsequent increase in the pH can be as a result of a basic product (ε-PL) being produced by the strain to counteract the toxicity of the acid conditions (Nishikawa, 2002).

In the first three days, the highest cell density was observed when the strain was grown in 2% glucose-containing medium followed by 1%, 2%, 5%, and 10% glycerol respectively (Figure 10). Medium containing 1% glycerol was the second best to be utilised in terms of growth rate. However, in industrial applications, 1% glycerol will not be an option as the carbon source will be exhausted before the harvesting of a product as shown by the less cell density as compared to all other concentrations at the end of day 4. Optical density readings of the glucose-containing medium were not reliable after 3 days due to formation of granules which

settled at the bottom of the cuvette leaving the broth less turbid. This could have possibly been a result of the exhaustion of the carbon source. The most applicable glycerol concentration, 2%, shows a typical exponential growth of bacteria with the last days of culturing showing a higher cell density despite being lower than 1% glycerol-containing medium in the first days, which can be due to availability of the carbon source after a long time and the viscosity of glycerol impeding oxygen transfer respectively. A higher cell density was observed in 5% glycerol containing medium during the last days of the culture despite a longer lag phase. The 10% glycerol containing medium showed a 4-day lag phage indicating that the strain had difficulty in growing in it.

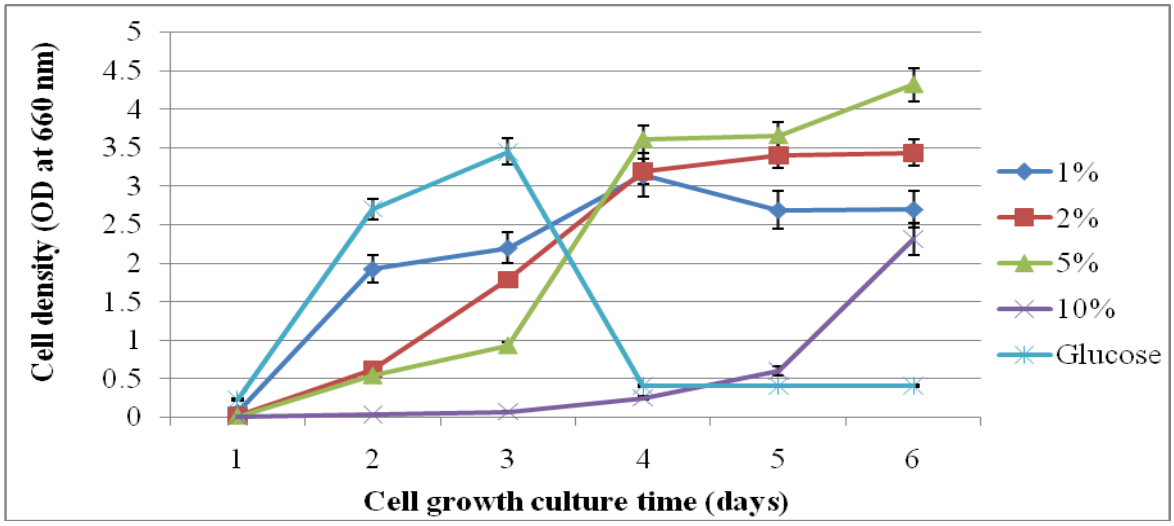


Figure 10. Cell density represented by OD readings of *S. albulus* grown in shake flasks with different percentages of glycerol and glucose as a control. Glucose was the best to be utilised before cell density fell after day 3 followed by glycerol medium containing 1%, 2%, 5%, and 10% respectively. 2% containing glycerol medium showed a typical bacterial growth curve and was chosen to be a standard.

After 6 days the culture entered log phase growth. It can be expected that the cell density would still increase in the following days. However the culture was stopped at this stage. It should be noted that experiments to estimate glycerol concentrations remaining in the fermentation broth were not performed because it was assumed glycerol was the only carbon source available for the observed growth of *S. albulus*.

3.4 Crude glycerol utilisation by *S. albulus*

3.4.1Effects of salts and other impurities on the growth

While pure glycerol was able to support growth of *S. albulus*, the feasibility of crude glycerol being used to grow the strain was investigated. The experiments were to investigate if the impurities in the crude glycerol could affect growth of *S. albulus*. However, when crude glycerol is used as a feedstock in microbial fermentations, growth inhibition is chiefly due to high levels of Na, K and methanol (Rumbold *et al*., 2003). Crude glycerol samples used in this study ranged from pale yellow to dark brown streams (see Figure 8) from 6 South African biodiesel producers and the analysis results were presented in Table 2.

Table 4 shows growth of *S. albulus* in different sources of glycerol. Sample 1 formed precipitates when the pH was adjusted to pH 7.3 and after autoclaving it formed a thick sludge which could be attributed to the fatty acids (analysis not done) and salts since this crude glycerol sample contained the highest amounts of potassium salts (486.9 $mgKg^{-1}$). The observed precipitation could also be as a result of the presence of unreacted glycerides and esters (Thompson and He, 2006). The sample was therefore not considered a proper medium for fermentation processes.

Sample 2, 3 and 4 were from the same producer, thus only 3 and 4 were chosen on the basis that they contained glycerol and methanol in the same range. Both samples had *S. albulus* growing in them but lower than the rate at which the strain grew in sample 6 and 7. Samples 3 and 4 had higher potassium salts as compared to the latter and this could probably had effect on the growth rate observed. Sample 5 and 8 were not used in this study because they were solid at room temperature.

Table 4. Growth of *S. albulus* in different sources of crude glycerol. No growth was observed in source 1 whereas slow growth was evident in sample 3 and 4. The fastest growth rates were observed in sources 6 and 7.

Source	Growth of *S. albulus*
1	-
3	+
4	+
6	+++
7	+++

Key: Degree of growth

+++ Very fast growth
++ fast growth
+ slow growth
- no growth

To investigate the effects of the salts and other impurities, sample 7 was chosen for the studies because it was less turbid as required for OD measurement for biomass estimation which was less difficult as compared to sample 6. The biodiesel producer used KOH as an alkali to catalyse the transesterification process of plant oils with methanol as evidenced by the high levels of potassium salts (see Table 2). The crude glycerol from this process contained 606.56g/l of crude glycerol which was diluted to 20g/l with water in this study.

The pH was adjusted to 7.3 (optimal condition for growth of the strain). Lowering the pH of crude glycerol allowed the soaps to be converted into free fatty acids (Pyle *et al*., 2008) hence reduced precipitation of the crude glycerol. The medium was subsequently autoclaved therefore evaporating the methanol. Cell growth in crude and pure glycerol was compared to a conventional carbon source, glucose (Figure 11). To the best of the author's knowledge this is the first report of growth of *Streptomyces* species on crude glycerol from biodiesel production.

The results show that there is general similarity of the growth rates (as indicated by changes in OD) of *S. albulus* in the carbon sources, with glucose being the best (OD = 0.425 per day) and crude glycerol being the slowest metabolised carbon source (OD = 0.395 per day).

Although, the growth rate of *S. albulus* was slow in crude glycerol compared to the other two carbon sources, the results show that the strain was capable of growth and cell maintenance on biodiesel-derived glycerol.

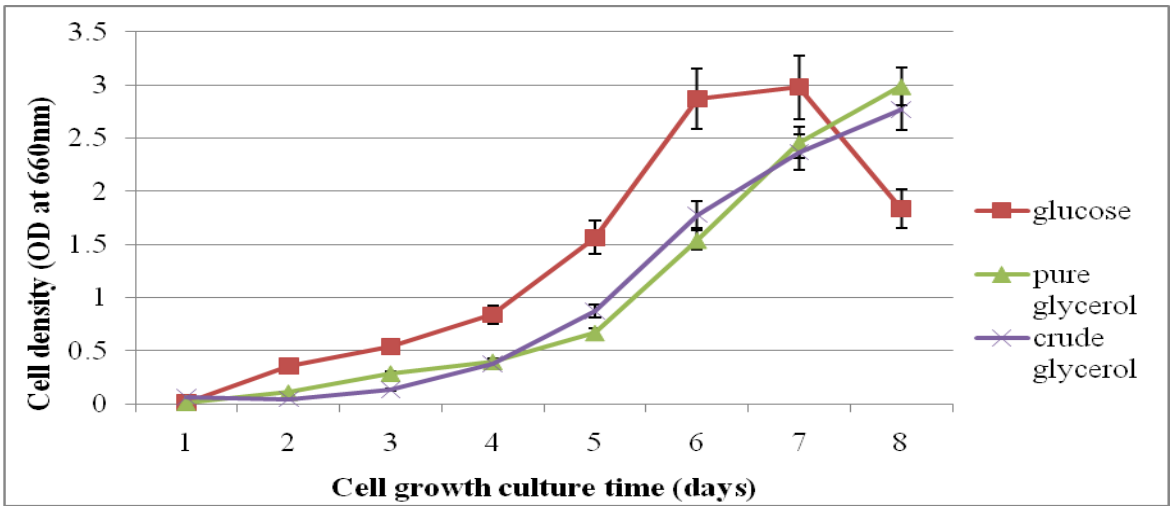


Figure 11. Comparison of conventional carbon source (glucose), pure and crude glycerol. There is a general similarity in growth glucose in pure and crude glycerol. A longer lag phase was evident in crude glycerol medium but higher cell densities recorded between day 4 and 7.Glucose as a control was the best utilised.

In general, glucose metabolism is faster than glycerol because it is a 6-Carbon molecule and therefore completes two cycles with one mole as compared to 2 moles of a 3-carbon molecule glycerol. In addition, the reason why glycerol is slower is that 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, Voegele, *et al*., 1993). *E. coli* has a glycerol facilitator protein, responsible for glycerol uptake whereas the uptake of glycerol by *Streptomyces spp* is not documented (Heller *et al*., 1980). It is this non-selective glycerol facilitator protein that allows *E. coli* to utilise glycerol at higher rate as compared to *S. albulus.*

It could be probably as a result of poor regulation of glycerol metabolism in general in

S. albulus. Glycerol enters the glycolytic pathway on the second stage after its conversion into dihydroxyacetone and then dihydroxyacetone phosphate catalysed by two enzymes glycerol dehydrogenase and dihydroxyacetone respectively (Da Silva *et al.*, 2009). Glycerol is also channeled to the hexose and pentose pathways which consume part of the glycerol in order to produce nucleic acids and $NADH_2$ necessary for cell growth and utilisation of glycerol as compared to glucose (Papanikolaou *et al.*, 2008). The enzyme responsible for the synthesis of $NADH_2$, $NADP^+$-malic enzyme, has been reported to be a slow enzyme thus it could be responsible for the slowed the assimilation of glycerol (Ratledge, 2002).

Comparing crude and pure glycerol, with respect to the effects of impurities on microbial growth, the results showed that initial stages up to the fourth day, crude glycerol was not as good a carbon source as shown by the low growth rates possibly due to impurities (salts) in the crude glycerol (Sneha *et al.*, 2009, Pyle *et al.*, 2008). Such impurities would lengthen the lag phase as the strain adapts to the new environment (Rumbold *et al.*, 2009).

After 4 days of growth, crude glycerol was a better carbon source than clean glycerol but the reverse was true for the last 2 days of growth, possibly due to optimum amounts of salts that can change the rate of growth independent of the salts. Additional nutrients from vegetable oils could explain the higher growth rates as they are reported to increase cell growth rates (Çelik *et al*, 2008). However, there is no significant difference between the growth of *S. albulus* in crude and pure glycerol as can be seen by error bars overlapping. In addition, the last stages could be explained by the exhaustion of these nutrients leaving other impurities and other factors to reduce the growth rates. Cell density ceased to increase in glucose medium well before both glycerol substrates possibly as a result of exhaustion of the glucose substrate. The biomass OD readings in glucose containing medium after 7 days were unreliable due to the granulation of the bacteria. The differences in growth rates of *S. albulus*

on crude and pure glycerol is not certain suggesting further experimentation should be done to find out the effects of other impurities excluding these analysed in this study.

In this study, it may be considered that impurities in biodiesel-derived glycerol (without methanol) do not affect the growth of *S. albulus*. Our results indicate that *S. albulus* can grow in concentrations up to 20 g/l of biodiesel-derived crude glycerol. The results of this study are in consensus to a different study by Papanikolou *et al*., 2009 in which he reported that industrial feedstock impurities did not affect the growth of *Yarrowia lipolitica*. However other reports show that *E. coli* and *Corynebacterium glutamicum* do not grow on crude glycerol at all, and *Saccharomyces serevisiae* grows very slowly (Rumbold *et al*., 2009; Jonhson and Taconi, 2007).

3.4.2 Effects of methanol on the growth of *S. albulus*

The effects of using methanol containing glycerol in the medium was investigated by comparing cell growth profiles of glycerol without methanol medium and glycerol medium with differing amounts of methanol. Crude glycerol in this work contained no methanol because autoclaving of the carbon source completely evaporates methanol (Sneha *et al*., 2009) and hence its effects had not been investigated. Methanol, as an impurity in crude glycerol is highly toxic to most microorganisms because it inhibits microbial growth as a result of its oxidation to a formaldehyde and formic acid which are toxic to microbes (Voysey, 1987). Synthetic crude glycerol medium containing, 2% pure glycerol and 0, 5, 10, 20, and 30 g/l of methanol sterilised by passing through a 0.2µm filter, were prepared to study the effects of methanol.

Figure 12, shows cell densities of *S. albulus* grown on varying concentrations of methanol with medium containing no methanol as a control. The results clearly indicate the negative effects of methanol on growth of *S. albulus*. However, the cell densities show that *S. albulus* can grow in methanol containing medium despite struggling as shown by the lower growth rates observed. Methanol causes burden to cells to be converted to a non-toxic carbon source as it requires higher amounts of oxygen (Çelik *et al.*, 2008). Cell growth decreased with increasing concentrations of methanol with 30g/l being the maximum (results of concentrations above 30 g/l not shown). Error bars overlapped showing that there was no significant difference in growth rates in different concentrations of methanol probably as a result of initial inhibitory effects which are of the same magnitude at first irrespective of the concentration. The limited amounts of oxygen available in shake flasks could probably have affected the metabolism of methanol leading to the decreased growth rates observed. If excess oxygen is supplied in a fermentation process, it would mean more carbon source in the culture and hence a higher growth rate will be anticipated. This was not the case possibly due to insufficient oxygen in shake flask fermentation.

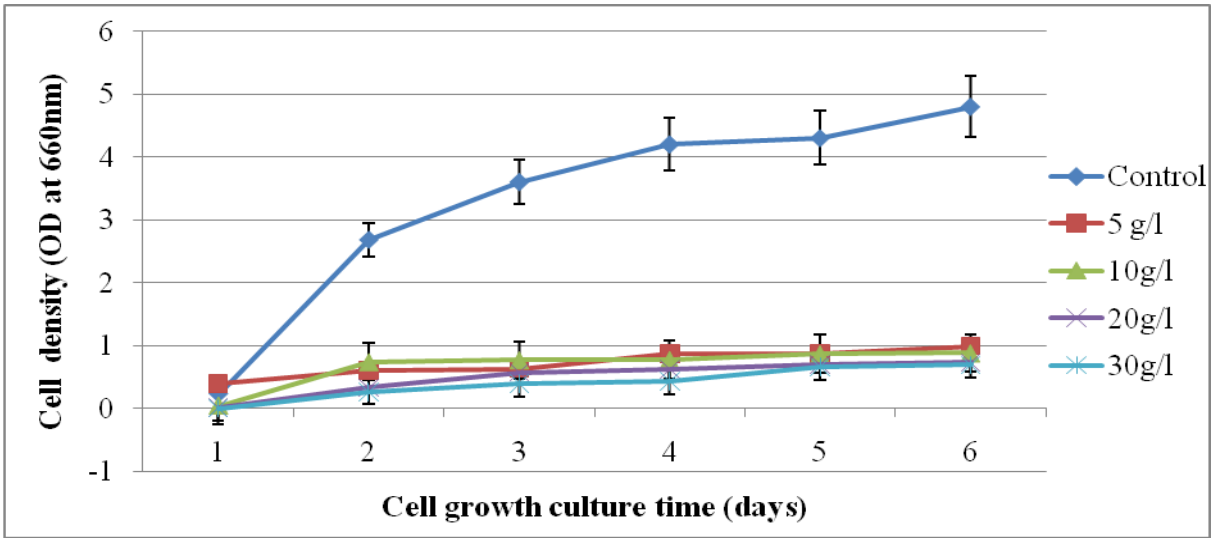


Figure 12. Effects of methanol on the growth rate of *S. albulus*. The figure shows that methanol has a negative effect on the growth rate of the strain. The effects of methanol were proportional to the amount of methanol in the medium, 30 g/l had a higher lag phase as compared to 20, 10 and 5 respectively.

Despite the inhibitory effects showing that the strain struggled as observed, the utilisation of methanol-containing glycerol as a carbon source is still possible as the levels of methanol in many crude glycerol streams characterised is within the limits (25 – 30g/l) (Thompson and He, 2006) *S. albulus* utilised. In a South African context where levels of methanol in crude glycerol anlysed were as low as less than 7%, applicability of using this stain after dilutions is not expected to be a cause of concern. In addition, companies that produce biodiesel are looking at ways of recovering methanol for reuse in the process to reduce the costs thus its concentration in the crude glycerol will be reduced remarkably and then valorisation of crude glycerol will be at relative ease. The trace concentrations that will be in the carbon source after this removal step will be removed by autoclaving the media availing a methanol-free or a less methanol-containing medium in which *S. albulus* can thrive. *S. albulus* has shown ability to grow in methanol despite the low growth rate and this attribute is impressive since it negates the methanol removal process. Çelik *et al*., 2008 reported that such organisms that tolerate high methanol concentrations, for example, *Pichia pastoris* have an alcohol oxidase 1 (AOX1) enzyme that converts methanol into formaldehyde and formate which allows them to utilise crude glycerol effectively. This utilisation efficiency could be as a result of energy that is generated when methanol is oxidised to carbon dioxide via formaldehyde and formate (Hazeu *et al*., 1983, Bystrykh *et al*., 1993). In similar studies where *Streptomyces coelicolor* was grown in methanol as a carbon source, *Streptomyces* spp are capable of utilising methanol as a carbon source (Kato *et al*., 1977).

3.5 ε-PL characterisation

3.5.1 Cation exchange purification and identification of ε-PL

Culture broth from a pure 2 % glycerol-containing medium (from section 2.4) was applied to an ion-exchange chromatography and the elution profile monitored using a JASCO UV

spectrophotometer at 210 nm.The absorbance of the eluted fractions was then plotted manually on a graph to show the elution profile (Figure 13).

The results show two peaks, first one with fraction 4 and 5 and the second one with fraction 7 and 8. Fractions 5 and 7 had the highest absorbance (1.778 and 1.936 respectively) recorded. To identify the product, thin layer chromatography (Figure 14) technique was applied and the first peak was identified as lysine and the second being ε-PL. *S. albulus* produces ε-PL at a pH between 4 and 5 but its immediately degraded by a ε-PL degrading enzyme at a pH above this range (Kahar *et al.*, 2001). In the present fermentation, there was no strict control of the pH and thus the polymer accumulated with its monomers in the culture broth. Nicholas *et al.*, 1995 reported the presence of metal endoproteases in *Mycobacterium* species that degrade α-PL.

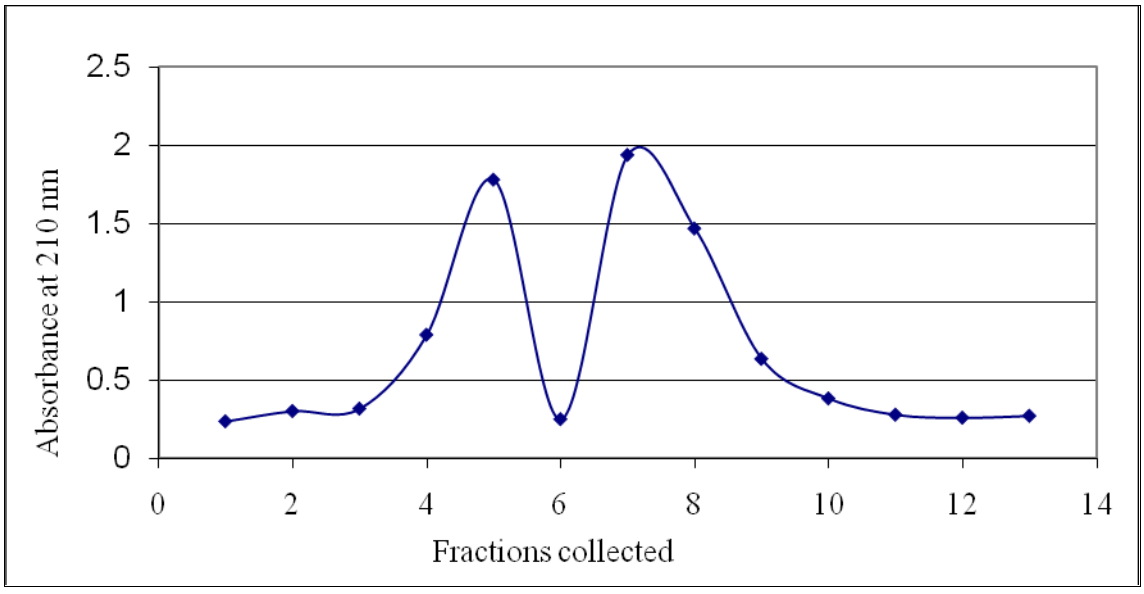


Figure 13. Elution profile of the product. Two peaks were evident with the highest absorbance recorded for fraction 5 and 7. The two peaks were assumed to be containing the eluted product. Fraction 6, at the depression to form the two peaks recorded absorbance in the same range with fractions 1, 2, 3, 10, 11, 12, and 13.

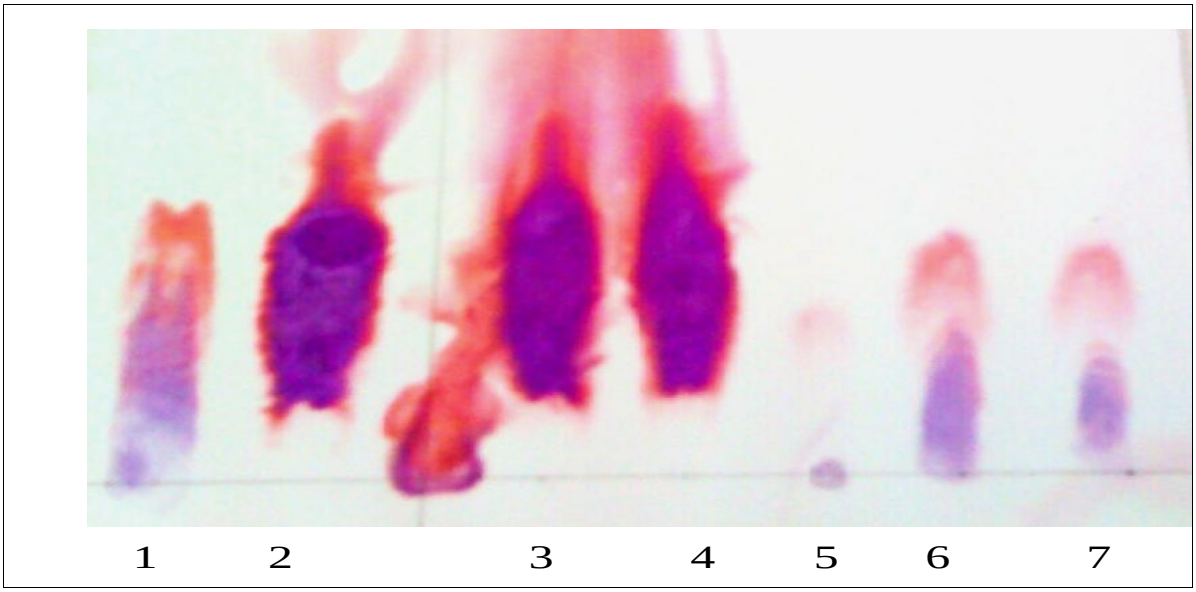


Figure 14. TLC of ε-PL produced by *S. albulus.* Lanes: 1, standard ε-PL (5 µl); 2, standard L-lysine (5µl); 3 and 4, fraction 4 and 5 respectively; 5, fraction 6 and; 6 and 7, fraction 7 and 8 respectively.

The elution profile logic follows the principle that the more positively charged a molecule is, the later it elutes the column (Dean *et al*., 1985). ε-PL polymer is more positively charged as compared to the single L-lysine residues, thus the elution profile observed in Figure 13. The TLC show a strong purple colour in the presence of lysine as compared to the ε-PL due to the presence of free amine groups which are more available for reaction with the ninhydrin when polymerisation has not occurred.

Samples from the second peak were pooled as they were identified to be containing ε-PL to estimate the concentration of ε-PL produced by *S. albulus* from pure glycerol as a carbon source using the Itzhaki method, 1972. Using ε-PL as a standard, a concentration vs absorbance standard curve was plotted (Figure, 15). Orange-coloured suspensions were evident in low concentrations of ε-PL whereas at high concentrations yellow solutions were formed when MeO was added.

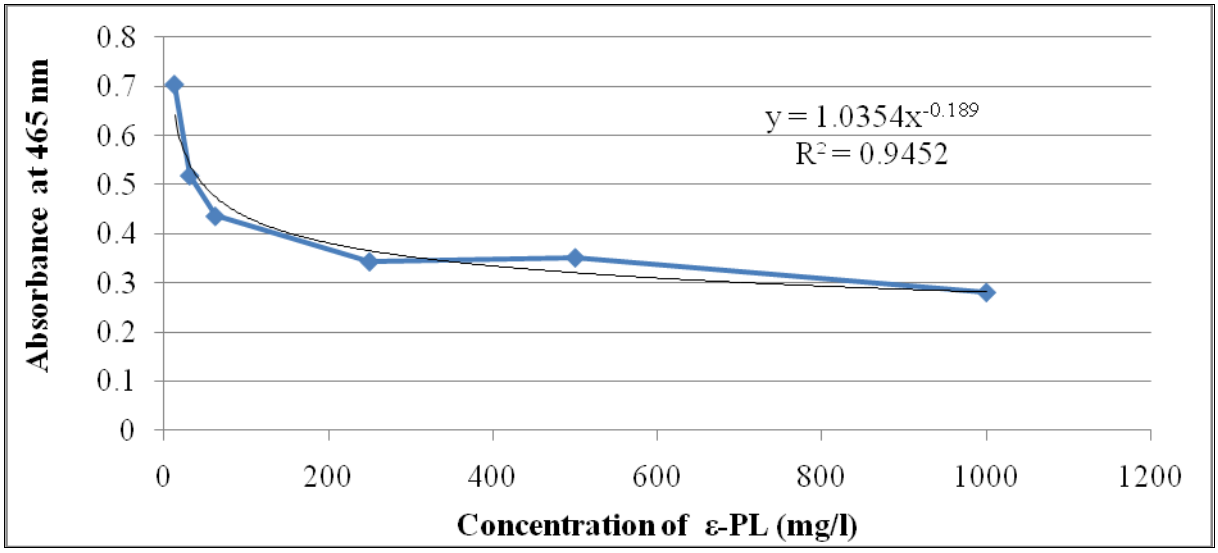


Figure 15. Calibration curve of mean absorbance vs concentration of ε-PL (mg/l).

The plot shows that absorbance decreased with increasing concentration of ε-PL. The same trend, stochiometric reaction of MeO and ε-PL, observed at higher concentrations (500mg - 1000mg/l) was expected as documented by Itzhaki, 1972. Lower concentrations of ε-PL gave a concave curve whereas higher concentrations gave a linear part of the graph (when 150, 500 and 1000 mg/l are joined leaving 250g/l point). The absorbance of ε-PL from eluted fractions read off a Novaspec II (Pharmacia biotech) spectrophotometer was 0.364.

Extrapolating from the curve, 219 mg/l was produced from a 200ml culture medium. ε-PL produced in this study is similar to the 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, Hiraki *et al.*, 1998 produced 1.2g/l after strain improvement by nitrosoguanidine treatment whereas a two stage process reported by Kahar *et al.*, 2001 involving a pH control strategy yielded 48,3g/l. The discrepancies in the amount of ε-PL produced may have been as a result of a lack of pH monitoring in this study. This was evidenced by the presence of the monomers of the polymer in the product eluate as the product depolymerises at a certain pH (Kahar *et al.*, 2001). As observed by the growth experiments glucose produced more biomass as compared

to glycerol thus the amount of product produced in this study was expected to be lower than cited in the literature.

3.5.2 HPLC analysis of ε-Poly-L-Lysine

Following TLC analysis, there was need to accurately determine the products of the cation exchange elution profile using an HPLC with a diode array detector. Authentic ε-PL (10mg/l) was prepared and run as a standard at 1.0ml/minute. An isocratic run profile (Figure 16), a single peak, eluted at 0.77 with 88% acetonitrile water: 12% ammonium formate showed that there was not enough separation probably because the retention time was too low such that the polymer did not stick to the stationery phase (column). The standard ε-PL, was a polymer of molecular weight ranging from 1000-5000 and we expected a number of peaks resulting from different residues. To increase the separation, retention time was increased, by lowering the flow rate to 0.6 ml/minute and there was no big difference as the profile remained the same despite that the standard was eluted at 2.27 minutes (Figure 17). The mobile phase was changed to A: methanol 95% and 5% water and B: methanol 60% and 40% water and the procedure was repeatedly run to determine the gradient elution profile and no positive results were obtained (Figure 18). The profile shows that the product could have been retained on the column or it was washed out by the immiscible mobile phase. It was concluded that the column was not suitable for the separation of the polymer. Due to time restrictions, other columns were not tested.

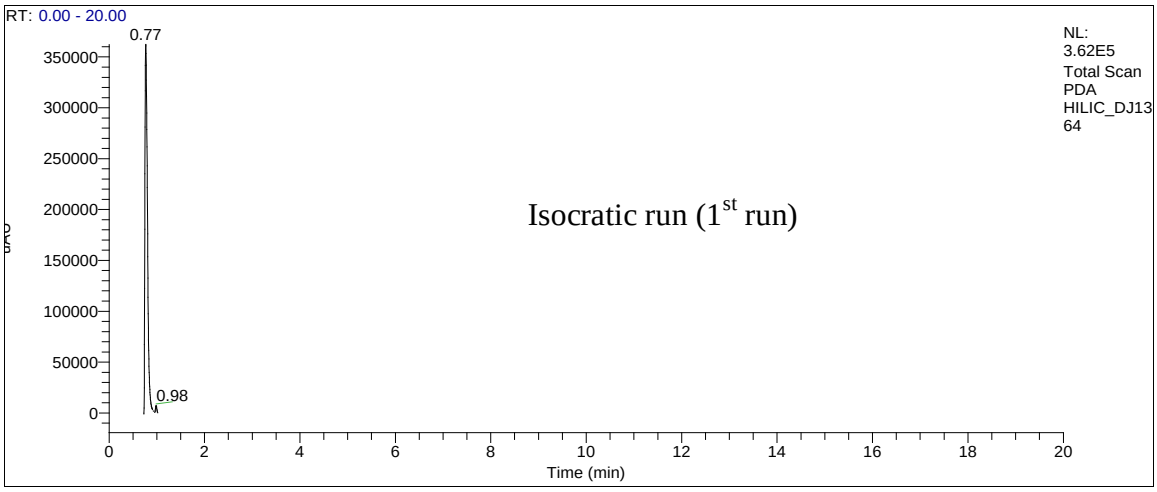


Figure 16. HPLC elution profile (1st isocratic run)

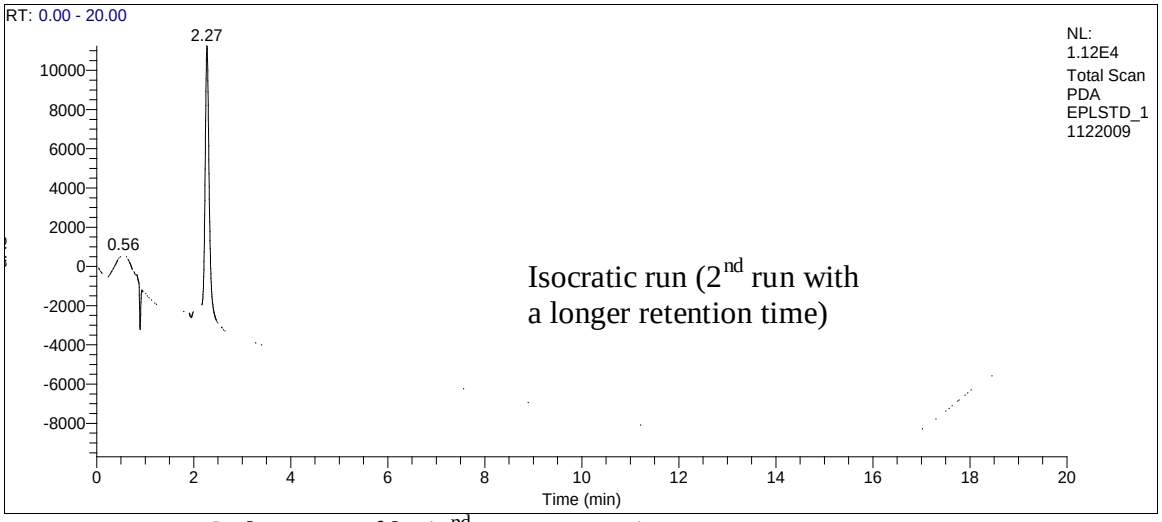


Figure 17. HPLC elution profile (2nd isocratic run)

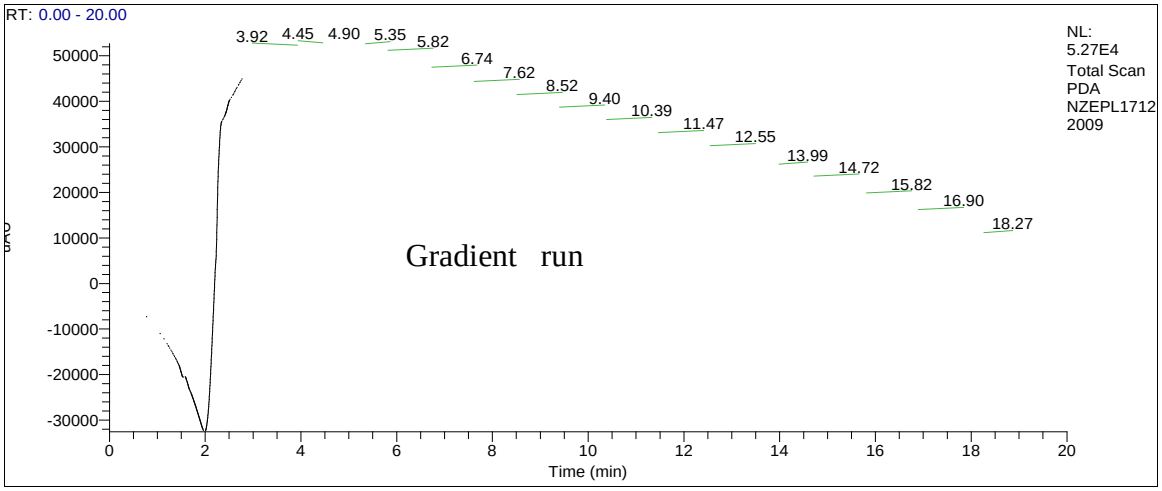


Figure 18. HPLC elution profile (gradient run)

3.5.3 Effects of impurities on the production of ε-Poly-lysine.

The results of this study show that methanol reduced the cell density of *S. albulus* by at least 5 times despite being concentration dependant and on the other hand crude glycerol lowered the biomass density. Their effect on biomass was suspected to lower polymer production. To investigate the effects of salts and other impurities in the absence of methanol, crude glycerol with methanol recovered was used as a carbon source and its effects on the production of ε-poly-lysine was investigated comparing it with pure glycerol and pure glucose as a control. Table 5 shows the results of elemental analysis of the two carbon sources. The results show that the crude glycerol used contained higher amounts of potassium as compared to pure glycerol. Crude glycerol contained about 3000 times as much potassium as pure glycerol suggesting that the biodiesel producer used KOH as a catalyst (Thompson and He, 2006). Pure glycerol contained no sodium whereas crude glycerol contained 6.44mgKg^{-1}. Both sources contained no sulphur and significantly the same amount of phosphorus. In addition, lead in crude glycerol was almost thrice as much as in pure glycerol whereas zinc was about one and a quarter times as much respectively.

Table 5. Elemental analysis of the two carbon sources used for polymer production. In general, higher amounts of salts were recorded in crude than in pure glycerol samples. Pure glycerol sample did not sodium and sulphur whereas crude glycerol had high sodium salts with no sulphur recorded.

Sample	Type	K	Na	P	S	Pb	Zn
Pure glycerol	mgKg-1	0.094	0	0.637	0	3.084	3.764
Crude glycerol	mgKg-1	373.200	6.440	0.803	0	9.826	5.131

Effects of the salts on the production of ε-PL were determined qualitatively using rapid ninhydrin test previously reported by Itzhaki, 1972. A drop of the eluate was spotted on a strip of paper and the presence of ε-PL was revealed by the purple colour formation (Ninhydrin test). The intensity of the colour of the ninhydrin/ε-PL complex (results of colour intensities not shown), which varied directly with ε-PL concentration, was used to estimate qualitatively the amounts of ε-PL produced. Table 6 shows that more ε-PL was produced when glucose was used as a carbon source as compared to pure glycerol and crude glycerol respectively. Growth rates of the strain, as previously described, were higher in glucose containing medium and the level of the product produced was expected to be higher as a correlation to the biomass density.

In addition, Nishikawa and Ogawa, 2006 reported the formation of an ε-PL-glycerol ester and hence reducing the concentrations of ε-PL reacting with the ninhydrin. The probability of ε-PL being produced in equal amounts when its production is compared between pure glycerol and clean glycerol is high as there was no significant difference between the biomass densities during the growth phases. It should be noted that experiments comparing product yield at varying pure and crude glycerol concentrations were not performed and therefore there is need to look into such experiments as concentration of the carbon source partially affects product yield and would be necessary if industrial applications are adopted.

Table 6. Qualitative results of ε-PL produced from crude glycerol as compared to other carbon sources. Levels of ε-PL produced from glycerol were higher than the two glycerol samples. However, crude glycerol sample produced about half the quantities of ε-PL probably as a result of inefficient downstream processing as the crude glycerol contains a number of impurities as compared to pure glycerol.

Carbon Source	**Levels of ε-PL produced**
Glucose	XXX
Pure Glycerol	XX
Crude Glycerol	X

Key: Level of production

XXX	High
XX	Low
X	Lowest
-	No production

The results show that biodiesel-derived crude glycerol is a promising alternative carbon source in the industrial production of ε-PL and merits further investigation.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

In this study, the aim was to investigate the feasibility of using crude glycerol, a by-product, waste stream during biodiesel production, as an alternative feedstock to the conventional carbon source, glucose, as a value-added bioconversion in the production of ε-Poly-L-lysine and its integration in the existing biodiesel business to increase profitability and sustainability of biodiesel. Moreover, crude glycerol and methanol were analysed and their presence did not have resounding effects on the growth and production of the polymer by *S. albulus*. This is the only work that characterised crude glycerol from South African biodiesel producers and subsequently studied its feasibility as an industrial carbon source in the production of biopolymers whose market value is unquestionably high.

The study concludes that crude glycerol is an attractive alternative carbon source which supported cell growth and maintenance of the strain in the study at hand despite the presence of impurities. Although the utilisation rates were lower than the conventional carbon source, we boast a proof of concept finding in valorisation of the waste crude glycerol in a South African biorefinery approach context.

However, it is very unlikely that this bioconversion route in this study can successfully convert this waste stream at a rate necessary to prevent its glut in the near future. Therefore, there is need to look at other utilisation routes of this waste stream in producing a wide range of products from different processes whether chemical or biological (integrated biorefinery approach). There is huge potential to exploit the booming South African biodiesel market to produce value added products with a higher market value. The bioconversion process

provides a significant improvement of the glycerol markets taking into account its current value on the market.

In addition biodiesel producers around South Africa dispose of crude glycerol containing less amounts of glycerol and methanol than European countries down the drain with some storing it in large containers behind their buildings. It is interesting to note that the strain in this study was able to utilise methanol-containing glycerol and thus this negates the methanol removal step reducing the expenses if the process is to be integrated in a biodiesel process. However it should be noted that the strain was battling to utilise the methanol-containing glycerol and hence further studies are necessary to reduce the metabolic stress. Although the polymer produced was lower as compared to the quantities reported in the literature the levels produced are significantly larger than glucose taking into account the abundant raw material.

Further studies are necessary to fully understand and optimise the process although the potential to produce ε-Poly-L-lysine is promising from this abundant waste stream. Crude glycerol contains a number of proteins, salts, lipids and other impurities which affect product formation and its distribution in the culture which can also decrease product quality and costs associated in downstream processing. It will be an added advantage if glycerol pre-processing stages are carried out to reduce the salts that could have lowered the product synthesis in this study. In addition, there is need to look at the tools of genetic engineering to increase the uptake of glycerol and the ability to utilise methanol as a carbon source. Introduction of glycerol metabolism and ε-Poly-L-lysine over-expression genes could be applied to *S. albulus* to increase the industrial production of ε-Poly-L-lysine from the crude glycerol waste stream.

Alternatively, optimisation of the bioprocess involves taking into account all the processes involved, to maintain the balance between quality and yield. For example, we need to improve fermentation and downstream processing technologies. It is necessary to design efficient control strategies to optimise fermentation systems for industrial applications. The control of pH elsewhere in the literature produced more than 50 times as much the polymer produced in this study. This pH control strategy allowed physico-chemical interactions of the cells and their environment which increased the yield. We would suggest that designing a fermentation system with an integrated downstream processing, for example a continuous fluidized-bed bioreactor would allow the polymer to accumulate in the broth as it is an extracellular product, which is harvested and the cells remain in the culture in a continuous process. In addition, there is need to study the enzymes responsible for the polymerisation process for the design of a cell-free bioprocess. The process entails the substitution of the microbial production step of L-lysine with the direct biocatalytic conversion of L-lysine residues using purified enzymes thereby allowing the use of readily available L-lysine to produce this highly-valued polymer. This process allows optimal production of the polymer because it increases predictability of product formation and negates the stress factors that can lower the polymer production.

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APPENDICES

APPENDIX A: Crude glycerol analysis.

Table A1. Results of HPLC analysis of 8 glycerol samples.

Sample	Glycerol (g/l)	Methanol (g/l)
1	251.285	42.618
2	349.208	42.692
3	207.042	43.190
4	219.050	62.786
5	628.356	14.849
6	342.600	44.378
7	606.560	48.521
8	289.960	31.274

Table A2. Elemental composition of crude glycerol streams as detected by ICP-OES.

Sample	Type	K	Na	P	S	Pb	Zn
blank	**$mgKg^{-1}$**	**0**	**0**	**0**	**0**	**0**	**0**
	rsd	0.995	0.972	11.809	2.046	10.36	1.462
Ref blank glyc	**$mgKg^{-1}$**	**0.094**	**0**	**0.637**	**0**	**3.084**	**3.764**
	rsd	0.916	1.144	1.266	4.856	10.66	3.089
1 glyc	**$mgKg^{-1}$**	**486.900**	**10.470**	**0.805**	**0.750**	**4.581**	**6.693**
	rsd	0.648	1.322	3.924	0.694	9.343	1.746
2 glyc	**$mgKg^{-1}$**	**173.400**	**48.990**	**1.338**	**0**	**7.081**	**6.014**
	rsd	1.107	2.177	2.061	1.219	8.466	1.525
3 glyc	**$mgKg^{-1}$**	**387.200**	**34.530**	**3.977**	**0.805**	**6.324**	**8.094**
	rsd	1.119	1.717	0.785	0.752	11.919	1.999
4 glyc	**$mgKg^{-1}$**	**449.000**	**28.470**	**4.104**	**0.917**	**5.765**	**7.308**
	rsd	0.837	1.303	1.547	1.347	9.79	1.947
5 glyc	**$mgKg^{-1}$**	**324.700**	**68.170**	**0.908**	**0.342**	**1.580**	**2.380**
	rsd	1.021	0.704	1.797	1.166	9.72	1.906
6 glyc	**$mgKg^{-1}$**	**175.700**	**50.580**	**1.174**	**0.181**	**4.535**	**5.877**
	rsd	1.033	1.549	0.721	2.744	9.91	0.781
7 glyc	**$mgKg^{-1}$**	**373.200**	**6.440**	**0.803**	**0**	**9.826**	**5.131**
	rsd	1.158	2.152	2.494	5.143	10.21	1.473
8 glyc	**$mgKg^{-1}$**	**7.767**	**27.050**	**1.512**	**2.030**	**0.4794**	**35.99**
	rsd	1.198	6.658	1.121	1.529	12.01	9.291

APPENDIX B: Growth of *S. albulus* on glycerol containing medium.

Table B1. CFU counts of *S. albulus* grown on solidified media with different amounts of glycerol

Sample	CFU counts in days X 10^4 per milliliter												
	0	1	2	3	4	5	6	7	8	9	10	11	12
1%	0	102	136	140	141	144	147	148	148	148	149	149	149
2%	0	20	28	30	43	68	75	93	100	101	102	102	102
5%	0	12	20	23	30	43	60	89	98	106	107	109	110
10%	0	10	13	17	33	59	80	100	109	115	130	141	161
Starch	0	1	3	6	8	15	23	30	30	33	38	49	61

Standard error: 5 %

Table B2.Cell density as a function of OD readings (at 660nm) in shake flasks.

Sample	Cell density (OD at 660nm)					
	1	2	3	4	5	6
1%	0.1	1.925	2.200	3.140	2.690	2.700
2%	0	0.622	1.785	3.190	3.400	3.430
5%	0	0.539	0.928	3.605	3.655	4.320
10%	0	0.037	0.070	0.250	0.601	2.320
Glucose (2%)	0.2	2.705	3.445	0.407	0.407	0.410

Standard error: 5%

Table B3. Comparison of growth in pure, crude glycerol and glucose as a control

Sample	**Cell density (OD at 660 nm)**							
Glucose	0.011	0.354	0.541	0.838	1.565	2.870	2.980	1.8345
Pure glycerol	0.013	0.109	0.289	0.400	0.670	1.540	2.456	2.987
Crude glycerol	0.060	0.045	0.132	0.378	0.870	1.780	2.367	2.768

Standard error: 5%

APPENDIX C: Growth of *S. albulus* in methanol-containing glycerol

Table C1. Effects of methanol in growth of *S. albulus*

Sample	**Cell density (OD at 660nm)**					
	1	2	3	4	5	6
Control	0.200	2.680	3.600	4.200	4.300	4.800
5g/l	0.400	0.600	0.628	0.865	0.873	0.990
10g/l	0	0.740	0.768	0.775	0.867	0.880
20g/l	0	0.326	0.569	0.624	0.701	0.730
30g/l	0	0.263	0.387	0.425	0.658	0.690

APPENDIX D: Product purification and characterisation

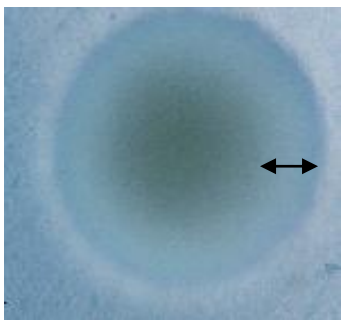
Table D1. Product elution profile as a function of absorbance at 210nm using a JASCO UV spectrophotometer.

Fractions eluted	1	2	3	4	5	6	7	8	9	10	11	12	13
Absorbance at 210nm	0.233	0.299	0.315	0.787	1.779	0.248	1.937	1.466	0.633	0.380	0.276	0.257	0.269

Table D2. ε-PL OD readings (465nm) for constructing a standard curve.

Concentration of ε-PL	**absorbance 1**	**absorbance 2**	**absorbance 3**	**Mean**	**Standard Error**
1000	0.280	0.284	0.281	0.282	0.00120
500	0.350	0.354	0.353	0.352	0.00120
250	0.345	0.341	0.347	0.344	0.00200
150	0.421	0.424	0.428	0.423	0.00150
62.5	0.439	0.433	0.436	0.436	0.00173
31.25	0.516	0.521	0.518	0.518	0.00145
12.63	0.700	0.703	0.706	0.703	0.00173

APPENDIX E. Detection of secreted polymer using methylene blue



A halo around a colony

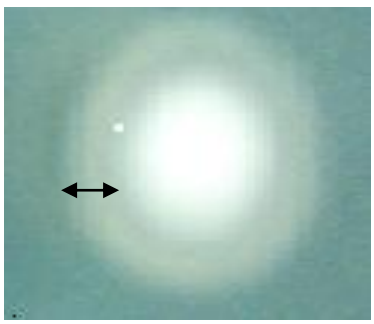


Figure E. A halo around colonies of putative positive ε-PL producers.